Efficiency in Plant Breeding

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W. Lange, A.C. Zeven and N.G. Hogenboom (Editors)

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Contents

Opening session

Address of welcome

H. Lamberts

President of Eucarpia

Ladies and Gentlemen,

On behalf of the Board of Eucarpia and the Organizing Committee, it is a great honour and a real privilege for me to welcome you here at the 10th triennial Congress of Eucarpia. I am sorry that I have to announce that the Minister of Agriculture and Fisheries of the Netherlands is not able to open this congress because of urgent matters elsewhere. His speech will be delivered by Professor Dr D. de Zeeuw, General Director of Agricultural Research.

Your Excellencies the Ambassadors and other official representatives of Austria, Finland, France, Hungary, Italy, Malaysia, Poland, Sweden and the United Kingdom, the Queen's Commissionar for the Province of Gelderland, the Burgomaster of Rhenen, the Rector Magnificus of the Agricultural University at Wageningen and the representatives of the sponsoring organizations, we are very glad to welcome you here at this opening session, so that you can witness our warm welcome to all participants of the congress.

Because some of you are unfamiliar with Eucarpia and plant breeding, I would like to give you a brief historical picture of the association. Eucarpia was founded in 1956, here in Wageningen where the first triennial congress was held. When it started, there were about 100 members. Nowadays we count more than a 1000, all closely connected with plant breeding and coming from practically every European country. From the very start, it was realized that the association was to stimulate the cooperation between breeders and research institutes, and especially between countries. This resulted in the establishment of specialized sections, mainly based on crops or groups of crops. We now have 12 sections. Each section works more or less independently of the parent association, in the organization of conferences, symposia and working groups.

The general congress, of which this is the 10th, meets every three years. It has become a tradition that this congress is held in the country of the president. At the end of the congress the president resigns and is replaced by someone from the country that will organize the next congress. The association is administered by a Board and Executive Committee. Let me, however, not trouble you with further details. I think that we can congratulate ourselves with the continuous growth in membership of Eucarpia and in the number of section meetings and other activities.

Since 1956, 27 years ago, plant breeding has developed drastically; new breeding

methods have developed in which innovation is practically a continuous process. Because of this, scientific contact is an absolute necessity. Joint efforts have led to an increase in productivity every year, for instance in winter wheat of at least 1% per year. About half of this increase can be attributed to plant breeding. So in those 27 years plant breeders have increased production by about 15%. But plant breeding aims not only to increase productivity, but also to obtain new varieties with better stability of yield. With the creation of varieties resistant to diseases and pests, plant breeding is helping to reduce pollution of water, air and soil, since the availability of resistant varieties reduces the need for pesticides. There also is an increasing interest in varieties that can grow under unfavourable conditions, as well as in varieties that need less energy to be grown, for instance at lower temperatures in greenhouses or at lower level of nitrogen fertilization. This is very important for the developing countries too. In general, the work done in breeding research and in practical plant breeding has been of enormous importance for the world food production and we can predict that this will continue to be so. It is a privilege for us breeders to work in a field that is clearly contributing to the welfare of the world.

Now, Dr de Zeeuw, it is an honour for me to request you to open this 10th Congress of Eucarpia.

Opening address[1]

G. Braks

Minister of Agriculture and Fisheries, The Hague, the Netherlands

Mr Chairman, Your Excellencies, Mijnheer de Commissaris van de Koningin van Gelderland, Mijnheer de Burgemeester van Rhenen, Ladies and Gentlemen,

We are honoured and pleased that the Netherlands has been selected as host of the 10th Eucarpia Congress. I have been told that the foundation meeting and the first Eucarpia congress took place in the Netherlands, and we appreciate that you have returned for your 10th congress.

I hope that each of you has had a pleasant journey and that the guests from abroad will enjoy their stay in this country.

In my address, let me refer briefly to the significance of plant breeding, to new developments in research, to plant breeders' rights, to cooperation between research workers and commercial plant breeders and, lastly, to the maintenance of genetic material.

The ancient Chinese scholar Shunce, who lived from 300 to 225 B.C., is reported to have said: 'Rather than allow things to propagate randomly, one should exercise one's skills and transform'. Knowledge that the quality of seed or planting material largely determines crop performance is probably almost as old as agriculture itself, and this quotation from the Chinese philosopher indicates that man's skills in improving cropping quality dates back to the pre-Christian era.

Vast experience in commercial applications and particularly in scientific research has made man's impact on the transformation of plants manifest. Undoubtedly plant breeding is one of the applied sciences that has had a major impact in agriculture and horticulture.

Plant breeding has been defined as 'controlled evolution of cultivated plants based on selection and combination or recombination of genetic properties'. Thanks to plant breeding there have been major improvements in production, in the stability of our cultivated crops and in the quality of our produce. For example, even in the Netherlands, with very high wheat yields, the average yield still increases annually by 0.5 to 0.75%, mainly thanks to plant breeding. However, the significance of plant breeding is even greater in the many countries with food shortages, where increases in food production will have to come primarily from increased yields.

1. Delivered by Prof. Dr Ir D. de Zeeuw, General Director of Agricultural Research.

Priorities in present-day plant breeding are, inter alia, curbing the application of chemicals by resistance breeding, breeding varieties that require less energy (in protected cultivation) and less nitrogen fertilizer (less energy and raw materials).

Plant breeding offers scope for innovations and diversification of products, which may boost sales and increase profitability of agriculture and horticulture.

New discoveries in basic sciences such as genetics and cytology, plant physiology and biochemistry, and development of sophisticated and advanced research techniques can result in major breakthroughs in plant breeding. Several research institutes in various countries are trying to apply recent developments in cellular biology and molecular genetics to plant breeding. Fruitful cooperation of the various programmes of basic research in biology with applied research on plant breeding are extremely important.

Moreover, the tasks in this new field are so complex and extensive that international cooperation, exchange of information and division of tasks will be indispensable. I am glad that some of these new developments in science and research techniques are on the agenda of your congress.

In the longer term, individual nations will benefit more from free exchange of information and from cooperation than from monopolizing newly developed breeding techniques. In our opinion, techniques should remain freely available to everyone in plant breeding.

On the other hand, plant breeders' rights, that is protection of the breeders' proprietary rights, have proved a major stimulus to breeders, whose activities are important for continuous development of the assortment of varieties. These days, capital outlay in a new variety can run into hundreds of thousands of guilders. So a breeder should have some guarantee that he will be repaid for his efforts.

In the Netherlands, the value of breeders' rights is beyond dispute. It should be observed, however, that legislation on plant breeders' rights should not be too rigid, in order to ensure that new varieties are easily accessible to all interested parties and, secondly, to prevent statutory regulations from hampering new developments.

With regard to plant breeders' rights, genetic manipulation raises a new problem. The Dutch Government has set up a study committee under the auspices of the National Council for Agricultural Research. The committee is to advise me on this matter.

My next point is the cooperation between plant breeding research and commercial practice. The Dutch agricultural research institutes are mostly foundations, in whose boards and advisory committees the breeders and growers play a major role.

In the plant breeding institutes, both breeders and growers accordingly have a say in research programmes. These institutes perform research into new breeding methods and into new properties or combinations of properties to be bred into our cultivated plants. They do not produce finished varieties, but supply breeding material at various stages of development to the plant breeding companies, where finished varieties are developed. The only exception is a small group of crops that are important for agriculture but not economically interesting for breeding companies.

The cooperation between research institutes and commercial plant breeders in the Netherlands has proved highly satisfactory and allows optimum utilization of the limited research capacity.

Finally I wish to mention the great importance of the preservation of genetic material. Genetic variation in each cultivated crop and in related species forms the basis for progress to be achieved by plant breeding. It is accordingly essential that this genetic variation is preserved and collected for storage in 'gene banks'.

In the last few years concern over the 'genetic erosion' has been increasing all over the world, and measures are being taken to prevent further loss of genetic resources. It is ironic that plant breeding itself has been a major cause of a considerable loss of genetic variation in the past: traditional varieties and landraces having been replaced by more uniform higher yielding varieties. Another cause is that agricultural development has replaced natural habitats of wild relatives of our crop species by arable land. In this way, natural plant resources are threatened with extinction and it has been truly said: 'Extinction is for ever'.

We are following with great interest the FAO initiative for an 'International Convention for the Conservation and Exchange of Plant Genetic Resources'. We welcome this initiative to focus international attention on an urgent problem. However, different types of material need to be distinguished:

- wild species, primitive cultivars and landraces,
- advanced varieties,
- material used in on-going breeding programmes.

Elementary for the preservation of genetic diversity is the conservation and exchange of the first group: 'wild species, primitive cultivars and landraces'.

Appropriately, the International Board for Plant Genetic Resources (IBPGR) restricts itself to this kind of material. We fully respect the establishment of a world network of gene banks and activities aimed at the conservation of natural habitats. We are, however, not in favour of the establishment of a Central World Gene Bank, as was proposed by the FAO. We do not regard this as a workable proposition.

To emphasize our concern, we in the Netherlands have decided to expand our activities: in the first place by establishing a national gene bank in Wageningen and secondly by extending our German-Dutch cooperation in the potato gene bank in Brunswick to include other crops.

For advanced varieties, there seem to be few problems as long as they are in use. There is a clear need to store old varieties that have gone out of use.

The proposed obligation to exchange material used in on-going breeding programmes, gives us great concern, however. These are the tools of the breeder and involve both intellectual and financial ownership: gene combinations resulting from human labour rather than a natural resource. Ultimately these gene combinations will become available in released varieties any way.

To include unfinished breeding material of on-going programmes in the convention is not compatible with the political systems of many countries and will make it more difficult to adhere to the convention. We are, however, confident that a workable solution will be found by the FAO working groups currently studying this issue.

Meanwhile let us realize that most genetic resources for our crop species are found in the third world. Hence we often depend on very poor countries to bear a major responsibility in the conservation of genetic resources. It is clear that we must share this responsibility, financially and technically, and we support FAO where it proposes to do something about that matter.

Ladies and gentlemen, I assume that such developments in plant breeding will be discussed at your congress, which I hope will be successful.

I hope there will be some time left for those among you who are visitors to this country to do some sightseeing. Our contry can offer you a lot of interest in culture, history and scenery.

Finally in expressing the wish that this congress may contribute to the international exchange of knowledge and research results and to international cooperation, I open this 10th Eucarpia Congress on 'Efficiency in Plant Breeding'.

Plant breeding in the Netherlands

C. Dorsman

Institute for Horticultural Plant Breeding, IVT, Wageningen, the Netherlands

Wageningen is the centre of agricultural research in the Netherlands, where both the Agricultural University and twenty autonomous research institutes of the Ministry of Agriculture and Fisheries are situated.

The Agricultural University incorporates a large number of disciplines, plant breeding being one of them. The Department of Plant Breeding of the University (IVP) aims at fundamental plant breeding research, as well as at education of scientific plant breeders. Research is centred on four main themes: (1) selection methods and natural selection; (2) ploidy variation, wild species and primitive forms; (3) resistance and resistance breeding; and (4) heterosis and hybrid varieties. The main crops involved are potato, barley, wheat, rye, maize and Petunia.

Most of the autonomous institutes were founded just after World War II, to support and to stimulate agriculture. Two are engaged in plant breeding research: the Foundation for Agricultural Plant Breeding (SVP) attends to field crops, the Institute for Horticultural Plant Breeding (IVT) to horticultural crops. Their policy has always been to stimulate private plant breeding and to serve as a bridge between universities and the private sector. Research is mainly aimed at development of new breeding and selection methods, as well as at development of breeding material. Such material, which is regularly made available to Dutch breeders, consists chiefly of half-products, i.e. populations containing new genetic characters or new combinations of characters, which may be incorporated into new varieties by the breeders of the private companies. The institutes develop commercial varieties only in crops that are not dealt with in private plant breeding in the Netherlands (e.g. strawberry, apple, pear, seed-poppy and some flower crops).

Research at SVP concentrates mainly on potato, wheat, barley, maize, grasses, sugar beet, and cruciferous crops. IVT covers fruits (apple, pear, strawberry), vegetables (e.g. tomato, cucumber, lettuce, bean, onion, carrot) and ornamentals (e.g. tulip, rose, carnation, lily, freesia, chrysanthemum, woody ornamentals). Moreover, both institutes have departments for discipline-oriented research (e.g. biometrics, cytogenetics, physiology, techniques in vitro, chemistry). All three breeding institutes have germplasm collections.

Research programmes of SVP and IVT are arranged in close consultation with other research institutes (including university departments) and with agricultural and horticultural interests. In the boards and advisory committees of both institutes, farmers, growers and commercial plant breeders are amply represented.

In the Netherlands, commercial plant breeding expanded strongly after World War II, being stimulated by scientific research, mainly in Wageningen, and by the regulations on plant breeders' rights (Act on Plant Breeders' Rights 1941; Seed and Planting Material Act 1967). Such developments have occurred in close dependence on the rapidly modernizing Dutch agriculture and horticulture. However, as the national market for seeds and young plants is relatively small for most crops, international trade has been a vital component of Dutch plant breeding.

In agriculture, commercial plant breeding covers mainly potato, wheat, barley, oats, maize, grasses, sugar beet, fodder beet, flax, peas and certain other fodder crops. The breeding firms employ over fifty Wageningen graduates.

In horticulture, the breeding of vegetable crops, which includes all vegetables grown in temperate climates, was the first to develop and to gain international acclaim. More than thirty graduate breeders operate in the horticultural seed firms. For ornamentals, apart from interests in traditional Dutch crops like tulips, commercial breeding on a scientific level is a recent development. It is directed mainly to carnation, chrysanthemum, alstroemeria and gerbera. For fruit crops, hardly any breeding is in commercial hands.

Commercial breeders are well organized in three unions: the Netherlands Breeders' Association (NKB) for agricultural crops, the Horticultural Seed Trade Association of the Netherlands (NTZ) for vegetables and seed flowers, and the Association 'CIOPORA Netherlands' for vegetatively propagated ornamentals.

Plant breeding is not an isolated activity. In accordance with regulations of the European Community, varieties of agricultural and vegetable crops are only admitted to the trade when individual identity, homogeneity and stability have been established. The Government Institute for Plant Variety Research (RIVRO), at Wageningen performs these tests (for vegetable crops assisted by the NAK-G), and the General Netherlands Inspection Services (NAK for field seeds and seed potatoes; NAK-G, NAK-S and NAK-B for horticultural crops) control seed quality. Moreover, RIVRO handles both technical research in the applications for breeders' rights and research on the value for cultivation and use of both agricultural and horticultural crops. Such activities result in a number of lists of varieties for these crops.

Improvement of selection methods

Effects of intergenotypic competition on selection

C.J.T. Spitters

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Summary

A quantitative analysis of intergenotypic competition and its effects on selection is given with a stochastic model and with a simple eco-physiological approach. The principles are illustrated with cereals.

In the stochastic model, expressions are derived for the components of variance in mixture and for the expected response to selection. Selection in a mixture of genotypes is considered to be an indirect way of selection for monoculture performance. Rules of thumb based on the model are developed for the expected response after single-plant selection and after progeny testing with different types of microplots.

On the basis of a simple crop growth model, a concept for the growth of genotypes in a mixed population is developed. In many cases, the relative differences among the genotypes remain about the same during their growth in mixture. Then, the competitive ability of a genotype can largely be explained by its starting position. This is confirmed by the results of an experiment with 12 wheat varieties grown in mixture.

Descriptors: competition, selection, breeding method, model, experimental design, cereals, wheat, *Triticum aestivum*

Introduction

In plant breeding, effects of intergenotypic competition are studied empirically. Often a genotype is grown in monoculture as well as in a mixture with other genotypes. The yield of the genotype in mixture differs from its yield in monoculture. Thus it is concluded that intergenotypic competition may bias the outcome of selection. But how large is that bias? Is it worth developing a method to reduce this bias? To arrive at answers we need to know how intergenotypic competition lowers the selection response, and what plant characters determine competitive ability.

This paper presents a theory about the effect of intergenotypic competition on the yields of genotypes in a mixed population and on the result of artificial selection. We will follow step by step the path by which selection of single plants or progenies leads to a certain response. This is examined with an empirical, stochastic model. To gain insight into the causal factors of competitive ability of a genotype in mixture, a simple model is presented that is based on principles of crop physiology. From both models, techniques are derived to reduce the competitional bias, with the effectiveness of the techniques being quantified. The principles of the competition effects are illustrated with cereals.

An empirical, stochastic approach for the selection response

The central question is: to what extent is the response to selection affected by competition between the genotypes in the population to which the selection is applied? Let us consider selection for yield. As the farmer grows his crops in genetically uniform monocultures, the yield of a genotype in mixture should be related to its yield in monoculture. Thus selection response should be measured by the progress made for yield in monoculture.

If we assume an expression for the expected yield of a genotype in mixture as a function of its yield in monoculture, we can then derive how the components of variance for yield alter with intergenotypic competition. We need to know that in order to derive an expression for the response of yield in monoculture to selection for yield in mixture.

The approach has been described fully in Spitters (1979). There the model was tested with the results of trials on single-plant selection and progeny testing in barley.

The stochastic model

Expected yield in mixture As genotypes are selected for their performance in monoculture, we express the yield of a genotype in mixture as a function of its yield in monoculture.

Many experiments show that competition is not additive but multiplicative, a conclusion supported by the physiological approach. This means that in a mixture of two genotypes, the percentage gain in yield per plant by a genotype equals the percentage loss in yield per plant by the other genotype, with gain and loss being expressed relative to their respective yields in monoculture. This proportionality between the expected yield of a genotype i in any mixture and its yield in monoculture is given by

$$Y_{i,\,\mathrm{mix}} = b_i Y_{i,\mathrm{mono}} \tag{1}$$

The proportionality factor b is a measure of the competitive ability of the genotype in the mixture and is estimated empirically by dividing its yield per plant in mixture by its yield per plant in monoculture.

Equation 1 implies that the yield of a plant depends on the genetic make-up of the entire population rather than on the genetic composition of its nearest neighbours. In cereals, this situation is approximated by individual plants where each plant affects to a considerable degree the plants that are farther than the nearest neighbours (diffuse competition). On the other hand, a row of plants influences only its adjacent rows (nearest-neighbour competition). Only diffuse competition among single plants is considered here. Corresponding expressions for nearest-neighbour competition, i.e. for testing of progenies in row plots were described in Spitters (1979, p. 57, 66-68).

Components of variance The yield of a plant in a population is a stochastic quantity, a function of the two random variables genotype and environment. The observed yield of a plant, its phenotype, is represented by the linear function

$$\underline{p} = \mu + \underline{g} + \underline{e}$$

where $\underline{g}$ is the deviation from the population mean μ due to genotype and $\underline{e}$ is the deviation from the population mean due to environmental and other uncontrolled factors. (Stochastic variables are underlined in the text.)

From Equation 1, we may derive yield of a plant in mixture

$$\underline{p}=\underline{b}(\mu+\underline{g})+\underline{e} \tag{2}$$

The aggregate environmental effect $\underline{e}$ is taken as additive in accordance with the usual approach in genetics.

For a normally distributed variable, the distribution can be characterized by the mean (the expected value) and the variance. Expressions for the components of variance may be derived from Equation 2. Due to the multiplicative form, only approximate results can be obtained (Spitters, 1979, p. 51-53, 68-69).

For the phenotypic variance in mixture, i.e. the variance among the single plants in the mixture, we obtain

$$\text{var } \underline{p}_{\text{mix}} \approx \text{var } \underline{g}_{\text{mono}} + 2\mu \text{ cov } (\underline{b}, \underline{g}_{\text{mono}}) + \mu^2 \text{var } \underline{b} + \text{var } \underline{e}_{\text{mono}} \tag{3}$$

where μ is the population mean of the monocultures. This phenotypic variance in mixture can be partitioned into the variance among genotype means in the mixture, the genetic variance

$$\text{var } \underline{g}_{\text{mix}} \approx \text{var } \underline{g}_{\text{mono}} + 2\mu \text{ cov } (\underline{b}, \underline{g}_{\text{mono}}) + \mu^2 \text{ var } \underline{b} \tag{4}$$

and the environmental variance

$$\text{var } \underline{e}_{\text{mix}} = \text{var } \underline{e}_{\text{mono}} \tag{5}$$

Response to selection The ultimate interest of the breeder is the progress to be expected with selection for yield. That progress is the progress for monoculture yield when selection is for yield in a mixed population. Let us distinguish two independent steps (Figure 1): (1) the extent to which the selected plants are the genotypes with the highest yield in the particular environment, i.e. in that particular mixture; (2) the extent to which the genotypes with the highest yield in the mixture, yield most in monoculture.

The first step measures the accuracy of yield testing and is represented in the right-hand quadrant of Figure 1. The selected plants have a mean phenotypic yield of $\bar{p}_s$. The difference between this mean and the population mean $\bar{p}$ is called the selection differential S_{mix}. If the regression of genotype on phenotype is rectilinear the response to selection is

$$R_{\text{mix}} = rc_{\text{R.S}} S_{\text{mix}}$$

where rc is the slope of the linear regression. Substitution of the statistical definition of the regression coefficient gives

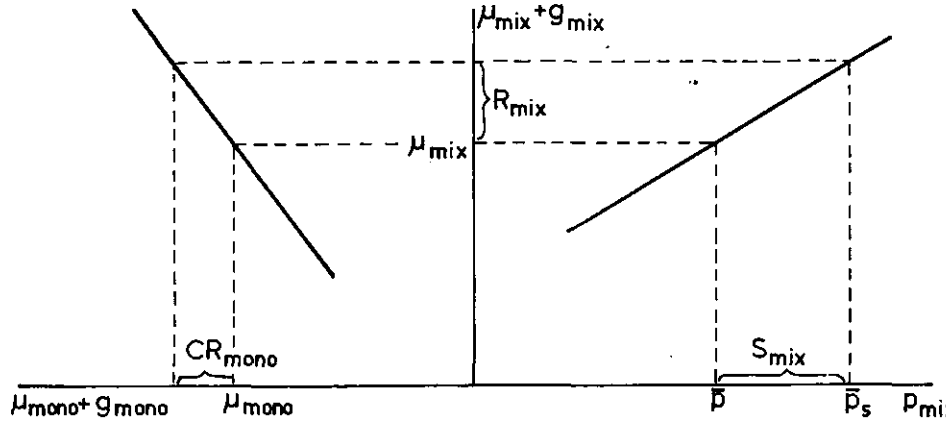


Fig. 1. Correlated response for yield in monoculture due to selection for yield in mixture. Right: Accuracy of yield testing represented by the regression of genotype on phenotype in mixture, showing the selection differential S_{mix} and the selection response R_{mix} for yield in mixture. Left: Interaction between genotype and competitional environment represented by the regression of genotype in monoculture on genotype in mixture showing the relation between selection response for yield in mixture (R_{mix}) and correlated response for yield in monoculture (CR_{mono}).

$$R_{mix} = \frac{\text{cov}(g_{mix}, p_{mix})}{\text{var } p_{mix}} \; S_{mix} = \frac{\text{var } g_{mix}}{\text{var } p_{mix}} \; S_{mix} = h^2_{mix} \; S_{mix}$$

The heritability h^2_{mix} measures that part of the total phenotypic variation which can be ascribed to genetic differences between plants.

Division of the selection differential S_{mix} by the phenotypic standard deviation transforms S_{mix} into a dimensionless parameter called 'intensity of selection': $i_{mix} = S_{mix}/\sqrt{(\text{var } p_{mix})}$. Falconer (1960, p. 194) showed that, with a normal distribution of the measurements, i_{mix} is a simple function of the percentage of individuals selected.

The response to selection can now be extended to

$$R_{mix} = h^2_{mix} \; S_{mix} = i_{mix} \sqrt{h^2_{mix} \; \text{var } g_{mix}} \tag{6}$$

The response R_{mix} represents the progress in yielding ability in that particular mixture, a character of no interest to the breeder. For this reason we need a second step: the correlated progress in yield in monoculture due to selection in mixture. This is represented in the left-hand quadrant of Figure 1. The correlated response for yield in monoculture is

$$CR_{mono} = r c_{mono.mix} \; R_{mix} \tag{7}$$

After elaboration of the regression coefficient and substitution of Equation 6, we obtain

$$CR_{mono} = R_{mix} \; r_g \sqrt{\text{var } g_{mono} \,/\, \text{var } g_{mix}} = i_{mix} \; r_g \sqrt{h^2_{mix} \; \text{var } g_{mono}} \tag{8}$$

where r_g is the correlation between the yield of a genotype in monoculture and its yield in mixture.

By extending Figure 1 with a third quadrant, allowance can be made for the drop in response from the generation of selection to the next generation due to the genotypes not being true to seed. With a fourth quadrant allowance can be made for the genotype × environment interaction arising from the deviation of the growing conditions (husbandry, location, year) where selection has been practised from average farm conditions.

To compare trials with different yield levels, the equations should be formulated in terms of dimensionless variables that are expressed relative to the yield level. This is achieved by expressing variances in terms of coefficients of variation with the coefficient of variation being the standard deviation divided by the population mean μ:

$$CV_e = \sqrt{\text{var } \underline{e}}/\mu \text{ and } CV_g = \sqrt{\text{var } \underline{g}}/\mu$$

The responses R_{mix} and CR_{mono} are also divided by the population mean μ. This approach with dimensionless quantities is adopted below. Table 1 summarizes the relevant quantities and equations. The symbols R_{mix} and CR_{mono} are also used for the relative responses.

Illustration of the model and order of magnitude of the competitional bias

The theoretical model was developed to elucidate the lines along which intergenotypic competition biases the outcome of selection. General principles will now be illustrated and an order of magnitude for the effects will be indicated.

Table 1. Coefficients of variation and predicted responses for selection on the basis of grain yield of single plants, 1-row plots, the centre row and all three rows of 3-row plots, and field plots 10 metres square. The values are computed according to Spitters (1979, p. 61, 57, 67, 69, 222). Inputs were $CV_{g,mono} = 0.05$, $\sqrt{(\text{var } \underline{b})} = 0.20$, $r_{b,g} = 0$, $i = 1.75$ (10% selected), $CV_{e,mono} = 0.40, 0.20, 0.12, 0.06$ for single plants, single rows, 3-row plots and field plots, respectively. $CV_{e,mono}$ is the CV_e in absence of intergenotypic competition. The values are considered to be representative for small-grain cereals. The row plots are 2 m long with rows 20 cm apart.

	Single plant	1-row plot	3-row plot		Field plot
			centre	3 rows	
CV_g	0.21	0.11	0.05	0.06	0.05
CV_e	0.40	0.21	0.20	0.12	0.06
$CV_p = \sqrt{(CV_g^2 + CV_e^2)}$	0.45	0.24	0.21	0.13	0.08
$h^2 = CV_g^2/CV_p^2$	0.21	0.22	0.06	0.20	0.41
$R = i \cdot CV_g \cdot \sqrt{h^2}$	0.17	0.09	0.02	0.05	0.06
$CV_{g,\,mono}/CV_{g,\,mix}$	0.24	0.45	1.00	0.83	1.00
$r_{g'}$	0.24	0.45	1.00	0.83	1.00
$CR_{mono} = R_{mix} \cdot r_g \cdot CV_{g,\,mono}/CV_{g,\,mix}$	0.010	0.018	0.021	0.033	0.056

Justification of the applied approach Results of experiments with barley have been used to illustrate the model (Spitters, 1979, p. 163-165, 213-218, 237-245). Any experiment, however, involves only one out of an infinite number of combinations of the population parameter values, which makes it hard to arrive in such an empirical way at rules of thumb about the orders of magnitude of the competition effects. Let us therefore discuss the output from the model, generated with input values that are considered to be realistic averages.

Order of magnitude of the input variables and calculation procedure Input values are given in the caption of Table 1. These estimates are mainly from five years of experiments with barley and spring wheat on sandy clay-loam at Wageningen (Spitters, 1979; Kramer et al., 1982).

The coefficient of genetic variation (CV_g) was that among F_5 or F_6 lines grown in pure stand. A range of 2 to 12% seems quite normal for CV_g. The values for the coefficient of the environmental variation (CV_e) were derived from trials where all the studied types of plot were present. Their ratios will have more general application than their absolute values. The standard deviation of the competitive ability $\sqrt{(\text{var } \underline{b})}$ is in the order of 20%. In other words, for 95% of the genotypes yield in mixture deviates less than 40% ($=100\times1.96\sqrt{(\text{var } \underline{b})}$ from yield in monoculture. On the whole, there is little relation between the competitive ability of a genotype and its yield in monoculture ($r_{bg}\approx0$).

From these input values, the components of variance and the response to selection were estimated with the model for several methods of yield testing: single-plant selection and progeny testing with different types of plot (Table 1). The estimates for single plants were obtained from Equations 4-8. As mentioned already, dimensionless variants of these equations were used. The computations for the other situations were based on equations presented in Spitters (1979, p. 57, 61, 67, 222).

To interpret the results, bear in mind that 'interplot' competition is most severe among single plants; it is smaller between 1-row plots; it is slight between 3-row plots and it is negligible between the centre rows of neighbouring 3-row plots and between large field plots. Not only effects of intergenotypic competition are involved but also effects of sample size. A larger plot size implies a greater sample size which reduces CV_e.

Genetic and environmental variance in mixture The genetic variance in mixture is greater than that in monoculture, provided that the correlation between competitive ability and monoculture yield is not too strongly negative (Equation 4). For the parameter values used in Table 1, the genetic variance CV_g^2 in the single-plant mixture was more than 17 times as large as that in monoculture.

On the other hand, the environmental variance, i.e. the variance among entries having the same genotype, is not affected by the diffuse intergenotypic competition among single plants and only very little by the nearest-neighbour competition between rows (compare CV_e for 1-row plots with that for the centre rows of 3-row plots in Table 1). The other differences in CV_e in Table 1 were caused by differences in sample size.

Response to selection As the genetic variance is enhanced while the environmental variance remains practically unchanged, the share of the genetic variation in the total phenotypic variation increases due to intergenotypic competition. Because of this increased heritability, together with the increased genetic variation, the direct response to selection will be greater in presence of intergenotypic competition than in absence of it (Equation 6; contrast 1-row plots with centre rows). The effect of competition may be such that selection of single plants in mixture results in even greater response than selection based on complete field plots with monocultures: 17% against 6% (R in Table 1). Thus discrimination among genotypes for yielding capacity is easier with than without intergenotypic competition.

However yielding capacity in mixture is of no interest because varieties are grown in pure stand. We have to deal with the correlated response for yield in monoculture CR_{mono}, which is the result of the selection for yield in mixture. This correlated response is smaller than the direct response for yield in mixture because the yields in the two environments do not perfectly correspond ($r_g < 1$) and because the useful genetic variation is smaller than that in the selection environment ($CV_{g,mono} / CV_{g,mix} < 1$). The more intense the competition, the greater the reduction from R_{mix} to CR_{mono} (Table 1). Thus, although competition acts as a magnifying glass (increased genetic variation), that glass has severe spherical aberration (correlation coefficient less than one).

The optimum type of plot for yield testing The progress after selection is measured by the correlated response for yield in monoculture CR_{mono}. The field plots show the greatest progress, followed by 3-row plots, single rows and single plants, in that order (Table 1).

A breeder aims to maximize his profits, i.e. the difference between the output (response) and the input (costs) of the selection process. Response and costs should be expressed in the same dimension, e.g. both in monetary values. Now the problem arises how the yield response, calculated with the model, has to be converted into a financial response. The relation between financial and yield response seems S-shaped, rather than linear. With small yield responses, the chance of identifying a genotype that produces a successful variety seems disproportionally small. With high yield responses the law of the diminishing returns will hold. This S-shaped relation is allowed for in an extreme way by assuming that at least the yield response attained with the unreplicated field plots is required to get any financial return. This approach will be worked out now, the more as some principles emerge.

The correlated responses of the different types of plot relate to each other as 0.18 : 0.32 : 0.38 : 0.58 : 1.00, respectively (CR_{mono} in Table 1). To achieve the same response as with the field plots, the response of the microplots should be enhanced. Equation 8 shows that this can be done by increasing $\sqrt{(h^2)}$ or i, that is (1) by testing each entry with a greater number of replicates, which reduces CV_e and consequently enhances the heritability or (2) by increasing the number of entries, which makes it possible to increase the selection intensity i.

For single plants and 1-row plots, $\sqrt{(h^2)}$ should be increased by a factor 5.6 and 3.1, respectively (Equation 8) to level the difference from field plots. Table 1 then shows that the heritability should be increased to more than one, an impossibility since genetic variance never exceeds phenotypic variance. Hence, these plot types are inferior to field

plots, given the input values studied in the model. Harvesting only the centre row of a 3-row plot is also inferior as it will be hardly cheaper than harvesting all three rows, and as it yields a substantial lower response.

To bring the response after selection for yield of all three rows of the 3-row plots to the level in field plots, either i or $\sqrt{(h^2)}$ should be enhanced with a factor 1.7. So, $\sqrt{(h^2)}$ should be magnified to 0.77. Given the CV_g of 0.06, this needs a decrease of CV_e from 0.12 to 0.05, which is achieved with 6 replicates of each entry. As an alternative, i may be increased by the factor 1.7 to a value of 3.0, which corresponds with a selection intensity of 0.4%. Compared to the 10% selection imposed on the field plots, it means a 25-fold increase in population size.

With respect to increase in number of replicates and with respect to stiffer selection, the law of the diminishing returns holds. So there is an optimum for the combination of the two parameters where the number of plots to be tested is minimal. Under the conditions studied, trial and error showed that the optimum was an increase in the number of entries to be tested by 35% and replicating each entry 4 times. Therefore, when the costs of using 1.35×4 ($=5.4$) 3-row plots are less than of one field plot, yield testing on the basis of 3-row plots would be more profitable. Values for a greater genetic variation in pure stand and for stiffer selection on the field plots are given in Table 2.

The above also suggests that it would be more profitable to evaluate a moderate number of (promising) entries accurately in replicated plots than to test many entries without replication. This trend is stronger, the smaller the heritability and the greater the selection intensity. Bos (1983) has worked out this balance between replication and intensity of selection in more detail.

Table 1 needs some comment. It is claimed that the values substituted for the input parameters were sufficiently accurate to generalize the conclusions. These conclusions were supported by experimental evidence (Spitters, 1979, p. 237-245; Kramer et al., 1982). Nevertheless, there will be situations where some of the findings become modified

Table 2. Increase in the number of replicates r and the number of entries to be tested by a factor n in 3-row plots to achieve the same response as in unreplicated field plots ($r = 1$, $n = 1$). Given is that combination of r and n that fulfils this requirement with the smallest increase in the total number of 3-row plots. The total number increases by the factor N. Calculations are for two levels for genetic variation of yield in monoculture and for intensity of selection in the field plots.

$CV_{g,\,mono}$	$i = 1.75$ (10 %)		$i = 2.06$ (5 %)	
	$r \times n$	$= N$	$r \times n$	$= N$
0.05	4×1.35	$= 5.4$	6×0.96	$= 5.8$
0.10	2×1.85	$= 3.7$	3×1.50	$= 4.5$

as there is a substantial variation in the parameters among populations and selection nurseries. Moreover, the prospects of selection on microplots will be somewhat overestimated if agricultural practice in the breeding nursery differs more widely from that in commercial farming. If lack of seed or shortage of land impose restrictions on the larger plot types, yield testing based on single plants or 1-row plots might become useful.

The major drawback, however, concerns the break-even ratio of the costs of a microplot compared to the costs of a field plot. This ratio was calculated on the assumption that at least the yield response attained with the unreplicated field plots was required to get any financial return from yield testing. This is an arbitrary assumption and only illustrates a way of thinking. It emphasizes that choice of optimum plot type will be achieved only when economic aspects are integrated into research on field plot technique.

A causal physiological approach for the characters determining competitive ability

In the preceding, yield in mixture was related to yield in monoculture in an empirical way (Equation 1). Such an empirical relation does not clarify the causal factors determining that relation. Understanding of the processes in a system is, however, prerequisite to intervene in that system in an effective and reliable way.

A simple model is presented below for the growth of a genotype in monoculture and in mixture. The model will be used to derive which characteristics give a high yield in mixture and which give a high yield in monoculture. Subsequently, measures are derived to reduce the bias due to intergenotypic competition. We recently elaborated on this approach (Spitters & Aerts, 1983; Spitters, 1984).

Simple model for growth in mixture

Growth in monoculture In the early stages after emergence, a plant increases almost exponentially in weight. This exponential increment results from an exponential increase of the growth rate, attributable to an exponential rise in the amount of light intercepted (Figure 2). When the canopy closes, plants start hindering each other in the extension of their light interception. Exponential growth ends and competition for light begins. From the time that the canopy has closed, the crop intercepts a constant maximum fraction of the incident light. If light is the main limiting factor, the daily growth rate becomes approximately constant so that the total dry weight increases almost linearly with time (Figure 2).

Growth in mixture We may extend the approach for monocultures to a mixture of two genotypes (Figure 3). Initially, there is no interplant competition. The plants grow almost exponentially, as in monoculture. Once the canopy has closed, nearly all the incident light is intercepted. The light is distributed over the genotypes according to their share in the total leaf area. If the genotypes produce an equal area of new leaf for each unit of absorbed light energy, their shares in the total canopy remain constant (horizontal lines in Figure 3 right). As growth rate is related linearly to the amount of intercepted light, growth in mixture proceeds linearly with time, but for each genotype at a different rate. Owing to these differences in growth rate, the differences between the genotypes

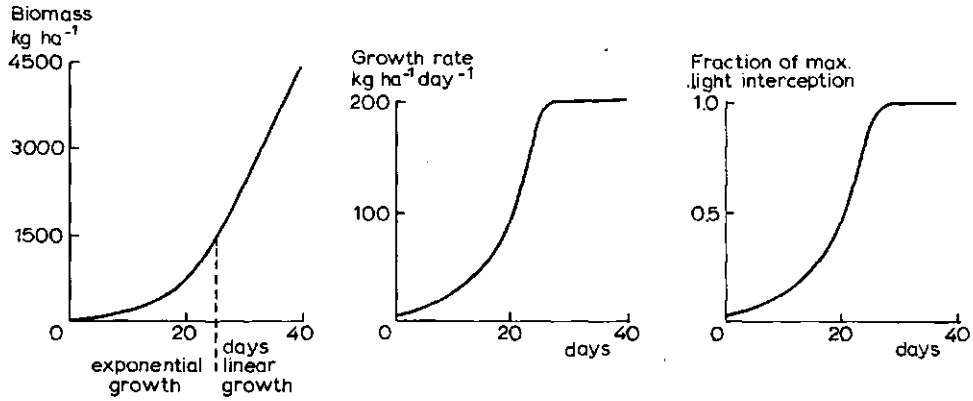


Fig. 2. Progression in time after emergence of total biomass, of daily growth rate, and of fraction of maximum light interception. Schematic example for a spring-sown cereal, illustrating the transition of exponential into linear growth.

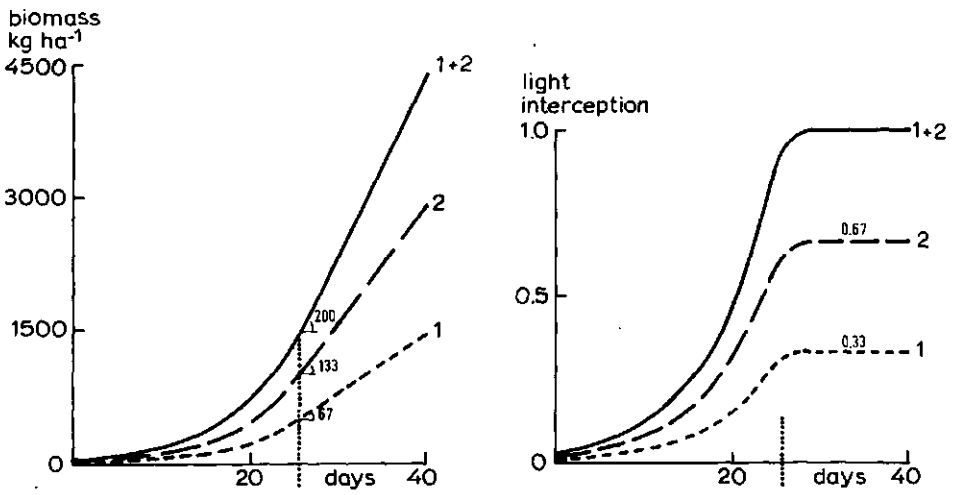


Fig. 3. Progression in time after emergence of biomass and of light interception of two genotypes in a mixed crop. Their totals are identical to those in Figure 2. The initial weight of Genotype 2 was supposed to be twice that of Genotype 1. The slope of the biomass curves represents the growth rate expressed in kg ha^{-1} day^{-1}.

increase with time (Figure 3 left).

If the share of a genotype in the total canopy remains constant, then this portion equals the share the genotype has at the time the crop is closing. When the genotypes grow at an equal percentage per day during the exponential phase, i.e. when they have the same relative growth rate (RGR), their shares can be fully predicted from their initial weights. This is so in Figure 3 where Genotype 2 possesses twice as heavy seedlings as

Genotype 1. Because the genotypes have the same RGR during their exponential growth (parallel lines on a logarithmic scale), the relative differences are maintained and Genotype 2 acquires twice as large a portion of the canopy. That enables it to grow in the linear phase at twice the rate.

Although the absolute differences between the genotypes in the mixture swell up with time, their relative differences remain constant. The double final biomass of Genotype 2 is fully explained by its seedlings being twice as heavy. This principle holds also when other factors than light limit growth.

Characters determining the competitive ability of a genotype

Deductions from the growth model From the previous concept, one may deduce the strategy a genotype leads to produce a large biomass in mixture. We may loosely define this as a high competitive ability.

The initial status appeared to be critical. A favourable initial status is achieved with a high initial biomass per m^2, which is attained either at many seedlings per m^2 or with heavy seedlings. Heavier seedlings are obtained from larger seeds and with earlier emergence.

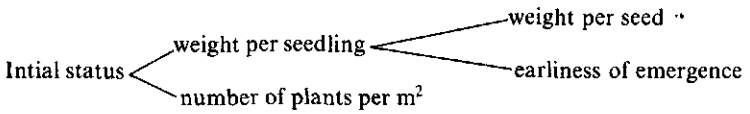
The relative differences among the genotypes in mixture are, by definition, only maintained if they have the same relative growth rate in course of time, in the exponential as well as in the linear growth stage. This was supposed in Figure 3. However the 'space' may be redistributed in the mixture in favour of the genotypes with the higher relative growth rate. Dry matter increment per unit biomass per unit time (relative growth rate, RGR) can be partitioned into dry matter increment per unit leaf area per unit time (net assimilation rate, NAR) and amount of leaf area formed per unit biomass (leaf area ratio, LAR). Thus, when light is the main growth limiting factor, a genotype will improve its share in the total canopy when it produces more dry matter per unit absorbed light (higher NAR), when it forms a greater leaf area per unit dry weight (greater LAR), or when its leaves are more favourably placed through a greater plant height (higher NAR). When factors other than light are limiting, the process of redistribution of the space becomes somewhat more complex, although a great leafiness is mostly advantageous. If space is redistributed, the relative differences between genotypes in mixture do not remain constant but change with time. This becomes more important the more prolonged competition is, as in perennials.

The characters that determine the competitive ability of a genotype are summarized in Table 3.

The dominating effect of initial status An experiment with 12 wheat varieties grown in mixture at 5×5 cm^2 plant $^{-1}$ confirmed that genetic differences in biomass production in mixture can be largely ascribed to differences in initial status, expressed as seedling weight ($r^2 = 0.71$, Figure 4). Thus, the simple approach of Figure 3 appears to be useful in the field too.

Differences in seedling weight were partly due to differences in seed weight ($r^2 = 0.64$) and partly to differences in earliness of emergence ($r^2 = 0.54$). Seedling weights predicted from both, by means of the exponential growth function with an RGR of 0.16 d^{-1}, fully

Table 3. Characters determining the biomass production of a genotype in mixture and with that also its competitive ability.



RGR in exponential phase

Redistribution of 'space'
- biomass produced per unit of limiting resource absorbed
- absorption capacity formed per unit biomass
- spatial position with respect to absorption of the limiting resource ('priority')

Length of (vegetative) growing period

explained the differences in weights of seedlings ($r^2 = 1.01$; Figure 4). (These correlations were obtained after adjustment for the error variation of the variety means.)

The genetic variation for initial status was very small: 10% for the genetic coefficient of variation for seed weight and 9 h for the genetic standard deviation of time of emergence. It is remarkable that such small initial differences between genotypes have such a strong influence on their final biomass in mixture. It was noted already that, with certain assumptions, the relative differences are maintained in mixture. On the strength of these assumptions a doubling of final biomass of a genotype in mixture (a 100% yield advantage) is gained with twice as heavy a seedling, either with seeds twice as large or with emergence 4.6 days earlier ($RGR = 0.15\ d^{-1}$).

Many authors have tried to relate competitive ability to morphological characteristics. They often failed, as is illustrated with the finding of Sakai (1961) that 'competitive

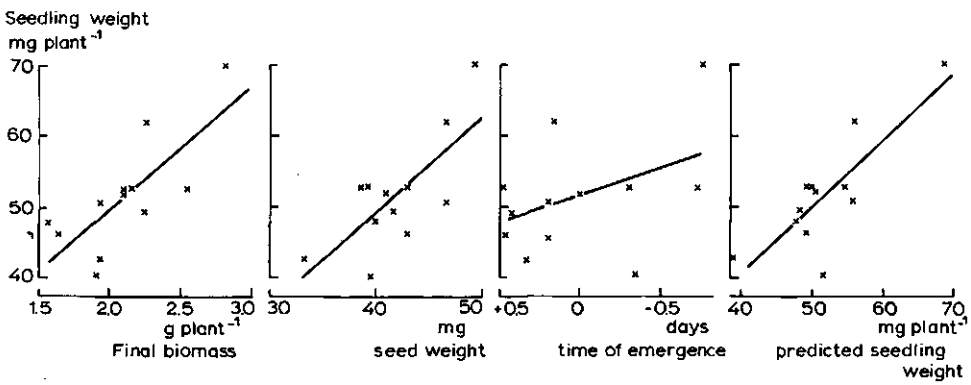


Fig. 4. The final biomass of 12 wheat varieties in mixture in relation to their seedling weights on day 22. The varietal differences in seedling weight are explained by differences in seed weight, time of emergence and the combination of both ($\hat{w}_{22} = w_0 \exp(0.16 \times 22)$). The genetic correlation, i.e. the correlation after adjustment for the error variation of the varietal means, is 0.84, 0.80, 0.73, and 1.00 respectively.

ability was not associated with morphological traits which might be supposed to favour competition'. This is quite understandable from the model.

Effect of competition on marketable yield and relation between yield in mixture and monoculture

Competition among genotypes for growth limiting factors is expressed in their biomass. That is why the previous approach deals with biomass. The farmer, however, is concerned with yield of some desired plant parts rather than with biomass. So the influence of competition on yield will be partitioned into its effects on total dry matter (biomass) and its effects on the allocation of the dry matter within the plant. The degree of correspondence between the yield of a genotype in mixture and its yield in pure stand will receive special attention in the discussion.

Biomass The supply of the growth limiting factors is the same for each monoculture, provided that the genotypes have a similar growing period. Moreover, there is little variation in the efficiency with which the genotypes harness the limiting resources for their dry matter production. Thus, genetic variation for biomass in monoculture is small in general, as often confirmed in the literature.

However in mixture, the genotypes have to draw on the same stock of limiting resources, they compete for the same 'space'. The unequal distribution of these resources between genotypes gives rise to different rates of dry matter production, which is the major cause of the biomass differences in mixture. So genetic differences in mixture will exceed those in monoculture (CV_g in Table 1). In the empirical approach, the competitive ability of a genotype was defined as the ratio between its production in mixture and its production in monoculture (Equation 1). This ratio measures the ability of a genotype to acquire the limiting resources in the mixture, to occupy 'space', with genetic differences in efficiency of utilization being removed from this measure. As a consequence, the competitive ability b of a genotype is proportional to the surface underneath the curve for its fraction of the available resources acquired (the curve for light interception in Figure 3).

Differences in the ability to monopolize the resources are attributable mainly to differences in starting position (Figure 4). If these differences affect monoculture production, their influence is small and in no proportion to their significance for competitive ability. That gives a rationale behind the finding that there is on the whole no relation between competitive ability and pure stand performance, $r_{b,g} \approx 0$.

Under this condition, the genetic correlation between productivity in monoculture and mixture (Spitters, 1979, Equation 4.57) simplifies to

$$r_g = \sqrt{\frac{CV^2_{g,\,mono}}{CV^2_{g,\,mono} + \text{var}\,\underline{b}}} \qquad (9)$$

Hence, the smaller the genetic variation for monoculture biomass relative to that for competitive ability, the worse the relation between the biomass of a genotype in monoculture and its biomass in mixture. Nevertheless, a positive correlation is expected because the genetic differences in yield in monoculture tend to be maintained in mixture.

Harvest index That fraction of the biomass that is located in the agronomically desired plant organs is called the 'harvest index' (HI). In most crop species, HI appears to be little affected by intergenotypic competition (Spitters, 1979, p. 189-191). Thus, the HI of a genotype in monoculture and its HI in mixture will generally show a correlation close to unity.

Marketable yield Yield is the product of biomass and harvest index. As competition little affects HI, the competitive ability of a genotype, as estimated by b in Equation 1, will be about the same, irrespective of whether the estimate is for yield or for biomass. Therefore, the competitional variance is also about the same for both traits.

On the other hand, the genetic variance for monoculture performance will be greater for yield than for biomass as there is substantial genetic variation in HI. For example, in cereals, the progress in yield due to breeding is associated with an increase in HI, with little change in biomass (e.g. Riggs et al., 1981).

Given the larger $CV^2_{g,mono}$ of grain yield and the equal competitional variance, Equation 9 shows that the genetic correlation between performance in monoculture and mixture is greater for yield than for biomass. For the same reasons, $CV_{g,mono}/CV_{g,mix}$ is greater for yield too. The coefficient of the regression of monoculture performance on mixture performance, the product of the two quantities (Equation 8), is therefore larger for yield. So selection for yield of some plant parts will in general be less biased by intergenotypic competition than selection for biomass itself.

Consequences for breeding practice

As we have seen, the ranking of the genotypes in mixture differs from that in monoculture. This genotype × population interaction is the mechanism by which intergenotypic competition biases the outcome of selection. The difference between the yield of a genotype in mixture and its monoculture yield is brought about by the unequal distribution of the growth limiting factors between the genotypes constituting the mixture. The share that a genotype gains in mixture is closely related to its initial status, its starting position.

Because initial differences between genotypes bear little relation to yield in monoculture, minimizing the initial differences seems to be an effective method in reducing competitional bias. Table 3 indicates how initial differences may be minimized:

- Differences in seed size are reduced by grading the seeds and sowing large and small seeds in separate plots. Grading seeds in order to decrease competitional bias is a technique advocated by several authors.
- Simultaneous emergence and establishment is promoted at one hand by favourable germination conditions, especially a fine seed-bed, and at the other hand by uniform drilling, especially in sowing depth.
- Differences in the numbers of plants with which the genotypes are present in the population are of importance when the unit of selection consists of several plants. That holds for progeny testing where progenies of plants selected in a previous year are evaluated in small plots. The number of plants within a plot strongly affects the ability of such plot to compete with its neighbours.

Minimizing variation in initial status not only reduces intergenotypic competition, but

also reduces the intragenotypic competition brought about by non-genetic variation in initial status. And this reduces environmental variation.

Several other methods of reducing the competitional bias were proposed for single-plant selection as well as for progeny testing in microplots (Spitters, 1979, p. 176-192, 225-232). The effect of plant spacing was worked out further in a recent paper (Spitters, 1984).

Plant breeding literature on field-plot technique is dominated by numerous papers presenting results of experiments where selection was practised according to various methods in segregating populations. Every time the results were different, leading to conflicting recommendations. The efforts would have been employed more efficiently, if some principles of technological research had been recognized. These start with the definition of a conscientious working hypothesis on the basis of a coherent theory. The hypothesis should be falsified or verified in a reductionistic way with experiments in which the studied methods differ in a minimum number of factors. Use of known pure lines, families or clones will be more fruitful in breeding research than use of unknown irreproducible genotypes of segregating populations. Apart from that, summarizing and quantitating a theory by a mathematical model facilitates the detection of conditions for which the results hold. In that way one comes to 'if . . ., then . . .' statements rather than to the empirical conclusions that 'sometimes method A is better than B and sometimes B is better than A'. Experiments may supply the orders of magnitude for the parameters needed as input in the model (Table 1). Experiments are also required to falsify the theory and are helpful in sharpening and extending the theory. The models presented in this paper are simple. They serve to clarify the broad lines of the effects of intergenotypic competition as well as to provide a frame for further research.

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Competition and selection for yield: a perspective from forestry

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Summary

Additional information is given on three topics raised by Spitters (1984) with reference to work on woody perennial crops.

First, prolonged and intense competition, because it is a spatial, neighbour-dependent process, can obscure, rather than magnify, differences among genotypes in mixtures. Competition can thereby decrease the effectiveness of phenotypic selection in tree stands for individual-tree competitive ability.

Secondly, the relative differences among tree genotypes in mixed-genotype stands commonly changes with age, depending on their initial size, their ability to 'capture' the site, their response to inter-tree competition after canopy closure, and their rate of aging.

Thirdly, some points are made regarding the debate on whether increases in individual-tree competitive ability will enhance per hectare growth rates in closed stands.

Descriptors: competition, selection, forestry, juvenile-mature correlation, heritability

Introduction

My intention is to broaden the discussion to woody perennial plants, where competition is more prolonged than in small-grained crops, and where the impact of competition on selection for yield is, in some respects, different from that described in Spitters' excellent paper (1984).

Few tree breeding programmes have yet progressed beyond the first of Spitters' two steps: the selection within mixed-genotype populations (wild or plantations) of genotypes which will grow rapidly both as spaced individuals and in mixtures. Tree researchers have only recently begun to consider the possible effects of this type of selection on production per hectare in plantations after canopy closure (Cannell, 1979, 1982; Talbert, 1981).

Competition obscures genotypic differences

In general, phenotypic selection for rapid individual-tree growth within mixed-genotype stands of trees has not been very effective. That is, heritabilities of individual-tree growth rate and competitive ability have been low (Samuel & Johnston, 1979; Green,

1971; Thomas & Stevens, 1977) and most tree breeders regard progeny testing for growth rate to be mandatory. It may be argued that prolonged, intense competition within the mixed-genotype parent stands has been one of the factors masking genotypic differences in competitive ability, because competition is a spatial neighbour-dependent process.

Consider a mixed-genotype stand in which inherently vigorous and competitive genotypes are randomly (not uniformly) dispersed. Then, in some parts of the stand, these vigorous genotypes will be immediate neighbours, and so some will eventually be suppressed. Conversely, in other parts of the stand some of the less vigorous genotypes will have equally unvigorous neighbours, so that some will grow large, often equally as large as the inherently vigorous genotypes. After a period of intense competition the outcome will be progressively more uniformly, or evenly, dispersed large plants and uniformly dispersed mortality (Figure 1).

Thus, the spatial, non-genetic, competition processes will obscure rather than magnify differences among randomly distributed genotypes. Of course, as competition proceeds there is increasing spread in the size-frequency distribution, and perhaps a move towards bimodality, but this is not necessarily a separation of inherently vigorous and unvigorous genotypes. Clearly, plant size becomes most uniformly dispersed as a result of competition when the environmental resources for which the plants are competing are also uniformly dispersed, like incident light.

Several theoretical models have described the conditions under which uniform dispersion in plant size is the inevitable consequence of neighbour-dependent growth (e.g. Diggle, 1976; Gates, 1978; Ford & Diggle, 1981), and evenly dispersed mortality and/or large plants have been detected in mixed-genotype populations of several species (Ford, 1975; Cannell et al., 1977, 1984). Within tea plantations, whereas bushes with strongly-

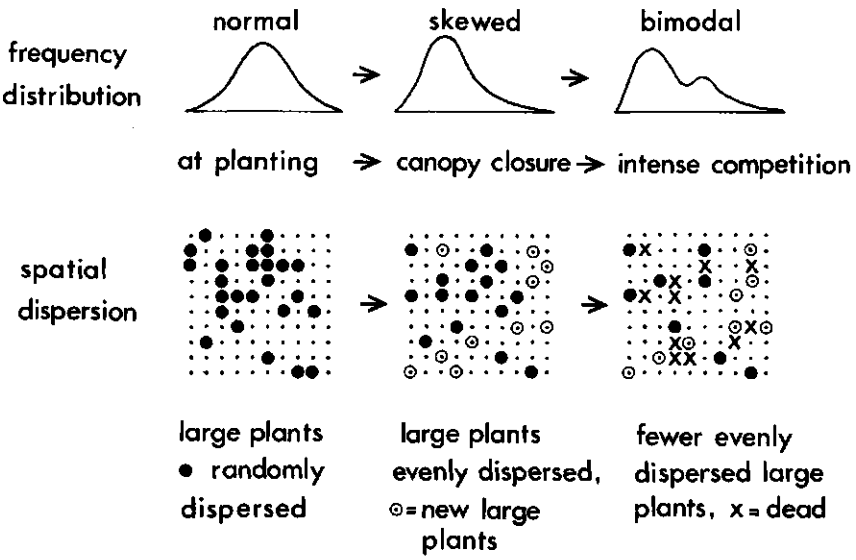

Fig. 1. Expected changes in the frequency of plant size and the spatial distributions of large plants, within populations in which there is progressively greater competition among individuals for evenly dispersed resources of light, water and mineral nutrients. Taken from Cannell et al. (1977).

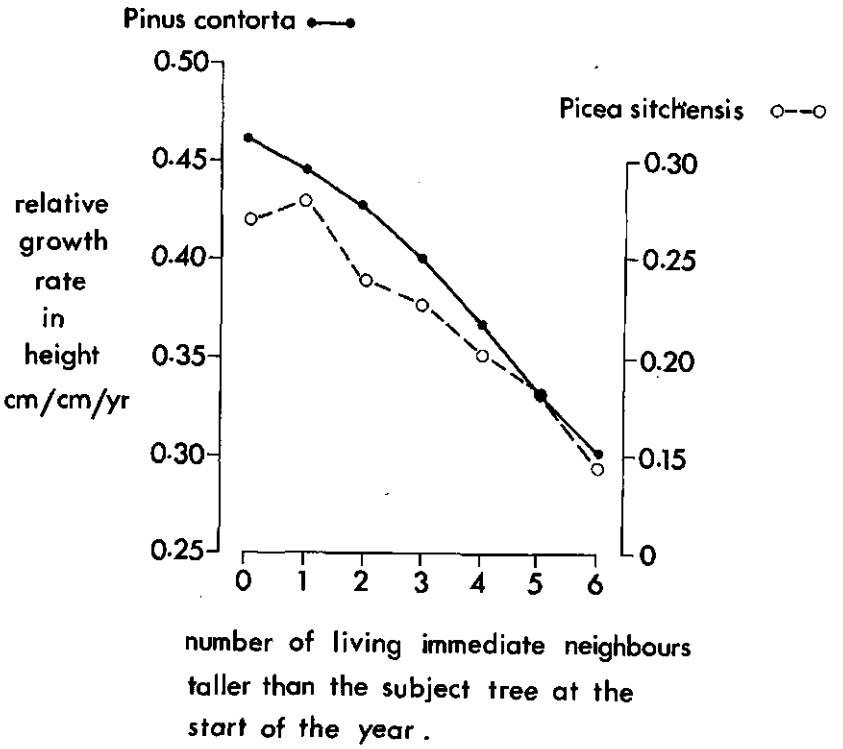

Fig. 2. Neighbour-dependent growth in tree stands. The relative growth rates in height of young *Picea sitchensis* and *Pinus contorta* trees during a year of intense inter-tree competition. The trees grew from about 100 to 140 cm tall in this year, and were planted in a nursery experiment 14 cm apart. The slopes are significant at $P<0.001$. Taken from Cannell et al. (1984).

inherited leaf colours occurred at random, bushes with large plucking table areas were uniformly (non-randomly) dispersed (Cannell et al., 1977). And within closely-planted stands of *Picea sitchensis* and *Pinus contorta* mortality became progressively more evenly dispersed as competition intensified, and the relative growth rates of the trees became more strongly dependent upon the height of their neighbours (Figure 2; Cannell et al., 1984). In the latter study, the 'competitive status' of the trees (defined by an 'overtopping' model, Ford & Diggle, 1981) accounted for about 30% of the variation in tree height.

Changes in rank during growth in mixtures

It is well-known for forest trees that the relative differences among genotypes can change markedly with time. Poor juvenile-mature correlations have often been reported in forest progeny tests (e.g. Nanson, 1968; LaFarge, 1975; Giertych, 1974). The progenies differ in initial size, in their abilities to 'capture' the site, in their response to the changed environment after canopy closure, in competitive ability, and in their inherent rate of aging.

The correlation between the genetic gain expected at maturity by selecting at the juvenile stage, $G_{M/J}$, is given by:

$$G_{M/J} = i_J (r_p - E_{pc}) \sigma_{PM}$$

where i_J = selection intensity for the juvenile trait, σ_{PM} = square root of the phenotypic variance of the mature trait, r_p = phenotypic juvenile-mature correlation, and E_{pc} = the product of 'environmental path coefficients' between the juvenile and mature phenotypes (Falconer, 1960). In forestry, we cannot assume that $E_{pc} = 0$, when spanning the environments before and after canopy closure (Cannell, 1979; Franklin, 1979; Ford et al., 1979).

Franklin (1979) found large, and remarkably similar, changes with time in the environmental and additive genetic variances within long-term progeny tests of *Pseudotsuga menziesii, Pinus ponderosa, Pinus taeda*, and *Pinus elliottii* growing in various parts of the USA (Figure 3). During the years before canopy closure some genotypes captured their microsites faster than others and, at that time, heritabilities were high. However, additive genetic variances remained small, while environmental variances increased, so that heritabilities decreased, up to the time of canopy closure. Thereafter, as inter-progeny competition occurred, some progenies proved to be more competitive

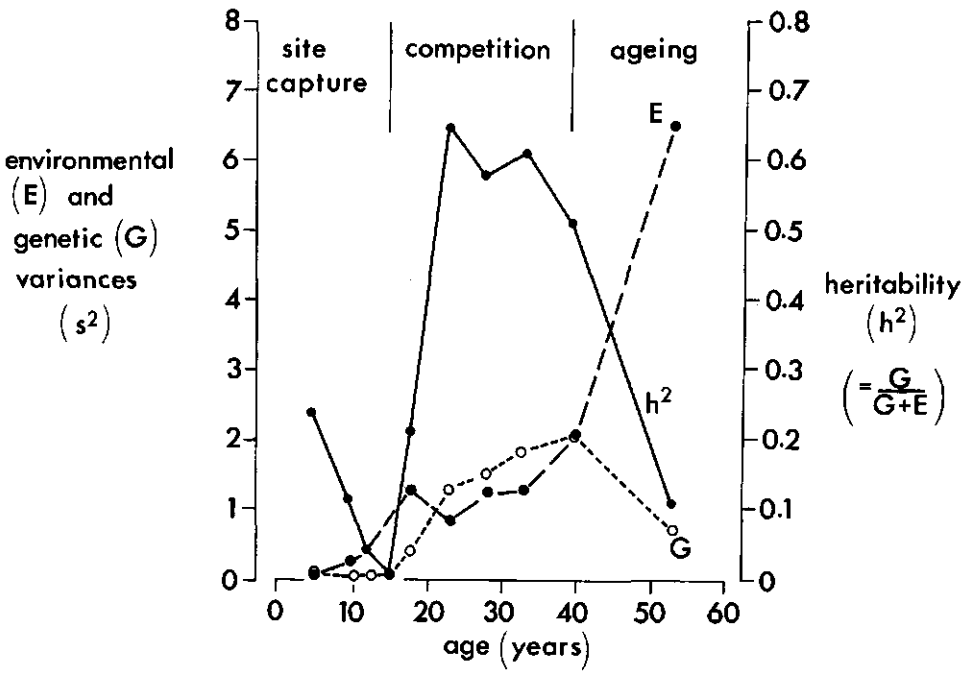

Fig. 3. Changes with age in the variances and heritability of height in a progeny test of *Pseudotsuga menziesii* at Wind River, Washington, USA. Phenotypic variance (σ_P^2) was assumed to be the linear sum of environmental (σ_E^2) and additive genetic variances (σ_A^2), and $h^2 = \sigma_A^2/\sigma_P^2$. Similar trends were found in other trials on other species. Taken from Franklin (1979).

than others, and there were abrupt increases in additive genetic variances, giving a second peak in heritabilities. Unfortunately, those progenies that were best at site capture were not necessarily those that performed best after canopy closure, so genetic correlations between progeny heights before and after canopy closure were low or negative. Adams et al. (1973) and Tauer (1975) have also shown that there are marked inherent differences in competitive ability within tree species. Clearly, in order to identify competitive progenies, they should not be evaluated before the onset of inter-progeny competition.

Correlation between competitive ability and yield in monoculture

Recently, forest researchers have questioned whether the competitive genotypes being selected for within forest progeny tests will, in fact, form closed stands with enhanced rates of stemwood volume production per hectare (Cannell, 1979; Talbert, 1981; Karki, 1983).

One view, based on an experiment in which 17 progenies of *Pinus taeda* were grown in pure blocks at three spacings (Wearstler, 1978), is that genetic improvement in tree growth rates will be sustained after canopy closure, equivalent to an increase in site quality (Talbert, 1981). An alternative view, based on an experiment in which 12 progenies of *Picea sitchensis* were grown in pure blocks at two spacings (Figure 4) (Cannell, 1982) holds that genetic gains in speed of site capture and perhaps in competitive ability, may disappear after canopy closure. A third view is that genetic gain in competitive ability,

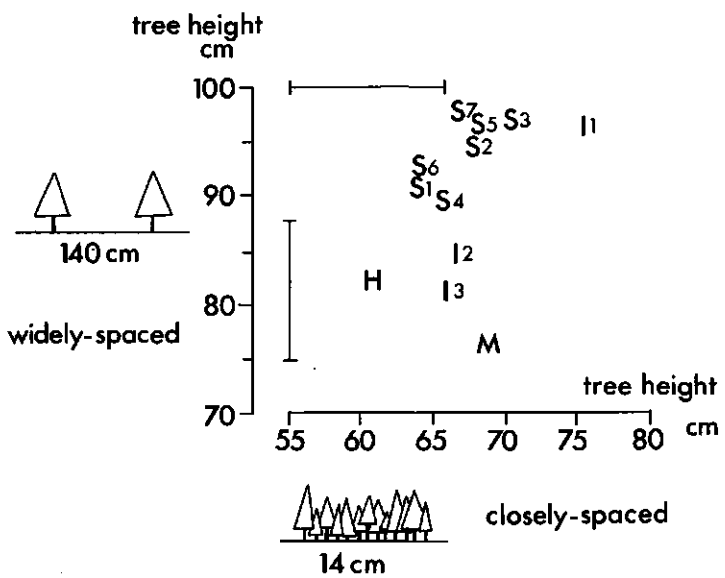

Fig. 4. Mean height of 12 progenies of *Picea sitchensis* at age 4 when grown in a nursery as either widely-spaced trees or in closed stands. M = Masset, and H = Hoquiam were provenance (ecotype) standards. S denotes a progeny with superior height at age 4-6 in forest trials relative to Masset; I denotes a progeny with inferior height at age 4-6 in forest trials. The bars are least significant differences at $P = 0.05$. Taken from Cannell (1982).

while not necessarily increasing total wood volume production per hectare after canopy closure, may help to concentrate the volume increment on to fewer trees, thereby making it easier to produce a high proportion of high-value sawlog timber (Cannell, 1979). Clearly this is an important problem that needs to be resolved.

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Effects of competition in the selection process

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Summary

Competition between plants interferes with the selection process: (1) by reducing genotypic differentiation and therefore progress through selection, (2) by imposing a limit on the number of environments and replicates when testing in early generations, both directly by reducing the number of seeds produced per plant, and indirectly through the establishment of field plots as experimental units, and (3) by making single-plant selection for yield unreliable, since competitive ability is usually negatively correlated with yielding ability. The detrimental effects of competition are overcome effectively by using wide spacings (i.e. 90 cm in wheat, 125 cm in maize) and the honeycomb field designs. With such designs a multisite and multireplicate screening of potential crosses and exceptional genotypes or families can be carried out in early generations. In this way annual progress will be maximized and the time needed to develop a new cultivar can be halved. Genes for adaptation and stability will be incorporated early in the programmes and both individual buffering and monoculture performance will be improved. Finally the scheme ensures a constant improvement of the cultivar, and paves the way for mechanizing and computerizing selection for yield.

Descriptors: honeycomb selection, competition, genotypic differentiation, yield, selection efficiency, early generation selection

Results from studies over fifteen years on the role of competition in selection can be summarized into three main effects and a general conclusion.

Effect 1 Competition between plants reduces genotypic differentiation and therefore progress through selection.

When sugar-beet and cotton varieties were tested in the presence and absence of competition, genotypic differentiation for sugar content in sugar beets, and mean number of days to maturity in cotton were reduced under competition by 57 and 49%, respectively. Competition also reduced yield differentiation when seven maize hybrids were tested in the presence and absence of competition, without any change in hybrid rank (Fasoulas, 1981). Similar reduction in the range of yield with increase in plant density was noticed by Baker & Briggs (1982) in barley, and Mitchell et al. (1982) in durum wheat, suggesting that single-plant selection would be most effective at low densities. In fact, Gogas (1983) obtained 40% progress in 4 years of mass honeycomb selection in the F_2 of a single maize hybrid when 0.7 plants were grown per square metre. Gardner (1978), using 2 plants per square metre and mass grid selection, needed 13 years

to obtain 40% progress, whereas Hallauer & Sears (1969) with 4 plants per square metre and grid selection, would have needed 26 years for the same progress. Although these results refer to different source populations, they are comparable, as the existence of additive genetic variance cannot be doubted, especially for the material used by Hallauer & Sears (1969), where studies have shown that 78 to 86% of the total genetic variance for individual-plant yield was due to additive genetic variance. Among the additional factors contributing to the superiority of honeycomb selection for population improvement and extraction of high-yielding inbred lines, should be mentioned the higher intensity of selection (2.5 against 10%) and the moving against the stable grid. In wheat, results from single-plant mass honeycomb selection with 1.4 plants per square metre showed the same gain as in maize (10.4%). This suggests that mass selection is equally effective in inbreeders as in outbreeders, if competition between plants is eliminated.

Effect 2 Competition between plants reduces the number of seeds produced per plant and indirectly establishes field plots as experimental units. This imposes a limit on the possible number of environments and replicates for all methods of family selection and prevents multisite and multireplicate screening for adaptability, yielding ability and stability in early generations.

With the honeycomb selection method plants are grown at 90 cm spacing in wheat and 125 cm in maize. Under such conditions the number of grains produced per selected plant is greater than 5000. This number is enough for progenies to be tested at more than 20 sites, with 100 plants per site, and two seeds per hill thinned later to a single plant. Thus, when evaluation is done in isolation, selection can be practised for high yield stability over many years and sites at an early stage. In this way, genes for adaptation and stability, instead of being irretrievably lost, are incorporated early in the programme. This makes regional tests unnecessary and halves the time needed to develop a new cultivar. Another important aspect of isolation is that it paves the way for mechanizing and computerizing selection for yield. Mechanization is transforming plant breeding techniques by increasing efficiency and by reducing labor, time and cost.

Effect 3 Competition between plants makes single-plant selection for yield unreliable, because competitive ability is usually correlated negatively to yielding ability.

Kawano & Thung (1982) and Kawano et al. (1982), studied the effects of intergenotypic competition in cassava on the efficiency of yield selection under different plant spacings. They found that intergenotypic competition was the major factor responsible for the low predictability of yield in a large plot from yield of a single plant. Because of the negative correlation between competitive ability and root yield in monoculture, they recommended that strong competitors should be eliminated from segregating populations in selecting high-yielding genotypes potentially adapted to productive environments.

Results presented in Table 1 from single-plant honeycomb selection for grain yield in the F_2 of two wheat crosses and two selection methods under two plant spacings (competition: 30 cm; isolation: 90 cm) substantiate the existence of the negative correlation (Fasoulas, 1978). The two F_2 wheat crosses were selected from a total of 83, for contrasting levels of heterosis in F_1 and yield in F_2. Relative genetic gain was assessed in F_3 and in isolation (90 cm), and is expressed as per cent of the F_2. For mass selection, the

Table 1. Relative genetic gain (in per cent over F_2) regarding grain yield in F_3 (plant spacing 90 cm) from single-plant honeycomb selection in F_2 of two wheat crosses, with two selection methods and at two plant spacings.

Selection method	Plant spacing (cm)	Sonora 64 × Farnese	Siete Cerros × Resistente
mass selection	30	−2 %	−9 %
	90	22 %*	2 %
best-family descent	30	27 %*	10 %
	90	79 %*	23 %*

* Significant at 5 % level.

average gain from selection under competition was −5.5% while that from selection in isolation was 12%. For the other selection scheme: the best-family descent (the pedigree scheme based on the best performing family and selection of individual plants within the best family only) the average progress under competition was 18.5% and in isolation 51%. Since comparisons were made in isolation (90 cm), verification under dense sowing in F_4, using as control the initial F_2, was indispensable. In this test (Table 2), the best four families from the high-yielding cross Siete Cerros × Resistente, two selected in isolation (I) and two in competition (C), were compared. Only the two families selected in isolation outyielded significantly (i.e. 12 and 14%) the F_2. After this verification, selection was continued in isolation up to the F_6. From mass honeycomb selection in the high-yielding cross Siete Cerros × Resistente, the variety Lesvos was derived, and from selection in the low-yielding cross Sonora 64 × Farnese the variety Cyprus. Although progress was much higher in the low-yielding cross (Table 1), the derived variety Cyprus was significantly inferior compared with Lesvos and with the leading Mexican variety

Table 2. Performance with dense sowing of four F_3 families of the wheat cross Siete Cerros × Resitente, two selected in isolation (I) and two in competition (C), and relative genetic gain (in per cent over F_2).

Best F_3 families	Yield[1] (kg/ha)	Genetic gain (%)
I_1 (90 cm)	6777[a]	14*
I_2 (90 cm)	6653[ab]	12*
C_1 (30 cm)	6173[bc]	
C_2 (30 cm)	5460[d]	
Check F_2	5859[cd]	

1. Different letters indicate significant differences.

* Significant at 5 % level.

Table 3. Performance with dense sowing of the wheat varieties Mykonos and Lesvos derived from mass honeycomb selection in isolation, in comparison with the best common parent Siete Cerros and the leading variety Yecora.

Variety	Yield[1] (kg/ha)	Improvement over Siete Cerros (%)
Mykonos	7350[a]	178
Lesvos	7088[a]	172
Yecora	5002[b]	121
Siete Cerros	4123[b]	100

1. Different letters indicate significant differences.

Yecora. To eliminate this defect, Cyprus was crossed with both Yecora and Siete Cerros. The cross Cyprus × Yecora showed up hybrid vigour and gave lines far inferior to the lines derived from the highly heterotic Cyprus × Siete Cerros. From the latter cross the variety Mykonos has resulted. In Table 3, results are given from a randomized complete-block experiment established in 1981-1982, where the two varieties Mykonos and Lesvos, both derived from heterotic crosses and from mass honeycomb selection in isolation, were compared with the leading variety Yecora and the best parent Siete Cerros. Due to outstanding performance, the two varieties were submitted to National List Trials. The results indicate three important things: (1) single-plant selection for yield is effective only when plants are grown in isolation; (2) the ideal cross to start selection is the one exhibiting the highest heterosis in F_1 and yielding ability in F_2; (3) the best-family descent is a very promising selection scheme. This method, applied in maize across sites to accumulate favourable adaptation and stability genes through a less restrictive form of inbreeding, may become a profitable way of obtaining highly productive and stable inbred lines.

Conclusions Because competitive ability is usually correlated negatively with yielding ability, the right condition to select for superior yielding performance in early generations is isolation. Direct selection for yield in isolation allows for all effects of competition and improves individual buffering and monoculture performance by improving various components of yield traits in a balanced way.

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Separating the effects of intra- and intergenotypic competition

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Summary

A method for measuring the separate effects of intra- and intergenotypic competition is described. Results from a competition experiment between three genotypes of perennial ryegrass (*Lolium perenne*) are given which illustrate the relative strengths of the competitive pressures operating in monocultures and binary mixtures. Recent work with chromosome assay lines of *Drosophila melanogaster* suggests that intra- and intergenotypic competitive ability may be inherited separately. If confirmed this would affect the breeding and selection of forage crops, which may be grown in practice as single variety pastures or in mixtures with other varieties or species.

Descriptors: competition, selection, regression analysis, perennial ryegrass, *Lolium perenne*, forage crops, coadaptation

Introduction and description of model

Intragenotypic competition is that unique form of competition which exists between individuals of a genotype or species when grown in monoculture, whereas intergenotypic competition occurs between individuals of different genotypes or species growing in a mixture, where it exists alongside intragenotypic competition. The problem arises therefore of how to separate and measure the effects of these two forms of competition so that a more accurate picture may be obtained of the relative strength of the intergenotypic pressures operating in a mixture. Recently Mather & Caligari (1981) have devised an experimental approach which does just this. Their approach covers both the substitution and addition designs commonly used for competition experiments, though the particular experiments referred to here were basically of the substitution type (De Wit, 1960). Typically a substitution experiment starts from a monoculture of competitor A with a known number of individuals per unit area. These are replaced – progressively – by competitor B to give a series of intermediate mixtures of known proportions of A:B, and ending with a monoculture of B. However, growing a monoculture at a single density does not provide a measure of intragenotypic competition over the range of densities at which a particular competitor occurs in the mixtures. To overcome this problem Mather & Caligari (1981) have modified the basic design by adding a series of monocultures to cover this range of densities. Table 1 shows the structure of an experiment between three genotypes of perennial ryegrass (*Lolium perenne*),

Table 1. Monocultures and binary mixtures grown in a competition experiment between three genotypes (B, C and E) of perennial ryegrass. Only genotypes B and C shown here. Those monocultures and mixtures enclosed within the solid lines represent a typical substitution design.

	Density (number of plants/unit area)				
B monoculture	20	15	10	5	
B/C binary mixture		15/5	10/10	5/15	
C monoculture		5	10	15	20

where the reference density was 20 plants/unit area (Mather et al., 1982).

Considering the monocultures first, the character being measured is defined in such a way that its expression shows a linear relationship over the range of densities used, that is the number of plants omitted where a substitution design is employed. In the perennial ryegrass experiment a square root transformation was necessary, suggesting that competition was multiplicative. However, no such transformation was required when Mather & Caligari (1981) analysed their data from inbred lines of *Drosophila melanogaster*, which indicated that the effects of competition were additive in their experiment. The slope of this regression line provides a measure of intragenotypic competition (b_m in Fig. 1) , against which the relative strengths of intergenotypic competition on this particular competitor can be gauged. This process is repeated using each competitor as indicator in turn.

How are these regression lines interpreted in a substitution experiment? When the replacement of A by B has no effect on the expression of A compared to its expression in monoculture, then B exerts no intergenotypic competition on A. If, however, the expression of A remains constant, despite replacement by B, then the intergenotypic effect of B equals the intragenotypic effect of A (b_0 on Fig. 1). A whole range of intergenotypic effects are possible, from facilitation where the slope of the regression line is more negative than the monoculture, that is A does better than in monoculture (bB), through intermediate effects (bC), to the point where genotype B competes more strongly with A than A does with itself (bE).

Experimental results and discussion

Three genotypes of perennial ryegrass (B, C and E) were grown in this experiment at a range of monoculture densities and in all three possible binary combinations, as shown in Table 1. Two treatments were imposed on this experiment, one half being cut frequently every three weeks during the growing season, the other being harvested every six weeks. For the purposes of illustration only the first years' results for genotype E will be presented here (Table 2 and Fig. 2). Clearly the regression coefficients for genotype E, as indeed for the other two genotypes, differ between the two cutting regimes, with the stronger competitive pressures occurring under the infrequent cutting treatment. Genotype B exerts only a weak intergenotypic effect on E, whereas the intergenotypic pressure exerted by C on E in both cutting regimes exceeds the intragenotypic pressure of E on

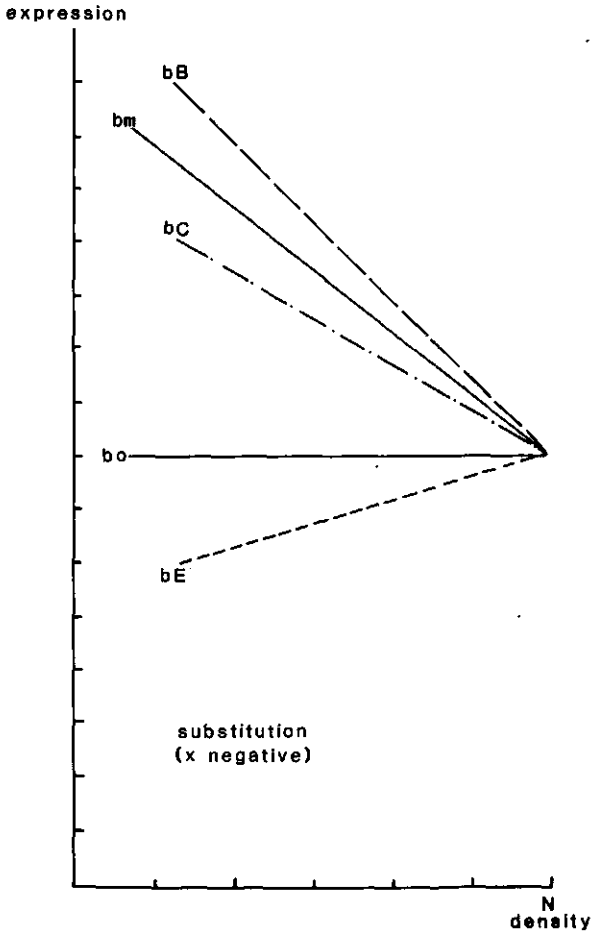

Fig. 1. The possible interrelationships of intragenotypic competition (b_m) to intergenotypic competition in a substitution experiment. The ordinate represents the expression of the character being measured whilst the abscissa (x) is the number of plants omitted from the monocultures or substituted by a second genotype in the binary mixtures (modified from Mather & Caligari, 1981).

Table 2. Estimates of the intra- and intergenotypic regression coefficients for genotype E during the first year.

Associate	Frequent	Infrequent
B	−0.0240	−0.0649
C	0.0105	0.0176
E	−0.0338	−0.0782
Standard error	±0.00691	±0.01286

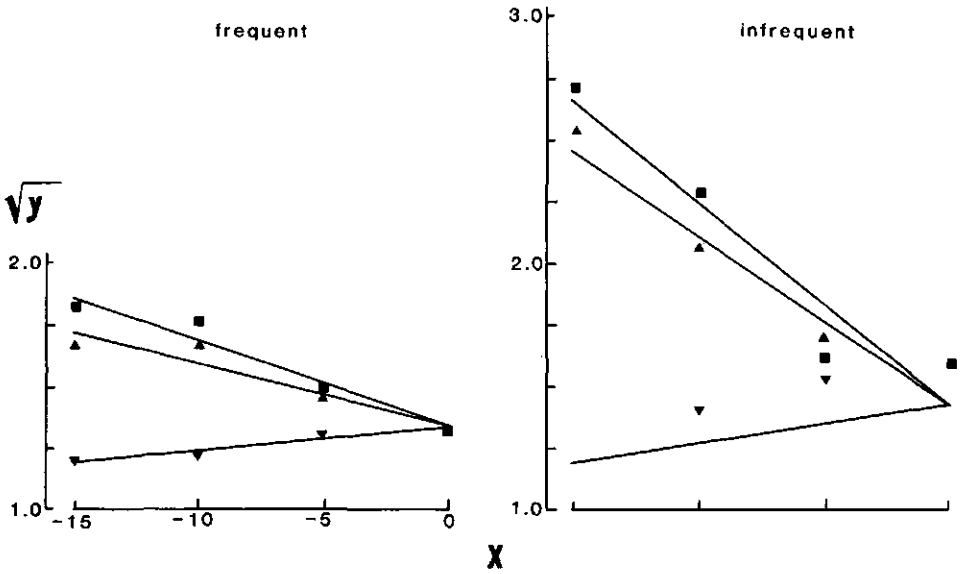

Fig. 2. The observed points and fitted regression lines of $\sqrt{y}$ on x with genotype E as indicator under the two cutting regimes (modified from Mather et al., 1982).

itself. Subsequent results from this experiment show that these pressures change from one growing season to the next.

Using this approach it has proved possible to quantify the separate effects of intra- and intergenotypic competition. Given that their effects can be distinguished apart, it is not unreasonable to suggest that they could be inherited as distinct characters. Recent results from an experiment with chromosome assay lines of *Drosophila melanogaster* tend to confirm this suggestion. If these effects are inherited separately this will have implications for the breeding and selection of certain forage crops, which possess strong incompatibility mechanisms and so cannot be easily selfed, and which may be grown in practice as single variety pastures or in mixtures with other varieties or species. Indeed, it is intended to set up a series of experiments, based on the modified substitution design described here, to examine those competitive interactions which occur within coadapted and non-adapted white clover/perennial ryegrass mixtures with a view to improving the productivity of the mixture as a whole.

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Use of indirect selection in plant breeding

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Summary

Selection is indirect when the breeder improves a primary economic character by selecting for one or several secondary characters. Indirect selection is commonly used in plant breeding because the primary characters are often difficult to measure during the selection process.

We consider the problem mainly in a biometrical aspect. For efficient application of indirect selection a secondary character must be highly correlated with the primary character and have a higher heritability. Experimental results tend to show that indirect selection is generally less efficient than direct selection, except if the primary character has a very low heritability. A possible greater selection intensity for the secondary character increases the effectiveness of single-trait indirect selection.

Consideration of several secondary characters correlated to the primary character increases also the efficiency of indirect selection. From the literature and from our experience on forage maize breeding different examples are considered. In forage maize, some combinations of morphological traits are expected as efficient as direct selection for dry matter yield. Generally, morphological components of yield are genetically correlated with yield and have a higher heritability. Indirect selection can also be applied through a selection index. Simultaneous consideration of the primary and of secondary characters generally is efficient and allows description of the ideotype.

The significance and limitations of the biometrical approach are discussed.

Descriptors: indirect selection, correlated response, selection index, genotypic value, ideotype, breeding method, selection criteria, selection theory

Introduction

Selection is indirect when the breeder improves a primary economic character by selecting for one or several other secondary characters. So progress is achieved by the correlated response of the primary characters. Indirect selection is used because primary characters are often difficult or costly to measure directly during the selection process.

The environmental conditions of tests are frequently different from the conditions of agricultural practice. For example, spaced plants or microplots are used in various species, because of both the breeding method (individual selection on phenotypic value) and the limited amount of seed for testing. But in such conditions the plant breeder should have selection criteria (secondary characters) for performance in dense stand (primary characters). For the same reasons, the macro-environmental conditions (sites) of selection are generally different from those of agricultural practice. The breeder

frequently selects in one environment (one site, one year) for a response in several environments. The primary character will be the average yield for a set of environments, and secondary characters the yield or criteria of yield in the site of selection.

To shorten the selection cycle, tests may also be conducted in juvenile or early stages for performance in 'mature' stages. This is important for perennial plants (fruit trees, forest trees, some important forage plants), but selection at juvenile stage can also be useful for other species as a preliminary selection with low intensity. Such selection may reduce the cost of selection by eliminating 'bad' plants or families and so allowing a better investigation of the remaining material, or to take in more material.

Another purpose of indirect selection is to find secondary (or component) characters genetically linked to the primary character (e.g. yield) and having a higher heritability. The breeder is also looking for secondary characters easier to measure than the primary character and applicable to a great number of genotypes (or families). So secondary characters can be used for preliminary screening with low selection intensity and mainly in the early generations of selection.

The use of indirect selection raises the problem of finding efficient selection criteria, i.e. defining the ideotype. We defined ideotype as an abstract genotype with an ideal 'profile' under the conditions during breeding which, under the conditions of agricultural practice will give to a maximum economic yield. The necessary physiological approach of this problem at the level of the whole plant will not be considered in this paper. It will undoubtedly be further elaborated in the section 'Development of better selection criteria' (Wilson, 1984).

From a genetic point of view, we must note that all selection is indirect in that the breeder always selects for genotypic values or for the economic value through phenotypic values. Recurrent selection is often indirect in the conventional sense. Indeed, the aim of population improvement is to improve varietal ability (Gallais, 1979). However the breeder does not directly evaluate it; he measures phenotypic value or value of some progenies (half or full sibs, S_1) to improve the ability of the population to give good varieties. For example, mass selection is used to improve general combining ability as well as general synthesizing ability or value per se. In early generations of inbreeding pedigree selection is also indirect: selection in heterozygous condition for performance in homozygous condition.

Let us further consider mainly which way of biometrical approach of secondary characters can be used to evaluate the genotypic value of primary characters. In the review of the literature, we will consider as primary characters mainly yield or an index of economic value obtained by weighting the genotypic value of different primary characters. In addition, we will mention some results we obtained in breeding silage maize (Gallais et al., 1983).

Genetic and environmental correlations

The study of secondary characters rests upon the study of correlations between primary and secondary characters. At phenotypic level such correlations may have two origins: genetic and environmental (Falconer, 1960).

Genetic correlation can be defined as the correlation between two characters in a given environment, because of variation in genotype. Environmental correlations can be

defined, as the correlation between two characters for a given genotype, because of variation in the environment. Genetic correlation is then related to an environment, but one generally assumes that it is the same whatever the environment. Similarly environmental correlation is related to a genotype but one generally assumes that it is independent of the genotype. For a given material (population), these two correlations can be very different, which is sometimes forgotten.

Genetic correlation between two characters is most relevant when determining the value of a secondary character; it may be caused by linkage and/or pleiotropy. If caused by linkage, a correlation can be changed as a result of selection: a negative correlation can be broken. If the correlation is due to pleiotropy it cannot be changed or broken. Then one of the two linked characters can be considered as a physiological marker of the other. A correlation caused by linkage may also have a physiological basis; natural selection tends to generate linkage disequilibrium resulting in an increased fitness of a population. So there will be a continuity between correlation due to linkage and that due to pleiotropy. However genetic correlation, whatever its origin, is dependent on the frequencies of genotypes in a population and thus can vary from one population to another. With pleiotropy, this correlation would be more stable than with linkage.

Environmental correlation may be considered the result of 'exploration' of the genotype by the environment. According to environment, different genes can be expressed. This correlation seems to be of greater interest to the physiologist than to the plant breeder. However if several predictor characters are used to evaluate the genotypic value of one character, knowledge of such correlation is also useful (see below).

Analysis of correlation is also of interest to get to know the causal relationship between two characters: if genetic and environmental correlations are strong enough and of the same magnitude, a physiological basis can be assumed. If they are very different, genetic correlation might be due to linkage.

Efficiency of single-trait indirect selection

Theoretical results For simplicity let us consider only the prediction of the genotypic value for a plant or a line; but the results may be extended to prediction of the genetic value of the progenies of such plants which resulted from random mating.

The expected response to direct selection of a primary character X_1 is:

$$\Delta G_1 = i\, h_1\, \sigma_1$$

and by indirect selection for a character X_2, the correlated response of character X_1 is:

$$i\, r_G\, h_2\, \sigma_1$$

where i is the selection intensity (in standard units), h^2 is the heritability, r_G the genetic correlation between X_1 and X_2 and σ^2 the genetic variance.

The ratio of these two genetic advances will represent the relative selection efficiency (RSE) of indirect selection to direct selection. If for both types of selection the selection intensity is the same, indirect selection will be more efficient than direct selection if:

Table 1. Conditions for superiority of indirect selection over direct selection. For a given combination of the genetic correlation (r_G) and of the heritability of the secondary character (h_2^2), the heritability of the primary character (h_1^2) must be inferior to the given value in the last column.

r_G	h_2^2	h_1^2
0.30	0.64	0.06
0.40	0.64	0.10
	0.50	0.07
0.50	0.64	0.16
	0.50	0.12
	0.36	0.09
0.60	0.64	0.23
	0.50	0.18
	0.36	0.13
0.70	0.64	0.32
	0.50	0.24
	0.36	0.18

$$r_G\, h_2 > h_1$$

thus if the product of the genotypic correlation (between primary and secondary character) multiplied by the square root of the heritability of the secondary character is greater than the square root of the heritability of the primary character. Such an equation was already discussed by Searle (1965).

Because the genetic correlation is less than 1, a first consequence is that the heritability of the secondary character (h_2^2) must be greater than that of the primary character (h_1^2). Since h_2 cannot exceed 1, r_G must also be greater than h_1. For the two parameters r_G and h_2 the one must be higher if the other is lower. So the breeder can easily check whether single-trait indirect selection is efficient (Hänsel, 1976).

Table 1 shows that indirect selection will be efficient only for low heritability of the primary character. Higher heritabilities of a primary character will require a high genetic correlation and also a high heritability of the secondary character. For instance, if the heritability of the secondary character is 0.80 and the genetic correlation is 0.80, and if the heritability of the primary character is greater than 0.40, direct selection will be more efficient.

Experimental results with morphological or physiological characters related to yield Very few of the published results on indirect selection for yield in various species, allow direct analysis regarding the three parameters: heritability of the primary and of the secondary characters and their genetic correlation.

Hallauer & Miranda (1981) described a complete situation for grain yield in maize. They studied various morphological characters (plant height, ear length, ear diameter, kernel depth), yield components (number of kernel-rows, kernels per ear-row, kernel

weight) and physiological characters (time to flowering). Average genetic correlation in several populations were stronger for ear traits than for other characters. Kernel depth had the strongest correlation ($r = 0.51$) with yield but heritability was not enough to make indirect selection more efficient than direct selection. Finally any secondary character was not heritable enough nor closely correlated to yield to allow efficient indirect selection, in spite of a rather low heritability of yield ($h^2 = 0.20$).

In several populations (synthetics) of forage maize, we have also studied various morphological or physiological characters in relation to dry matter yield, in a restricted range of variation of earliness. The characters included height of the plant, ear height, length and width of ear leaf, vigour at the 6-8 leaf stage, acid detergent fiber (ADF) and protein content (Gallais et al., 1983). Among these characters ear and plant height as well as leaf length were consistently related to yield and had a higher heritability (Table 2). However on average, indirect selection was no more efficient than direct selection for yield. Nevertheless, examination of results for each population suggested that with very low heritability of yield on a plot basis ($h^2 < 0.20$), indirect selection by using plant height might be more efficient.

Published results considering grain yield of various species do not allow analysis of the situation, but suggest the relative ineffectiveness of indirect selection.

In rapeseed (*Brassica campestris*), Richards & Thurling (1979) did not find any of the characters studied (harvest index, 1000 grain weight, number of grains per siliqua) of interest for indirect selection in dry climate. A slightly different result was observed in *Brassica napus,* where indirect selection for flowering time was as efficient as direct selection for yield.

From results of Rosielle & Frey (1975 a, b) and of Rosielle & Eagler (1977) in oats (*Avena sativa*), the same conclusion can be drawn. In spite of high genotypic correlation

Table 2. Usefulness of secondary characters in selection for dry matter yield in forage maize (Gallais et al., 1983). r^2_{GG} is the coefficient of determination for true genetic value by the predicted value: (1) with only morphological characters and vigour at the 6–8 leaf stage, (2) = (1) + yield + dry matter content, (3) = (2) + acid detergent fiber content + protein content.

Heritabilities and correlations		Synthetics					
		Syn 19	Syn 3.13	Syn 21	Syn 5.9	Syn 20	Syn 4.14
h^2 dry matter yield (Y)		0.23	0.42	0.27	0.32	0.12	0.54
h^2 ear height		0.61	0.51	0.51	0.46	0.57	0.50
h^2 plant height		0.64	0.50	0.54	0.55	0.57	0.57
h^2 leaf length		0.71	0.52	0.56	0.53	0.48	0.41
r_G (Y-ear height)		0.75	0.58	0.46	0.54	0.52	0.82
r_G (Y-plant height)		0.79	0.52	0.52	0.57	0.65	0.86
r_G (Y-leaf length)		0.88	0.34	0.42	0.12	0.74	0.71
r^2_G h^2 for plant height		0.36	0.13	0.15	0.17	0.24	0.42
r^2_{GG}	(1)	0.94	0.21	0.32	0.31	0.58	0.51
	(2)	1.0	0.52	0.48	0.46	0.68	0.68
	(3)	1.0	0.70	0.74	0.48	0.68	0.72

between several characters (harvest index, plant weight, plant height, heading date) with grain yield, direct selection was always more efficient.

Cases where single-trait indirect selection is clearly superior to direct selection for yield, with the same selection intensity, are rare. In maize the example of prolificacy is sometimes given. Indeed with the same population, Gardner (cited in Lonnquist, 1967) gives a genetic advance of 3.8 % per cycle of mass selection for yield and Lonnquist (1967) gives a genetic advance in yield of 6.3 % per cycle of selection for prolificacy. This greater effectiveness of selection for prolificacy in increasing productivity was partly due to greater selection intensity. However this was not sufficient to explain the observed results. So, in this case, indirect selection was competitive with direct selection at the same selection intensity and was applicable with less effort to a larger number of plants. Prolificacy may be indicative of plant vigour; it can increase sink capacity but also source. Further results have shown that it is related to nitrate reductase activity (Boyat & Robin, 1977).

Other results can be found illustrating the efficiency of indirect selection. From an experiment of Moll et al. (1975) in grain maize, indirect selection for ear height seems more efficient than direct selection for yield. In soybean, Harrison et al. (1981) showed that in some crosses selection for seed yield through selection for apparent rate of photosynthesis in the canopy could be as efficient as or more efficient than direct selection. Heritability of apparent photosynthesis is greater than heritability of yield and the genetic correlation between the two characters is strong enough ($r = 0.55$). However their results tend to confirm predicted results, according to which indirect selection will be more efficient than direct selection if heritability of the primary character is low ($h^2 < 0.20$). In wheat, Ledent (1979, 1982) reassessed some morphological characters, and reached no clear conclusion.

To summarize this review of several experiments on the application of single-trait indirect selection for yield, morphological components of yield are genetically linked to yield and have greater heritability. The same seems to be true for size or earliness characteristics. However single-trait indirect selection is scarcely more efficient than direct selection, in spite of variation in heritability and in genetic correlation from one population to another. If only one secondary character is being applied with the same selection intensity as in the primary character, the most effective method for yield improvement is direct selection for yield, if this primary character can be measured. Are physiological characters more efficient than morphological characters? There is no evidence for this, and the first are often more difficult to measure than the second. Furthermore it seems an illusory to find only one physiological character explaining genetic variation in yield.

Testing at early stages The advantage of early screening is obvious. Early screening is clearly indirect selection. If genetic correlation, heritability and cost of testing are such that the early screening can be considered efficient, it can be used as a definitive test for the primary character. This is the case for genetical characters which seem early expressed, such as several disease resistances, or adaptation to cold or to drought. The situation is less clear for the prediction of forage or grain yield.

In many crops, particularly cereals and forage grasses, seedling tests under controlled conditions have been developed to assess hardiness, cold tolerance (leaf growth at

moderately low temperature) and tolerance to water stress (Wilson, 1981). However direct methods of screening for water stress should be developed. Wilson (1981) points out the interest of smaller stomata or fewer stomata per unit of leaf area in forage grasses. In wheat, Blum & Ebercon (1981) found that growth of seedlings on a nutrient solution with polyethylene glycol (PEG) allowed screening for tolerance to water stress.

In forage grasses, one can select at seedling stage for some components of dry matter yield. Jones et al. (1979) showed the efficiency of selection for yield per tiller in tall fescue. In sugar beet, the ratio of taproot to leaf weight was predictable in 21-days-old seedlings (Snyder & Carlson, 1978); hypocotyl diameter at that stage might predict root yield (Doney & Theurer, 1976).

It was attempted to approach yield potential through enzyme activities (for instance nitrate reductase activity, Boyat & Robin (1977); various allozymes, Stuber et al. (1982), and mitochondrial activities (Hanson et al. (1975)). The correlations with yield are generally low, so such tests can only be used for first screening with a low intensity of selection.

Testing at a favourable or unfavourable site? Selection is generally aimed at identifying genotypes that have high average value for a number of environments. However tests will be performed at only one or a few sites. Performance at one site can be considered as a particular character, and so the efficiency of one site can be measured as the efficiency of a secondary character (Allen et al., 1978).

One of the questions raised by the plant breeder about the choice of selection site or environment is to know whether one can better select in a poor or a rich environment. Environmental and genotypic variances are usually higher when growing conditions are favourable. However, heritability and genetic gain do not appear to be consistently associated with yield level. In wheat, Gotoh & Osanai (1959) found an environment with low fertility more efficient. Byth et al. (1969) in soybeans and Frey (1964) in oats did not find any differences between rich and poor environments. However Frey (1964) pointed out that lines selected under stress from the environment showed more genotype × environment interaction.

An interesting experiment by Arboleda-Rivera & Compton (1974) on grain maize showed that selection in poor (dry) environment was more efficient for that situation (2.5%/cycle) and induced a closer correlated response in a good (rainy) environment (7.6%/cycle). Direct selection in a favourable environment was more efficient for that situation (10.5%/cycle) but was not associated with a significant advance in a poor environment (0.8%/cycle).

There is no reason to expect a general answer. Under stress, selection will be more for 'adaptation' and in 'rich' environments for 'potential'. Both aspects should be considered by the plant breeder.

Indirect selection with a greater selection intensity The rather low response of indirect selection for a complex character like yield does not mean that such selection is inefficient. With the situation generally observed for secondary characters with higher heritability than yield and significantly correlated with it, there will be a significant response to indirect selection. If indirect selection can be applied more easily, it allows a greater intensity of selection which can counterbalance its genetic inefficiency. However

Table 3. Selection intensity necessary in indirect selection to have the same genetic advance by indirect selection as by direct selection for a given selection intensity of direct selection and a given relative selection efficiency of indirect over direct selection (RSE).

RSE indirect/direct	Selection intensity of direct selection (%)			
	30	20	10	5
0.50	2.6	0.6	<0.1	
0.75	15.4	8	2.6	0.8

this will be possible only if the relative selection efficiency (RSE) of indirect selection to direct selection is not too low, and with a low intensity of selection for the primary character (Table 3).

Our experiment in forage maize shows that the inefficiency of indirect selection for yield through selection for plant height could be compensated by a greater selection intensity. If direct selection for yield has a selection intensity of 20% and 10%, the same advance would be expected for indirect selection with a selection intensity of 9% and 3%, respectively, which is realistic. This would be the conclusion for most of the examples discussed.

So, the efficiency of indirect selection increases if one can apply a greater selection intensity. This is generally so for secondary characters that can be easily and cheaply measured on a great number of plants.

Effect of breeding method Searle (1978) has shown that the relative efficiencies of indirect selection to direct selection will be greater with individual selection than with progeny testing, since progeny testing increases the heritability of the primary character. This was observed by Williams et al. (1965) who considered full-sib and individual selection. With full-sib family selection, indirect selection for yield by selecting for ear diameter was expected to be 20% less efficient than direct selection for yield. However their data indicate that individual selection for ear diameter would be 8% more efficient in improving yield than individual selection for yield.

Extension of indirect selection with several secondary characters

Indirect selection can be carried out with several secondary characters, as is more commonly practised by plant breeders. In their conditions of selection, they select for one or several primary characters through secondary characters. For example in the breeding of forage plant, where selection for yield in dense sward is done by evaluation of spaced plants, it is effective to consider vigour, growth habit and length of leaves simultaneously. Independent culling level is often used for selecting simultaneously for several characters. Such a method does not exploit information to the full and does not take into account that for two characters that are genetically linked, one of them give genetic information on the value of the other.

The best way of predicting the genotypic value (G_I) of a primary character X_1,

knowing the phenotypic values of several secondary characters ($X_2 \ldots X_n$) is to use a linear predictor such as:

$$G_1 = b_2 P_2 + b_3 P_3 + b_4 P_4 = b' P$$

where b is the coefficient of regression of a genotypic value G_1 onto phenotypic values P. In matrix notation the coefficients can be expressed as:

$$b' = W \underline{P}^{-1}$$

W being the row vector of the genotypic covariance of the primary character with secondary characters, and $\underline{P}$ the variance-covariance matrix of phenotypic values.

The problem is strictly equivalent to the classical problem of predicting genotypic value from phenotypic value, which introduces the concept of heritability both as a fraction of genetic variance in phenotypic variance, and as a coefficient of determination for true genetic value by estimated genetic value. Here too, the accuracy of prediction can be evaluated with this coefficient of determination, which is the square of the correlation between both values $(r_{G\hat{G}})^2$. It is the equivalent of a heritability, such as $(r_G h_2)^2$ in the first part of this paper.

The difficulty is to obtain estimates of $\hat{b}$ of the coefficients of regression (b). If the units of selection are in an experimental design with replicates, one can carry out a multivariate analysis of variance leading to estimates of $\hat{W}$ and $\underline{\hat{P}}$ and then to $\hat{b}$. Such estimates of b could also be known a priori from experiment. However such an experiment must involve material highly related to the material used in selection and evaluated in the same conditions, because such estimates depend on the material and the environment (however in the absence of genotype × environment interaction they are independent of the environment).

This approach has been used mainly for yield selection based upon morphological components of yield, because they are easy to measure, generally have higher heritability than yield, and are genetically linked with it.

In selecting for dry matter yield of *Lolium perenne,* Glenday & Fejer (1956) found an advantage (7 to 15%) of selection by a linear combination of tiller length + tiller number, and of tiller length + fresh weight of tiller per unit of length. From the experiment of Richards & Thurling (1979) in rapeseed (*Brassica campestris*), a linear combination of the number of branches, number of siliquae and seed weight, appeared to be 10% more efficient than direct selection. For lentil Nandan & Pandya (1980) found an advantage of 22% for an index combining grain/pod, branches/plant, number of pods/plant and seed weight, over direct selection for yield.

If it is necessary to measure the yield to obtain a measure of the yield components, the cost of selection will increase and may create a problem of allocation of means. The question arises: is it better to measure yield components and yield with a certain number of replicates or to measure only yield of a greater number with replicates? We will discuss this problem in the last part of this paper.

Our experiment with forage maize is an example of efficient use of secondary characters to select for dry matter yield without yield measurement. Observation of vigour at a young stage (6-8 leaves) with measurement of morphological characters gives

at least as much information as direct selection for yield (Table 2). In only one out of six populations indirect selection was inferior: in this case the heritability of yield on a plot basis was relatively high. The advantage of such indirect selection is that it can be done just after flowering, before any damage (lodging, maize borer) could occur that could disturb yield measurement.

Multitrait indirect selection can be useful also to allow greater selection intensity than direct selection for a complex character. For sugar cane Miller et al. (1978) used stalk length, stalk diameter and stalk number as secondary characters, and found indirect selection to be 89% as efficient as direct selection for yield. If selection was for yield of sucrose, inclusion of Brix value (a measure of sugar concentration) in the index gave 92.1% of the direct selection. So indirect selection was inferior to direct selection, but it was easier and cheaper, mainly at early stage of selection, such that more material could be studied. This is undoubtedly the justification for use of multitrait indirect selection in many species not mentioned here (wheat, barley, etc.).

The situation when selecting for several secondary characters genetically related to the primary character is about the same as for the use of only one secondary character. Multitrait indirect selection should be efficient if heritability of the primary character is low. However its application will be more efficient than by using only one character, and any low effectiveness can be compensated by a higher selection intensity. Its potential will be fully used if selection is through a biometrical index and not by independent culling level.

Simultaneous consideration of secondary and primary characters

The most general situation in plant breeding is that of simultaneous selection for several primary characters, sometimes using also secondary characters correlated to the primary characters. One particular primary character is then both primary when it is selected for and secondary when selection is for another correlated primary character. The information can best be exploited with a general index for the economic value (Gallais, 1973).

Theoretical aspects with one primary character To simplify, let us first consider selection for one primary character with measurement of this character combined to a correlated secondary character.

Measurement of one or more secondary characters correlated to a primary character gives genetic information on the primary character. Thus simultaneous consideration of these two types of characters must increase selection efficiency. This problem was explained by Falconer (1960) and elaborated by Searle (1965). With only one secondary and one primary character, (X_1), the genotypic value (G_1) of the primary character will be predicted by a linear regression of the genotypic value onto the phenotypic variables P_1 and P_2:

$$G_1 = b_1 P_1 + b_2 P_2$$

As before the coefficients b are a function of the covariances between genotypic value of the primary character and phenotypic value of the two characters, and of phenotypic

variance and covariance for the two characters. Such parameters can be estimated by analysis of variance and covariance of the two characters, or more generally by multivariate analysis of variance.

For equal selection intensity, the relative selection efficiency (RSE) of this method to direct selection depends only on genetic (r_G) and phenotypic (r_P) correlations between the two characters and on the ratio of the two heritabilities $t^2 = h_2^2 / h_1^2$:

$$RSE = \sqrt{1 + (r_P - t\, r_G)^2 / (1 - r_P^2)}$$

Such a quantity can only be superior to 1. So combination of primary and secondary characters must improve the efficiency of both direct and indirect selection, but the degree of improvement depends on the parameters. The efficiency of such a method depends on both genetic and environmental correlations. There might be situations where genetic correlation is zero and where observation of secondary character gives some information, i.e. if environmental correlation is significant enough. The index allows correction of phenotypic value for environmental bias.

Figure 1 shows the effectiveness of such a selection in terms of genetic (r_G) and phenotypic (r_P) correlations. It is limited by an ellipse for a given RSE and a given ratio of heritabilities. A significant RSE requires some divergence between genetic and phenotypic correlations, i.e. between environmental and genetic correlations. Furthermore, the effectiveness of the index decreases with the heritability of the primary character. For a ratio of heritability of a secondary character to the primary character between 1 and 2, environmental and genetic correlations must be widely different (Vincourt & Gallais, 1981).

The effectiveness of the method can be illustrated by the number of replicates required to obtain the same genetic advance (by selection in plots) by indirect selection. If one or more replicates can be saved and if one looks to the cost of selection, there is a problem to optimize means. It will sometimes be better to decrease the number of replicates and to measure several secondary characters, and in other cases, it will be more efficient to increase the number of replicates and to measure only primary characters.

Some results with one primary character (yield) From the results of Richards & Thurling (1979) with rapeseed (*Brassica napus*), an index of yield + number of siliquae + flowering time should be 16% more efficient than direct selection for yield. Selection for other characters along with yield and flowering time, like number of seeds per siliqua, number of siliquae and harvest index, would not markedly increase the efficiency of yield improvement. For *Brassica campestris*, (Thurling, 1974) addition to yield of various yield components always increases the efficiency of selection for yield. The greatest increase was expected with yield + harvest index + seed weight + number of seeds per siliqua. In lentil (Nandan & Pandya, 1980), the use of a discriminant function with grain yield gave a relative efficiency over straight selection as high as 22%.

In grain maize (Moll et al., 1975), a combination of yield + ear height was 46% superior to yield alone and 30% to indirect selection by ear height, which was more efficient than direct selection.

In our experiment on forage maize, we have already stressed the efficiency of secondary characters measured before harvesting. On average with morphological characters

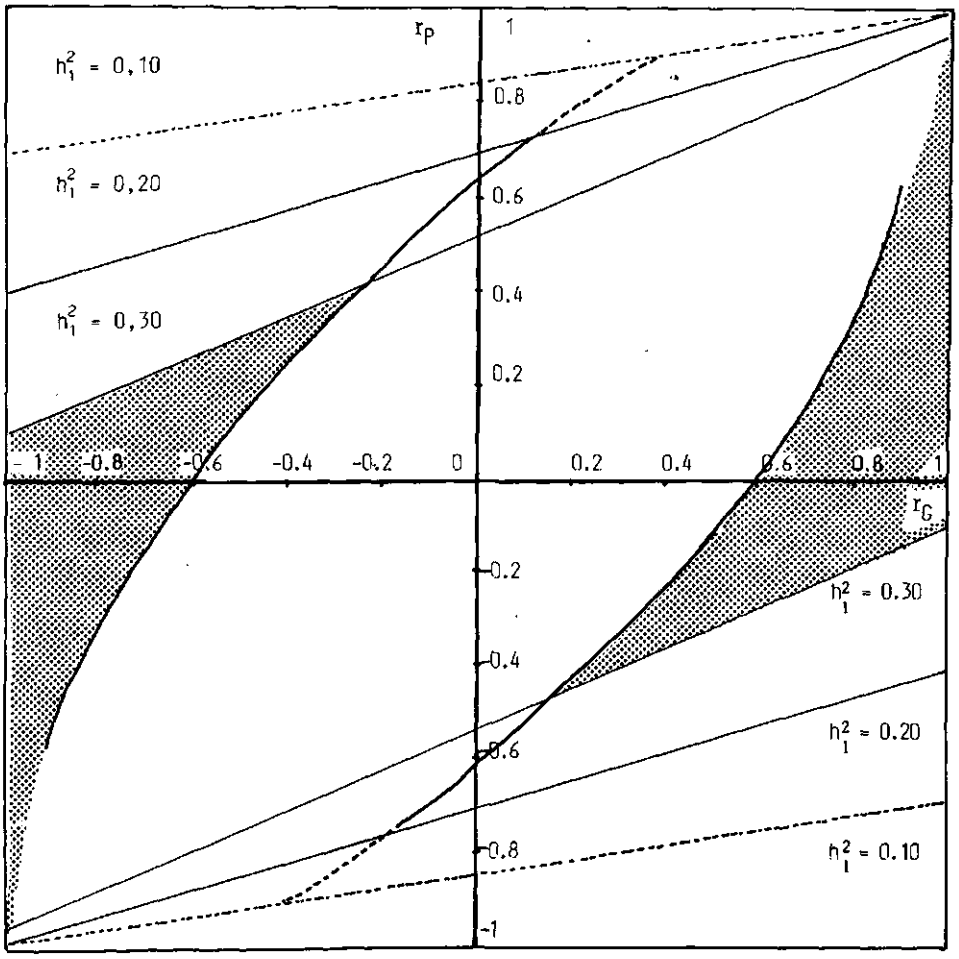

Fig. 1. Field of effectiveness of simultaneous selection on one primary character and one secondary character relatively to direct selection on the primary character, in terms of the phenotypic (r_P) and genotypic (r_G) correlations and for a ratio of heritability of secondary character (h_2^2) on heritability of primary character (h_1^2) of 2. Situation within the hatched area corresponds to a relative selection intensity (RSE) of 1.3.

and vigour at young stage (6-8 leaves), the coefficient of prediction of genotypic value for yield (equivalent to a heritability) was 0.49 with two replicates. Adding yield and dry matter content increased 'heritability' of yield to 0.64 (corresponding to an advantage of 32% over direct selection). Such an increase in heritability was equivalent to a saving of five replicates.

The evaluation of the gain resulting of measuring secondary characters could also be made for previous reported results, and would lead to similar conclusion according to the selection intensity: simultaneous consideration of secondary characters correlated with yield together with yield itself in an index of selection increases the efficiency of selection for yield, sometimes greatly. It seems also to increase the efficiency in the

presence of genotype × environment interactions (Miller et al., 1958, for cotton; and our experiment with forage maize).

In some crops, consideration of yield components was not expected to be efficient (Davis & Evans, 1977, in navy beans; Pritchard et al., 1973, in soybeans; Shankar et al., 1963, in pearl millet; Sikka & Jain, 1958, in wheat). Indeed, 'combined' selection can be ineffective if genetic correlations are low and not so different of environmental correlations and if heritability of the secondary characters is not high enough. Furthermore such parameters have only a statistical meaning and they would vary within a species from one population to another. Combination of certain secondary characters with the primary character can then be efficient for some populations and not for others. Inaccuracy in estimation of regression coefficients of genotypic values of observed characters, could also explain some 'erratic' responses to this type of selection. This is one of the main problems to solve if we are to exploit secondary characters to the full. However the plant breeder can only gain from combined use of secondary and primary characters.

Extension to several primary characters The interest of the previous approach with several primary characters is that their phenotypic values can be used in addition to those of secondary characters to predict the genotypic value of each primary character. It is the best way of using the data recorded. Predicted genotypic value can then be weighted according to the relative economic value of each primary character. This is the classical selection index. If economic weightings are not applicable the method of desired gains (Pesek & Baker, 1969) may be applied.

In our work on forage maize, we considered several primary characters: dry matter yield, dry matter content, acid detergent fiber (ADF) content, and protein content. Simultaneous consideration of such characters to predict genotypic value for yield greatly increased the 'heritability' of yield (Table 2).

Results of Davies & Evans (1977) with navy beans showed also the interest of such an approach. The biometrical weighting of primary characters measured alone increased the efficiency of selection by nearly 50% over direct economic weighting. If in their experiment consideration of yield components did not appear to increase selection efficiency, observation of some plant type characters (total number of nodes and inflorescences, and hypocotyl diameter) gave a further increase of nearly 10% in the efficiency of selection for economic value.

Examples of systematic use of selection index in plant breeding are rare. It requires computing facilities, but if such facilities are available there is no serious argument against use of selection index when reliable estimates of parameters can be obtained from experimental design and from pooling data over two or three cycles of selection. Although such index is efficient even with independent characters, let us emphasize here only its interest due to genetic or environmental dependence between characters, i.e. the interest due to the cumulation of direct and indirect selection.

Limitations of indirect selection

The main limitation to indirect selection is any negatively correlated response to selection for some characters. For example, selecting forage maize for plant height as criterion of dry matter yield will increase lodging susceptibility. Similarly using ADF-

content as a criterion of feeding value will also increase lodging susceptibility. A solution is to consider lodging susceptibility as a separate criterion, or to find a criterion like digestibility in vitro, that is related to feeding value but is not so strictly related to lodging susceptibility. Another good example is given by Barocka (1976) for sugar beet, in which selection for sugar content with a refractometer increased the content of soluble dry matter in the residue.

To limit such negatively correlated response, the general solution is to impose some constraint on the correlated character, thus to measure it. This can increase the relative cost of indirect selection over direct selection, and sometimes it can be more efficient to use direct selection. However measurements of secondary characters and of their correlated unfavourable characters may be efficiently used in the biometrical approach to genotypic value of the primary character, as discussed previously.

Another limitation of single-trait indirect selection is if the genetic correlation is due to linkage disequilibrium without physiological basis. Such a correlation may disappear during the selection process and so genetic advance might become very poor and even zero. It is then necessary to re-estimate (or verify) genetic correlations during the selection process. Such a re-estimate is inherent in the biometrical approach to multitrait selection.

Conclusion

The plant breeder is always faced with indirect selection. Finding indirect criteria for efficient selection is finding efficient selection criteria. The immediate approach for a plant breeder to assess such criteria is to study phenotypic correlations, which reflect genetic correlations. The physiologist can be of great help in choosing characters to study. Direct selection within a population for a high and low expression of a primary character is also a plant breeder's approach to finding selection criteria. It allows study of changes due to selection at the level of the whole plant, so that unfavourably correlated characters can be identified. However conclusions, like correlations, apply only to the material studied.

The criterion of choice of secondary characters is essentially their operational value:
- They must be genetically correlated to the primary character, at sufficient level, and have higher heritability than it. Environmental correlations are also involved in efficient multitrait indirect selection.
- They must allow handling of a large number of units of selection rapidly, reliably and preferably early in the life cycle. Technical progress may affect selection criteria. For example, the application of near infrared reflectance (NIR) may change completely selection for chemical composition.

A purely biometrical approach can be developed and efficient selection criteria can be found without physiological analysis. However with such an approach, one must bear in mind that genetic correlation does not necessarily have a physiological origin.

For a complex character, such as yield, several secondary characters or criteria can be considered simultaneously. They allow definition of the ideotype in the test conditions. If so, a biometrical approach to multitrait indirect selection seems generally efficient. It takes into account genetic and environmental correlations among characters, and thus allows better prediction of genotypic value of the primary character. This biometrical

approach may save the equivalent of several replicates and creates questions about allocation of resources to maximize genetic advance.

Knowledge of the physiological basis of yield and of adaptation to environment should improve the ability of plant breeders to identify more accurately their material; however a significant advance can already be expected from efficient use of currently available data. To increase the effectiveness of plant breeding, physiologists, biometricians, geneticists and plant breeders must work together.

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Selection for a complex characteristic by a subtrait ('Tables of indirect selection')

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Summary

Falconer's (1961) postulate for efficient indirect selection was demonstrated for different levels of heritability (h^2) of the main trait and of the subtraits and for different levels of genetic interdependence between them. An attempt was made to elaborate a 'Table of indirect selection' for yield in early generations by various single subtraits in winter wheat.

Descriptors: indirect selection, wheat, *Triticum aestivum*, heritability, yield

A complex characteristic (main trait) like yield or quality results from interactions between components (subtraits) of the main trait. The subtraits may be known or unknown, and the type and amount of interaction depends on genotype and environment.

In indirect selection as discussed here, a single trait or index is used to select for a mostly more complex main trait. In principle, indirect selection by a subtrait is possible for each phene, because each phene is complex within a hierachy of complexity. Indirect selection may be preferred to direct selection: (a) when the heritability of the main trait is too low for efficient direct selection, e.g. on the basis of individuals or small samples; (b) when the selection procedure for the subtrait is simpler, less time consuming or cheaper than for the main trait.

Falconer (1961) postulates that selection for Trait A by Trait B is more efficient than direct selection for A, if the square root of the heritability of A (h_A) is less than the product of h_B times the genotypic correlation between A and B (r_{AB}), assuming the same intensity of selection for the two traits. The same is valid for h_A^2, h_B^2 and r_{AB}^2, whereby $h_B^2 \times r_{AB}^2$ cannot exceed h_B^2 or r_{AB}^2, whereas $h_B \times r_{AB}$ can exceed them, suggesting a gain, where there is a loss in determination (Hänsel, 1976). Accordingly indirect selection should be more efficient than direct selection if

$$h_B^2 \times r_{AB}^2 > h_A^2$$

where A is the main trait and B the subtrait.

In Table 1 numerical examples for this postulate are given, 0.70, 0.35 and 0.17 being 'high', 'medium' and 'low' h^2 and r^2, respectively. Under these assumptions only combinations 'high' × 'high' and 'high' × 'medium' and vice versa would justify indirect selection,

Table 1. Efficiency of indirect selection for Trait A (main trait) by Trait B (subtrait) with different levels of heritability of A and B and of genotypic interdependence between them.

h^2_B	r^2_{AB}	$h^2_B \times r^2_{AB}$	h^2_A		
			0.17	0.35	0.70
0.70	0.70	0.49	+	+	–
	0.35	0.25	+	–	–
	0.17	0.12	–	–	–
0.35	0.70	0.25	+	–	–
	0.35	0.12	–	–	–
	0.17	0.06	–	–	–
0.17	0.70	0.12	–	–	–
	0.35	0.06	–	–	–
	0.17	0.03	–	–	–

+ , $h^2_B \times r^2_{AB} > h^2_A$: indirect selection is efficient

– , $h^2_B \times r^2_{AB} < h^2_A$: indirect selection is not efficient

and this only, when the heritability of the main trait is less than 0.17, and less than 0.49 for the extremely rare case that both the heritability of the subtrait and its correlation with the main trait are 'high'.

Given a complex characteristic of a certain species and considering each of its subtraits separately, one may elaborate a specific 'Table of indirect selection' (Hänsel, 1976). In Table 2, a corresponding attempt is made for yield in winter wheat (main trait) and a number of subtraits, which are evaluated in F_4 and F_5 progenies, when breeding for the drier region of eastern Austria.

Table 2. 'Table of indirect selection' in early generations.
Crop: winter wheat
Main trait: canopy yield
Breeding area: eastern Austria

h^2_B	r^2_{AB}	Subtraits
high	high	*rh* dwarfing
	medium	high disease resistance, harvest index, non-competitive growth habit
	low	days to ripening, awns, leaf angle, plant height (except *rh* dwarfing)
medium	high	winter hardiness, drought resistance
	medium	lodging resistance, leaf area duration, spike weight (sink capacity)
	low	grain weight, flag-leaf area, tillering capacity
low	high	–
	medium	medium disease resistance, seedling vigour
	low	net assimilation rate

Since heritability and genotypic correlation depend on genetic variation within the breeding material, environmental conditions, competition effects, plot size, number of replicates per trial and number of trials, their estimates in early generations may differ from those derived from a set of field trials. Nevertheless the latter (e.g. State trials) may indicate trends and comparative magnitudes of these parameters under less exacting conditions. The ranking of the subtraits of winter wheat in Table 2 is based on the literature, on analysis of State trial results and on breeding experience. Table 2 suggests that the subtraits *rh*-gene dwarfing, stress tolerance (cold and drought), high (mostly vertical) disease resistance, a higher harvest index and perhaps a less competitive plant habit (Evans, 1981) could be used efficiently in indirect selection for yield in early generations. Indirect selection based on one of the 'physiological' traits whose contribution to yield is well established would not be more efficient than direct selection for yield on a ear-to-row or plant-to-microplot basis. The reason for this is their comparatively low heritability as well as their interaction (Planchon, 1979), which partly counteract their correlation with yield.

The assignment of a subtrait to a particular level of heritability and of correlation may also change with the yield level already reached by breeding and with selection conditions. For instance the correlation of the *rh* genotype with yield is valid only in crosses between *rh* genotype with rather long, more 'extensive' wheats and when the selected lines with the *rh* gene are cultivated under 'intensive' growing conditions. The 'high' correlation of the various types of stress tolerance with yield and their 'medium' heritability is valid only at certain natural or artificial levels of stress. Beyond an 'optimum', harvest index cannot improve yield potential. Furthermore subtraits assigned a 'medium' to 'low' heritability and correlation to yield in Table 2 could be efficient in indirect selection, if the level of other subtraits were much higher or much lower than in the most advanced winter wheats bred for the region. Subtraits, like days to earing, that are of minor importance for yield selection in Central Europe, may become of primary importance in marginal areas of cultivation (Hänsel et al., 1980). Similar 'tables' could be constructed, for instance, for baking quality (Hänsel, 1976), winter hardiness or drought resistance as main traits and different morphological or physiological characteristics, or physical or chemical properties as subtraits.

For each breeding project, a specific and at first tentative design of a 'table of indirect selection' could provide useful information. A 'table' once elaborated could be improved by new results, methods and experience or even adapted to a specific parental combination, and could become an additional tool for the breeder in the delicate process of making decisions.

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Morphological characters: a physiological analysis

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Summary

Morphological characters and yield components are determined sequentially during plant development. Correct interpretation of how correlations among morphological characters arise may indicate processes determining yield. The mechanisms giving rise to correlations among morphological characters are briefly reviewed. Limitations to biometrical studies of relationships among morphological characters are mentioned as well.

Descriptors: yield, correlated response, morphological characters

Introduction

Gallais (1984) gives valuable informations about biometrical studies involving morphological characters. Let me comment as a physiologist on the following aspects of Gallais's paper. First, he gives encouraging comments about the usefulness of morphological characters in breeding: if such characters are easy and cheap to measure, and if they are genetically related to yield (or the primary character selected), but more heritable, they are useful for efficient multitrait indirect selection. The approach may be purely biometrical and does not require physiological analysis. Second, he comments on the reasons (physiological or not) why correlations among morphological characters (including yield components) arise. Although many studies have beed devoted to the subject of morphological relationships, few of them explicitly discuss these reasons. Third, he reminds us of the limitations of such studies (which are often overlooked).

Usefulness of physiological studies on morphological characters

The direct role of physiologists in plant breeding has as yet been limited. I have no knowledge of a predominant contribution of plant physiologists for the release of a single new variety. Nevertheless Gallais (1984) notes that, although their role is not essential, physiologists may help in the choice of morphological characters to be used as secondary traits in indirect selection. Without their contribution, this choice might be a lengthy trial and error process. Moreover knowing whether a genetic correlation has a physiological basis may be relevant. Breaking of negative associations among traits by breeding is easier if the correlation has no physiological basis.

On the other hand, if genetic correlations lack a physiological basis, they have to be reassessed during selection, since they may disappear (Gallais, 1984) by disruption of linkage groups or recombination.

Usefulness has to be sought also in other ways. Grain yield, yield components, and morphological characters are determined sequentially during development, for a great part before anthesis. This sequential determination of yield has been discussed by many (Evans, 1975). The same applies to morphological characters other than yield components. Limitations during critical periods of the development leave their mark on yield but also on morphological characters. An understanding of how plant shape (as defined by relationships between size of organs) is determined might therefore be useful. Independently of the problem of yield in crops it has been recognized for a long time that science of growth is concerned primarily with three variables: size, age and shape (Medawar, 1945). This statement places yield development in the general context of earlier studies on allometry, relative growth, shape, etc. (Gould, 1966).

Mechanisms giving rise to correlations among morphological characters

Correlations among morphological characters may have physiological basis or not. Non-physiological correlations may be due to gene linkage (Adams, 1967; Das, 1972; Gallais, 1984). They are more likely to be found in a study across genotypes, with progenies from crosses among a few parents or in species with few chromosomes. Quantitative morphological characters are, however, probably polygenic, and this may complicate the problem.

Correlations due to linkage may have some physiological basis if the linkage groups were influenced by selection: naturel (Gallais, 1984) or man-made.

Other correlations may involve the complex interrelationships between physiological processes during development. Both genetics and physiology of development are concerned: developmental interdependency; intertwining of fate of organs, even though each may be affected by different environmental stresses and gene systems (Hamid & Grafius, 1978); genetic correlations arising from developmentally induced relationships that are only indirectly the consequence of gene action (Adams, 1967).

As an attempt to disentangle the various underlying processes, let me enumerate some mechanisms that may play a role.

Pleiotropy (Das, 1967; Adams, 1967) A gene triggers a sequence of action through the multiple interrelationships and chain reactions in the physiology of the plant.

Indirect relationships between size of organs Such relationships may occur in organs growing simultaneously and be due to responses to sequential variation in internal or external environment (e.g. temperature, nitrogen, assimilates, hormones). There is no causal direct relationship (e.g. competition) between characters. The sequence of variation may be environmental (e.g. season) or dictated by genes regulating plant development. This may explain at least partly the relationships between organs determined during rapid stem elongation (Ledent, 1978; 1983).

Direct causal relationships between organs Organs may directly influence the growth of other organs in the following ways:

- competition for limited resources (e.g. nitrogen, hormones, assimilates);
- correlative growth (e.g. hormones produced by one organ affect another organ);
- pleiotropic effect of common meristem, from which various organs originate (Hamid & Grafius, 1978). There are also relationships between meristem size of parts of ear primordium and grain production in corresponding ear parts at maturity (Kirby, 1974).
- functional interdependence (in the broad sense).

One example is the relationship between the size of a plant part acting as sink (e.g. grain) and the source of assimilates (e.g. flag leaf area, leaf area duration (LAD)). This is the most common interpretation (implicit or explicit) used (or abused) for relationships of morphological traits with yield. It applies if variations in source (e.g. leaf area, LAD) across the material used are wide (broad range of nitrogen fertilization, crop density, water status, contrasts between years and between sites, and variation in diseases).

However, variations in leaf area or LAD may not always explain variations in yield. In varieties grown in high-yielding conditions, the relationships between yield components and morphological characters were not found to be due to associations between assimilate-producing surfaces and plant parts using the assimilates in a predominantly source-limited situation (Ledent, 1983). Clearly determination of grain yield in a set of genotypes normally found in a breeding program involves more complex processes than those determining carbohydrate production (Grafius & Wiebe, 1959).

Sink-source relationships do not apply only for carbohydrates, but also for nutrients such as nitrogen or minerals. The size of organs serving as final or temporary storage may limit the relations due to source. Redistribution of nitrogen during senescence may also play a role.

Relationships between composite characters and their components Examples of composite characters are yield, leaf area duration, specific leaf weight and leaf area. These relationships may be considered as trivial and purely formal at first sight. The problem is the real physiological significance of the composite character. Genetically these characters are artefacts (Grafius & Wiebe, 1959). The relationships most commonly discussed are those between yield and its components. For some physiological reasons, there seems to be a balance to be achieved among components to obtain high yields (Fischer & Hille Ris Lambers, 1978), hence the concept of an optimum geometry of yield (Adams & Grafius, 1971) varying with the environment and genotype.

Limitations to biometrical studies of relationships between morphological characters

The results of this type of studies always have limited application. The conclusions depend indeed on various factors. They are limited

- to the plant material used (genetic make-up of entries, range of environments);
- to the plant characters measured and their variation. (Lack of correlation with a character may be due to its limited variation, Adams & Grafius, 1971);
- to environmental conditions at various stages of development: there is no physiological reason for all genotypes to achieve balance among components in the same way always.

Conclusions

The shape of a plant is the result of the sequence of events affecting its growth and development. It is a visible message left by the plants to those who try to understand how yield is determined. The problem is to interprete it without undue simplification. Whether this will be possible and how far it will be helpful to breeders remain open questions.

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Unconventional uses of indirect and index selection

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Summary

Some special situations in which indirect selection is known to be efficient are described. Attention is drawn to the fact that the rate of response to selection of any type is always limited by the genetic variation in the primary character.

A secondary variate can be used to correct phenotypic observations of the primary character for either environmental or genetic sources of error. Two examples of the latter use are given.

Descriptors: indirect selection, synthetic variety, reciprocal recurrent selection

Factors affecting gain in different selection systems

The gain expected to follow selection of any type can be expressed by the general formula:

$$R_{y/x} = i_x \, \sigma_{gy} \, D_{gy \cdot px}$$

where i_x is the selection intensity in standard units when using selection criterion x, σ_{gy} is the standard deviation of breeding values of y, the single character or complex which is the objective of selection, and $D_{gy \cdot px}$ represents the square root of the coefficient of determination of breeding values of y by the phenotype of x. If the selected units are varieties or clones which are not to undergo further sexual cycles, then the term genotype can be substituted for breeding value in these definitions. With simple direct selection, then $D_{gy \cdot px}$ is h_y, the square root of the heritability of the character, and with indirect selection using a single character x, it is $r_g h_x$, as already noted by Gallais (1984). In general, however, when several characters are to be improved by selection on a further set of secondary characters, as in index selection, then $D_{gy \cdot px}$ is a more complex statistic. No matter what form of selection is practised, however, it should be noted that this coefficient can never take a value greater than unity, representing perfect measurement of breeding values.

Thus indirect or index selection of any type will have an advantage over direct selection if $i_x D_{gy \cdot px}$ exceeds $i_y h_y$, and in general any two systems of selection can be compared in the same way. Some examples of common methods of increases in $D_{gy \cdot px}$ which are effectively methods of indirect selection have already been mentioned, for

example:
- the use of highly correlated traits with high heritability, possibly components of yield itself or traits which are pleiotropic to it and measured with less error, such as physiological characters,
- the use of progeny tests or family selection instead of mass selection,
- the use of environments which accentuate yield or disease response differences, or of samples of environments,
- the use of selection among inbred lines for the improvement of synthetic variety or hybrid performance, or of monoculture assessment for the selection of mixture components.

This final example illustrates a particularly favourable principle which applies to indirect selection in cases where the objective of selection is a performance unit (e.g. synthetic variety) which is made up of several selection units (lines). Selection to isolate superior K parent synthetics can be carried out on the population of synthetics themselves, or on the population of candidate lines. The second option has the disadvantage that any genetic interactions among the K lines which would only come into play in the expression of the synthetic itself cannot be assessed, so that the genetic correlation between lines and synthetics (r_g) is reduced. However, it has a distinct advantage in terms of the total variation expressed, as the varieties show only $1/k$ as much additive genetic variation as do the lines. This means that line selection has a higher heritability than variety selection, and with large environmental effects and large synthetics, indirect selection for line performance will be the more efficient. This principle of reduced variation among the primary units due to their composition as random associations of elements also applies to the question of selection of components for variety mixtures, for which purpose the monoculture assessment of potential components may often be the most efficient method.

It was noted above that the value of $D_{gy \cdot px}$ can never exceed unity, no matter what system of selection is used. The limit to the rate of response achievable is always set by σ_{gy}, a statistic to which insufficient attention is often paid in this context. If there is inadequate genetic variation available in the primary character(s), then all attempts to find efficient selection criteria will fail to increase rates of selection response to an economically acceptable level. Such a lack of variation may be due to a negative correlation, for example among components of yield. Many published reports of low heritability for yield may in fact be due to low genetic variance rather than high error variance, and so be unresponsive to all types of selection. If the assessment of selection criteria is carried out on a purely empirical level, without any attempt to estimate the contributions of the three components in the above response equation, then this cause of failure could remain undetected and unsuspected.

The use of a secondary variate to correct for non-additive genetic effects

Purser (1960) showed that when a secondary trait (x_2) has a zero additive genetic correlation with the primary character to be improved (x_1), then the optimum index is given by

$$I = x_1 - b_p x_2,$$

where b_p is simply the coefficient of phenotypic regression of x_1 on x_2, a statistic which can usually be easily and accurately estimated. The secondary character here performs the function of correcting the primary trait for environmental effects, and the technique is similar to the familiar covariance adjustment used in field experimentation.

This type of index can be used to correct, not only for environmental effects, but also for non-additive genetic effects which would otherwise bias the estimation of breeding values. An example of this use can be suggested for the improvement of mass selection in field beans and other crops in which the mating system is a mixture of self and random pollination and the natural population is a mixture of individuals with different coefficients of inbreeding. The phenotypic value of an individual, upon which selection is normally based, is influenced both by the alleles it carries which specify its true breeding value, and by the degree to which it is inbred. A second character, known to have no additive genetic correlation with the first, but showing marked heterosis, could then serve to correct the observed values for the differential effects of inbreeding, as well as environment.

A second, more complex example, is the suggestion by Moreno-Gonzalez & Hallauer (1982) to enhance the efficiency of full-sib reciprocal selection. This procedure is designed to improve the reciprocal combining abilities of a pair of populations A and B by selecting individuals from A on the basis of the yields of their full-sib progeny after crossing each with a randomly chosen member of population B. The suggested modification is that the S_1 (or S_2 or other convenient generation) of the partner should also be measured and these values used exactly like a secondary character (x_2). In this way, the observed full-sib value is corrected by the index to make some allowance for the fact that the randomly chosen partner happens to be a particularly low or high yielding individual. An exactly similar procedure would also be applied to the selection of members of population B. A zero additive genetic correlation is assured by the random pairing of plants from the two populations, but the efficiency of the index may be impaired because the S_1 value of a plant need not correlate with, and therefore give a good prediction of, its contribution to the value of a family of full sibs.

These two examples serve to illustrate a novel function for the use of a secondary variate, and to emphasise the versatility of indirect and index selecion methods, and can be added to the long list of applications already given by Gallais (1984). Breeders should be constantly searching for such applications to enhance the efficiency of their selection procedures.

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Selection in early generations

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Summary

Selection in early generations is practised for inbred lines in which inbreeding is not yet completed and that are used in breeding of pure lines and in hybrid breeding. Selection of dihaploids in potato, before returning to the tetraploid level, is another example.

In early generations, not much seed is available, and hill plots often are used. Experimental results for yield showed that the correlation between hill and row plots was usually high enough as long as one environment was regarded. Nevertheless, selection in early generations was not generally superior.

Studies with theoretical models indicate, that the reduced genetic variation in lines derived from plants in early generations is no hindrance to effective selection. Epistasis can be neglected. Combined and two-stage selection are superior to simple selection.

Descriptors: early generation selection, hill plots, row plots, autogamous species, yield, combined selection, two-stage selection

Introduction

Briggs & Knowles (1967) used the term 'selection in early generations' for selecting inbred lines in which inbreeding is not yet completed, as in the breeding of pure lines in the first generations after crossing and in hybrid breeding during the development of inbred lines as parents for the hybrid variety. Selection in early generations is conducted on lines that are not yet fixed.

The term also can be used for other cases, for example selection at another level of ploidy. In potatoes, selection on the diploid level is being considered, but the selected diploid genotypes must ultimately be returned to the tetraploid level.

There is no sharp line between selection in early generations and indirect selection. Characters like yield often cannot be assessed directly on single plants or hills. The direct measure is replaced by visual screening or by measuring a correlated character. In this paper only selection in early generations for self-fertilizing species will be treated in more detail.

Selection of superior crosses

Several authors have estimated the value of different crosses in early generations. Nass (1979) selected crosses in wheat on the basis of F_1 and F_2 population means. Such populations have a high degree of heterozygosity, whereas pure lines are homozygous.

Other authors investigated the variance of segregating generations. A superior cross must have a large variance, combined with an acceptable mean. In barley, Choo et al. (1982) estimated the variance of lines derived from single seed descent (SSD) and of doubled haploids derived from F_1 plants. Snape (1982) used the triple-test cross in wheat, by crossing a sample of F_2 plants with both parents and the F_1. The same procedure was used by Jinks & Pooni (1980) in *Nicotiana rustica*. The easiest way to test variation is to derive lines from F_2 plants and to test them in the F_3 generation. In this situation, however, the variance is influenced by the dominance variance, which cannot be used in the selection of pure lines. But with one generation of self-fertilization, the dominance variance will be reduced to a quarter and may safely be neglected. However, since only crosses and not lines are being selected at an early stage, this topic will not be discussed further.

Mostly lines from selfed seed of single plants are tested in stead of the single plants themselves. Such a line will be denoted here as an F_t-derived line, where t stands for the generation number of the single plant, and where the plants in the test are already in generation $F_{t'}$ (t' being $t+1$, $t+2$, etc.). Other authors relate the line generation to the test generation and consequently denote the test generation as $F_{t'}$. Since only a few seeds are normally available in early generations, tests are often conducted in hills or single rows and only in one environment. In this paper, the only characteristic considered is yield.

Size of units in the trials

Several trials are reported in the literature, in which hill and row plots are compared. The correlation coefficients of some of these trials are given in Table 1. The correlation

Table 1. Correlation coefficients between hill and row plots. r_g, genotypic correlation; r_p, phenotypic correlation.

Crop	Source	Genotypes	Correlation
wheat	Baker & Leisle, 1970	varieties	$r_g = 0.87; 0.99$
	o'Brien et al., 1979	F_2-derived lines in F_3	$r_p = 0.40$ to 0.79
durum wheat	Baker & Leisle, 1970	varieties	$r_g = 0.81; 0.86$
barley	Ross & Miller, 1955	varieties, advanced lines	−0.241 to 0.946
oats	Ross & Miller, 1955	varieties, advanced lines	0.241 to 0.948
	Jellum et al., 1963	F_2-derived lines in F_4 and F_5	0.469 to 0.956 (2 sites, 2 years)
	Frey, 1965	varieties	$r_g = 0.98$
soya	Torrie, 1962	varieties	0.26 to 0.90
	Garland & Fehr, 1981	lines	$r_p = 0.84$ to 0.93

coefficient depends on the number of replicates and the size of hill and row plots. With one exception, all correlation coefficients are positive and most of them are larger than 0.5. Often, however, varieties were used and tested in only one environment, so the results must be treated with caution. Nevertheless they indicate that hills can be used for selection for yield.

Response to selection in early generations

Results are summarized in Tables 2-4. Table 2 gives correlation coefficients between lines derived from plants of different generations. Most are low, and the authors deny that selection in early generations is effective.

Whan et al. (1981) tested at two sites and in two years, and found that the genotype-year interaction was the most important factor. If the tests are conducted in the same year, the correlation between lines from different generations is still large enough to justify selection, but for different years, the correlation is poor, even for lines from the same generation.

The results on selection in early generations are summarized in Table 3. Most of the authors conclude that selection in early generations is not effective enough to justify the extra work. Thakare & Qualset (1978) found that combined selection in wheat, using results of testing F_2-derived lines and F_3-derived sublines, was especially efficient.

Table 4 presents some results of Whan et al. (1982) on wheat. The selected fraction was 0.10. They compared lines derived from F_2, F_3, F_4 and F_5, at two sites in two years. Selection was effective, as long as one year was considered, but the response to selection was weak, if measured in another year.

Further investigation compared different selection methods like early testing, pedigree with visual selection and SSD (single seed descent). Agble (1978) found that early testing was more successful in tomatoes. Boerma & Cooper (1975) preferred SSD in soybeans, since the results of all methods were similar and SSD required less effort. Knott & Kumar (1975), Knott (1979) and Seitzer & Evans (1978) found that in wheat testing in early generations was not worthwhile.

In conclusion, results do not give a clear answer to the question whether selection in early generations should be practised, nor whether it should be executed as visual screening of single plants or hills or as exact testing in hills, single rows or larger plots.

Table 2. Correlation coefficients between lines derived from plants of different generations.

Crop	Source	Generation		Type of test in early generation	Correlation
		early	late		
wheat	Knott & Kumar, 1975	F_2	F_5	single rows, 3 replicates	0.29; 0.14
	Whan et al., 1981	F_2, F_3, F_4	F_3, F_4, F_5	2 and 4 rows, 1 rep., 2 sites, 2 years	−0.17 to 0.65
barley	McKenzie & Lambert, 1961	F_2	F_5	3 replicates	0.313; 0.543

Table 3. Selection response in early generations.

Crop	Source	Lines derived from early generation	Selected fraction	Selection response (%) ($\bar{x} = 100$)	Number of top lines
wheat	Knott & Kumar, 1975	F_2 (single row, 3 replicates)	0.20	–	17 from 36
	Thakare & Qualset, 1978	F_2	0.33	101 to 107	–
	Whan et al., 1982	F_2, F_3, F_4 in F_3, F_4, F_5	0.10	79 to 178 (details in Table 4)	–
barley	McKenzie & Lambert, 1961	F_2	0.10	–	1 & 5 from 10
	Hanson et al., 1979	F_3 (single plants, visual selection)	0.20 to 0.30	102	–
	,,	F_5 (single row)	0.17	104	–
soya	Wilcox & Schapaugh, 1980	F_2, F_3, F_4	0.10	96, 97, 101	–

Table 4. Some results from selection trials on wheat by Whan et al. (1982). Selected fraction 0.10.

Type of selection	Selection response in later generations (%, $\bar{x} = 100$)		
	F_3-derived lines	F_4-derived lines	F_5-derived lines
Same site, same year			
F_2-derived lines	125	111	113
F_2 (mean of F_3-derived lines)	–	119	135
F_3-derived lines	–	123	134
F_3 (mean of F_4-derived lines)	–	–	132
Different sites, same year			
F_2-derived lines	117	120	126
F_2 (mean of F_3-derived lines)	112	117	123
Same site, different years			
F_2-derived lines	106	104	101
F_2 (mean of F_3-derived lines)	101	91	111

Sneep (1981) recommended that as many seeds as possible should be obtained from F_2 plants to allow exact tests, as soon as possible.

In plant breeding selection is practised in several stages of the programme and at these stages also several factors like size of population, screening procedure and selection intensity may differ. Trials in which all factors that can be varied are investigated require too much time, space and labour, and even such trials cannot give a general answer. So let us consider in theory the efficiency of selection in early generations.

Genetical model and expected response to selection

The following model for an F_j-derived line is used (where j stands for a certain generation number).

$$p_j = g_j + e$$

In this model p_j is the phenotypic effect, g_j the genotypic effect, and e the sum of the environmental effects, including the genotype-environment interaction. We assume that e does not depend on generations, and also that g_j, with variance $V(g_j)$ and e, with variance V_e, are not correlated. The phenotypic variance is $V(p_j)$. If also sublines are included in the model $V(g_{j(i)})$ is the genotypic variance among the F_j-derived sublines within a certain line of generation F_i.

The genotypic variance can be devided into additive, dominance and epistatic variance. The dominance variance is being neglected, since this variance among lines will be reduced to a quarter with each generation of selfing and therefore will be small, even among F_2-derived lines. Only the additive variance and the epistatic variances of 'additive' type are taken into account. The covariance $C(p_j, g_\infty)$ between the phenotypic value of an F_j-derived line and the expected genotype of a single homozygous line sampled from the F_j-derived line by succesive generations of selfing equals $V(g_j)$. The selection response in the standardized form is given to

$$R = i\rho_{p_j g_\infty} = i\, V(g_j) \,/\, \sqrt{V(p_j)\, V(g_\infty)}$$

with i = selection intensity. V_e is related to plot size and is expected to be smaller with larger plots.

Simple selection

When the total number of plots is fixed, the breeder can test many lines with one replicate or fewer with several replicates. Table 5 shows the optimum number of replicates for selection between F_2-derived lines, F_3-derived lines and homozygous lines

Table 5. Optimum number of replicates (r) and response to selection (R) between lines derived from F_2, F_3 and F_∞; genotype per 100 plots selected.

V_A	V_{AA}	V_e	F_2-derived lines		F_3-derived lines		F_∞-lines	
			r	R	r	R	r	R
2	0	1	2	1.56	2	2.00	2	2.38
1	0.5	1	3	1.28	2	1.84	2	2.38
0	1	1	3	0.99	2	1.66	2	2.38
2	0	10	7	1.04	6	1.38	5	1.69
1	0.5	10	8	0.84	6	1.26	5	1.69
0	1	10	10	0.62	7	1.13	5	1.69

(F_∞-lines). The genotypic variance among homozygous lines was set to 4 and one line was selected from 100 plots. With only additive variance (V_A) and additive by additive epistatic variance (V_{AA}) the genotypic variances in early generations are: $V(g_2) = V_A + V_{AA}$ and $V(g_3) = 1.5\ V_A + 2.25\ V_{AA}$. Three extreme cases are taken: only additive variance ($V_A = 2$, $V_{AA} = 0$), additive and epistatic variance ($V_A = 1$, $V_{AA} = 0.5$) and only epistatic variance ($V_A = 0$, $V_{AA} = 1$). The non-genetic variance was taken as small ($V_e = 1$) or large ($V_e = 10$).

The response to selection in early generations is less effective than in late generations. But if the model is correct, the response to selection in early generations is large enough to justify selection. The number of replicates must be increased, since the genotypic variance is smaller, and more replicates are necessary to identify good genotypes. The influence of epistasis on the optimum number of replicates is small.

Combined selection

Sublines can be derived from lines in later generations. If we consider F_2-derived lines and F_3-derived sublines, the information can be combined in an index.

$$I = b_2\bar{p}_{2,k} + b_3(p_{3,kl} - \bar{p}_{2,k}),$$

where $\bar{p}_{2,k}$ is the mean of the k-th F_2-derived line and $p_{3,kl}$ is the phenotypic value of the l-th F_3-derived subline. This type of selection is called combined selection. We looked for the optimum number n_2 of F_2-derived lines and n_3 of F_3-derived sublines within each line, if $N(=n_2n_3)$ is held constant. Only one replicate was considered. England (1977) found that in family selection the optimum number of replicates should be as small as possible.

The response to selection depends on the quotient b_2/b_3 of the index weights. The optimum quotient maximizes selection response. In studies with models, the genetic and non-genetic components of variance are known and the index of Smith (1936) can be

Table 6. Optimum number of sublines (n_3) for combined selection, using F_2-derived lines and F_3-derived sublines. n_2, number of F_2-derived lines; n_3, number of F_3-derived sublines; one replicate; selected fraction 0.10; N, number of plots $= n_2.n_3$.

N	V_A	V_{AA}	V_e	Optimum n_3	$n_3 = \frac{\sqrt{N\ V_e}}{\sqrt{V(g_2)\,(V(g_{3(2)}) + V_e}}$
120	2	0	1	2	5.5
	0	1	1	4	7.3
	2	0	10	12	23.4
	0	1	10	20	32.7
1200	2	0	1	15	17.3
	0	1	1	20	23.1
	2	0	10	60	73.9
	0	1	10	100	103.3

used. When n_2 and n_3 are both large, the optimum quotient n_2/n_3 equals $V(g_2)(V(g_{3(2)})+V_e)\ /\ V_e^2$ (Weber, 1982).

However it is not usual that both n_2 and n_3 are large, and selection intensity i is smaller and depends on n_2 and n_3. The optimum was calculated for two different values of $N(=n_2n_3)$, two non-genetic variances, the extreme cases of absence of epistasis or full epistasis and a selected fraction of 0.10 (Table 6). As comparison n_3 was also calculated with the formula given above.

The optimum n_3 was smaller for small populations; if V_e is large, n_3 must also be large. Selection between lines is then more important than selection within lines, and a larger number of sublines is necessary to estimate the phenotypic mean with reasonable precision.

In a practical breeding programme the breeder must choose values of n_2/n_3 and b_2/b_3. The optimum quotients depend on the components of variance which the breeder does not know exactly. In Table 7, the response to selection for the optimum quotients is compared with other values. For combined selection n_3 was doubled. For simple selection, three cases were considered: between lines only, between sublines only and between lines, if only one subline is derived from each line.

Combined selection is more effective than simple selection. The value of n_2/n_3 is not critical and must not be exactly optimum.

For simple selection, it is always better to raise only one subline per line, if selection is practised between sublines, although the difference in response to selection is small. If selection is practised between lines only, n_3 is the number of replicates and the optimum depends on the non-genetic variance (Table 5).

The breeder must have some information on components of variance to choose index weights. If epistasis is small, the optimum of $b_2/b_3 \approx 1+2\ V_e/(V(g_2)+V_e/n_3)$.

Table 7. Response to selection for combined selection or simple selection, using F_2-derived lines and F_3-derived sublines. n_2, number of F_2-derived lines; n_3, number of F_3-derived sublines; one replicate; selected fraction 0.10; N, number of plots $= n_2 \cdot n_3$.

N	V_A	V_{AA}	V_e	Combined selection		Simple selection		
				a	b	c	d	e
120	2	0	1	1.30	1.30	1.24	1.28	1.29
	0	1	1	1.19	1.19	0.88	1.07	1.08
	2	0	10	0.96	0.92	0.93	0.72	0.72
	0	1	10	0.70	0.67	0.59	0.56	0.56
1200	2	0	1	1.36	1.36	1.22	1.30	1.31
	0	1	1	1.25	1.25	0.86	1.20	1.20
	2	0	10	1.17	1.16	1.11	0.73	0.73
	0	1	10	0.86	0.84	0.75	0.56	0.56

a. n_2/n_3 and b_2/b_3 at optimum
b. n_3 doubled, b_2/b_3 at optimum
c. n_2/n_3 as under a), between lines only, $b_3 = 0$
d. n_2/n_3 as under a), between sublines only, $b_2 = b_3 = 1$
e. $n_2 = N, n_3 = 1$, only one subline per line

Table 8. Optimum number of F_2-derived lines (n_2, at first stage; n_2' selected and entering the second stage) and F_3-derived sublines (n_3) for selection in two stages (one out of N plots selected).

N	V_A	V_{AA}	V_e	First stage	Second stage	
				n_2	n_2'	n_3
20	2	0	1	12	2	4
	0	1	1	10	2	5
	2	0	10	11	3	3
	0	1	10	8	2	6
100	2	0	1	58	2	21
	0	1	1	46	2	27
	2	0	10	45	5	11
	0	1	10	32	4	17

Selection in two stages

In a breeding programme, selection is practised in several stages. Cochran (1951) described a method of calculating the expected response to selection in two stages. He assumed that the genotypes were already fixed. Schnell (1958) and Utz (1969) used this approach and included the genotype-environment interaction and three stages of selection.

For the selection in two stages in early generations the approach of Cochran cannot be used directly. Genotypic variance is reduced by selection, but in the next generation it increased again, as sublines are developed. As an example let us consider selection between F_2-derived lines at the first stage and between F_2-derived lines and F_3-derived sublines at the second stage.

The response to selection can be approximated as follows. At a first step, the best F_2-derived line is selected in two stages. For this step, the approach of Cochran can be used. At a second step the best F_3-derived subline is selected within the best F_2-derived line. This procedure was used independently by Weber (1980; 1981) and Utz (1981; 1982). As for combined selection the number of plots per selected subline (N) was fixed and the number of replicates was one.

Table 8 contains the optimum number n_2 of lines at the first stage, the selected number n_2 entering the second stage and the optimum number n_3 of sublines at the second stage, for two values of both N, and the non-genetic variance V_e, and for the extreme situations with or without epistasis. The influence of epistasis is limited. If V_e is large, the proportion of plots at the second stage increases.

Combined or two-stage selection

Table 9 compares combined selection with two-stage selection. Since response to selection for two-stage selection is overestimated with the approximation, the results in Table 9 are based on simulations. The gain due to two-stage selection is remarkable, especially if non-genetic variance is large.

Table 9. Response to selection with combined or two-stage selection, using F_2-derived lines and F_3-derived sublines. N, number of plots.

N	V_A	V_{AA}	V_e	Combined selection	Two-stage selection	Relative gain with two-stage selection (%)
20	2	0	1	1.36	1.46	7.4
	0	1	1	1.14	1.26	10.5
	2	0	10	0.76	0.88	15.8
	0	1	10	0.59	0.66	11.9
100	2	0	1	1.86	2.19	17.7
	0	1	1	1.54	1.84	19.5
	2	0	10	1.09	1.43	31.2
	0	1	10	0.83	1.04	25.3

Conclusions

Studies with models showed that selection in early generations is effective, provided that non-genetic variance, including genotype-environment interaction, is not too large. This point is critical.

Selection is done in several stages, and selection intensity at the first stage must be low, to decrease the danger of losing good lines. If the interaction with years is the major source of error, a test in one year is weak, regardless if it were executed through visual screening, or testing in hill plots or row plots.

Reduced genotypic variance of lines derived from plants in early generations is no hindrance for an effective selection, and epistasis has little influence on optimum strategy of selection.

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Early generation selection and rapid generation advancement methods in autogamous crops

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Summary

Rapid generation advancement using single seed descent or by doubled haploid production enables varietal selection to be carried out on homozygous rather than segregating material. However, in the absence of efficient early generation selection, genetic drift may cause the loss of desirable alleles. Nevertheless, in practice, transgressive segregants can still be obtained.

The efficiency of these techniques can be improved by introducing early generation selection, particularly at the F_2. Methods of increasing selection efficiency for qualitative and quantitative characters are discussed.

Descriptors: selection, single seed descent, doubled haploids, barley, *Hordeum vulgare,* wheat, *Triticum aestivum*

Introduction

In autogamous crop species the pedigree method of breeding predominates. In this system methods of improving selection efficiency in early generations have obvious merit. However, selection for characters of low heritability, in particular yield, is only likely to be effective in early generations if sufficient replication is available and an efficient selection system, such as combined selection or two-stage selection, is applied (Weber, 1984). In practice these systems will require an input of resources which may not be justifiable in terms of the gains obtained. An alternative strategy is to rapidly advance the segregating generations to homozygosity without conscious selection prior to evaluation. The procedures of single seed descent (Brim, 1966; Snape & Riggs, 1975) from the F_2 generation, or doubled haploid production from the F_1, are both used for this purpose. This paper discusses the advantages and disadvantages of rapid generation advancement procedures relative to the pedigree system and the use of early generation selection to improve their efficiency.

Advantages and disadvantages of rapid generation advancement procedures

There are two advantages of rapid generation advancement techniques over the pedigree system (Snape, 1982). First, they allow a time saving in varietal development by enabling homozygous material to be available for large scale yield testing and stock multiplication at an earlier date – a saving of two years is possible in winter barley

breeding (Simpson & Snape, 1981). Secondly, more efficient selection is possible relative to early filial generations because of the greater contribution of additive effects to the variation, and the absence of within family genetic segregation. This allows better discrimination between genotypes within any one generation of field testing and a better response to selection across generations.

The major disadvantage of these techniques is the possible irretrievable loss of desirable alleles due to the random sampling of genotypes – the problem of genetic drift (Sneep, 1977). However, unless selection is particularly efficient this will also occur in a pedigree system because of the stepdown in numbers from F_2 plants to F_3 families. For example, in wheat Bingham et al. (1981) report that from a population of 1.2 million plants only about 45 000 F_3 families are grown – thus only 4% of the total F_2s are retained.

Although genetic drift undoubtedly takes place in randomly advanced populations, experimental evidence shows that sufficient variation is retained to enable transgressive segregants to be isolated. For example, Figure 1 shows the distributions for yield obtained in a random F_6 population of a wheat cross derived by single seed descent and a population of F_1 derived doubled haploid lines of barley. In both populations segregants yielding 10%+ more than the best parent could be isolated.

Selection prior to generation advancement

Major genes The selection of alleles at major gene loci in the F_2 is the most effective method of improving the frequency of useful genotypes, since only $(\frac{1}{2})^k$ lines (where k is the number of segregating major gene loci) have desired combinations. Numerous tests are now available to facilitate such selection. The inoculation of F_2 plants with disease isolates is the most obvious approach. However, with the increased understanding of the genetical control of important agronomic characters through genetical analysis, efficient screening techniques for a range of other characters is possible. In wheat for example, dwarfing genes can be selected for by their insensitivity to applied gibberellic acid (Gale & Marshall, 1976), combinations of endosperm protein sub-units with desirable effects on bread-making quality can be selected by SDS polyacrylamide electrophoresis on half-grains (Payne et al., 1981), and isozyme variants can be identified using isoelectric focusing (Hart, 1979). With the development of the newer techniques of molecular biology, further selection may be possible for desirable chromosomal segments containing many genes. Already, large quantitative differences in gene numbers for repeated DNA sequences can be detected by in situ hybridisation of DNA probes onto chromosomes (Hutchinson et al., 1981), as can structural differences by detecting polymorphisms in restriction enzyme digests of such sequences (Flavell, 1982). Development of these techniques further may enable single copy genes to be screened. Although, at present, these techniques require relatively large amounts of plant tissue and are time consuming, progress is continually being made in improving their efficiency. It is conceivable that future refinements will enable these systems to be automated to handle the numbers required for a breeding programme.

Quantitative characters The effect of selection for a quantitative character in the F_2 on the mean performance of their F_∞ progeny derived by single seed descent or doubled

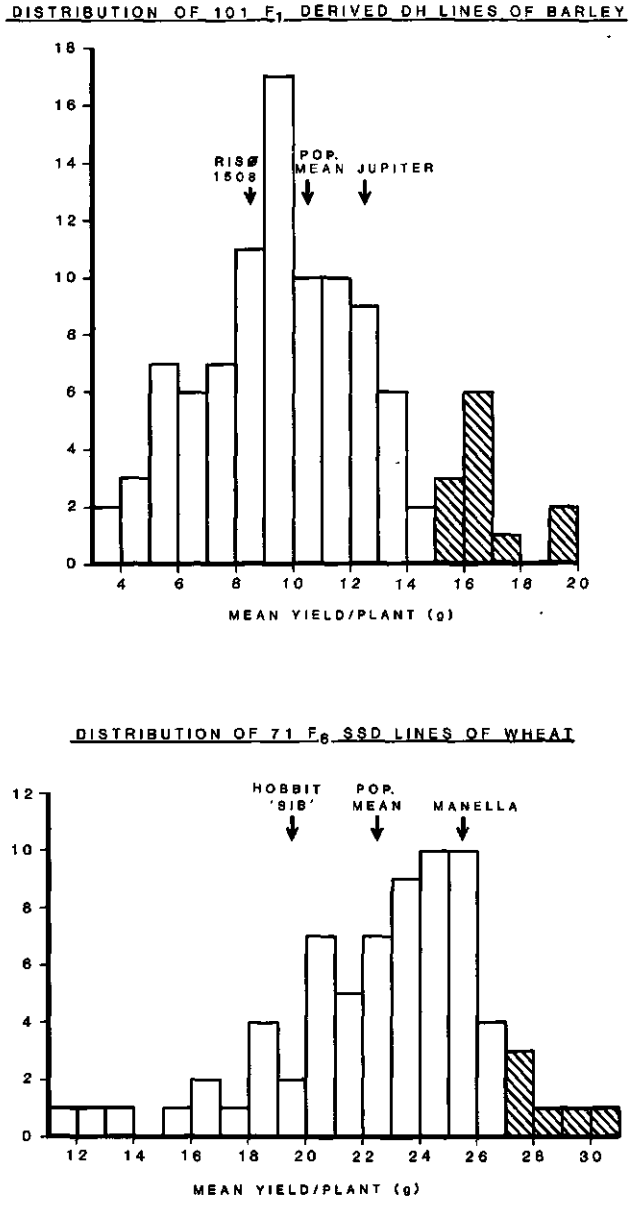

Fig. 1. Yield distributions of doubled haploid (DH) lines of barley (upper) and of single seed descent (SSD) lines of wheat (lower): shading indicates lines significantly higher than the best parent at the 5% level.

haploid procedures is given by the response equation:

$$R = i\, b_{F_\infty F_2} \sigma_{F_2}$$

where

R = response
i = selection intensity
b = regression of F_∞ means on F_2 phenotypic values
σ = F_2 phenotype standard deviation

This reduces to

$$R = i \frac{V_A + V_{AA}}{(V_A + V_D + V_{AA} + V_{AD} + V_{DD} + V_E)^{1/2}}$$

where the variance components are defined relative to the F_2.

Thus response depends on the narrow heritability of the character, measured in the F_2 population. With quantitative characters of high heritability, such as flowering time or height, good response can be expected. However, as discussed by Weber (1984), selection for characters of low heritability, such as yield will produce little or no, direct response. Only by selection for a correlated character of high heritability – due to linkage or pleiotropic effects – can response be improved. In wheat for example, Law et al. (1978) advocate selection for increased plant height because of the strong positive genetic correlation between height and yield in the segregating generations of crosses, and the strategy of yield improvement by selection for 'tall dwarfs' has been developed (Gale & Law, 1977). The identification of new genetic markers, in particular isozyme variants, and the characterisation of the effects of allelic variation on agronomic characters could be particularily useful in this respect if advantageous effects can be associated with particular alleles.

Discussion and conclusions

There is increasing evidence that rapid generation advancement procedures can increase the efficiency of breeding programmes. Doubled haploid systems have attracted most interest although on practical considerations single seed descent is easier and cheaper to perform. However with winter crops where vernalisation is required and generation turnover is slow, doubled haploid systems are to be preferred. The introduction of selection at the F_2, although it reduces the time saving component of these systems, should, nevertheless, increase their overall efficiency by eliminating undesirable genotypes for selectable major gene systems. The development of new marker systems and the characterisation of their effects on agronomic traits may enable efficient selection systems to be developed for quantitative characters of low heritability at the F_2. The procedures themselves may also provide environments where selection occurs. Thus interplant competition during single seed descent may eliminate less vigorous genotypes which have lower yield potential. Similarily the embryo culture and tissue culture

systems employed to develop doubled haploids may selectively eliminate less vigorous individuals, and unconsciously, improve mean F_∞ performance.

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Is selection on quantitative characters between F_3 lines in small grains feasible?

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Summary

Selection for yield between F_3 lines in barley and wheat is only possible with mini-plots of about 1–1.5 m^2 in area and without replicates. In this situation variation due to error is very high. Though use of moving means and/or many standard plots in the trial can be a help, the source of error makes the testing of F_3 lines for yield questionable.

Descriptors: barley, *Hordeum vulgare*, wheat, *Triticum aestivum*, F_3 lines, yield, selection, mini-plots

Genotype x environment interaction

When testing early generations of self-fertilizing crops, plant breeders always deal with unselected segregating lines. Numerous experiments have been conducted to study ways of selection with mini-plots. Many of these experiments, however, had existing varieties for material. Weber (1984) was quite right in warning that data obtained with varieties should not be unconditionally be compared with data derived from working with unselected impure lines. Indeed, varieties have been selected precisely for insignificant genotype × environment interaction and many a variety-to-be has foundered on this very property when the haven was already in sight.

When selecting early generations, we must therefore be aware that they show considerably greater genotype × environment interaction than suggested by experiments with varieties.

Mini-plots

For many crops including wheat and barley, seed yield per plant is low, even though the plants to produce seed for sowing are given a wide spacing.

When with F_2-derived F_3 lines of these crops, field plots are laid out for yield experiments at a sowing density as commonly practised in Western Europe, such field plots can at most be 1.5 m^2 in area. For reasons of field design, the allotted minimum area of a plot will be about 1 m^2, if many lines are to be sown. Field design is however not the only limiting factor. There are more reasons that make it undesirable to use a net area of less than 1 m^2, as will be indicated.

All this implies that it is impossible to lay out replicates when F_2-derived F_3 lines are sown. As a result, the reliability of such a yield plot with F_3 lines will be lowered seriously. In trials with cereals very high variations due to error occur for mini-plots of about 1 m². Even for mini-plots with a standard variety, the coefficient of variation ranged from about 11 to 19%.

In tests, 6-row plots correlated better with real yield than 3-row plots (Sneep, 1981). It would therefore be recommendable to adopt 6-row plots also for mini-plot design, with a greater plant distance within the row and a smaller plant distance between the rows. This will also lower the variation from competition, at least where the neighbouring plots are concerned.

Some breeders (Townley-Smith & Hurd, 1973) separate mini-plots with a plot or a row sown with another crop, for instance border rows of winter wheat between plots of spring wheat. Such has its advantages at the time of harvest. However the variance from competition is not removed, nor would it be removed by leaving one row open between plots. We would then see genotypic differences between plants to occupy the open space. We could speak of differences in ability to compete against space (Spitters, 1979). This phenomenon is also observed at the ends of plots where these are adjacent to paths. Because of crop care, observations during growth and harvesting, the paths cannot be left out.

Selection in a trial field without replications

Because of the small size of mini-plots, variation due to error is already large. This reduces the reliability of the differences in yield. In a yield trial without replication, there is the added difficulty of confounding of yield and, for instance, soil fertility. Is a high-yielding line genotypically a good yielder or did it chance to grow on a fertile spot?

Townley-Smith & Hurd (1973) advocate the application of a moving mean. With such a moving mean, neighbouring plots are included in the evaluation. This way cuts down variance due to error.

Moving means also have their disadvantages:

- There must always be complete randomization, and certain types cannot be sown by side on purpose.
- All plots must be harvested, including those that could already be rejected on visual assessment. The yields of all plots must be measured.

Inclusion of standard plots has the following advantages:

- One has a good and known standard for comparison.
- The trend of soil fertility can be better estimated.
- Not all plots need be harvested.

If several standard varieties are included, it becomes possible to gain insight into genotype × environment interaction. The disadvantage is that a larger trial field is needed.

Genotype x year interaction

For young lines, the genotype × year interaction is often quite large, on average larger than for existing varieties. Before introduction, existing varieties have been tested for

several consecutive years to avoid too large a genotype × year interaction.

About the difficulties that crop up in testing of early generations because of this, some people say: the lines to be selected must perform well in all years. It does not therefore matter much if only the very best are maintained in a given year with non-average conditions.

Such a point of view is not correct, if 90 or even 95% of F_3 lines are culled. In such procedure, some of the discarded lines may not achieve maximum yield in the given year, but produced reasonably well and would have yielded well in coming years.

The correlation between performance of the F_3 mini-plots and performance of F_2-derived F_4 lines (with replications of 7.0 m × 1.5 m) is low: for a generation of wheat, 0.23 and for two trails with barley even negative; in all trials, however, not significant.

Conclusion

Testing early generations for quantitative characteristics has its attractive sides from the viewpoint of population genetics. From a technical point of view, the culling of F_3 lines can be faulted in many ways:

- Most crops do not produce enough seed to set up adequate trails so that mini-plots can rarely be replicated.
- Mini-plots result in a high variation due to error because of the inevitable errors in design, which derive from the impossibility of adapting the size of the plots to the variation in soil fertility and from the variation in competition.
- With the lack of replications in this type of field design, the genotype × environment interaction cannot be estimated.
- Soil fertility can be allowed for to some degree by use of a moving mean or by use of many standard plots.
- Using not just one but several standard varieties can provide some insight into genotype × environment interaction.
- The genotype × year interaction is hard to estimate. Some indications could be provided by the behaviour of standard varieties.

The sources of error mentioned raise the question whether early generations (F_3 lines) of crops, with only a limited amount of seed per plant, can be tested reliably despite the theoretical advantages and prospects.

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Selection in early generations

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Summary

Aspects of early selection in hybrid breeding and in multitrait situations are discussed. In hybrid breeding, heterosis and inbreeding depression play a decisive role in early selection for yield. S_1-family selection has proved recommendable in many situations. If, besides yield, other characters with higher heritability are important, early selection should be performed in successive stages, starting with the traits requiring the smallest effort of evaluation.

Descriptors: early generation selection, hybrid breeding, performance per se, combining ability, multistage testing

Weber (1984) has limited his paper to line breeding in self-fertilized crops and to yield as the only character under selection. The objective of this short contribution is to extend discussion to

- early selection in hybrid breeding of cross-fertilized crops;
- early selection in multitrait situations.

In hybrid breeding, the S_0, S_1, and S_2 generations are generally considered as 'early' generations. The goal of selection in these generations is to enhance the chances of detecting superior inbred lines in later generations (Figure 1). In this respect, there is no difference between line breeding and hybrid breeding. However the genetical and operational situations deviate considerably. In cross-fertilized crops, heterosis and inbreeding depression are far more important than in self-fertilized crops. This dependence of performance on heterozygosity markedly affects the genotypic correlation between the selection units used in early generations and the final inbreds and hybrids in later stages. As can be seen from Table 1, the effectiveness of early selection may increase and decrease from S_0 to S_2, depending on the selection unit and the target criterion. According to theoretical and experimental results, selection based on S_1 performance per se is a good compromise, if both combining ability and line performance per se (inbreeding minimum) are to be improved (Hallauer & Miranda, 1981). Early test-cross selection can be considered superior to S_1-family selection, if combining ability for yield is the main goal. However if the tester is not identical or closely related to the final hybrid partner, test-cross selection may well be inferior, depending on the genetic properties of the breeding population and of the tester (Wilde & Geiger, 1983). For characters revealing less heterosis than yield, inbred-family selection should generally be more

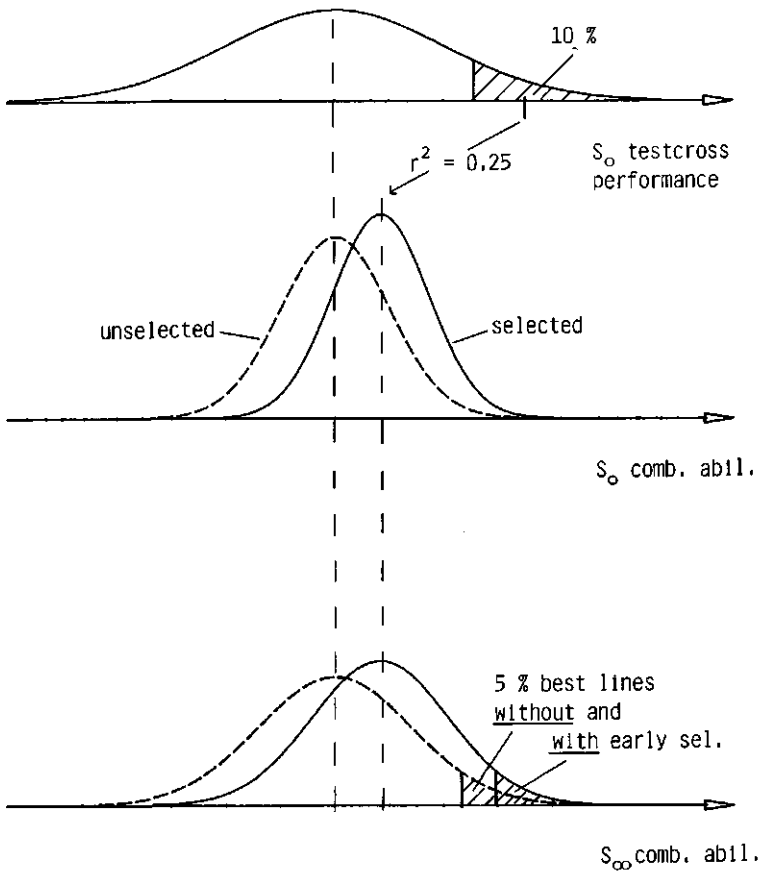

Fig. 1. Effect of early (S_0) test-cross selection on frequency of high-combining inbred lines in S_∞ (r = correlation between the phenotypic values of the test crosses and the combining-ability effects of the candidates).

Table 1. Trends of genotypic correlations between selection unit and target criterion for yield in cross-fertilized crops.

Selection unit in early generation (S_0, S_1 or S_2)	Target criterion in late generations	Genotypic correlation trend $S_0 \rightarrow S_2$
Test cross	combining ability	increase
	line performance per se	no experimental evidence
S_0 plant or inbred family per se	combining ability	decrease
	line performance per se	increase

Table 2. Example of early selection in successive stages.

Number of entries	Evaluation unit	Main selection criterion
100 000	plant	disease resistance
10 000	hill or row	lodging resistance
1 000	plot	yield

efficient than test-cross selection (Klinger, 1984).

In most breeding programs, apart from yield many other characters such as disease resistance, quality traits, lodging resistance, maturity, sterility-maintaining ability and fertility-restoring ability, are important breeding objectives and consequently have to be considered in early stages of selection. Characters with high heritability require less intensive testing and display closer genotypic correlations between early and late generations than those with low heritability. So the higher the heritability of a selection criterion the more effective is early selection. However, selection units and testing intensities optimum for one trait may be far from optimum for another (Utz, 1982). Economic use of breeding facilities is therefore possible, only if selection is performed in successive stages, whereby the optimum for each type of character is taken into account.

Furthermore, multistage selection is dictated by the large number of entries to be handled in early generations. Assume the breeder wants to improve disease resistance, lodging resistance and yield by selecting the best 10% for each character. If the characters are genetically unrelated and the breeder wants to save 100 entries for evaluation in later generations, he has to start with 100000 entries (Table 2). Clearly, testing of such large numbers is most efficiently done in successive stages, starting with the character(s) requiring the least effort for evaluation.

Since the candidates in 'early' generations are usually heterozygous, the breeder may take advantage of the variation arising from segregation within the selected fraction, thereby reducing the number of initial entries needed. On the other hand, the number would increase, if the traits were negatively correlated, if more traits were included or if higher selection intensities were being applied. Thus in multitrait situations, no rational alternative to selection in successive stages exists and early testing for yield can be recommended only in the last stage of the procedure.

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Improvement of harvest index

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Summary

Grain yield of the main cereal crops has doubled during the past 30 years in Europe. The increase in yield has been mainly due to increases in harvest index and, to a lesser extent, to increase in total biomass. By introducing semidwarf varieties or hybrids resistant to diseases, harvest index has reached 45-52% for wheat, barley and oats, and 50-60% for maize. These values represent the maximum now practical but not the biological maximum of the harvest index. Cereal breeders might increase the harvest index from the present value of 50% to about 60%. If there is also an increase in biological yield, a genetic gain in grain yield of around 20-25% could be achieved.

Harvest index can be considered as a promising yield selection criterion, because of the genetic variation in this trait, the moderate sensitivity to environmental conditions (e.g. disease stress, year effect), acceptable heritability and positive correlation with grain yield.

According to recent evidence, harvest index alone fails to be a perfect selection criterion in early segregating generations, because of heterosis, competition and segregation. Increase in biological yield and the maintenance of the maximum harvest index, or the parallel increases in harvest index and growth rate seems to be most promising for selection in early generations if yielding ability cannot be evaluated by precise yield tests.

Descriptors: harvest index, cereals, yield, biological yield, growth rate, biomass, selection

Introduction

The term 'harvest index' was proposed by Donald (1962) for the ratio of grain yield to biological yield. Other definitions like 'migration coefficient' (Baven, 1920) or 'coefficient of effectiveness' (Niciporovic, 1956) are identical in meaning with harvest index, but the later is accepted and used by plant breeders and plant physiologists. Another term closely related to harvest index and used by cereal agronomists is the grain/straw ratio, or, for maize, the grain/stover ratio. It can easily be converted to harvest index or vice versa, but has some drawbacks compared to harvest index. Values like 1 : 1 or 1 : 1.5 are difficult to handle: as an alternative, straw-to-grain ratio is sometimes used, raising confusion. To avoid such confusions, the following terms will be used here:

biological yield × harvest index (HI) = grain yield

Grain yield of the main cereal crops has doubled during the past 30 years in Europe. The increase in yield has been mainly due to increase in harvest index and to a lesser

extent to increase in biological yield. A number of studies indicate this. Van Dobben (1962), comparing old wheat varieties arising since the turn of the century with the leading wheat varieties of the 1960s, found a constant increase in harvest index from 34 to 40%.

A similar trend was found by Vogel et al. (1963). They found the new semidwarf wheat varieties to be superior in grain-to-straw ratio over the taller ones, with a range in harvest index from 32 to 38%. Fischer & Kertész (1976), examining spring wheat, durum wheat and triticale varieties at CIMMYT, found that harvest index ranged from 32.9 to 48.8% when the grain yield varied from 4.85 to 7.64 t/ha, and the new varieties had the highest harvest indices and highest grain yield.

Harvest index of old Hungarian wheats ranged between varieties from 29 to 33% (Papp, 1960). Twenty years later, Balla & Szunics (1978) found that the best varieties attained a harvest index of 39 to 40%.

The most striking change can be seen in maize, whose harvest index changed from 24% in 1950 to 43% in 1970, while the yield increased from 3 t/ha to 8 t/ha (Zelitch, 1975).

Donald & Hamblin (1976) gave a detailed survey of harvest index, analysing the relationships between biological yield, grain yield and harvest index, the response of HI to nitrogen dressing, to irrigation, spacing or competition. Finally they discussed how HI can be used as a selection criterion. I agree with their main conclusions. Until we can understand the detailed physiological and biochemical aspects of grain production, plant breeders must use biological yield and harvest index as two simple but valuable criteria for the assessment of the performance of cereals. Let us see what advances have been made during the past 10 years in this field.

Results and discussion

Variation in harvest index The main question is whether HI can be used during selection process and what its significance is for efficient production of varieties.

Harvest index varies markedly between varieties of particular cereal crops (Table 1). The maximum values under commercial production is for wheat 51%, for barley and oats 52%, for rye 29%, for triticale 50% and for maize 60%.

A general conclusion can be drawn that HI of recent varieties reaches 40% in almost all the crops mentioned. The possible ceiling is seen from the final values. The highest feasible HI for maize would be around 60%, but this is also possible for wheat.

According to Dubinin (1972), the 2:1 grain-to-straw ratio (HI 66%) is not a biological plateau. Lukjanenko (1966) also said that a HI of 48-50% was a realistic aim. Balla & Szunics (1978), on the basis of a series of experiments, confirmed Lukjanenko's statement. Austin et al. (1980) are even more optimistic in saying that breeders may be able to increase harvest index from the present value of 50 to about 60%, achieving a genetic gain in yield of some 25%.

My experiments (Table 2) on a series of widely grown European wheat varieties confirm the view of Austin et al. (1980), Lupton (1982) and others. Yield increases of modern varieties are mostly due to increases in HI and only to a lesser extent to increases in biological yield. With one exception (Mironovskaja 808) the high-yielding varieties e.g. Sadovo S, Ogosta, Novosadska Rana 1 have high HI. The varieties can be grouped

Table 1. Variation in harvest index of cereal crops.

Crop	Harvest index (%)		Reference
	average	range	
Wheat	37	34–40	Van Dobben (1962)
	39	28–46	Singh & Stoskopf (1971)
	42	31–50	Syme (1972)
	41	33–48	Fischer & Kertész (1976)
	36	28–40	Balla & Szunics (1978)
	38	33–43	Kertész (1981)
	44	34–51	Austin et al. (1980)
Barley winter	45	44–47	Singh & Stoskopf (1971)
spring	48	35–52	Singh & Stoskopf (1971)
spring	43	33–49	Riggs et al. (1981)
Oats	41	31–52	Singh & Stoskopf (1971)
	38	31–44	Takeda & Frey (1976)
Rye	27	27–29	Singh & Stoskopf (1971)
Maize open-pollinated	24		
hybrids	42	38–47	Hanway & Russel (1969)
	48	47–60	Bálint (1983)
Triticale	41	40–42	Fischer & Kertész (1976)
		10–34	Sapra & Hughes (1977)
	40	30–50	Kiss (1983) pers. commun.

Table 2. Biological yield, grain yield and harvest index of some wheat varieties and lines grown in Eastern Europe. Szeged, 1977–1979, 3-year average.

Cultivar	Biological yield (g/m^2)	Grain yield (g/m^2)	Harvest index (%)
Sadovo S	1877	756	40.3
Jubilejnaja 50	1865	716	38.4
GKF 2	1695	700	41.3
Ogosta	1677	696	41.5
Mironovskaja 808	1886	662	35.1
Lutescens 38.72	1697	655	38.6
Novosadska Rana 1	1595	646	40.5
Mv 4	1698	642	37.8
Sava	1621	632	39.0
Rubin	1579	622	39.4
Mv 1	1644	615	37.4
Bezostaja 1	1606	607	37.8
Jacometti	1482	581	39.2
Grana	1641	581	35.4
Partizanka	1511	568	37.6
D1	1450	567	39.1
Kavkaz	1572	536	34.1
Rannaja 47	1401	531	37.5
F 26.70	1490	514	34.5
GKT 8001	1401	514	36.7
Mutant 48	1552	506	32.6
Rannaja 12	1398	495	35.4
Average	1611	605	37.6
LSD 5 %	128	35	2.1

by HI and yield (Figure 1).

Tall varieties represent high biological yield and low harvest index, whereas semidwarf ones show an increasing HI with a high level of biological yield. The extremes are some of the full dwarfs with a low biological yield. They cannot produce a high grain yield, even if HI is high. These results can be summarized by one of the models constructed by Donald & Hamblin (1976) (Figure 2), according to which the grain yield increases proportionally with HI as long as maximum biological yield is maintained.

The HI of wheat hybrids (F_1s) can be even higher. In a detailed study, 5 female and 5 male parents were mated factorially. The hybrids and their parents were examined at high and low plant densities over three successive seasons. Moderate heterosis was found in harvest index (Table 3). The same result was achieved in another diallele experiment in 1981. Some hybrids gave high heterosis in both grain yield and HI. So parents which behave in this way should be used in cross breeding as they are valuable.

Harvest index and plant density As plant density increases, HI decreases (Donald & Hamblin, 1976; Fischer & Kertész, 1976; Borojević, 1978). In a number of experiments over several years the HI was measured in high plant density (400 m^{-2}) and low density

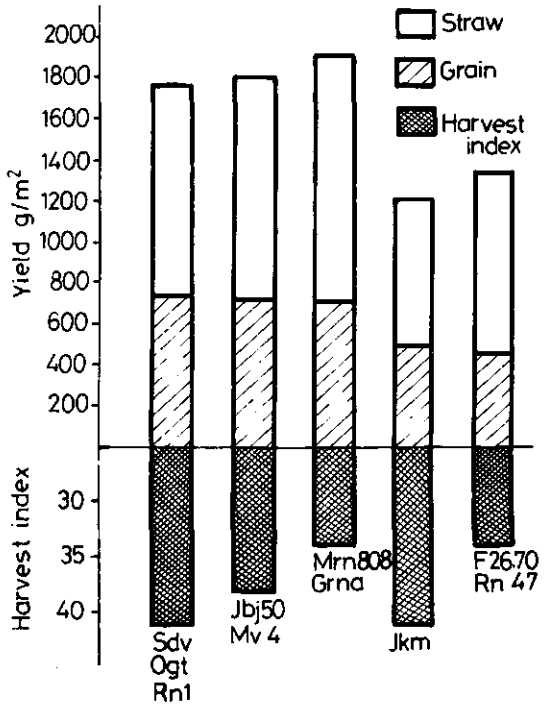

Fig. 1. Changes in biological yield, grain yield and harvest index.

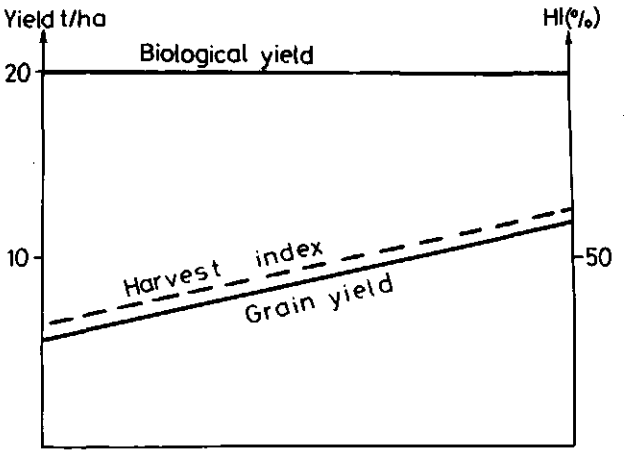

Fig. 2. Model of relationships between biological yield, grain yield and harvest index (after Donald & Hamblin, 1976).

Table 3. Harvest index of wheat lines and hybrids. Szeged, 1977–1979, 3-year average.

Entries		Harvest index (%)
Exp '77–'79		
Parental lines	$n = 10$	38.2
F_1 hybrids	$n = 25$	40.0
LSD 5%		0.7
Exp '82		
Parental lines	$n = 10$	46.6[1]
F_1 hybrids	$n = 45$	48.0[1]
LSD 5%		0.6

1. One-year average.

(20 m^{-2}). The HI of varieties and hybrids was found to be significantly higher in spaced plantings (Table 4).

Borojević (1978) examined five contrasting wheat varieties and found that as the head density increased from 300 m^{-2} to 1000 m^{-2} the HI decreased from 41 to 35%.

In maize, the tendency was the same with a few exceptions (Figure 3). In an experiment of Menyhért & Ángyán (1969), cited by Bálint (1983), some of the hybrids maintained their HI with an increase in density and gave a corresponding increase in grain yield. So in these hybrids, the yield can be further raised by increasing crop density. This can be attributed to breeding. These results fit in with a statement by Hanway & Russel (1969).

Relationships between harvest index and yield Studies on homozygous varieties showed a correlation between HI and grain yield (Table 5). The strong correlations for cereals imply the significance of the increase in harvest index in producing high-yielding cereals. Experience of breeding and these correlations suggest that by increasing HI we can select higher-yielding lines. But during the selection process, especially in F_2 and F_3 generations, this relationship is confounded by segregation, spacing and the changing environmental conditions from year to year. Moreover we need to consider the heritability of the HI.

Table 4. Harvest index of wheats for different plant densities. Szeged, 1977–1979, 3-year average.

	Harvest index (%) for density		
	400 m^{-2}		20 m^{-2}
Parents ($n = 10$)	38.2		46.8
F_1 hybrids ($n = 25$)	40.0		47.2
LSD 5%		2.5	
Varieties ($n = 21$)	38.0		46.0
LSD 5%		3.2	

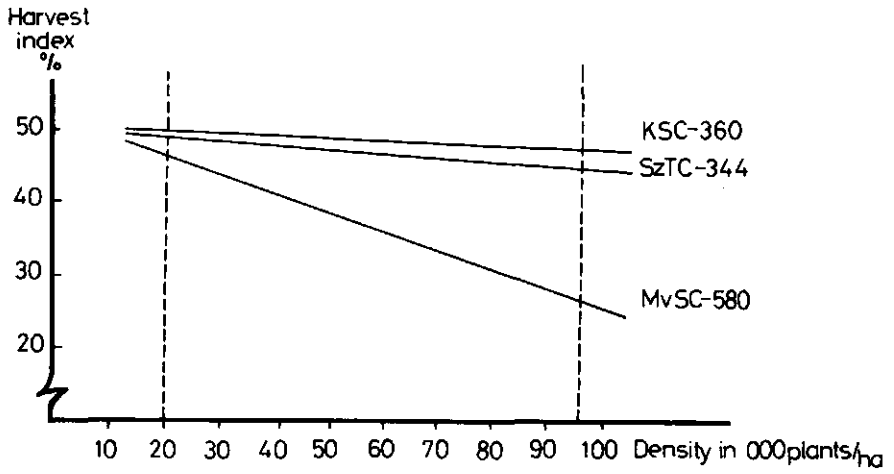

Fig. 3. Harvest indices of different maize hybrids in several plant densities (drawn from data of Menyhért & Ángyán, in Bálint (1983)).

Harvest index and environmental conditions Results of earlier studies suggested that harvest index decreased with increasing nitrogen dressing (Donald & Hamblin, 1976) or with increasing plant density (Fischer & Kertész, 1976; Borojević, 1978; Kertész, 1981; Baker, 1981) as long as soil, water and other conditions were adequate. Biological yield would then increase almost proportionally with grain yield, unless other factors were deficient. This depression in grain yield and harvest index can be severe in severe disease epidemics. Barabás & Matuz (1983) examined yield loss of wheat after heavy leaf rust infection and found that the susceptible varieties reacted with a yield loss of 21.8% though the grain yield of resistant ones decreased by only 7.5% relative to the plots in which the disease was controlled with fungicides. In the same experiment, I found that

Table 5. Phenotypic correlations (r) between harvest index and grain yield in some cereal crops.

Crop	r	Reference
Wheat	0.65	Thorne et al. (1969)
	0.62	Singh & Stoskopf (1971)
	0.85	Syme (1972)
	0.75	Nass (1973)
	0.71	Bhatia (1975)
	0.65	Fischer & Kertész (1976)
	0.55–0.85	Balla & Szunics (1978)
Barley	0.89	Hamblin (1971)
	0.66	Singh & Stoskopf (1971)
Oats	0.50	Singh & Stoskopf (1971)
	0.47–0.71	Takeda & Frey (1976)

Table 6. Grain yield and harvest index of wheat with leaf rust and controlled with fungicides. Szeged, 1982.

	Resistant varieties			Susceptible varieties		
	controlled with fungicide	uncontrolled	difference (%)	controlled with fungicide	uncontrolled	difference (%)
Grain yield (t/ha)	7.60	7.07	7.5	7.54	6.19	21.8
Harvest index (%)	37.9	38.1	0.1	34.9	31.9	9.4

only susceptible varieties had an associated decrease in harvest index (Table 6). So harvest index is less sensitive to disease stress than the grain yield itself.

Comeau & Barnett (1979) and Cooper & Sorrels (1983) used harvest index to measure barley yellow dwarf virus (BYDV) resistance of oats on the assumption that infection in susceptible lines would be reflected in a decreased harvest index. They found that tolerance index (HI infected/HI uninfected) ranged between 91-98% and 45-73% for resistant and susceptible varieties, respectively.

These data confirm the view that harvest index is under genetic control and its heritability should be higher than that of grain yield or biological yield (Bhatt, 1976; Fischer & Kertész, 1976; Thakral et al., 1979).

Inheritance of harvest index Few detailed studies have appeared on the heritability of harvest index, though Singh & Stoskopf (1971) found that some wheat lines had consistently high or low harvest index over successive seasons, indicating the genetic control of this trait. Rosielle & Frey (1975) studying the performance of oat selections estimated h^2 values of 0.59, 0.61 and 0.64 for biological yield, grain yield and harvest index, respectively. Bhatt (1976) examined F_1 and F_2 wheat populations and found that HI was controlled primarily by additive gene actions, which agreed with similar results of Rosielle & Frey (1977). Some data on h^2 of biological yield, grain yield and harvest index are given in Table 7. Moderate to high heritability estimates were obtained in each study. It is worth noting that high estimates of heritability accompanied by high estimates of

Table 7. Heritability (h^2) of biological yield, grain yield and harvest index.

h^2 (%)			References
biological yield	grain yield	harvest index	
59	61	64	Rosielle & Frey (1975)
–	–	70	Bhatt (1976)
92	45	87	Thakral et al. (1979)
–	48	60	Austin et al. (1980)
40	56	42	Srivastava et al. (1981)

Table 8. Components of variance (mean square values) for different traits at high and low plant densities. Szeged, 1977–1979. GCA and SCA = General and Specific Combining Ability, respectively.

Trait	High density			Low density		
	GCA ♀	GCA ♂	SCA	GCA ♀	GCA ♂	SCA
Biological yield	1323.4***	4663.7***	487.4^{ns}	196.4***	399.4***	108.2*
Grain yield	297.7**	1094.5***	108.8^{ns}	30.1*	180.0***	32.7*
Harvest index	10.4**	29.0***	5.4^{ns}	6.5**	45.8***	6.4**

*, ** and ***, significant at 5, 1 and 0.1 % probability levels, respectively.
ns, not significant.

genetic coefficient of variation would result in high genetic advance in harvest index.

My results support these conclusions in some respects. A detailed study on almost 100 F_1 and F_2 populations over three years show that gene effects controlling harvest index were mostly additive (Table 8) and the ratio between variances of General Combining Ability (GCA) and Specific Combining Ability (SCA) was found to be high and SCA variances were not significant at high seed rate. Studies should apparently be conducted at plant density in line with commercial practice.

The studies cited indicate that harvest index has a predictive value in selecting parents with complementary physiological habits and favours yield improvement by exerting selection pressure for harvest index in early generations.

Harvest index as a selection criterion for yield Measurements on spaced plants that reliably predict a genotype's yielding ability at commercial crop densities would be useful, especially in breeding programs aimed at raising yield, in which early generations are space-planted and then subjected to heavy selection. The main problem for the plant breeder is to predict yielding ability from one plant arrangement to another, from isolated plants to field plot, from segregating population to pure stand, or from micro-plot to field plot. Rosielle & Frey (1975), in an extensive study on oats, found that indirect selection through the harvest index was only 43 % as efficient as direct selection for yield. Anyway selection by HI helped in selecting early and dwarf forms, and 'lines selected on the basis of HI may be agronomically superior to those selected for grain yield'. When undesirable traits were held constant in unrestricted selection, selection for grain yield was only 57% efficient. If HI was added as a secondary selection trait, efficiency rose to 70%. Nass (1980) used HI as a selection criterion for grain yield in F_2 populations grown at two densities. The F_4 lines selected in F_2 for high HI yielded about 63 g more grain per plot in 1978 than F_4 lines having low HI in F_2. Another conclusion was that selection for high HI at high density was more promising in achieving superior F_4 lines. This opinion contradicts that of Donald & Hamblin (1976), in that HI measured in a spaced planting of F_2 had a higher value for predicting yielding ability.

Whan et al. (1981) studied the effect of direct and indirect selection from F_2 to F_5 on wheat. In certain combinations, direct selection for yield proved effective. Selection for harvest index in early generations in order to improve yield was ineffective when the response was measured at the same site, either in the same year or in different years.

Table 9. Effect of direct and indirect selection in F_2 on yield of F_3 lines in dense stand in two crosses.

Selection criterion in F_2	Grain yield per plot in F_3 (g)	
	Ogosta × GK2	Ogosta × Partizanka
Biological yield per plant	790.0	680.0
Grain yield per plant	772.6	679.6
Number of heads per plant	796.7	686.1
Number of grains/head	708.1	686.8
1000-grain mass	737.0	730.7
Harvest index	697.0	668.6
Number of grains per plant	801.9	681.8
Grain yield per head	738.2	697.1
Plant height	691.1	731.1
Mean of the F_3 lines ($n = 300$)	700.5	700.0
Mean of the best 10% of F_3 lines	898.6	835.4

As regards this key point I have been conducting a series of selection experiments during the past 10 years. In five F_2 populations (3000 plants each), the best 10% was selected on the basis of nine characteristics related to yield. From the selection differentials, we would expect a significant advance in each trait if the inheritance of the traits was good.

After measuring the offspring's yield in stand we found in a standard block experiment that neither indirect selection for different traits (including HI) nor direct selection was effective (Table 9). In the cross Ogosta × GK 2, the mean yield of the F_3 lines was 700.5 g/plot. Positive response of about 10% was achieved when grain yield, biological yield or head number were the selection criteria.

Selection for number of grains per plant gave the best response of 14%. Selection for other traits gave no prediction of yield in the successive generation. The low efficiency of this type of selection can be shown by our failure to select the best 10% of the F_3 lines. Their average yield was 898.6 g/plot, 28.3% better than the average for the 300 F_3 lines.

Results of this type of experiment suggest that selection is dubious without detailed knowledge of the interrelationships between the traits. So we started to look for a general

Table 10. Effect of 'index selection' through principal-component values (PC value) of F_3 lines on yield of F_4 progeny of wheat relative to standard varieties. Szeged, 1979–1980.

	F_3 generation		F_4 generation
	rel. grain yield (%)	PC value	rel. grain yield (%)
Best 5%	126.0 ± 3.20	3.600 ± 0.22	127.0 ± 2.70
Best 10%	122.0 ± 2.20	2.387 ± 0.29	125.0 ± 2.16
Worst 10%	72.5 ± 1.82	−1.873 ± 0.29	104.8 ± 4.10
Worst 5%	71.0 ± 0.22	−2.980 ± 0.34	100.0 ± 3.70

law existing among the F_2 spaced versus F_3 stand generations by principal-component analysis (PCA). Theoretically PCA helps to clarify the interrelationships between traits and groups the traits according to their contribution to the 1st or the 2nd principal component. After calculating PC values for each plant, these values will serve as selection criteria in F_2.

Results showed that even PC values were not sufficiently effective selection criteria (Kertész et al., 1980b), though if selection with PC values started in F_3 they were effective (Table 10). Selecting the best 5% on the basis of PC values in F_3 resulted in a yield 27% higher in F_4 than the best control varieties (Kertész et al., 1980a).

Improved harvest index represents increased physiological capacity to mobilize and translocate photosynthetic products to organs of commercial value. For selection, not only harvest index but also a number of physiological and quantitative characteristics should be considered as criteria, especially in early generation selection.

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The effect of plant interaction on crop harvest index

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Summary

The problem of extrapolating from single plant harvest index (HI) to crop HI is discussed with regard to the effect of plant interactions within the crop. Data are presented for crop and population responses of two dried pea genotypes which differ for biological and seed yield and crop HI. The difference in crop HI for dried peas is shown to be almost solely due to the composition of the populations and not to genetic differences in HI potential. Suggestions are made that changes in the plant populations may be also important in cereals and that recognition of this importance should be reflected in changes in selection criteria within breeding programmes.

Descriptors: harvest index, population responses, dried pea crop, *Pisum sativum*, selection criteria

Kertész (1984) has identified in his lecture what we consider to be the main problem when using a concept such as harvest index for improving crop yields. This is the prediction of yielding ability from isolated plants to field crops, and it is this point which we would like to expand, using some of our own data on the dried pea crop as an example.

Breeding new varieties can be considered as two interconnected processes. Firstly there is the initial selection, which is usually made from large numbers of genetically unique single plant segregants. After several selfed generations and some degree of multiplication there is the assessment phase, when the performance of a new genotype is determined in a simulated crop environment. This assessment of a genotypes performance, however, invariably occurs without knowledge of any changes in the composition of the plant population, due to plant interactions which have occurred during the development of the crop. It is this lack of information on the relative level of interaction between individuals which makes it almost impossible to extrapolate from HI measurements made on the crop to the potential of individual segregants. It is, for example, possible to obtain a low crop HI because a genotype is inherently poor at partitioning assimilate into seed, or, because the interactions between individuals within the crop have induced poor partitioning within a proportion of the plants within the population.

It is very important to define for the breeder the extent to which low crop HI is due to a shift in the population compared with a genuine difference in the genetic potential for partitioning, since his selection criteria will change accordingly. If there is only a small effect due to interaction between individuals, then a high HI will become manifest in the

crop, following selection for high HI in the plant. If there is a high level of plant interaction then little or no correlation will exist between selections and assessment. Such a high level of interaction will demand a change in selection criteria towards plants which have characteristics which will make them less antagonistic towards neighbours when grown as a crop and as such better crop plant models. We are beginning to determine such characteristics for the dried pea crop, and from these studies we will formulate crop plant 'ideotypes' for use in dried pea breeding programmes (Hedley & Ambrose, 1981).

As part of our studies for improving the dried pea crop we have assessed the relative importance of genetic differences in plant HI compared with the effect of plant interaction on the crop HI. We have compared pea genotypes which were known to differ widely for crop yields and HI, using precision sown replicated microplots over a range of densities. In addition to measurements of crop yield and HI, the resulting plant populations were also analysed to determine the biological and seed yield of individuals within each population. An example of some of these data for a relatively high and a low yielding pea genotype is presented in Figure 1a-d. In this example the high yielding genotype (BS.4) shows a relatively stable biological and seed yield and HI at a normal (100 plants/m^2) and high (277 plants/m^2) planting density. The low yielding genotype (BS.151) shows a more extreme reduction in these three parameters over the two planting densities (Fig. 1a-b). The crop harvest index of BS.4 exceeds that of BS.151 at both planting densities by approximately 20%. Virtually all of the difference in harvest index between these two genotypes and within each genotype between densities can be accounted for in differences in the population structures (Fig. 1c-d). At both planting densities most BS.4 plants achieved HI of 40-70% with only small proportions of barren plants at the high density. The individuals in the BS.151 populations, however, were distributed over the whole range of plant HI with a high proportion of barren plants at both densities. The upper limit, for plant HI, however, was similar for both genotypes at both densities (65-70%) and this represents the plant potential for HI.

From such experiments we have found that by far the greatest factor affecting yield and harvest index is the structure of the population. Genotypic differences in the potential for partitioning assimilate were found to be minimal. Likewise, the effects of planting density on crop harvest index in peas are due solely to shifts within the plant population. In high population densities this shift often results in an increase in the number of barren plants in the population.

Although the population responses which we have observed for peas may not be so extreme between cereal cultivars, they may nevertheless explain the poor correlations observed between the HI of selected individuals and the HI derived from corresponding cereal crops. Genotypic differences in the level of plant interactions in crops may also be a major factor determining differences in HI between the old and new wheat varieties. The main advantage of the new varieties could be a reduction in the mutual antagonism between individuals in the crop rather than any great improvement in potential HI.

Our studies with the dried pea crop have led us to consider that the susceptibility of a genotype to develop a high variance for HI within a population is a genetically determined character in its own right. If this is true then it should be possible to select against such a character using suitable selection environments, or, by association of a predisposition to high variance with specific morphological characters. To make the selection of

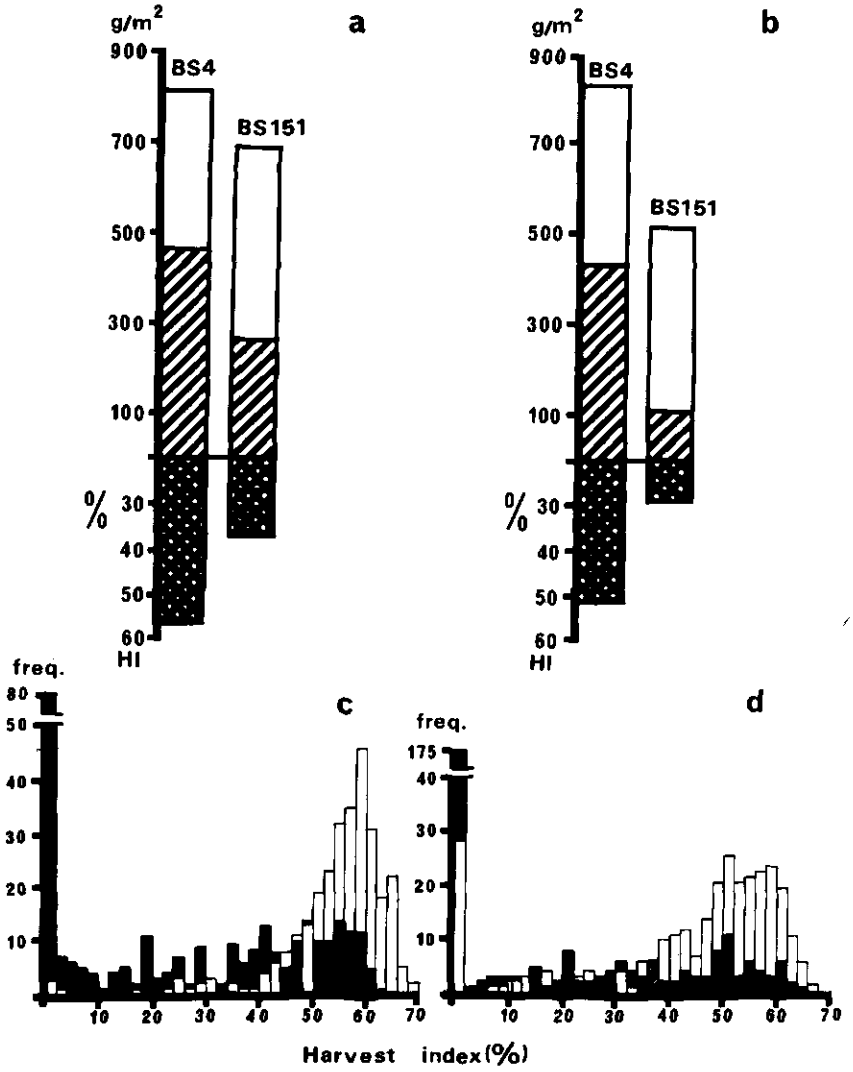

Fig. 1. Changes in biological yield, seed yield and harvest index of two dried pea genotypes (BS.4 and BS.151) grown at two planting densities.
1a: crop responses at 100 plants/m²
1b: crop responses at 277 plants/m²
Biological Yield = □ Seed Yield = ▨ Harvest Yield = ▩
1c: plant frequency distribution for HI at 100 plants/m²
1d: plant frequency distribution for HI at 277 plants/m²
□ = BS.4 ■ = BS.151

such individuals easier it would be advantageous to delay selection until the performance of segregants can be assessed in a plot composed of like individuals. This could be achieved by using some form of single seed descent breeding system where the selection phase is delayed until after a preliminary microplot assessment has been made.

We would conclude from our studies that without information on the behaviour of plants within crops harvest index will be of limited use as a selection criterion in segregating populations.

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Harvest index and grain yield as selection criteria in plant selection

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Summary

In two plant-selection trials with spring wheat cultivars, harvest index (HI) and weight of grain per plant were compared for their effectiveness in improvement of yield in monoculture. The plant density in the cultivar mixtures ranged from 6.25 to 400 m^{-2} (5×5 to 40×40 cm^2/plant). Heritability for HI was higher than for grain yield per plant, with two exceptions. In Trial 1, the coefficient of genetic correlation r_g between HI and monoculture yield was higher than r_g between grain yield per plant and monoculture yield. In Trial 2, the opposite was found. In the absence of differences for biomass yield in monoculture, HI proved a good selection criterion. A drawback was the time required for each measurement. This prevents a high selection intensity and counters its advantage over grain yield per plant as selection criterion.

Descriptors: harvest index, yield, selection, indirect selection, wheat, *Triticum aestivum*, heritability

Introduction

In his paper on the improvement of harvest index (HI), Kertész (1984) has raised two major issues that I would like to comment on. They are the value of HI as a selection criterion in selection for grain yield in monoculture under normal farming conditions (monoculture yield) and the influence of plant density on HI and on the selection response for monoculture yield. Kertész's treatment of the subject matter was empirical. I will consider the parameters which determine the selection response from a more theoretical approach.

The paper deals with plant selection in spring wheat. The response was determined for yield in monoculture. The value of plant HI will be compared with that of grain yield per plant. Both HI and grain yield are considered as auxiliary traits in selection for monoculture yield.

Analysis

The target trait is grain yield in monoculture. If the auxiliary trait (HI or weight of grain per plant) is correlated with the target trait, a correlated response (CR) for the target is expected. It can be shown that $CR = i_a h_a r_g \sqrt{\text{var } g_t}$ (Falconer, 1960, p. 318; Spitters, 1979, p. 61). The subscripts a and t refer to auxiliary and target traits. The

relative effectiveness (RE) of HI in relation to grain yield per plant for the response in the target trait is equal to the ratio of the correlated responses. Thus,

$$RE = \frac{h_{a,HI}}{h_{a,y}} \frac{r_{g,HI}}{r_{g,y}}$$

if the selection intensity i is set equal for the two traits. The subscripts HI and y (= weight of grain per plant) indicate the auxiliary traits under consideration. The procedure to estimate r_g was described by Kramer et al. (1982). Knowledge of the order of magnitude of these parameters and of their response to change in plant density may permit prediction of the value of the two auxiliary traits for improvement of the target trait, yield in monoculture.

The parameters h_a and r_g were estimated in two trials, with two sets of cultivars or breeding lines planted in mixture at different densities and in monoculture. Details of these trials will be reported in Kramer (1984).

Results and discussion

The results are given in Table 1, derived statistical quantities per plant in Tables 2 and 3. Harvest index decreased with plant density (Table 1). It remained almost constant as long as the mixture yield did not decline with decreasing plant density. This was the case at 5×5, 10×10 and 15×15 cm²/plant (Kramer, 1984). The coefficient of environmental variation CV_e for HI was highest at the high density (Table 2). This is mostly due to the

Table 1. Average harvest index of 15 wheat cultivars at various plant densities in Trials 1 and 2.

Cultivar	Plant density (cm²/plant)						
	Trial 1					Trial 2	
	5×5	10×10	15×15	20×20	40×40	6×6	40×40
Adonis	0.457	0.441	0.452	0.426	0.406	–	–
Arkas	0.446	0.434	0.434	0.418	0.392	–	–
Bastion	0.413	0.406	0.416	0.404	0.396	–	–
Ceb. 7857	0.409	0.400	0.425	0.412	0.395	–	–
Ceb. 7958	0.439	0.430	0.446	0.434	0.386	–	–
Gaby	0.439	0.417	0.429	0.414	0.370	–	–
Melchior	–	–	–	–	–	0.478	0.403
Minaret	0.446	0.455	0.460	0.453	0.440	0.520	0.502
Pitic	–	–	–	–	–	0.467	0.451
Ralle	0.417	0.407	0.409	0.397	0.372	0.481	0.394
Selpek	0.397	0.395	0.405	0.367	0.353	0.456	0.416
Sicco	0.414	0.414	0.424	0.400	0.393	0.491	0.460
TK 1937	0.401	0.411	0.433	0.421	0.427	0.494	0.507
TK 2832	–	–	–	–	–	0.534	0.498
TK 6126	0.397	0.406	0.423	0.408	0.414	0.491	0.465
Mean	0.422	0.418	0.429	0.412	0.394	0.489	0.456

Table 2. Estimated coefficients of environmental variation (CV_e) and genetic variation (CV_g) for HI at several plant densities in Trials 1 and 2. n, number of plants sampled.

Estimated quantity	Plant density (cm^2/plant)						
	Trial 1					Trial 2	
	5×5	10×10	15×15	20×20	40×40	6×6	40×40
CV_e	0.161	0.114	0.066	0.085	0.093	0.156	0.124
CV_g	0.052	0.042	0.038	0.051	0.060	0.046	0.084
n	48	48	48	48	48	96	36

small sample for measuring HI (average grain yield/plant 0.89 g at 5×5 cm^2 against 32.5 g at 40×40 cm^2). In Trial 1, CV_e was lowest at 15×15 cm^2. At wider spacings, the additional trial area required may slightly increase CV_e.

The coefficient of genetic variation CV_g for HI tended to increase slightly with area per plant. In Trial 2, this tendency was strongest. In other words, genetic differences for HI were greater at low plant density. This may relate to the absence of a decline in HI at low density of some cultivars (e.g. Minaret, Pitic and TK 1937 in Table 1). As a consequence of the changes in CV_e and CV_g, the heritability for HI in mixture was highest at low plant density (Table 3). Therefore, the recognition of genotypic values on the basis of phenotypic values is better at low density. For comparison the square root of the heritability for grain yield per plant in the same experiment at all densities is also presented in this Table. These quantities have been taken from Kramer (1984).

In Trial 1, the coefficient of genetic correlation r_g between HI and monoculture yield increased with a widening of the plant spacing. From Trial 2, no conclusion could be drawn, both coefficients were too low. The reason for this difference must be sought in the choice of cultivars. In Trial 1, all cultivars were selected for uniformity in growth duration, resulting in small and insignificant differences for biomass yield in monoculture ($CV_{g,mono}$=0.028). A good correlation between HI and monoculture yield under these conditions would be expected (Donald & Hamblin, 1976). However, in Trial 2,

Table 3. Estimates of the square root of the heritability of the auxiliary traits HI ($h_{a,HI}$) and grain yield per plant ($h_{a,y}$) and of the coefficients of genetic correlation (r_g) of these traits with grain yield in monoculture. (The monoculture yields will be reported in Kramer, 1984). RE refers to relative effectiveness of HI in relation to grain yield per plant.

Estimated quantity	Plant density (cm^2/plant)						
	Trial 1					Trial 2	
	5×5	10×10	15×15	20×20	40×40	6×6	40×40
$h_{a,HI}$	0.30	0.35	0.50	0.51	0.54	0.29	0.56
$h_{a,y}$	0.35	0.29	0.33	0.28	0.37	0.45	0.52
$r_{g,HI}$	0.19	0.40	0.61	0.59	0.58	0.17	-0.25
$r_{g,y}$	0.10	0.40	0.04	0.64	0.33	0.84	0.74
RE	1.14	1.21	23.1	1.68	2.56	0.13	-0.36

cultivars were chosen on the basis of large differences in growth duration. Unfortunately biomass yield in monoculture was not measured, but with differences in growth duration a low correlation between HI and grain yield in monoculture would be expected. Again, for comparison, r_g between grain yield per plant and monoculture yield are presented in Table 3.

Similar results were obtained by Bos (1981, p. 118) in rye. He found a heritability of 0.10 for grain yield per plant and of 0.46 for HI. In addition, the coefficient of phenotypic correlation r_p between HI of the parent and yield of the offspring was higher than r_p between the parental and offspring yields (0.42 and 0.28 respectively). The plant density used by Bos was in between 10×10 and 15×15 cm^2/plant.

In conclusion, HI was a better selection criterion than grain yield per plant, in the absence of differences in biomass yield in monoculture, especially at a low plant density. However, HI has a major drawback: it is more time-consuming to measure than grain yield per plant. If a certain number of plant progenies can be accommodated in a breeding program, the intensity of selection i for grain yield can be set higher than for HI, counterbalancing the advantages of the HI in selection. When nothing can be presumed about yield differences in biomass in monoculture in the breeding material, use of HI is risky.

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Stability of the harvest index

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Summary

After presenting the position of the harvest index among the subtraits of yield that are easily measurable by the breeder in his effort to select high-yielding types, a survey is given of the changed situation concerning grain : straw relationships of old and new winter wheat varieties. With a simple graphical method, the optimum values for harvest index are indicated and the stability of this index in different environments is demonstrated.

Descriptors: harvest index, yield, heritability, correlation stability, environments

From the paper of Kertész (1984), it is obvious that harvest index (mass fraction of grain in grain and straw) varies significantly between varieties in cereal crops. Besides that, the increase in economic yield of cereals is mainly but not exclusively due to increased harvest index. In present breeding strategies, segregating material is rarely selected consciously for a higher harvest index, but is still consciously selected for shorter stems – within a given range – and simultaneously for higher grain yield. This concept leads in practice to a higher harvest index.

The improvement of harvest index from about 0.35 to 0.50 without deliberate selection indicates that, from a physiological point of view, it has been a much easier route than increasing biomass, but it will find limits in the near future. These limits will be due not only to physiology but also to decreasing correlation between harvest index and yield performance. This decreased correlation will directly influence the selection efficiency of this character in the search for high-yielding genotypes.

Among the theoretically possible combinations of the degrees of heritability (h^2) and of correlation to yield (r) of plant characters (Hänsel, 1975), harvest index seems to be exceptional, often having a medium heritability and a high correlation to yield. In the search for easily measurable subtraits of yield, the plant breeder has to deal with the following combinations:

h^2	r
high	high
high	low
low	high
low	low

Whereas the first combination occurs only exceptionally and the last one is of no value for the breeder, he has to work with characters with either high h^2 and low r, such as ripening time or ear weight, or a low h^2 and high r, as in certain crosses when using harvest index as subtrait.

From the extensive survey of the literature in the field of operational value of the harvest index (Kertész, 1984), one gets the impression that harvest index often has the ideal combination for selection of high-yielding lines because of its high heritability plus its often observed high phenotypic correlation with yield, particularly in cereals. This relationship, determined in harvest index studies of homozygous varieties, can be rather different in early generations of breeding material, influenced by segregation, spacing and changing environmental conditions from year to year.

Recent investigations (Brooking & Kirby, 1981) have shown that the capacity of a variety to give a high harvest index is determined at an early stage in ear development, as evident in a high mass ratio of developing ear to stem. The trend towards earlier flowering and lower biomass at anthesis by using dwarfing genes changed the growth pattern of modern varieties remarkably and widened the interactions of breeding material under different environmental conditions. So the strong impact of high harvest index on the remarkably improved yield was not accompanied by an equally improved stability of this character.

In two trials at Cambridge, England, Austin (1980) compared the grain-to-straw ratio of old varieties with new ones after different rates of nitrogen dressing, viz. 28 and 104 kg ha^{-1}. The results (Fig. 1) show that the new varieties outyielded Little Joss, the oldest variety tested by almost 40% with either low or high nitrogen dressing. The newer high-yielding varieties were shorter, so increased grain yield was, on average, associated with a striking decrease in straw yield. So far, this trial fits in reasonably well with those of similar trials elsewhere.

Looking carefully at the grain : straw ratio of these ten diverse winter wheat varieties at different dressing rates, one can see that these rather different genotypes can be separated according to stability of their harvest index. By drawing lines through the observed yielding performance of each variety with low or high rate of nitrogen dressing, one can detect a change of the ratio between grain and straw within some varieties (Type I and III). In other varieties (Type II), this ratio did not change substantially despite remarkable differences in yields of grain and straw (Svab & Ruckenbauer, 1982).

To Type I belonged the old and tall varieties Little Joss and Holdfast, which are characterized by a low biological yield and a rather low harvest index. These varieties changed with a higher nutrient supply their grain : straw ratio in favour of grain.

To Type II belonged all varieties with a moderate to higher harvest index: Cappelle Desprez, Maris Widgeon, Maris Huntsman, Mardler and the 'taller dwarf'. The harvest index was maintained almost constant within a variety under the two environments. The genetically controlled relationship, specific for each variety, is seen from the different angle from the common point of origin but maintained under the two conditions of cropping.

Type III included the semidwarfs and dwarfs: Hobbit and the two dwarf selections. Their high harvest index even with low nitrogen dressing (above 0.60) decreased with high nitrogen dressing slightly in favour of straw. The surprising stability found in Type II cannot be maintained in Type III varieties.

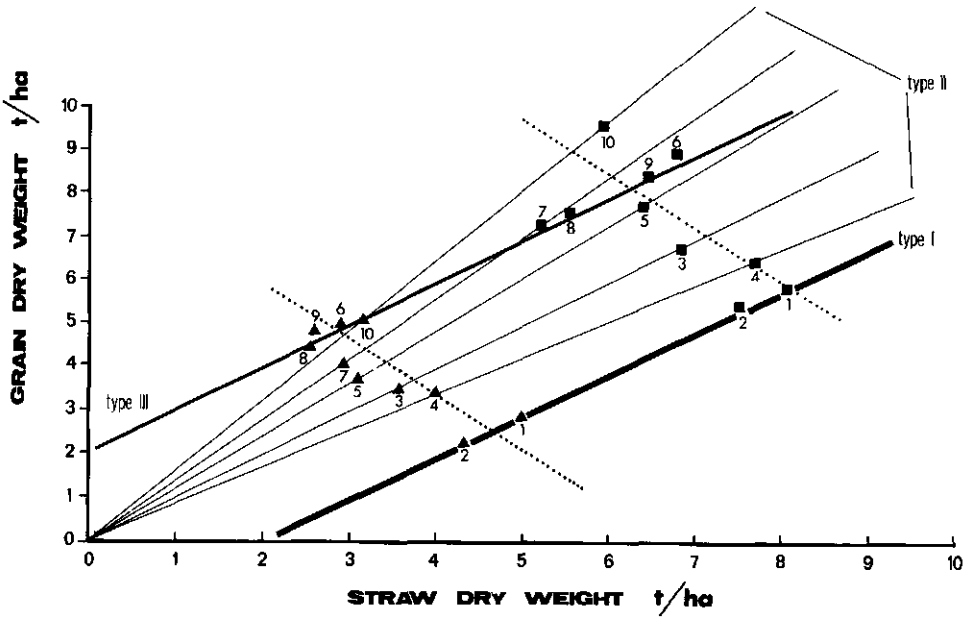

Fig. 1. Grain : straw ratio (dry weight) for ten varieties of winter wheat grown with two rates of nitrogen dressing (▲ = low, 28 kg.ha^{-1}; ■ = high, 104 kg.ha^{-1}).
Varieties, as numbered, were: *(1)* Little Joss – 1908, (2) Holdfast – 1935, (3) Cappelle Desprez – 1953, (4) Maris Widgeon – 1964, (5) Maris Huntsman – 1972, (6) Hobbit – 1977, (7) Mardler – 1978, (8 & 9) semi-dwarf advanced selections, (10) a 'taller' dwarf line. The harvest index of the Type II varieties was maintained almost constant in the two environments. In varieties of Type I and Type III the harvest index increased or decreased, respectively, with high nitrogen dressing. Modified from Austin (1980).

The guarded conclusion to be drawn from such studies is that the optimum harvest index has been reached in some wheat types. This could be particularly true when using dwarfing genes such as *Rht*1 and *Rht*2 (Gale, 1981). The only option further to increase yield in short-straw material will be to increase biomass production.

Continuous investigations about this valuable character in other breeding material with natural limits for culm reduction is still helpful to the plant breeder in the selection of parents, the prediction of possible merits for genetic combinations, and formulating more ideal plant types for selection in segregation progenies. The large number of variables adds to the complexity of the harvest index but points to the broad genetic variability available to the plant breeder interested in developing higher-yielding varieties.

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Development of better selection criteria

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Summary

Better understanding of the physiological basis of crop production allows selection criteria to be proposed and tested in experimental selection programmes. Mechanisms of yield formation, adaptation and other agronomic traits can then be quantified and evaluated and incompatibilities amongst breeding objectives revealed. In some crops, apart from increasing harvest index, yielding capacity can be increased by manipulating components of canopy structure to improve photosynthesis and, in forage grasses by reducing respiratory losses. Yield may also benefit from improvements in photosynthetic adaptability or longevity of individual leaves and in length of the period of active growth and period of filling of the economic 'sink'. Certain traits affecting responses to climatic constraints can also now be specified and selection criteria or techniques proposed. However, for traits to be of value in breeding the extent of any genetic correlations with other crop features must be known and it must be possible to establish reliable and rapid screening procedures.

Descriptors: selection criteria, yield, genetic variation, physiological traits, photosynthesis, respiration, stress tolerance, harvest index.

Introduction

In crop species, genetic variation seems to exist in most physiological and morphological traits examined (Wallace et al., 1972; Evans, 1975; Wilson, 1981a). However, this is of little value unless the breeder knows which characteristics can most beneficially be incorporated into a new variety and has the physiological and genetic methodology to combine these together. Model characters, such as awns in wheat and barley, short stems in small-grain crops or habit of growth in grasses, usually influence the breeders approach to selection but use of functionally-related traits as criteria depends on advances in knowledge of the physiological basis of crop performance. Selection based on production from nursery plants does not always detect those likely to perform best in a crop. However, predetermined relationships between inherent physiological, biochemical or morphological characteristics of spaced plants and agronomic traits of the crop can make selection more accurate and reliable. The present paper discusses this approach in improving yield and stress tolerance.

Criteria for selection

Useful selection criteria or screening procedures must meet certain requirements. They must be reliable, more accurate and/or faster than simple observation in the nursery. There must be a good correlation with crop yield or quality and a reasonably high heritability without strongly unfavourable correlated responses. The possibility of screening very young plants would be an additional advantage.

Means of assessing the effect that variation in particular physiological or morphological features may have on yield and other crop characteristics include the use of mathematical crop models, physiological analysis of contrasting varieties or plant collections, and experimental selection programmes.

Crop models can provide predictive information, applicable in particular assumed circumstances (De Wit, 1965; Monteith, 1977; Sheehy et al., 1979; Austin, 1982). Although only as accurate as the information used in their construction, and therefore often lacking precision, models can indicate and quantify likely effects of changing certain traits such as canopy structure, leaf photosynthesis or respiration. Testable hypotheses can, therefore, be developed. Their value for breeders will clearly increase with increasing knowledge of the comparative physiology of crops.

Many present ideas of desirable traits derive from analyses of contrasting varieties or ecotypes, from which it is often difficult to do other than observe useful correlations between, say, yield and a number of traits. Even so, hypotheses can then be proposed and tested with ideotypes or experimental populations constructed by selection for contrasting expression of specific traits from within an existing variety (outbreeders) or segregating population. This allows the direct effect of the character in the field to be assessed, the extent of any correlated responses to be observed and heritability to be estimated. It is also a powerful physiological tool in providing unique material for physiological analyses (Rhodes, 1975; Wilson, 1981a, 1982; Robson, 1982a, b).

The use of single specific, quantifiable traits in selection is a modest and practical first step in applying physiology to breeding (Wallace et al., 1972; Cooper, 1974; Wilson, 1981a). The development of selection indices presents greater prospects. Simultaneous selection for several physiological characteristics which do not seem to be mutually exclusive seems possible in wheat (Austin, 1982), field beans (Adams, 1982) and forage grasses (Wilson, 1981a). However, there can be no universal ideotype. Although physiological processes occurring in all crops might suggest some common criteria, their diversity in morphology, physiology, economic end-product and growing environments dictate that many aspects of the design of 'models' be unique to a particular crop or environment. Even as agronomic practice changes so must the model.

Two important breeding aims are to improve yielding capacity and minimise the effects of environmental constraints on yield. Consequently, their physiological bases in major crop species has been the subject of much research. To what extent can breeders now exploit results from these studies?

Yielding capacity

'Yield' is that part of the crop biomass which is allocated to the plant organs constituting harvestable yield. In forages economic yield is further diluted by the

conversion efficiency of harvestable plant matter to animal product. Functionally-related selection criteria for increasing yield must, therefore, be concerned with improving the overall carbon balance (photosynthesis, respiration, leaf area duration), or the pattern of allocation of assimilates (harvest index) (grain : stem ratio; shoot : root ratio; leafiness) or nutritive value in forages. Scope for increasing yield by improving these, without penalties for other important traits, depends on the economic end-product and the existing degree of improvement (Wilson, 1981a).

Much past increase in yields of crops which have a well defined storage organ results from increase in harvest index (Watson, 1971; Donald & Hamblin, 1976; Lawes, 1977; Austin, 1980). However, the upper limits of harvest index of many of these crops may soon be reached and further yield advances will then depend on increasing assimilate supply and storage capacity in concert (Evans, 1975; Austin, 1982). In forage grasses and clovers, where most of the shoot system is harvested and harvest index is high (60% in a well-grown crop), increase in biomass is even more important if harvestable dry matter is to be increased without prejudice to other agronomic requirements (Wilson, 1981a).

By definition, crop dry matter production is determined by level and duration of net photosynthesis. This can be affected by the maximum rates, adaptability and longevity of individual leaf photosynthesis, by morphological features of the crop canopy which influence light interception, and by respiratory losses. Some of these display additive genetic variation (Wallace et al., 1972; Cooper, 1981; Wilson, 1981a).

Individual leaf photosynthesis Leaf photosynthesis is complex and responds to diffusive processes and biochemical systems affected by different environmental stimuli. At low light, photochemical processes are limiting but as light increases CO_2 diffusion or utilisation becomes more important until, at light saturation, the CO_2-limited rate of photosynthesis (P_{max}) is reached. Within a dense crop, limitations of both light and CO_2 may therefore exist. In practice, increasing the efficiency of use of CO_2 may be the more realistic aim for breeders. In several crops, yield increases have resulted from atmospheric CO_2 additions, indicating P_{max} limitations (Evans, 1975). Moreover the significance of genetic variation in photosynthesis in weak light is not established for field crops and heritability seems low (Wallace et al., 1972; Wilson, 1973; May, 1975). Furthermore, lower leaves in poor light are usually older and potentially less efficient than the upper leaves, which in some grain crops are largely those responsible for grain filling (Evans, 1975). Improving the light environment within the crop may be a more practical way to alleviate any problem of density-related light inhibition (below).

Apart from the major distinction between C_3 and C_4 species (Troughton, 1975) large differences in P_{max} per unit leaf area exist between and within species (Wallace et al., 1972; Shibles et al., 1975; Wilson, 1981a). However, with some exceptions (Berdahl et al., 1972), regular correlations with yield have been difficult to demonstrate, nor has yield been increased by selecting for high P_{max} (Evans & Dunstone, 1970; Wilson & Cooper, 1970; Moss & Musgrave, 1971; Hart et al., 1978). This seems to be because P_{max} per unit leaf area and leaf size are often negatively correlated (Wilson & Cooper, 1967; Rhodes, 1971; Hart et al., 1978; Austin et al., 1982) although yield is not always source limited (Evans, 1975).

To exploit differences in P_{max} it will therefore be necessary to break this negative correlation. Using some unit other than leaf area on which to express rates may be more

helpful. The rate per mesophyll cell, chloroplast, or even thylakoid unit may be appropriate, but the practicalities for breeding difficult (Eagles & Othman, 1974; Jellings & Leech, 1982). Possible yield benefits from greater P_{max} for the same leaf area would depend on crop light interception and have been quantified in models. A semi-erect graminaceous crop might increase dry matter production (in a N-European summer) by about 25% if P_{max} were 50% greater (Monteith, 1977). Grain yield from a winter wheat crop in England might be increased by about 10% if P_{max} were increased from 30 to 37 mg CO_2 dm^{-2} h^{-1} (Austin, 1982).

Although leaves of many temperate species can reach P_{max} of around 30-35 mg CO_2 dm^{-2} h^{-1} in practice only few leaves in field crops seem capable of these levels at any one time. In grass crops, potential rates are usually only 10-15 mg even when leaves are brightly lit (Robson, 1981). This discrepancy could be caused by leaf ageing (Loach, 1970; Aslam & Hunt, 1978) or by poor light and temperature conditions during leaf expansion (Wilson, 1981b).

Time of onset and rate of decline in photosynthesis with age after complete expansion are genetically variable (Loach, 1970; Stoy, 1975; Aslam & Hunt, 1978). Although effects of this variation on yield are difficult to quantify some crops would be more affected than others. In grasses the decline may not start until two to three weeks after full expansion (Jewiss & Woledge, 1967) and older leaves may only intercept 10% of the available light (Leafe, 1972) even when still capable of high P_{max}. By contrast, in wheat, genotypic differences in ageing pattern of flag leaf photosynthesis may be of greater significance because of the central role of that organ in grain filling (Austin, 1982). In sugar beet, leaf longevity seems to contribute to high yields (Loach, 1970) so that selection for reduced leaf senescence could be useful.

The inhibiting effect of poor light during leaf expansion on subsequent P_{max} is important in grasses since many leaves develop in shade but are exposed to full light later. This effect can ultimately reduce crop photosynthesis by 30% (Robson, 1973). However, there is potentially useful genetic variation in this trait (Wilson & Cooper, 1969).

Canopy structure Size, distribution and presentation of leaves in canopies control light availability and therefore influence photosynthesis directly and through effects on the light environment in which leaves and plants develop. There may also be more subtle effects on yield since distribution of CO_2, water vapour and temperature through the crop can be affected (Evans, 1975). However the possibility of increasing yielding capacity by genetic modification of canopy structure depends on the crop, its environment and management (Wilson, 1981a). Prostrate, low-growing canopies reach complete light interception rapidly but erect, taller, arrangements are more effective in the long-term and develop greater maximum leaf area index (L_{max}), crop photosynthesis and potential dry matter production (Cooper & Breese, 1971).

Changing from relatively prostrate to relatively erect forms can theoretically increase dry matter yield in mid-summer in Britain from 17 to 29 g m^{-2} day^{-1} (Monteith, 1977), a difference observed in practice among ryegrass genotypes (Sheehy & Cooper, 1973). In the same species yield increases of up to 30 percent have been recorded after three generations of mass selection for long leaves, with some benefit from erect tillers (Rhodes, 1975). To realise these yield benefits it was necessary to allow maximum L to develop. However, leaf posture needs caution in some grasses since certain structural

features which lend strength and rigidity to leaves may be deleterious to nutritive value or stress tolerance (Wilson, 1981a).

In grain crops, other assimilate supplying organs are important in canopy designs (specific leaves, awns, spikes) (Wallace et al., 1972; Yoshida, 1972; Austin & Jones, 1976). In wheat and barley a high L at anthesis is important (Austin, 1982) and the uppermost leaves and spikes are major assimilate suppliers. Although more vertically inclined leaves can give greater photosynthesis in wheat this is not always reflected in yield (Austin et al., 1976). In contrast, the highest yielding rice varieties have good light transmission qualities with their short, erect leaves, short culms and upright tillers (Yoshida, 1972). In that crop, lower leaves are more important than in wheat or barley. In all grain crops, erect upper and lax lower leaves might be the most suitable model (Tanaka et al., 1968; Austin et al., 1976) and there is likely to be an optimum height which combines lodging resistance and high harvest index with the positive correlation which usually exists between height and grain yield (Yoshida, 1972; Rosielle & Frey, 1975; Law et al., 1978).

Theoretically, however, erect leaves may be of greatest advantage for crops with axillary inflorescences (Evans, 1975).

Respiratory losses C_3 crops lose substantial amounts of carbon by photorespiration and in the dark (Biscoe et al., 1975; Monteith, 1978; Pearman et al., 1981; Robson, 1981; Austin, 1982). Is any of this unnecessary and, if so, can yield be increased by decreasing respiration? At present, the possibilities of reducing or eliminating photorespiration are not promising and in any case the extent to which yield might be increased without other penalties is not clear (Wilson, 1981a). However, within the concept of 'biosynthetic' and 'maintenance' components of dark respiration carbon-use efficiency might be improved by reducing 'maintenance' respiration (McCree, 1974; Penning de Vries, 1974; Thornley, 1977). 50-55% of the total current assimilate can be lost in maintenance respiration in well-grown crops (Robson, 1973; Biscoe et al., 1975) so that any reduction might significantly affect yield (Robson, 1981; Wilson, 1981a; Austin, 1982; Wilson & Jones, 1982).

Negative correlations between dark respiration of fully grown tissues and growth of different cereal species and varieties (Scheibe & Meyer zu Drewer, 1959), contrasting maize inbreds (Heichel, 1971) and ryegrass genotypes (Wilson, 1975) suggested the dark respiration to be a useful approximate estimate of maintenance respiration. Selection for slow mature tissue respiration in ryegrass ($h^2 \approx 0.50$) (Wilson, 1982) has produced populations which have consistently outyielded the original variety by up to 13% annually and 20% in summer (Table 1). The improved carbon use efficiency of slow respiration selections (Robson, 1982a, b) is also associated with a greater advantage at high N levels (Robson et al., 1983). No agronomically deleterious effect seems to be associated with the selection but genetic stability is presently unknown.

Leaf area duration At a meeting of breeders it is relevant to stress the dependence of crop yields on length of the growing season (Watson, 1947; Monteith, 1977; Sibma, 1977). Apart from drought (below), temperature and photoperiod usually set the limits to the period of active growth but responses to these factors can usually be selected for. For example, there are marked differences between sugar beet varieties in rate of leaf

Table 1. Herbage dry matter yields (kg ha^{-1}) from sequentially harvested plots of *Lolium perenne* cv. S23 and of a population (GL112) selected from S23 for slow dark respiration of mature leaves. Plots sown 1979, harvested 1980. Data from Wilson & Jones (1982).

Cultivar	April		May	June		July	Sept		Oct	Total
			21			30			30	
	10	30		10	27		1	30		
S23	1199	1303	865	1144	1049	1555	1280	1026	336	9757
GL112	1379	1401	920	1186	1174	1880	1512	1191	366	11009
s.e.d.	44	77	78	78	56	62	85	68	48	391

expansion at low temperature (Milford et al., 1980) and selection for lower temperature threshold for leaf expansion of grasses in controlled environments can give earlier spring and later autumn growth. Selection from within grass and clover hybrids between N-European and Mediterranean types has achieved broader temperature optima and longer active growing season than in either parent (Barclay, 1970; Borrill et al., 1973). Although such growth responses to temperature extremes can be linked to reduction in stress tolerance (Cooper, 1974) suitable selection techniques and strategies can help to avoid this (below).

The role of photoperiod sensitivity is often important. For example, suitable day-length responses can delay flowering and usefully extend the growing season in crops grown for maximum vegetative growth (tobacco, forage). In wheat, delayed initiation can improve yield by increasing opportunities for lateral spikelet development (Rawson, 1970). Extending the duration of grain filling is a major objective in cereals (Evans, 1975; Austin, 1982). Indifference to daylength can allow some normally short-day species (e.g. maize) to be cultivated at higher latitudes (Aitken, 1977).

Assimilate partitioning (harvest index) Improvement of harvest index is the topic of a separate paper (Kertész, 1984) but it is also relevant to present discussions. In spite of the high harvest index of some crops (Bingham, 1971; Watson, 1971; Lawes, 1977) and even if parallel increase in photosynthetic capacity is necessary, optimising allocation of assimilates to the economic end-product is essential to maximise yield. However, the biochemical and physiological bases of assimilate translocation and partitioning to different sinks are not well understood and present selection methods are, therefore, unsophisticated. Harvest index itself is a relatively crude criterion (Rosielle & Frey, 1975; Donald & Hamblin, 1976) and has often been increased incidentally to some other aim such as lodging resistance or drought tolerance (below). Even so, selection for components such as root or sugar yield in beets, straw length, tillering and grain sink capacity in cereals, and onset and rate of tuberization in potatoes are important objectives (Moorby & Milthorpe, 1975; Donald & Hamblin, 1976). In forages improvements in digestibility or intake, or presentation to the animal can be considered to increase harvest index (Wilson, 1981b). However increases in harvestable proportion of perennial forages might place them at risk during periods of stress since reserves may then be inadequate for survival. Long-petiole clovers can suffer in this way (Rhodes, 1981).

Climatic constraints

Selection for tolerance or avoidance of stress can be made by observation of individual plant responses in the field or in controlled environments, or by measurement of functionally-related traits. In practice it is often necessary to develop environmental simulation techniques using, for example, rain shelters, irrigation control, or cold chambers. However, criteria based on knowledge of the physiological or biochemical bases of adaptability are likely to be most useful. Two of the most common causes of yield shortfall are water shortage and temperature extremes.

Water shortage Restrictions imposed by water shortage can occur anywhere that the evaporative demand exceeds water supply during the growing season. Periods of water shortage can be transient or long-term, predictable or unpredictable, and can be accompanied by other extremes such as high temperature. Selection criteria should therefore be based on an understanding of the climatic conditions in which the variety is to be grown. A variety most suited to face stress only during a well-defined predictable period will differ from one most suited to facing unpredictable intervals of water shortage occurring throughout its life.

Many characteristics which might be used to improve yield in certain drought environments have been suggested (e.g. Jones, 1976, 1979; O'toole & Chang, 1979; Turner & Begg, 1981; Wilson, 1981a, b). These include, the timing of crop development, often providing the most useful method of drought avoidance (Derera et al., 1969; Namken et al., 1974), developmental plasticity (Turner & Begg, 1981), exploration and transport capacity of roots (Derera et al., 1969; Troughton & Whittington, 1969; Passioura, 1972; Hurd, 1974; Willat & Taylor, 1978), control of transpiration (Jones, 1976; Wilson, 1981a, b; Turner & Begg, 1981), preferential allocation of reserves (Arcioni et al., 1980; Turner & Begg, 1981), osmotic adjustment (Morgan, 1980; Turner & Begg, 1981; Thomas, 1982), and stress metabolites such as proline or abscissic acid (Quarrie, 1980).

Morphological variation in specific components of root systems might allow selection for efficient and extensive water and nutrient extraction (large, deep system) or for conservative use of a finite supply (smaller system with high hydraulic resistance; small root xylem vessels) (Troughton & Whittington, 1969; Passioura, 1972; Hurd, 1974; Troughton, 1974). Modifications to provide better control of transpiration, and so reduce wasteful use of water, include increasing harvest index in grain crops by reducing tillering (Blum, 1973; Jones & Kirby, 1977; Innes et al., 1981) selecting for improved stomatal control either directly using leaf porometry (Jones, 1979; Gay & Wilson, 1981) or indirectly using correlated leaf morphological features (Wilson, 1981b; Nerkar et al., 1981) or considering traits such as waxiness, size and posture of leaves, or presence of awns (Wilson, 1981a).

Improved stomatal control can lead to better water-use efficiency in cool temperate conditions (Table 2). In higher temperature environments, maintaining stomatal opening when vapour pressure deficit is high may be beneficial (Turner & Begg, 1981). Maintenance of leaf turgidity when leaf water potential declines in response to stress may well promote drought tolerance (Atsmon, 1979) but present measurement techniques are laborious. Even so, crop photosynthesis in dry conditions often declines mainly due to leaf area reduction and genotypes do vary in this response (Silcock & Wilson, 1982).

Table 2. Soil moisture (mg g^{-1}) at 20 cm depth and dry matter yields (g m^{-2}) of plots of *Lolium perenne* cv. Grasslands Ruanui and of a population selected from Ruanui for low theoretical maximum leaf conductance (GL_5). First year, 1975. Data from Wilson (1981b).

Cultivar	July							Aug 8	Yield
	2	4	7	14	18	25	31		
Ruanui	144	150	142	163	158	161	147	141	103
GL_5	146	163	150	176	163	165	151	148	126
s.e.d.	2.5	3.5	3.9	3.5	1.0	0.9	1.0	1.5	9.0

Clearly, there is no lack of plausible traits to test. It is lack of techniques for rapid screening that is often more limiting to the testing of model varieties.

Temperature

Temperature usually limits leaf growth and photosynthesis outside optima of 15-25 °C in most temperate (C_3) crops and 25-35 °C of C_4 crops. However, those optima are subject to genetic and physiological adaptation and even within species populations often differ in their temperature response (Cooper, 1976). Consequently, apart from the possibilities of increasing leaf area duration (above) there is scope to extend latitudinal or altidudinal limits. Criteria need simply be based on leaf area expansion in given temperatures but growth at very low or high temperatures can be correlated with increased vulnerability to even greater extremes or to long-term survival (Cooper, 1964; Arcioni et al., 1980).

Crops survive freezing either by avoiding winter or by developing sufficient cold hardiness, which may be associated with dormancy the timing of which is often influenced by response to factors such as photoperiod. The plant can then become dormant prior to severe conditions (Østgard & Eagles, 1971; Bauer et al., 1975). Although chemical and physical factors in freezing tolerance are known (Breese & Foster, 1970; Levitt, 1972) its fundamental bases are insufficiently well understood to propose specific selection criteria. However, artificial freezing tests which take account of lethal temperature, light and temperature requirements for hardening, and propensity to deharden have been developed and successfully applied (Hides, 1979; Fuller & Eagles, 1980, 1981).

The effects of high temperature are often difficult to separate from those of drought but there are non-developmental adaptations in C_3 species which can help by reducing leaf temperature. These include morphological or physiological features which increase evaporative cooling and traits such as leaf hairs which reduce radiation income (Ehleringer & Björkman, 1978).

Conclusion

Improved understanding of the morphological, developmental and physiological bases of yield and other agronomic traits can suggest selection criteria but confidence in their use depends on practical demonstrations of the results of selection. The net effect of

the many ways in which even a single characteristic may influence final yield depends on environment, agronomic practice and relationships with other traits. Moreover, there may be many and subtle counter-productive features associated with exaggeration of particular features such as leaf size or leaf inclination, shorter stems in wheat, rapid photosynthesis per unit leaf area or extreme stress tolerance. There may also be problems such as genetic instability, only revealed at seed multiplication, which require solution before criteria can be applied.

Development of better criteria to improve yield potential will doubtless continue to pre-occupy physiologists and breeders but efficiency of use of scarce resources such as water, nitrogen or phosphorus will almost certainly become increasingly important.

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An evolutionary concept of breeding objectives and selection criteria

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Summary

The yield potential of most crop plants has increased considerably with domestication and plant breeding. This increase in yield potential is primarily related to plant morphology and development, whereas the efficiency of physiological processes in the plant cell has not changed. It is proposed that the potential for genetic improvement of yield components is inversely related to the evolutionary age of the characteristic. This implies that characteristics such as canopy structure, and size and number of sink organs are easier to modify than old processes such as photosynthesis.

Descriptors: yield, breeding, selection criteria, physiology, morphology, evolution, natural selection

Wilson (1984; also 1981) shows that yield formation is a complex process. Yield is the end-result of interaction of environmental factors and several plant characteristics. The yield potential of most crop plants has increased considerably with domestication and subsequent plant breeding. The biological principles underlying this increase in yield potential presumably also apply to current and future plant breeding. An indication of the relevance of selection criteria can be obtained by comparing modern varieties with low-yielding land races and wild ancestors. It appears that modifications of morphological characteristics (e.g. leaf area, leaf thickness, canopy structure, size and number of sink organs, and grain/stem ratio) and developmental characteristics (e.g. length of the growing season, duration of the sink-filling period, photoperiod sensitivity, and leaf longevity) have contributed much more to increase in yield potential than have improvements of basic physiological processes such as photosynthesis and respiration.

I propose that the potential for genetic improvement of plant characteristics is inversely related to the evolutionary age of the characteristic. This applies not only to yield components but also to other characteristics such as plant-pathogen relationships, nitrogen fixation, optimum temperature for growth, and resistance to environmental stress (Miedema, 1982). The following arguments support the hypothesis. The efficiency of cell metabolism cannot be improved (Penning de Vries et al., 1974). Processes such as the photochemical reactions in photosynthesis, CO_2 assimilation, and aerobic respiration developed very early in the evolution of the plant cell. So it may be assumed that most of the possible positive genetic variation has been sorted out already by natural selection. In the early stages of the evolution of higher plants, genotypes with an efficient

regulation of such processes would have survived because of their selective advantage in plant competition. During domestication and subsequent breeding, selection for high yield would probably eliminate genotypes with inefficient carbon economy. Several attempts to improve yield potential by increasing leaf photosynthesis or by decreasing photorespiration have been unsuccessful. However Wilson (1982) has shown that in the perennial ryegrass cv. Aberystwyth S23, selection for decreased maintenance respiration increased yield potential. Further research should be carried out to determine whether this is generally applicable, or restricted to forage grasses grown with heavy nitrogen dressings (Robson & Stern, 1982).

The evolutionary concept of selection criteria proposed here is based on the assumption that major environmental limitations to plant growth (light, CO_2, water) apply to both wild plants and crop plants, and that biochemical and physiological processes coping with these restrictions have attained a high efficiency. The concept does not exclude development of novel biochemical characteristics such as resistance to herbicides or heavy metals. Such characteristics can arise as a result of mutation, because they concern novel environmental constraints.

There is little reason to change selection criteria in practical plant breeding if the evolutionary concept is valid. Plant breeders screen for yield potential by estimating economic yield or simply by assessing plant performance in the nursery. They also select for yield-related morphological or developmental characteristics, but usually neglect physiological characteristics such as leaf photosynthesis. Screening for separate physiological characteristics is laborious and usually the data have no clear relationship to economic yield (Mahon, 1983). As the expression of physiological plant characteristics depends on environmental factors, screening has to be done under various conditions of light, temperature and water supply. So screening for economic yield in combination with desired morphological and developmental characteristics is a more efficient approach to selection of superior genotypes than is screening for separate physiological characteristics. Screening for economic yield should be done under a range of environmental conditions, and would include all known and unknown yield components. Thus it would not require time-consuming physiological research.

Finally, it should be emphasized that the evolutionary concept of selection criteria should not be considered as a general recipe for plant breeding, but rather as a working hypothesis for further research on the physiological and morphological basis of genetic variation in yield potential.

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Breeding for better climatic adaptation using physiological criteria

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Summary

Selection of varieties well-adapted to their environmental conditions may be based on physiological criteria. Although physiological responses to climatic constraints are complex and correlated, essential parameters can be determined. Differences in photosynthetic responses and translocation of assimilates observed at low temperature between cultivars allow selection criteria to be proposed for a better adaptation of soybean to cold climates. The capacity of some wheat genotypes to maintain their stomata open and to maintain active photosynthesis during a water stress, when leaf water potential is low, may be considered as a factor of resistance to drought. Selection of wheat varieties adapted to dry areas may be considered through analysis of relations between stomatal resistance and leaf water potential.

Descriptors: selection, physiological traits, photosynthesis, temperature tolerance, water stress tolerance, soybean, *Glycine max*, wheat, *Triticum aestivum*, *Triticum durum*

Introduction

Under different growth conditions, climatic parameters are not necessarily optimum for plant activity. Factors like water status, temperature and light condition photosynthetic activity and translocation of the assimilates, which are directly related to the economic productivity of crops. A better understanding of the genotypic capacity of adaptation which is essential in selection of varieties well-adapted to various regional environments, may be achieved through the study of responses of basic physiological processes to climatic constraints.

To carry out varietal selection, it is necessary to consider factors affecting productivity under limiting conditions, and to partition them into elementary parameters or to use measurement methods allowing a rapid screening. In the investigations reported here, soybean adaptation to low temperature and response of wheat to water stress were more particularly studied.

Soybean response to low temperatures

Low temperatures affect CO_2 fixation processes, translocation and plant growth in a complex way, where causes and effects are difficult to assess. Reduction in photosynthesis may be attributed partly to starch accumulation in chloroplasts due to an impaired

Table 1. Responses of some physiological factors to low temperatures in soybean. Growth temperature: 25 ° C, temperature applied during 5 days: 15 ° C, controls: 25 ° C.

	Physiological responses at		Measurement methods
	25 ° C	15 ° C (6)	
P_N* (1)	14.1 → 20.1	− 4 → −45	Infrared analyser CO_2
Translocation* (2)	30 → 45	− 32 → −55	Measured 24 h after assimilation of $^{14}CO_2$
Fresh weight increase (3)	0.08 → 0.11	− 8 → −51	Weight of plants grown on nutrient solution
L.S.W.* (4)	2.21 → 3.06	+25 → +56	Dry weight
W.L.* (5)	2.00 → 3.07	+18 → +63	Dry weight

(1) Net photosynthesis (mg CO_2 h^{-1} dm^{-2}).
(2) Translocation in % of products migrating outside the leaf.
(3) Fresh weight increase (g g^{-1} of fresh weight day $^{-1}$).
(4) L.S.W.: leaf specific weight (g dm^{-2}).
(5) W.L.: weight of the length unit of the petiole (g cm^{-1}).
(6) Response in % of control.
→ extreme values between genotypes.
* measurements on the 4th leaf at the R_1 stage.

transport of assimilates, leading to an increase in intracellular resistance to CO_2 transfer (Thorne & Koller, 1974).

In order to improve adaptation to variable climatic conditions, responses of physiological parameters (net photosynthesis, translocation, weight increase) to long periods of low temperatures, different from those occurring during leaf growth, were investigated. Low temperatures affect particularly translocation of assimilate in soybean (Stella & Thrower, 1965; Thomas et al., 1981). The results (Table 1) show that the effect of low temperatures is expressed by a more important reduction in translocation than in net photosynthesis, leading to accumulation of glucids and starch in leaves and petioles, followed by an increase in specific leaf weight and weight per length unit of petiole.

The weight increase of the whole plant is well correlated with these two phenomena. A large genetic variation exists for the responses of all the characters studied to low temperatures. Breeding for genotypes less affected by low temperatures at different stages may be carried out through the non-destructive evaluation of the fresh weight of plants grown on nutrient solutions under phytotronic conditions. The value of the specific weight of leaves first grown under favourable temperature conditions, then exposed to low temperatures during several days, corroborates the first screening.

Wheat response to water stress

Various physiological mechanisms related to growth and plant development are influenced by water stress (Hsiao, 1973; Boyer & Mc Pherson, 1975; Begg & Turner, 1976): photosynthesis, translocation, leaf, root and flower development. For each genotype, responses to water stress are closely interdependent and the study of plant

resistance to drought is complex. Turner (1979) determined three main types of resistance to water stress. Tolerance to low leaf water potential allows the maintenance of the main physiological processes in relation with productivity. Tolerance to water stress was assessed through two parameters: photosynthesis, as it is related with productivity, in particular in case of water stress (Kaul & Crowle, 1974; Kaul, 1974), and stomatal resistance, which controls gas exchange in the plant. Leaf water potential (ψ_w) reflects the plant water status and is expressed in pressure units (bar); as dessication increases, the potential value becomes more negative.

Net photosynthesis decreases steadily with leaf water potential. In Figure 1, linear regressions $P_N = A + B(-\psi_w)$ are compared for the six genotypes studied (three durum wheats and three bread wheats).

Haurani 27 and Baalback, two drought-resistant varieties, maintain active photosynthesis beyond a leaf water potential of -30 bar. But Capitole has a null photosynthesis when this potential reaches -17 bar.

Stomatal resistance to transpiration and CO_2 diffusion are only slightly different at the beginning of the water stress, then increase suddenly when the water stress reaches a

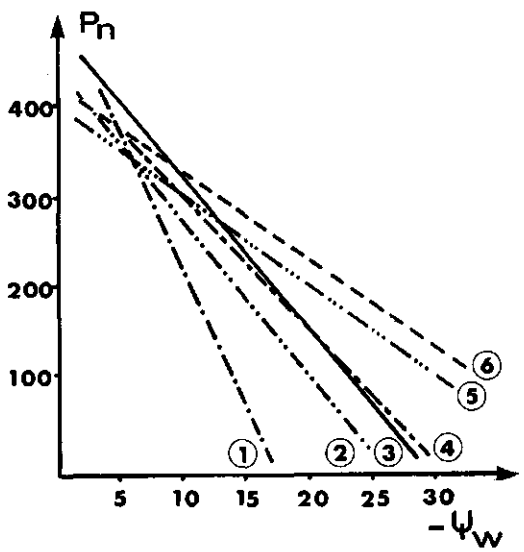

Figure 1. Relationship between net photosynthesis per unit area (P_N, 10^{-9} kg m^{-2} s^{-1}) and leaf water potential (Ψ_w, bar). Stage 7–8 leaves.

Regression lines

(1) Capitole[1] $P_N = 523 - 30.5 \quad (-\psi_w) \quad r = 0.38^{**}$
(2) Sakha[1] $P_N = 455 - 17.5 \quad (-\psi_w) \quad r = 0.37^{**}$
(3) Bidi 17[2] $P_N = 506 - 17.0 \quad (-\psi_w) \quad r = 0.40^{**}$
(4) Jori[2] $P_N = 461 - 15.0 \quad (-\psi_w) \quad r = 0.43^{**}$
(5) Baalback[1] $P_N = 417 - 10.4 \quad (-\psi_w) \quad r = 0.37^{**}$
(6) Haurani[2] $P_N = 440 - 9.87 \quad (-\psi_w) \quad r = 0.38^{**}$

1. Bread wheat.
2. Durum wheat.

** Significant at $P < 0.01$

Table 2. Critical values of leaf water potentials ψ_w for stomatal closure in wheat (stage 7–8 leaves).

Species	Durum wheat			Bread wheat			L.S.D.
Varieties	Haurani	Bidi 17	Jori	Baalback	Sakha	Capitole	
Ψ_w (bar)	-38	-29	-24	-34	-25	-16	2

critical phase corresponding to almost complete stomatal closure. ψ_w values for stomatal closure differ between varieties (Table 2). If stomatal closure is impeded, drought-resistant genotypes may carry on photosynthesis as well as dry matter production. This aspect may be considered as a positive element of productivity under dry conditions (Turner & Begg, 1981) but, probably only for limited water deficits.

Leaf water potential (ψ_w) and stomatal resistance (r_s) can be measured using simple techniques (Scholander pressure chamber, diffusion porometer). Classification of genotypes may be considered from $\psi_w - r_s$ relations, particularly from the critical ψ_w value for stomatal closure. This value may vary according to growth conditions and plant age but genotypes are eventually listed in the same order (Aboussouan, 1982).

Conclusion

Physiological criteria for selection of cultivars better adapted to climatic constraints are difficult to find out. For each genotype, most physiological characters interfering in adaptation phenomena are highly interdependent in their response to environment. But some parameters, such as photosynthesis and translocation seem to determine yield capacity under limiting temperature and water supply conditions. They can have great value in selection if simple methods can be used for screening of genotypes. Analysis of genetic correlations between characters are liable to show the effect of selection for a major parameter on the other yield components (Ecochard & Ravelomanantsoa, 1982).

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Development of better selection criteria – a comment

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The title of this session: 'Development of better selection criteria' prompts the question 'Better than what?' The nature and content of any talk has to depend on the answer to such a question. In consequence, I decided simply to emphasize that a 'different' or 'new' selection criterion may be 'better' or 'worse', depending upon a whole set of conditions – with these encompassing such aspects as

- the target area of the breeding programme, i.e. the environment considered over both space and time;
- the germplasm used in the breeding programme, i.e. the species and the range of variation within the species;
- the procedures used in the programme.

Selection criteria and set of germplasm which may be appropriate to one environment may not be appropriate elsewhere. It can thus be expected that some traditional criteria – spaced plant performance for example – may be appropriate for some germplasm-environment conditions (e.g. where drought or temperature responses are of more significance than light utilization), but not for other combinations.

Equally it can be expected that some of the newer and more novel selection criteria – those based on some physiological trait – may be useful in some situations, but not in others. Some feeling for where a particular selection criterion may be of value can be obtained from a consideration of the factors determining yield in the target area. To illustrate this, let me recall that crop yield depends on

- the speed with which a closed canopy is achieved, i.e. the speed of building up a system capable of intercepting radiant energy that would otherwise be wasted at the soil surface;
- the efficiency of conversion of intercepted energy into dry matter;
- the partitioning into harvestable product of the accumulated dry matter.

For some crops, interception of radiant energy may be more important; for others, efficiency of conversion into dry matter. Criteria which relate to utilization of radiant energy (e.g. photosynthetic rate of an individual leaf, erect canopy structure) will be of limited or negative value if interception is the most important aspect. This latter situation may apply with many crops – indeed, physiological changes along with crop evolution suggest that this situation has applied widely. Care will thus have to be taken to ensure that criteria relating to utilization of intercepted energy are likely to improve crop productivity, and that criteria in crop canopy development receive sufficient attention.

'New' selection criteria may thus be 'better' or 'worse', depending on the biological

characteristics of a specific breeding programme. They may be 'better' or 'worse', depending on the resources necessary for application of the criteria. Techniques of measurement have not received much attention in this session; they should perhaps receive as much attention as the 'straight' physiology when considering the development of new criteria for application in breeding.

Descriptors: photosynthesis, canopy development, radiant energy, yield, selection criteria

Blocks of prolamine components as a selection criterion in breeding and seed production

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Summary

90 and 120 allelic variants of stable blocks of prolamine components have been identified for six loci of Chromosomes 1A, 1B, 1D, 6A, 6B and 6D in wheat and for six loci of Chromosome 5 in barley, respectively. These blocks serve as effective genetic markers of the variation of such quantitative characters as productivity, grain quality and resistance to biotic and abiotic stresses.

Descriptors: selection criteria, wheat, *Triticum aestivum*, barley, *Hordeum vulgare*, prolamine, gliadin, hordein, chromosome marker

Analysis of a great number (>100) of wheat hybrid combinations, with the use of aneuploid lines obtained from Dr E.R. Sears (Columbia, Missouri, USA) has shown that gliadin components are inherited as stable blocks. This may be the result of rare occurrence of recombinations within a block or because the majority of recombinants may be eliminated (Sozinov et al., 1970; Sozinov et al., 1975). A catalogue of blocks of gliadin components in the world collection of wheat varieties has been prepared, including 18, 18, 8, 13, 12, 12, allelic variants for Chromosomes 1A, 1B, 1D, 6A, 6B and 6D, respectively. Six hordein-coding loci of Chromosome 5 have been identified in barley. Loci *Hrd* A, *Hrd* B and *Hrd* F are represented by 55, 56 and 6 allelic variants of blocks, respectively. A method of recording prolamine genotypic formulae has been proposed, and prolamine-coding loci in wheat and barley have been mapped (Sozinov et al., 1978; Rybalka & Sozinov, 1979; Sozinov & Poperelya, 1979; 1982).

Allelic variants of prolamine-coding loci have been found to be associated with the variation of economically valuable characters. For instance, the comparison of allelic variants of the 1A chromosome locus in hybrid populations (F_4, F_5) showed that the productivity of lines with the GLD 1A1 block was significantly higher (about 5-8%) than that of genotypes with the GLD 1A4 block. The clearest relationship between variants of blocks and the level of expression of a character is observed for such characteristics of grain quality as bread volume, sedimentation number, physical properties of dough, and mass of 1000 grains. Allelic variants of blocks are ranged by their influence on these characters. The presence of blocks GLD 1B3, GLD 1B6, GLD 1D3 and GLD 1A1 in a genotypic formula is associated with a considerable deterioration in physical properties of dough. All frost-resistant wheat varieties have, as a rule, blocks GLD 1A1, GLD 1A2, GLD 1D5, GLD 6A3, GLD 6D2. The GLD 1B3 block serves as a reliable marker of

1B/1R translocations, and, consequently, of resistance to stem rust. The relationship between different variants of blocks and the variation of other characters has also been demonstrated (Sozinov & Poperelya, 1980).

In winter barley, the allelic variants of blocks HRD A3, HRD A4, HRD F3 are associated with frost resistance. The variants of blocks have been found to have a relationship with the productivity, mass of 1 000 grains, resistance to powdery mildew, grain extractivity, and composition of protein fractions (Netsvetaev & Sozinov, 1982).

Prolamine blocks are actively used in winter wheat, spring and winter barley breeding programmes for choosing parental forms, selecting genotypes with a favourable combination of allelic variants of blocks, tracing the origin of varieties, studying regularities of the inheritance of genetic material in intraspecific and interspecific crosses. The study of protein polymorphism also gives opportunity to control the process of reproduction of varieties in the production of elite seed.

The relationship of allelic variants of blocks to the variation of quantitative characters is accounted for, in our opinion, by the existence of steady associations of certain prolamine genes and main loci influencing the variability of the quantitative character. Allelic variants of blocks serve as genetic markers of these associations.

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Efficient use of energy, nutrients and water

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Summary

Efficient use of energy (low temperature), nutrients (low availability of nutrients or excess salinity) and water (drought) in crop production will depend on the effect of these stress factors on growth, energy metabolism, mineral nutrient uptake, and structure and functioning of plant cell membranes. The use of such physiological parameters for selection will be discussed with special emphasis on selection for a flexible response of the plant to suboptimal conditions of temperature, nutrients and water.

Descriptors: nutrient stress, low temperature, salinity, drought, membranes, genetic differentiation, phenotypic plasticity

Introduction

When plants are grown under suboptimal conditions, crop production or 'population fitness' is reduced. Two ways are open for improvement of such a situation. Firstly, one may try to obtain new genotypes for which the 'suboptimal' conditions are optimally. Curves A, B, and C (Fig. 1) represent genotypes with different optima for an environ-

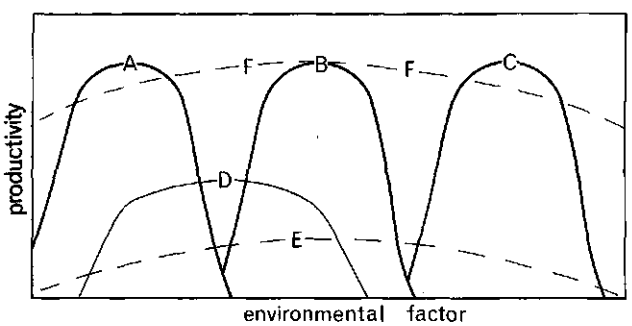

Fig. 1. Effects of genetic differentiation and phenotypic plasticity on plant productivity as affected by the environment. A, B, and C, genotypes of similar flexibility, but differing in optimum of the environmental factor for growth. D, flexibility greater than for A, B, and C, but productivity reduced. E, extreme degree of flexibility, in combination with a very low productivity. F, combination of extreme flexibility with high productivity.

mental factor, which could be temperature, salinity, water supply or nutrient supply. All three genotypes have in common, that crop production is affected similarly by suboptimal conditions: they show the same flexible response to the environment.

The second way is to select genotypes with a greater physiological plasticity (Fig. 1; Curves D, E and F). Curve D represents increased phenotypic plasticity at the expense of productivity. Sugar beet inbred lines constitute a good example of such options for selection. In a growth experiment (Marschner et al., 1981) the commercial variety Monohill shows optimum fresh weight at 50 mM NaCl concentration (650 g; Curve B), the salt-sensitive line ADA at zero concentration (450 g; Curve A) and the salt-tolerant line FIA shows constant growth between 0 and 150 mM NaCl concentration (170 to 180 g; Curve D).

In several plant species, an extreme plasticity has been realized in combination with a very low productivity (Fig. 1; Curve E). Specialized and slow-growing species provide many examples. Several tundra species show a growth rate which is hardly affected by temperature and nutrient supply (Stuart Chapin, 1980). In contrast to other *Plantago* species, growth of *Plantago coronopus* is only slightly affected by nutrient supply (Kuiper & Kuiper, 1979). The relative growth rate of *Carex limosa* was unaffected by concentration of K^+ within a thousandfold range (Veerkamp & Kuiper, 1982). The above examples indicate that in nature a coupling between a plastic growth response and low productivity of the genotype (species) exists. The coupling could be explained by the assumption that a plastic response to the environment is associated with a high energy demand for all the necessary biochemical and structural requirements of such a plastic response. Exceptions exist: *Spartina townsendii,* a species that appeared rather recently and originated on the English coast, shows vigorous growth in a range from freqently flooded salt-marsh up to infrequently flooded salt-marsh, suppressing numerous native halophytes over a wide range of frequency of flooding by sea water (Fig. 1; Curve F). This last example clearly sets the goal for selection of genotypes of crop plants under suboptimal conditions: a more flexible response to a specific environmental factor in combination with a high productivity by efficient use of energy, nutrients, water and other natural resources. One may choose for selection on biochemical or physiological parameters which are easily recognized at the cellular level. Several contributors to the symposium on breeding at the cell level deal with this topic. Parameters at the level of tissue, organ or the intact plant may be of equal importance for selection of highly productive and flexible genotypes by a specific strategy to cope with the suboptimal condition.

Low temperature stress

Plants from tropical regions such as cucumber, rice, corn and tomato are injured by low temperature above 0 °C, a phenomenon known as chilling injury. Temperatures below optimum but above the limit for injury are suboptimally. In that range, germination, vegetative growth and reproduction are retarded or arrested. Conditioning (hardening) by exposure to a low but non-chilling temperature may help the plants to function at lower temperatures (Mazliak, 1981). For selection of genotypes adapted to low temperature, three factors should be considered, i.e. the temperature range of chilling injury, the capacity for hardening, and the productivity of the genotype.

Within the temperature range between optimum and minimum for growth, at which temperature visible chilling damage first appears, several physiological processes may limit production. So selection for a single process may be successful, only if a genotype has been selected in which the various temperature-dependent processes are, in genetic terms, structurally related to each other. The following procedure for selection of physiological parameters is recommended.

In several tropical species, root function limits crop production at suboptimal temperatures. Shoot growth of cucumber can be largely maintained at low temperature by grafting onto the more chilling-resistant rootstock *Cucurbita ficifolia* (Den Nijs, 1980). Soil temperature may limit growth and leaf area expansion of young tomato plants (Harssema, 1977). In maize, relative growth rate decreases rapidly with root temperature, mainly because of decreased leaf growth. Net assimilation rate is affected only below 12 °C (Grobbelaar, 1963). Uptake of water and ions, and synthesis of cytokinins in the roots may all contribute to arrest of growth in thermophilic plants at low root temperature (Harssema, 1977).

Membrane functioning at low temperature is critical for the physiology of an organism, whether plant or animal. Membrane disfunctioning may lead to reduced enzyme activity and growth at low but non-chilling temperature. Compositional and structural changes in the various membranes of plant cells are essential in adaptation to low temperature. The physical state of the lipid matrix of membranes is significant in the functioning of chilling-sensitive species. Lowering of the temperature leads to a change in physical state of the lipid matrix from liquid-crystalline (with a certain rotational mobility of the fatty acid chain of the lipids and with diffusional mobility of the lipid molecules within the lipid matrix) to crystalline state (lipid molecules arranged in a crystalline order and immobile). The transition between the liquid-crystalline phase and the crystalline phase can be measured by physical methods, such as differential scanning calorimetry (DSC), electron-spin resonance (ESR), and X-ray diffraction. Indirect evidence may be obtained from sudden changes in the activity of membrane-dependent processes with temperature. In various crop plants, a correlation has been observed between the temperature at which lipid-phase separation occurs and the temperature dependence of various membrane-bound enzymic reactions (McMarchie & Raison, 1979; Pike & Berry, 1980; Mazliak, 1981). The response curve of succinate oxidation by mitochondria against temperature of the chilling-sensitive sweet potato, tomato and cucumber shows breaks indicating a change in membrane structure. Such breaks are absent in mitochondria from chilling-resistant sugar beet, potato and cauliflower (Fig. 2; Lyons & Raison, 1970). Water and ion uptake in roots also depend on functioning of the plasma membrane of the endodermal cell, where temperature response seems similar to that for succinate oxidation (Fig. 3; Kuiper, 1964; Doddema, 1978). Hardening of the plant results in a shift of the water uptake curve to lower temperatures. It is likely that such a shift represents a change in membrane-lipid composition. In *Passiflora,* the membrane fluidity of the extracted polar lipids has been determined by ESR; the change in spin-label motion increased from 1 °C for the most chilling-resistant species to 9 °C for the most chilling-sensitive species (Patterson et al., 1978). Electron-spin resonance of phospholipids extracted from cucumber leaves shows clear effects of treatments like grafting onto the chilling-resistant *Cucurbita ficifolia,* hardening the plants at low but non-chilling temperature, soil heating and genotype differentiation on the transition

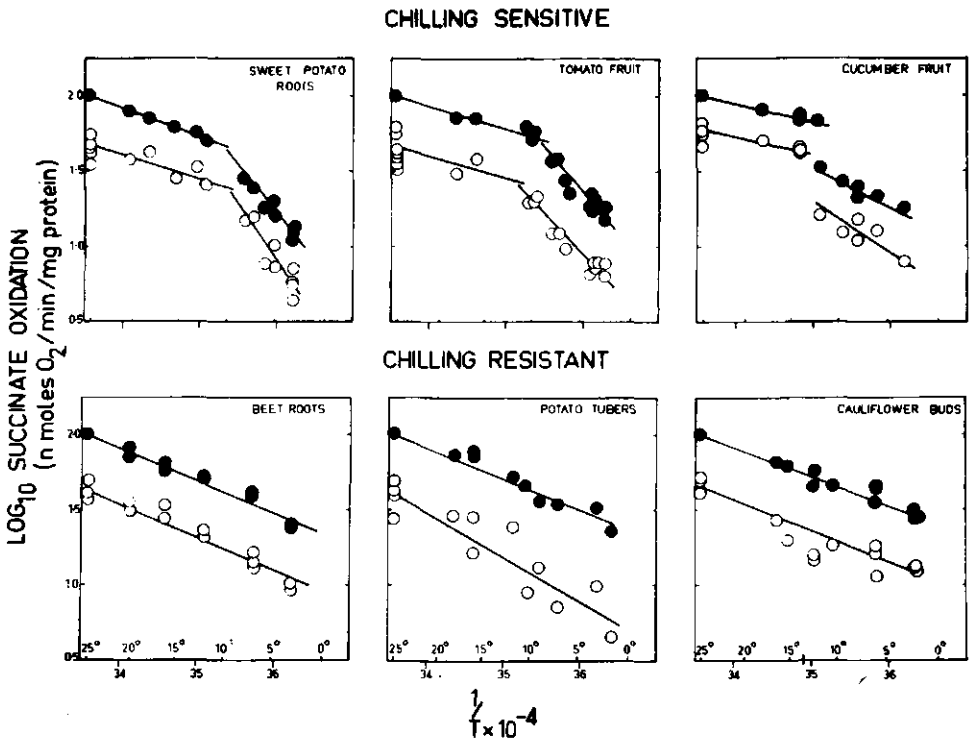

Fig. 2. Effect of temperature upon succinate oxidation by plant mitochondria. ●, State 3 respiration; ○, State 4 respiration. After Lyons & Raison (1970).

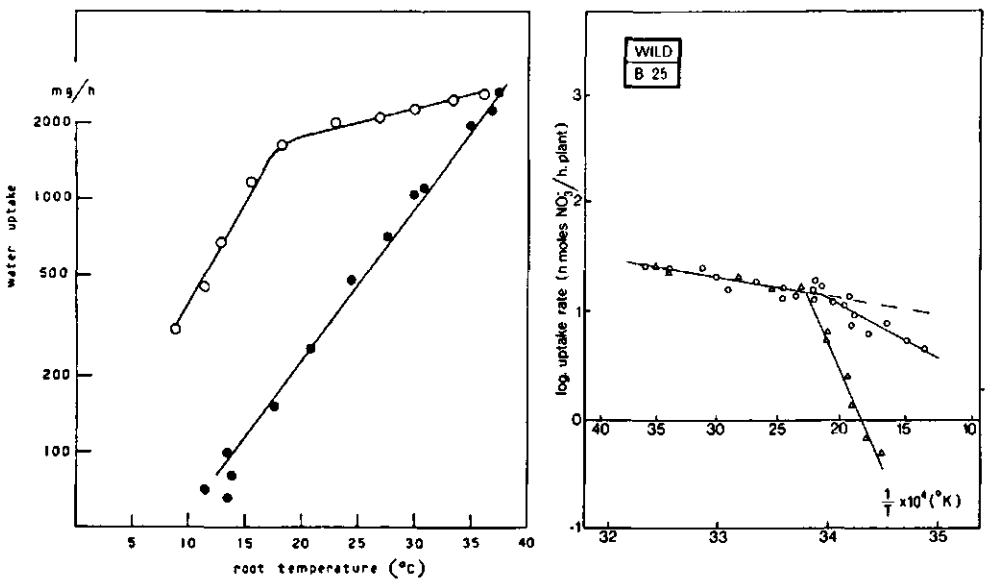

Fig. 3. Effect of root temperature on water uptake by intact *Phaseolus* plants (left: Kuiper, 1964) and on nitrate uptake of *Arabidopsis* (right: Doddema, 1978). Left: ●, grown at 24 °C; ○ grown at 17 °C. Right: ○, wild type; △, mutant B25.

Table 1. Transition temperature between liquid-crystalline and crystalline phase of phospholipids extracted from cucumber leaves. Detection by electron-spin resonance. Cv. Corona, temperature-sensitive genotype; lines 2, 3 and 4, more resistant genotypes. Grafted onto chilling-resistant *C. ficifolia* (Horváth et al., 1982).

Cultivar	Temperature regime (°C)		Other treatment	Phase-transition temperature (°C)
	day	night		
Corona	23	20		12.5
Corona	20	10		4.7
Corona	20	10	heated soil	6.9
Corona	20	10	grafted	<0
Line 2	20	10	heated soil	8.3
Line 3	20	10	heated soil	9.7
Line 4	20	10	heated soil	8.0

temperature of the lipid phase, all in line with the observation in *Passiflora* (Table 1; Horváth et al., 1982). Interestingly, in the more resistant genotypes, a higher transition temperature seems to be correlated with higher relative growth rate at suboptimum temperature. Similar results on temperature adaptation of *Nerium oleander* and fluidity of polar lipids from the chloroplast thylakoid membrane were reported by Raison et al. (1982). It is therefore attractive to replace time-consuming ESR measurement by fluorescence measurements of chlorophyll to monitor temperature-dependent chloroplast functioning in intact leaves, to study heat sensitivity (Schreiber & Berry, 1977) or low-temperature sensitivity (Smillie, 1979; Van Hasselt et al., 1982). Fluorometry is fast and non-destructive. The first results are promising: effects of hardening and differences between genotypes of cucumber are reflected as breaks in the temperature response of fluorescence (Van Hasselt et al., 1982).

Changes in the physical state of membranes are based also upon changes in chemical composition. The liquid-crystalline state of the lipid matrix of membranes may be maintained to lower temperatures by numerous modifications of the chemical composition of the lipid matrix. It is not surprising therefore, that membrane fluidity is rarely simply related to a single lipid parameter like fatty acid composition (e.g. Raison et al., 1982). In several species, hardening to low temperature is correlated with increased unsaturation of lipids. This may be due to increased unsaturation of particular lipids such as phosphatidyl choline (de la Roche et al., 1975; Smolenska & Kuiper, 1977; Willemot et al., 1977). In other species lipids with esters of relatively saturated fatty acids are replaced by other lipids with esters of more unsaturated fatty acids (Kuiper, 1970; Yoshida & Sakai, 1973; Horváth et al., 1980). While fatty acid composition may be determined relatively quickly by gas-liquid chromatography, the phospholipid composition by two-dimensional thin-layer chromatography is more time-consuming. Hardening to low temperature is correlated with a raised content of phosphatidyl choline in cold-resistant species (according to the same sources), as well as in chilling-sensitive species (Horváth et al., 1983). The content of this lipid is also greater in more chilling-resistant genotypes; the ideal bilayer structure of this lipid species (De Gier et al., 1982)

undoubtedly contributes to adequate functioning of plasma membrane and tonoplast, also at suboptimum temperature. Possibly, the simplified measurement of total phospholipids may be useful in selection of suitable genotypes for growth at suboptimal temperatures. A marker for chloroplast functioning may be *trans*-3-hexadecenoic acid, which is found exclusively in phosphatidylglycerol of chloroplasts, linked to the functioning of Photosystem 2 (Duval et al., 1982). In cucumber, this fatty acid may correlate with improved photosynthesis and production of the crop, as determined by genotype and conditions of growth (Horváth et al., 1982). Other lipids such as free sterols and α-tocopherol are very effective in fluidizing membranes (Blok et al., 1977; Pohlmann & Kuiper, 1981). Application of α-tocopherol to young rice seedlings improves fluidity of root-cell membranes, several membrane functions such as enzyme activity (membrane ATPase) and active transport (K^+), and vegetative growth, but only at suboptimum temperature (Tànczos et al., 1982).

Nutrient stress and salinity

In many wild species the strategy of the plant to cope with nutrient stress is a slow growth rate associated with a metabolism adapted to the low-nutrient environment. Often the slow growth rate is genetically fixed. Increased supply of nutrients results in luxury consumption without accelerated growth (Stuart Chapin, 1980). In fast-growing species the nutrients absorbed are utilized in metabolism and incorporated in new plant material. Metabolism of such plants when exposed to nutrient stress is often disturbed; growth is severely reduced or plants even die. An intermediate strategy is required for selection of productive genotypes of crops able to withstand limited periods of severe or moderate nutrient stress and with a flexible response to nutrient stress.

As an example, the reaction of genetically uniform lines of *Plantago major* L. to nutrient stress will be discussed (Fig. 4; Kuiper, 1983). Within the genus, *Plantago major* is a fast-growing species (Kuiper & Kuiper, 1979). The typical ssp. *major* continues growth at the same rate for seven days after transfer from full nutrient supply to a fifty-fold dilution (Fig. 4A). On the other hand, plants transferred from diluted solution to full nutrient supply cannot take advantage of the adequate nutrient supply by accelerated growth. Plants of the typical ssp. *pleiosperma* (Fig. 4B) show a quick and flexible response of shoot growth upon change in nutrition in both directions. This flexible response does not occur at the expense of growth, since plants of ssp. *pleiosperma* show faster growth than those of ssp. *major*. As a consequence the shoot/root ratio is unaffected by mineral nutrition in ssp. *major* and a flexible response is observed in ssp. *pleiosperma* (Fig. 4). In *Pinus resinata,* balanced growth between roots and shoots is realized by episodic fluctuations in leaf and root growth together with depressions in net assimilation. This species shows adaptive behaviour for shoot and root growth to different growth temperatures (Drew, 1982). Growth reduction by nutrient stress may be caused by reduced net assimilation rate, by morphogenetic changes or by a combination of the two. The contribution of each of these factors to growth reduction under nutrient stress depends on plant species (genotype) and ion species. Under P stress, the morphogenetic component is in general dominant (Loneragan & Asher, 1967) and the effect on net assimilation rate asserts itself only under severe P stress (Andreeva & Persanov, 1970; Veerkamp et al., 1980). Maintenance of relative growth rate during an extended

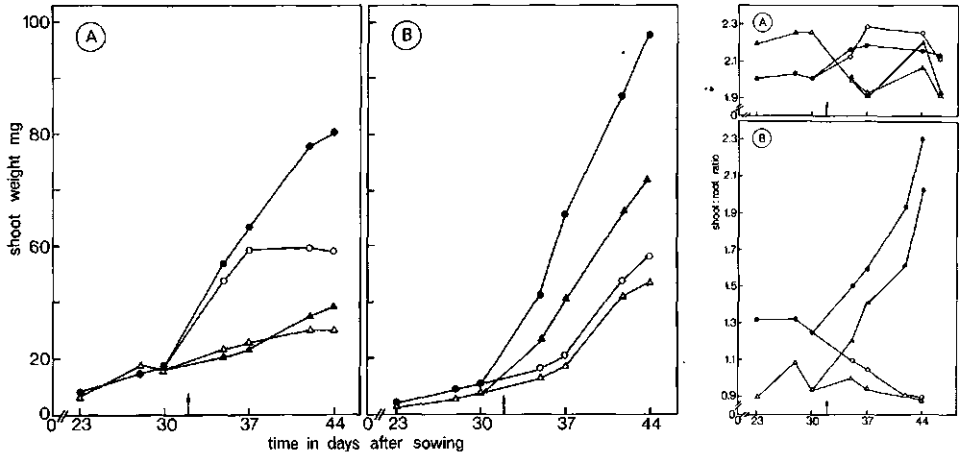

Fig. 4. Dry matter accumulation in shoots and shoot-to-root ratio of two inbred lines of *Plantago major*. A: ssp. *major*. B: *ssp. pleiosperma*. ●, full nutrients; ○, full nutrients transferred to nutrient stress (arrow indicates time of transfer); △, nutrient stress; ▲, nutrient stress, transferred to full nutrients. After Kuiper (1983).

period of P stress is only possible if shoot growth is relatively low in comparison with root growth, as shown in comparative studies on species of nutrient-rich and nutrient-poor habitats (*Carex,* Veerkamp et al., 1980; *Agrostis,* Clarkson, 1967).

Under K stress, both net assimilation rate and shoot development may be affected, depending upon the species. Net assimilation rate of *Carex* spp. from nutrient-poor habitats was less affected by K stress than that of species from nutrient-rich habitats, because of a more efficient K utilization, expressed as net assimilation rate relative to uptake/content of K^+ and better storage of K^+ in vacuoles (Veerkamp et al., 1982). Investment of energy in shoot growth was equally affected in all *Carex* spp. studied. In this group of plants different mechanisms have clearly been developed to cope with K^+ and P stress. Differentiation in the uptake mechanism is more clearly expressed in adaptation to K^+ stress than to P stress. Adaptation to P stress seems to be more regulated by formative effects, whereas in adaptation to K^+ stress effects of net assimilation rate and uptake mechanism should be taken into account. Uptake of K^+ by plant roots is regulated by the K^+ content of the roots in such a way that decreased K^+ content leads to increased affinity of the roots for K^+ (*Lolium perenne* and *Trifolium repens,* Dunlop et al., 1979; barley, Glass, 1976, 1978; *Carex,* Veerkamp & Kuiper, 1982; sunflower, Petterson & Jensen, 1979). In barley, genetic differentiation in K^+ uptake has been demonstrated (Glass & Perley, 1980); in *Carex* the flexible response to K^+ stress is more clearly expressed in eutrophic species than in oligotrophic species (Fig. 5).

Uptake of nutrients often requires metabolic energy. Ion-stimulated membrane ATP-ases act as carriers for ion transport as part of the regulation of ionic concentration in cytoplasm of the plant cell. In young oat roots without any nutrient stress, (metabolic) energy-dependent Mg^{2+} uptake requires one ATP molecule per ion of magnesium absorbed (Table 2; Stok et al., 1981). Uptake by plants grown under nutrient stress

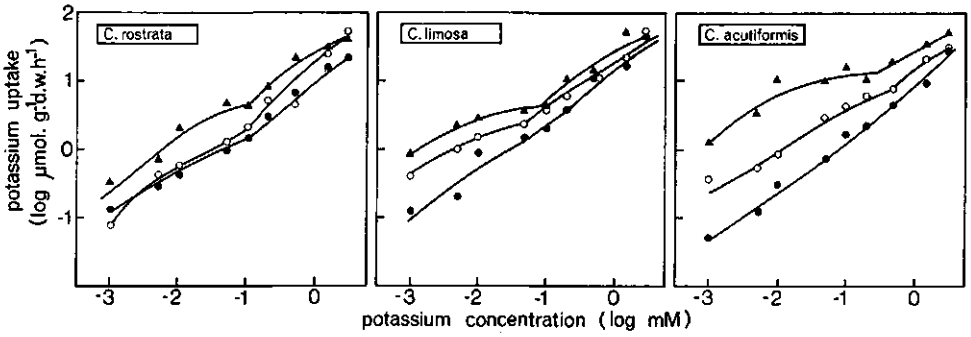

Fig. 5. Effect of potassium concentration on potassium uptake (both axes log scale) of *Carex* species from nutrient-poor environment (*C. rostrata, C. limosa*) and nutrient-rich environment (*C. acutiformis*). ●, 2 days starvation for K^+; ○, 14 days; ▲, 28 days. After Veerkamp & Kuiper (1982).

requires more energy, and ageing of the roots also tends to increase the energy demand for uptake and translocation.

Energy metabolism, including respiration, may limit productivity of crop plants under suboptimal conditions. Glycolysis and Krebs cycle are essential for formation of compounds necessary for cell growth and maintenance. In addition, glycolysis and Krebs cycle produce a small amount of ATP and of electron donors, which are subsequently oxidized in the electron-transport system, producing ATP. Part of the electron transport in mitochondria occurs through non-phosphorylating alternative pathway, acting as an energy overflow under conditions of limited ATP demand by the cell (Lambers, 1980, 1982). For the breeder it is of interest to try to select for genotypes with only a small proportion of the alternative pathway in total respiration. Observations on negative correlation between dark respiration and growth of *Lolium perenne* lines (Wilson, 1982) indeed suggest that reduction in the alternative pathway of respiration may improve growth. However Lambers (1980) presents several examples for the physiological significance of the alternative pathway for developmental changes in plants and for an adaptive

Table 2. ATP requirement for energy-dependent Mg^{2+} uptake by roots of oat seedlings. Calculation is based upon the ratio of Mg^{2+}-stimulated ATP hydrolysis of microsomal fractions of the roots (plasma membrane + tonoplast) and (Phase 2) Mg^{2+} uptake of intact roots. Transfer of plants to the other nutrient condition on day 5 (Stok et al., 1981).

Nutrient regime	Age of seedlings (days)	Number ratio of ATP to Mg^{2+}
Nutrient stress	5	3.5
Nutrient stress	6	8.7
Nutrient stress → full nutrients	7	2.6
Full nutrients	5	1.0
Full nutrients	7	1.7
Full nutrient → nutrient stress	7	5.5

flexible response to introduced environmental stress. When *Plantago coronopus* was exposed to salinity, the activity of the alternative pathway was reduced and the amount of sugar thus saved, was stoichiometrically transformed into sorbitol. This component functions as an osmoregulator in this halophyte (Lambers et al., 1981). The contribution of the alternative pathway to respiration is small

- in plants grown on NO_3 in the nutrient solution instead of NH_4,
- in pea plants which develop active *Rhizobium* root nodules,
- in plants which develop storage organs like taproots,
- in plants under conditions of a low supply of photosynthates to the roots.

In conclusion, the alternative pathway allows a flexible response of the plant, when exposed to suboptimal conditions (Lambers, 1980). The comparative study on inbred lines of *Plantago major* (Kuiper, 1983) shows that growth is positively correlated with the (phosphorylating) cytochrome pathway of respiration. The genotype with the highest flexibility (and highest productivity with full nutrients) shows the quickest reduction in the alternative pathway when the plants are transferred from nutrient stress to full nutrients: an adaptive response, since more energy (ATP) is needed after transfer for accelerated growth.

Control of permeability of root cell membranes is essential for prevention of passive leakage of ions from the roots back to the soil. Free sterols, as membrane compounds, are important in this respect. They are characteristic for plasma membrane and tonoplast. Through their 'condensing' effect, they are effective in restricting passive ion transport. The different sterols differ in degree of control of permeability of plant-cell membranes, cholesterol being most effective and sitosterol being least effective. In experiments with *Plantago* species roots were much higher in cholesterol and lower in sitosterol content in plants under nutrient stress (Fig. 6; Kuiper & Kuiper, 1978). Clearly, sterol metabolism is a factor in adaptive regulation of ion permeability in plant roots under nutrient stress. Membrane lipids are possible parameters for breeding for salt tolerance in crop plants (Table 3; Kuiper, 1984).

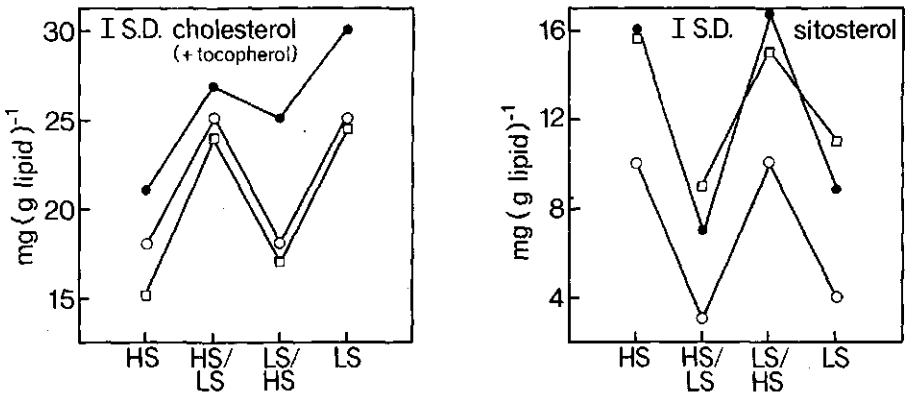

Fig. 6. Replacement of sitosterol by cholesterol in roots of *Plantago*. ●, *P. major* ssp. *major*; ○, *P. major* ssp. *pleiosperma*; □, *P. media*. HS, full nutrients; HS-LS, full nutrients, transferred to nutrient stress; LS-HS, nutrient stress, transferred to full nutrients; LS, nutrient stress. After Kuiper & Kuiper (1978).

Table 3. Genetic differentiation for salinity and for phenotypic plasticity, induced by salt stress, expressed as lipid metabolism and composition. For references: Kuiper (1984).

Lipid class	Genetic differentiation for salinity	Genetic differentiation for phenotypic plasticity induced by salt stress
Sterols	sugar beet, grape, yeast	*Plantago, Citrus*, yeast
Phospholipids	grape	apparent effects due to morphogenetic changes
Galactolipids	grape	sugar beet
Sulpholipid	*Plantago*	sugar beet, *Plantago*

Drought

As for nutrient stress and low temperature, various physiological parameters for selection of a desirable flexible response of the crop to drought may be useful. Special attention should be given to the role of stomata in drought tolerance and its regulation by abscisic acid (Mansfield & Wilson, 1981). Also the effect of drought on photorespiration seems to be an important parameter (Keys & Whittingham, 1981).

Genetic basis for a flexible response of plants to stress factors

Flexible responses of physiological processes in plants may be modified by selection and such a modification is probably independent of flexible responses of other physiological processes (Bradshaw, 1965). Specific genes would determine the flexibility (plasticity) of each physiological reaction. Flexible responses may be observed in heterozygous as well as homozygous plants. The genetically uniform *Plantago major* lines present genetic differentiation for several flexible physiological reactions in plants exposed to nutrient stress. Two extreme and two intermediate genotypes may be distinguished in the material used. The flexible response of Ca^{2+} and Mg^{2+}-stimulated ATPase activity in the (tonoplast + plasma membrane) fraction of the roots (Kuiper, 1982), growth response, and respiration (cytochrome and alternative pathway; Kuiper, 1983) were strongly correlated to each other, indicating a high degree of structural genetic control of the flexible response of the physiological processes studied to nutrient stress. In this species plasticity of the studied parameters was directly related to number of seeds per capsule, a simple parameter for further selection for a flexible response.

A high phenotypic (physiological) plasticity (Fig. 1; Curve E and F) may be expected in self-incompatible species (SI) and to a lesser extent in self-compatible species (SC). The self-incompatible *Plantago* species are characterized by a high plasticity (*P. maritima*, C. Blom, private communication; *P. lanceolata*, Antonovics & Primack, 1982), higher than in *P. major*. Investigation is needed whether an SI population of a given species consists of more flexible individual plants than an SC population. In *Thymus vulgaris* (SC), populations with a large proportion of male-sterile plants are observed in unstable environments, with fluctuating nutrient conditions. Male-sterile plants are lacking in stable environments (Dommée et al., 1978). Heterozygosity will be higher in a population with a large proportion of male-sterile plants and a possible significance of male sterility as a factor in phenotypic plasticity under suboptimal conditions needs investigation (Van Damme, 1983).

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Phenotypic plasticity and breeding for tolerance to Dutch stress

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Summary

Dutch stress is a combination of suboptimal growth conditions in greenhouses in winter and variation for these conditions during the productive phase of the plants. The relevance of physiological plasticity and ways of improving it by breeding are discussed. The importance is indicated of searching for limiting factors in growth under stress and of development of quick large-scale techniques for selection.

Descriptors : physiological plasticity, breeding, Dutch stress, limiting processes, genetic variation, selection techniques

Introduction

Phenotypic plasticity (Bradshaw, 1965) is the ability of a genotype to vary its expression in different environments. It is species-specific and population-specific, under genetic control, and can be modified by selection. Plants with a high plasticity have particular morphological or physiological characters whose expression can be altered by environmental factors. High-plasticity genotypes are likely to be found in unstable habitats.

The agricultural interest of the phenomenon is based on the necessity of minimum alterations in output of products in a highly fluctuating environment. Crops and varieties may differ in their ability to respond to optimum and suboptimum conditions. Plasticity of physiological characters is required for stability in productivity, i.e. constancy of genotypes in different environments (including stressful ones) for economically valuable characters, based on buffering in an individual or population.

Dutch stress

Energy of natural radiation in Dutch winters is far from optimum for cultivation in greenhouses. Decades ago, breeders started to develop varieties with the ability to tolerate this stress. For more than 10 years, rising energy prices have incited breeders to adapt crops to an environment with not only little radiant energy but also low temperatures. The relevance of stress for radiant energy and temperature is not the same for the different crops although both play a role in every crop. The aim of breeding research for

more efficient use of energy is to decrease the quotient of energy expenditure to amount of product.

The most important greenhouse crops in the Netherlands (e.g. tomato and cucumber, rose and carnation, with a total value close to 2 milliard guilders) are kept for one year or more and the plants are exposed in their productive phase to extremely variable environmental conditions. Irradiance (areic energy rate) varies from about 25 W/m^2 to 125 W/m^2, day temperatures from 19 to 35 °C and night temperatures from 14 to 20 °C (for tomato) and 13 to 35 °C and 6 to 20 °C (for carnation), daylength from 8 to 16 h. Such variation requires crops with high plasticity to allow growers to profit from year-round crops. The additional advantage of improved plasticity is that it gives the greenhouse grower a choice: current production at lower temperatures or higher production at current temperatures.

Dutch stress and the feasibility of a plastic response

High plasticity is achieved by removing factors of the genotype that are limiting under stress. The breeder can – at least partly – achieve this without knowing the limiting factors. A genetically variable population is put under stress, the best performers are selected and used for the production of the next generation and this cycle is repeated.

This approach has been rather successful, as is demonstrated by the success of breeding for earliness in different greenhouse crops, which is mostly an adaptation to low radiant energy. For example, early production of tomato and cucumber has increased considerably and lettuce breeding has allowed year-round production.

Breeding could be made more efficient, however, if for each stress situation the most limiting factors could be worked on directly. Illustrations of such a direct approach are well known (e.g. Wilson, 1982; Wilson & Jones, 1982), but much more physiological research will be needed to indicate the most relevant limiting properties in different crops. Such research is hampered by lack of isogenic material and by many unclarified aspects of plant metabolism, for example, interactions of source and sink. Further, methods have to be developed to measure separate processes in large-scale experiments, so that genetic variation can be exploited. Selection may concern properties of the intact plant in its various developmental stages or of single cells. Selection may concern biomass production or one process in it. Effective selection of cells for less limiting levels of significant processes is still an ideal, but developments are promising (Zelitch, 1980; 1982). In a joint project of IVT and the Vrije Universiteit van Amsterdam, these developments will be applied in a research on adaptation of tomato to low light intensity and low temperatures.

An example of a limiting factor at low temperatures, which is being studied at IVT, is root functioning in cucumber (Den Nijs, 1980). As in tomato, rooting and shooting capacities are studied separately in grafting experiments. Grafting cucumber onto *Cucurbita ficifolia* shows the relative inability of the cucumber root system to function at low temperature (Table 1) and indicates that selection for improved rooting capacity is useful.

If the root system fails through membrane disfunction, development of techniques for quick and large-scale measurement of the physical state of membranes deserves high priority. This is even more so if membrane capacities play a central role in limiting the

Table 1. Average number of fruits and total fruit weight (kg) per plant in the first 5 weeks of harvest of 20 lines and cultivars of cucumber, in relation to treatment and temperature.

Treatment	Temperature (°C)		Fruit	
	day	night	av. number	total wt (kg)
Ungrafted	23	20	5.2	1.4
Ungrafted	20	15	2.7	0.8
Grafted	20	15	6.7	1.8
Ungrafted	20	10	2.1	0.7
Grafted	20	10	5.7	1.6

plasticity of different processes. If root failure causes membrane disfunction in the plant, it would be more useful to search for the causal factor and select root genotypes for maximum range of the liquid-crystalline state of membranes.

At IVT, we concluded that it would be possible to improve certain physiological characters in order to increase productivity under stress. Enough genetic variation occurs for several processes, and its genetic control is sufficient for exploitation. However despite extensive knowledge of physiological processes, the breeder's request is still for the most relevant plant properties which can be rapidly measured in the different crops and under the various stress factors.

For tomato, a group of IVT workers are trying to exploit the genetic variation found for various physiological characters of growth in weak light and at low temperature, by independent selection and controlled recombination of physiological capacities. This work is concentrating on net photosynthesis, dark respiration, diffusive resistance and root capacities. It aims at revealing the agricultural relevance of processes and at development of improved genotypes. It is very laborious through lack of simple and rapid routine methods for determining physiological expression.

Conclusion

Genetic variation occurs that can be exploited by breeding for adaptation of crops to Dutch stress conditions and for improvement of a plastic plant response. Development of simple and quick routine techniques of measurement is urgently needed as well as a better insight of which physiological processes need improvement in order to increase production under stress.

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Efficient use of energy

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Summary

Data of the literature and own experimental results have proved, that leaf area ratio and specific leaf area are important growth components in adaptation to low temperature. But for efficient use of energy many factors are involved and a comprehensive selection can only be achieved under conditions which will prevail during production.

Descriptors : growth components, adaptation, temperature, irradiance, selection

The interesting lecture of Kuiper (1984) and some literature on this topic (e.g. Hogenboom, 1981; Van de Dijk, 1982) raise the crucial question whether there is any master reaction or criterion to allow selection for growth efficiency or adaptation to a specific stress factor. For some processes such as photosynthesis or nitrate reductase activity, good correlations have been found in some trials but not always. The explanation is that many processes are involved in growth or stress. The process that is limiting may depend on the preceding or present environment as well as on genotype.

Trials at Hannover on effect of light (Sylvania Cool-White VHO 215 W+48% (irradiance) incandescant lamps) and temperature on growth of cucumber transplants illustrate the topic (Fig. 1). We analysed the main growth components as follows:

Relative growth rate	=	leaf area ratio		×	net assimilation rate	
R	=	LAR		×	NAR	
R	=	LWR (leaf weight ratio)	× SLA (specific leaf area)	×	1/LA	× dW/dt

In winter with poor light (irradiance 33 W m^{-2}, 300-2500 nm) for 9 h and temperatures of 18, 21 and 24 °C, net assimilation rate showed no significant temperature reaction. This means that even at 18 °C, which is about the minimum temperature for growth, full photosynthetic capacity was reached (Lorenz, 1980).

In other experiments with lettuce grown at 18 W m^{-2} (300-2500 nm), full photosynthesis was attained at 6 °C and after growth in very low temperature even at 2 °C (Lorenz & Wiebe, 1980). Similar results were obtained with radish (Krug & Lorenz, unpublished)

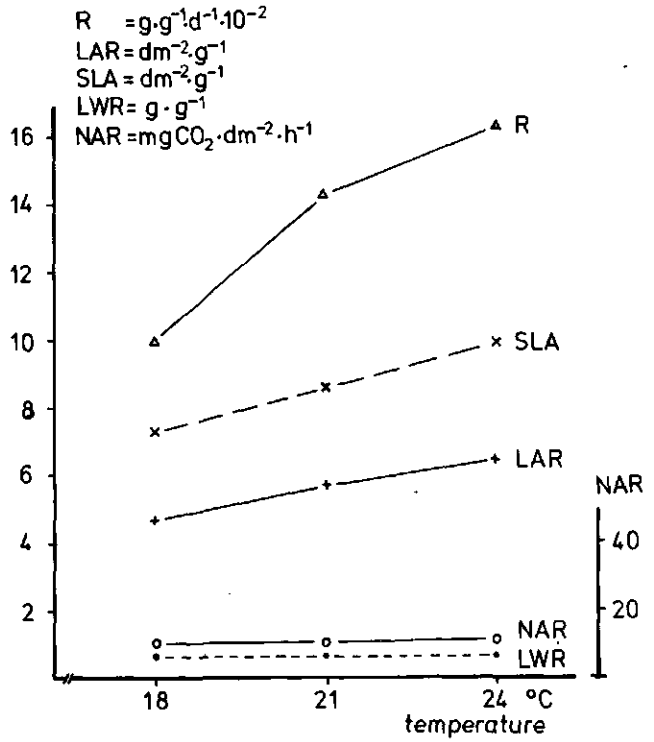

Figure 1. Temperature reactions of growth components of cucumber plants (5-leaf-stage), grown at a low irradiance level (33 W m^{-2}). After Lorenz (1980).
Relative growth rate: R (g g^{-1} d^{-1} 10^{-2})
Specific leaf area: SLA (dm^2 g^{-1})
Leaf area ratio: LAR (dm^2 g^{-1})
Net assimilation rate: NAR (mg CO_2 dm^{-2} h^{-1})
Leaf weight ratio: LWR (g g^{-1})

and maize (Miedema & Sinnaeve, 1980). These figures indicate that net assimilation rate of our crops is adapted to rather low temperatures and its reaction to low temperature will not be an overridingly limiting factor in winter.

A strong temperature reaction, however, is obvious for leaf area ratio, caused mainly by specific leaf area (area divided by mass). If this character decreases with low temperature, light perception is reduced and may become a limiting factor. This finding is confirmed by Dutch selections for low temperature adaptation with lettuce (Smeets, 1974; Groenwold, 1983) and cucumber (Den Nijs, 1980), which are characterized by high leaf area ratio. (Specific leaf area was not measured.)

Apart from specific leaf area or leaf area ratio, the feasibility of screening for efficient use of energy by biochemical, physiological or morphological criteria seems to be rather small. Moreover we have to consider that in greenhouses plants are exposed for only a limited time to very low temperatures. So reactions to medium and high temperature and other adaptations for efficient use of energy in protected culture should not be neglected. Breeding for reaction curves like b and c in Figure 2 will always give good progress

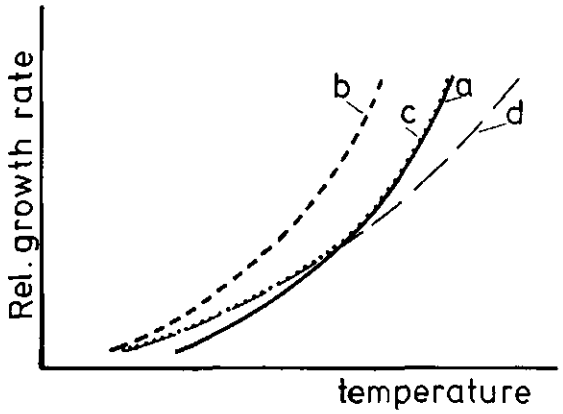

Fig. 2. Possible adaptations of relative growth rates to low temperature (hypothetical presentation).

Table 1. Desirable adaptations for efficient use of energy in protected culture.

1. Efficient use of low temperature
– tolerance (chilling injury)
– high growth rates
– bolting resistance for vernalization-sensitive plants
2. Efficient use of medium and high temperatures
– high growth rate with high irradiance (spring–summer)
– high growth rate with high temperature through heating (low irradiance in winter)
3. Efficient use of fluctuating temperature
– utilization of short-term favourable conditions
– buffering of short-term unfavourable conditions
– adaptation to high diurnal temperature amplitudes
– adaptation to higher night than day temperature (thermal screens)

compared with the standard curve a, but a reaction curve like d will be a questionable improvement.

Some desirable adaptations that may contribute to more efficient use of energy are listed in the Table 1. This diversity makes clear that comprehensive selection can only be achieved under 'natural' conditions, that means in environments prevailing in practice.

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Effects of low temperature on photosynthesis and growth characteristics

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Efficient use of energy is related to a high rate of crop photosynthesis over the whole growing season. Plants under most field conditions conserve far less than 5% of the solar energy they intercept.

Several environmental and endogenous factors are responsible for this low rate. Low temperatures often limit growth in temperate climates. Genetic differences in photosynthetic capacity at 3 °C were found for altitudinal ecotypes of white clover (Mächler & Nösberger, 1977). More important than reduction in photosynthesis with decreasing temperature were the negative effects on rate of leaf appearance, stolon characteristics and partitioning of dry matter (Boller & Nösberger, 1983). Genetic variation for several leaf and stolon characters existed among genotypes from the same site and could be exploited by plant selection. Growth rates in temperate climates could probably be increased by higher meristematic activity and a higher rate of photosynthesis at low temperatures.

At low temperature, the partitioning of photosynthetic products is influenced even at the chloroplast level. Temperature seems to affect the ratio between CO_2 fixation and assimilate export, which is related to transfer of inorganic phosphate (P_i) across the chloroplast membrane. Mächler measured CO_2 fixation and P_i transfer in wheat chloroplasts at 5 °C and 25 °C in our laboratory. Transfer of P_i showed a greater response to temperature than CO_2 fixation, so that carbohydrates accumulated in the chloroplasts. These observations with chloroplasts and observations with leaves suggest that measurements of total non-structural carbohydrates or of water-soluble sugars may be useful in screening for response of translocation to low temperature.

Descriptors: low temperature, photosynthesis, growth characteristics, chloroplast, CO_2 fixation, transfer of assimilates

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Breeding at the cell level

Manipulation of gametophytic populations

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Summary

A large part of the structural genes which are expressed in the sporophyte are expressed also in the pollen. For these, it is possible to select desired qualities in either portion of the life cycle. However, pollen exhibits two characteristics which make it a particularly promising system for selection programs: microscopic size and haploid genotypes. The first of these means that selection can be practiced within extremely large populations. With haploidy, selection can reach, not only rare recessive alleles, but even adaptations which involve too many loci to be selected for in the sporophyte. The utility of pollen selection and screening for lines which are resistant to cold, heavy metals, or phytotoxins has already been demonstrated. Further evidence indicates that even complex qualities such as yield are responsive to pollen selection. Both the theory and the methods of pollen selection will be discussed.

Descriptors: pollen, gametophyte, haploids, selection, cold resistance, salt tolerance

Dramatic advances in industrial microbiology and the speed with which pathogenic bacteria develop resistance to antibiotics are both due to characteristics of the organism which are subjected to selection: they are haploid and they exist in extremely large populations. The thesis that I would like to present to you today is that, by exhibiting these same characteristics, pollen confers upon the flowering plants a comparable selective system. The effects of this system is not limited to the gametophytic portion of the life cycle, however. A significant portion of the genome appears to be expressed in both phases of the life cycle, and thus, sphorophytic qualities which are controlled by such genes will respond to haploid, as well as diploid, selection.

The significance of pollen selection

The potential influence of pollen selection depends on the relative effects of three separate groups of genes. These three are: genes which are transcribed and translated only in the sporophyte, only in the gametophyte, or in both the sporophyte and the gametophyte.

For genes which are transcribed and translated only in the sporophyte, there will obviously be no genetically determined differences between pollen grains from a single plant. For such genes, gametophytic selection itself, not to mention its effect upon the sporophyte, is nonexistent. However, such genes may have a significant effect upon the

gametophytic generation, influencing both the quantity (Stephens, 1956; Done & Macer, 1978) and the quality (Johnson & Mulcahy, 1978; Yâmada & Murakami, 1983) of pollen. These effects, especially in open pollinated populations, are known to be highly significant, allowing one sporophytic genotype to outcompete another through pollen competition. However, with sporophytically determined pollen qualities, pollen competition is merely another manifestation of sporophytic competition and will not be considered further in this paper.

Genes which are transcribed and translated only in the gametophyte will have no direct influence upon the sporophytic portion of the life cycle, aside from the effects of linkage between these and genes which are expressed in the sporophyte. However, gametophytically limited genes may have significant effects upon pollen. This is almost certainly demonstrated in inbred lines, where it may explain the unexpected competitive superiority of self pollen, versus other pollen types (see Jones, 1928; Pfahler, 1967; Hornby & Li, 1975; Johnson & Mulcahy, 1978; Currah, 1983; and discussion below).

The final group of genetic factors, those which are transcribed and translated in both portions of the life cycle, could play a special role in adaptive processes, particularly in the angiosperms. Only the angiosperms possess a style, a structure which, although it undoubtedly evolved in response to other selective pressures, provides that group of plants with greatly intensified pollen tube selection (Mulcahy, 1979). This is no trivial advantage since pollen, as a haploid system, allows the expression (Nelson, 1957) and selection (Pfahler, 1983) of even rare recessive genes. Furthermore, with haploidy, adaptive polygenic complexes can be assembled far more easily than in diploid systems (Zamir, 1981). Additionally, these two features of haploidy are further enhanced in pollen by extremely large population sizes. Each of these three advantages, inherent in pollen selection, are available also to all sporophytic genes which are expressed in both haploid and diploid phases of the angiosperm life cycle.

Before considering the consequences of genes which are expressed in both phases of the life cycle, it is necessary to ask some fundamental questions. Are genes expressed in the pollen? If so, how common are such genes, and are these same genes ever expressed also in the sporophyte? These questions are far from trivial since Haldane (1932), Heslop-Harrison (1979), and even one school of mendelian genetics have interpreted the facts as indicating that much of the genome is suppressed in the gametophytic portion of the life cycle. Furthermore, those genes which are expressed in the gametophyte are often presumed to be different from those which are expressed in the sporophyte. Haldane (1932), recognizing the great potential of haploid selection within enormously large populations of pollen genotypes, said, 'Clearly a higher plant is at the mercy of its pollen grains. A gene which greatly accelerates pollen tube growth will spread through a species even if it causes moderately disadvantageous changes in the adult plant.' It was perhaps because of this concern that he went on to describe the pollen grain as '. . . the suppressed haploid generation of the higher plants . . . [which] has a physiology of its own, influenced by special genes.' This implied that relatively few genes are expressed in the pollen and also that any exceptions are, 'special', that is, not expressed in the sporophyte.

More recently, Heslop-Harrison (1979), although recognizing that there is some gene expression in pollen, conveys some concepts quite similar to those of Haldane (1932). 'But any contemplation of the differences between sporophyte and gametophyte in

angiosperms leads to a further conclusion, namely that much the greater part of the total genome of the species must be repressed in the gametophytes. They are not only simpler morphologically, but also biochemically: they lack photosynthetic capacity; and, in the main, they do not produce lignins, pigments, alkaloids and a host of other secondary plant products.' Further on, he suggests that '. . . one might expect selection to ensure that the test of the genome imposed by the haploid generation would be kept to a minimum . . .'

Another consideration that cannot be neglected is the concept of random fertilization, an observation upon which much of mendelian genetics is based. If, for example, the products of a heterozygous pollen source (*Aa*) do not deviate from an expected 1 : 1 ratio, it might logically be considered that this proves that the segregating locus has no effect upon pollen tube growth rates. Such an interpretation could be far from correct. Failure to detect a deviation from expectations does not prove that none exists. An examination of current literature indicates that few studies of genetic segregation include as many as 10000 observations. This number of observations would be sufficient to allow a 90% chance of detecting a true ratio of 52:48. Smaller samples, or smaller deviations from mendelian expectations, would show no significant deviation from expectations. This suggests that, individual genes which have small effects upon gametophytic (or sporophytic) vigor would, in most cases, be assumed to have no effect at all (Mulcahy & Kaplan, 1979). The cumulative effect of many such genes could, however, be quite substantial although unexpressed in segregation ratios. The question of gene expression in pollen is thus left undecided by these considerations.

Gene expression in pollen

Several lines of evidence suggest that genes are transcribed, translated, and exposed to selection in the pollen. Well known examples include the genes *waxy* and *alcohol dehydrogenase* in *Zea mays* and gametophytic self-incompatibility in many angiosperm families. However, it might be argued that the *waxy* and *Adh* loci are exceptions to a general rule of gametophytic genetic silence, and perhaps gametophytic self-incompatibility is a 'special' case which requires gametophytic genetic expression.

Thus it may be much more informative to consider examples which involve fairly complex metabolic systems. These are likely to encompass large numbers of loci and can hardly be considered to be either aberrations or special cases. For pollen, viability, germinability, and growth rate may thus be appropriate systems to examine for evidence of post-meiotic genetic transcription and translation.

In *Solanum tuberosum,* for example, two types of diploid pollen grains may be produced by means of different meiotic abnormalities (Simon & Peloquin, 1976). The first of these abnormalities, 'parallel spindles', restores diploidy, after the second meiotic division, by combining non-sister chromatids. This results in diploid microspores which are highly heterozygous. A second meiotic abnormality restores the diploid condition by inducing a premature cytokinesis. The resulting diploid microspores contain two sister chromatids and are thus largely homozygous. Fortunately, neither of these meiotic abnormalities shows complete penetrance and thus, plants exhibiting them, produce both diploid, and, as a built in control, haploid, pollen grains. This allows for a comparison of haploid and diploid pollen grains. It was found that heterozygous diploid

pollen (resulting from parallel spindles) exhibited a higher percentage of germination and more resistance to storage than did haploid pollen produced within the same anthers. However, in the same comparison, homozygous diploid pollen grains (from premature cytokinesis) failed to exhibit corresponding superiorities toward their haploid counterparts. These results are almost certainly expressions of heterozygous superiority in pollen and, since these would be impossible without the transcription, translation, and expression of genes within the pollen, this study provides strong evidence for gene expression in the pollen.

A similar set of examples also deals with growth rates in pollen and the effect that selection has upon them. Recalling Johannsen's classic study (1909) which demonstrated that, without genetic variation, there can be no response to selection, we must accept the corollary that, whenever we observe a genetic response to selection, there must have been genetically determined variation. Consider what happens if, within inbred lines, equal quantities of self and non-self pollen are mixed together and applied to stigmatic surfaces. This has been done with *Zea mays* (Jones, 1928); *Lycopersicon esculentum* (Hornby & Li, 1975); and *Allium cepa* (Currah, 1983). In nearly every case, self pollen accomplishes significantly more fertilizations than would be expected on the basis of its representation in the pollen mixture. However, when mixtures of self and other pollen were made up for F_1 hybrids of *Zea mays,* there is generally no predictable outcome of such competition. This difference between inbred and F_1 lines could be due to the fact that, with inbred lines, the pollen produced represents a highly selected population, the product of many generations of inbreeding. In each of these generations, excessive quantities of pollen must have created intense selection for rapid passage through that particular stylar environment. When this pollen type is mixed with that from a different inbred line, the highly selected self pollen generally wins. In F_1 hybrids, both self and other pollen is unselected and then the outcome of pollen competition is unpredictable. This hypothesis was eventually substantiated by the demonstration that, in *Zea mays,* self pollen exhibits, with each generation of inbreeding, an increased competitive ability against a standard pollen line (Johnson et al., 1976). The ability of pollen from individual plants to respond to selection for increased growth rate, as Johannsen would affirm, stands as good evidence for genetic expression in the pollen.

A final example of gene expression in the pollen is based on microelectrophoresis, pioneered by Ruchel et al., (1974) and modified by Mulcahy et al., (1981). This method allows the analysis of enzymes from single pollen grains. If the pollen grains under examination come from a single flower and fit into segregating electrophoretic patterns, the most logical explanation for this result is genetic segregation. More precisely, it is proof positive of post-meiotic gene expression. Figure 1 illustrates results obtained by acid phosphatase stains on zymograms of single pollen grains. All pollen grains were obtained from a single flower of the interspecific hybrid *Cucurbita moschata* × *C. palmeri.* Since each of these pollen grains came from a single flower, the bands marked by arrows very likely indicate the expression of a segregating locus. In a recent study of 13 different interspecific *Cucurbita* hybrids, it was found that 31 out of 70 clearly defined bands (=44%) were segregating (Miller & Mulcahy, 1983). Since this method detects gametophytic gene expression only at loci which are heterozygous, even this figure must be an underestimation. Apparently, at least for acid phosphatases, a very substantial fraction of the gene products found in pollen are the result of genes which are expressed

Fig. 1. Zymograms of acid phosphatases from single pollen grains of the F_1 hybrid, *Cucurbita moschata* × *C. palmeri*

in the pollen. Three sets of evidence thus indicate that there is significant gene expression in the pollen.

Are gametophytically expressed genes expressed also in the sporophyte?

Since many genes are expressed in the gametophyte, we must ask if these same genes are expressed also in the sporophyte. This is an essential consideration for, without substantial overlap between the two portions of the life cycle, gametophytic selection can modify the sporophyte only through genetic linkage. The possibility of nonoverlapping gene expression is clearly illustrated by the phenotype, *waxy*, well studied in *Zea mays*, but found also in several other taxa (Ericksson, 1969). Pollen which carries the wild type allele, *Wx*, contains both amylose and amylopectin whereas pollen carrying the mutant allele *wx* lacks amylose. (The *waxy* phenotype is expressed also in the endosperm, but that is irrelevant to the present discussion.) Three different genotypes are possible for the sporophyte, *Wx/Wx*, *Wx/wx*, and *wx/wx*, but all three are apparently identical, containing both amylose and amylopectin (Akatsuka & Nelson, 1969; Nelson & Tsai, 1964). If most of the pollen expressed genes were like *waxy*, that is, not expressed in the sporophyte, then gametophytic selection, for all its potential, would modify only the gametophytic portion of the life cycle.

Two studies have so far presented quantitative evidence on the extent to which genes which are expressed in the sporophyte are expressed also in the gametophyte. Meinke (1982) isolated 6 developmental mutants which were expressed in the embryos of *Arabidopsis thaliana*. Two of these showed disturbed ratios in segregating pollen.

Obviously these mutants were expressed in both portions of the life cycle. In another study, Tanksley et al. (1981) surveyed electrophoretic variants in nine different enzyme systems of *Lycopersicon esculentum*. These included a total of 30 different isozymes. Of these, 18 (=60%), were shown to be transcribed and translated also in the pollen. Conversely, 18 of 19 (=95%) pollen isozymes were found to be translated and transcribed also in the sporophyte. If this degree of overlap between the gametophytic and sporophytic portions of the life cycle is observed in other taxa, then the possibility of using gametophytic selection to modify the sporophyte will be seen to be very good.

Demonstrations of pollen selection

A series of studies has demonstrated that pollen selection can have a significant effect upon the resultant sporophytic generation. In each of the cases described below, pollen has been obtained from a single plant, and thus each pollen grain has the same sporophytic origin. All observed effects are therefore due to gametophytic selection. Furthermore, associated changes in the resultant sporophytes must indicate that gametophytically expressed genes, selected in the pollen, are expressed also in the sporophyte.

Ter-Avanesian (1949, 1978) was the first to select among pollen grains from single plants and did so by varying the quantity of pollen applied to the stigmas of *Gossypium hirsutum, Vigna catjang,* and *Triticum aestivum*. When very limited quantities of pollen were used, the variation in the resultant sporophytic generation was greater than when excessive quantities of pollen were used. These important observations are presumably explained by Meinke's (1982) finding that sporophytic developmental abnormalities tend to be eliminated by gametophytic competition. Working with *Lycopersicon esculentum,* Matthews (see Lewis, 1954) found similar reductions in sporophytic variation (leaf length, plant height, flower number, and fruit weight) when excessive quantities of pollen from F_1 plants were used in backcrosses. Again, using the technique of varied pollen quantities, Mulcahy et al. (1975, 1978) found that, in *Petunia hybrida,* plants produced under conditions of intense pollen competition, grew significantly faster than did other plants. In order to avoid the variation in seed size and number which sometime results from varying pollen quantity, a similar study was performed with *Dianthus chinensis*. This species has elongated stigmatic surfaces and, following Correns' (1928) method, the intensity of pollen competition was varied by modifying the distance pollen tubes have to travel. Pollinating the stylar tip maximizes the opportunity for rapidly growing pollen tubes to surpass those growing more slowly, and thus gametophytic selection will be relatively intense. Applying the same quantity of pollen to stylar bases will allow less opportunity for fast and slow growing genotypes to be separated and the intensity of selection is reduced. Also this study showed that intense gametophytic selection resulted in sporophytes which germinated more rapidly, and seedlings which grew more vigorously than did those produced with reduced gametophytic competition. Subsequently it was demonstrated also that the sporophytes produced under conditions of intense gametophytic competition are competitively superior against *Lolium perenne* than are other plants (McKenna & Mulcahy, 1983).

Other studies have demonstrated that, in *Zea mays,* speed of pollen tube growth is positively correlated with several important aspects of the resultant sporophytic generation: weight of seedling dry matter, ear weight, kernel weight per plant, and mean kernel

weight (Ottaviano et al., 1980). In *Lycopersicon,* gametophytic selection is an effective means of obtaining genotypes for tolerance to cold (Zamir et al., 1981; Zamir & Vallejos, 1983) and salt (Sacher et al., 1983). The multiple demonstrations that pollen selection can modify the sporophytic generation suggest that a significant portion of the genome must be expressed in both phases of the life cycle. It remains only for us to put this system to work. The angiosperms already have.

Conclusion

Some genes are expressed only in the gametophyte, others only in the sporophyte, and still others are expressed in both phases of the life cycle. Determining the proportions of the genotype which fit into each of these three categories is an important task, one presently being undertaken by several different groups. As for the effects of pollen selection, by using pollen from a single flower or plant, it is clearly possible to separate sporophytic and gametophytic effects upon pollen phenotype. Furthermore, in the several species so far tested, gametophytic selection is seen to have a highly significant effect upon the resultant sporophytes. This fact indicates that, however many or few genes are shared by the gametophyte and the sporophyte, the sharing is apparently sufficient to provide the angiosperms with powerful selective systems once thought to exist only in microorganisms. Very likely, although there are obvious differences between the morphologies, physiologies, and secondary metabolites for gametophytes and sporophytes, there must be enough aspects of primary metabolic processes that are essential to, and expressed in, both to provide a correlation between their respective metabolisms. Plant breeders may thus find that pollen selection provides a useful system for manipulating the genome of crop species. It has certainly been highly adaptive in natural populations. With this in mind, I would suggest that, Haldane (1932), instead of believing that 'A higher plant is at the mercy of its pollen grains,' would perhaps have been closer to the truth if he had realized that, 'A higher plant owes a great deal to its pollen grains.'

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Selection of gametes

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Summary

Selection mechanisms which function as sieves for gametes in the fertilization process of higher plants are: the incompatibility barrier, incongruity, gone competition and selective fertilization. Insight in the mechanisms is the condition for the possibility of manipulation of gamete selection in breeding.

Descriptors : selection, gamete, self-incompatibility, incongruity, gone competition, selective fertilization

In higher plants, the male gametes (encased as they are in the cells of the pollen grain and the pollen tube) do not randomly reach their ultimate goal, the female cells, during the process of double fertilization. There are several selection mechanisms, some of them highly selective. They all function as sieves, which gives the breeder instruments for the manipulation of the gametic populations. These mechanisms are under definite genetic control.

One of these mechanisms is sexual self-incompatibility. The many investigations on the incompatibility mechanisms in higher plants may be valuable from a cytological and biochemical point of view, and have provided insight into gene-controlled developmental processes. However, they lack a basic genetic prerequisite: they were executed on plant material without preceding analysis of the *S* allele garment. However a multitude of careful genetic studies have elucidated that this highly effective system of gamete selection is genetically controlled by a finite number of loci with multiple alleles. At times, though, this clear genetic system seems strongly linked to environmentally sensitive gene groups that result in varying pseudo-self-compatibility, of which a breeder wishing to exploit self-incompatibility must be aware.

Clear distinction is needed between incompatibility, formerly called self-sterility, in both its expression as gametophytic and sporophytic determination based on the fitting together of sameness, and incongruity, a mechanism which acts to prevent interspecific mixing by lack of fit of physiological mechanisms.

Pollen selection during maturation in the anther, earlier called gone competition and demonstrated in *Oenothera,* depends on genetic factors, for there is clear evidence that differences in rate of development are controlled by the genome as well as the plasmon. But the physiological condition of the sporophyte, which strongly influences competi-

tion, sometimes hides the different starting conditions of pollen grains of the same anther. Besides gone competition, we can recognize selective fertilization, which results in deviations from random combinations between pollen tubes and ovules. This has been studied, for instance, in *Oenothera,* tobacco, pea, cotton and *Salpiglossis.* The lack of explanation for the biochemical mechanisms of this selection through chemotropism should not allow us to question the phenomenon as such.

As for gamete selection by coupled gametophytic-sporophytic genetic activity, the plant breeder has probably been exploiting this phenomenon of selecting 'genes' expressed in both the gametophytic and sporophytic generation for some time when he has bred his crops in the environment in which they will be asked to produce, for instance if it be an area of drought or saline soil.

Gametophytic transcription and translation probably occurs before pollen is shed, since lethal doses of ionizing radiation to mature pollen seems not to affect either normal incompatibility or compatibility in the lily style, as measured by pollen-tube growth, or competitive ability of *Nicotiana* pollen to accomplish fertilization.

A fruitful area of interspecific crossing is application of the sieve of incongruity barriers and ways of handling it. Besides male sterility, incongruity can be used in hybrid seed production. There are two strategies to overcome incongruity. One is to lower the barrier capacity by inactivation of the interspecific barrier genes, e.g. removal of substances by which the gene is 'recognized' by physical or chemical treatments. The other way is elevation of the penetration capacity of pollen also by physical treatment. Even more efficient, at least in some cases, is application of mentor pollen. Mentor pollen can even be replaced by the purified pollen wall proteins from congruent pollen, or by treatment of the pollen with solvents, thus rendering it congruent, or by treatment of stigmas with solvent, to make them receptive for pollen tubes.

All this means that 'sieve pores' responsible for reproductive selection in angiosperm gametes can be widened and thus manipulated. Sexually asymmetric fertility selection and selective fusion of the produced female and male gametes, including partial self-fertilization and prezygotic incompatibility are ways of selecting gametes; we should also not forget postzygotic viability selection either.

Gametophytic selection and haplo-diploid gene expression in maize

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Summary

Gametophytic selection was studied in maize (*Zea mays*), by using growth of pollen tubes as selection criterion. Male-gametophytic competitive ability was clearly affected by selection and for several characters positively correlated responses were observed in the sporophyte, indicating genetic overlap. By means of electrophoretic analysis of polymeric enzymes it could be revealed that some enzymic systems show haplo-diploid transcription and others are pollen specific.

Descriptors: maize, *Zea mays*, gametophytic selection, correlated response, sporophytic traits, polymeric enzymes, haplo-diploid transcription

In maize, gametophytic genetic variation due to haploid gene expression is known with regard to gametophytic competitive ability controlled by single genes (*ga* factors), for a few biochemical traits (*wx, ae, Adh*) and for pollen-tube growth in vitro (reviews: Pfahler, 1975; Ottaviano & Sari Gorla, 1979; Ottaviano, 1983). Here we have summarized some of our results that are relevant to the paper by Mulcahy (1984).

The first set of data concerns gametophytic selection. For this type of study, maize has a very suitable structure: silk length varies according to the position of the flower on the ear, increasing from the top to the base. This structure allows selection of gametophytes according to silk length, because the probability of fertilization of the most competitive gametophytes is to be expected to increase with the distance to be covered by the pollen tube in the stylar tissues.

This criterion can be used to test whether genetic variation is expressed in a population of pollen from a single heterozygous plant and is consequently due to haploid genetic control. First, heterozygous plants are selfed and two samples of kernels are chosen from each of the resulting ears, one from the base and one from the apex. The procedure is repeated for several generations, so that two groups of lines are produced: 'base lines', resulting from high-intensity gametophytic selection and 'apex lines' from low-intensity selection. Finally the lines are evaluated for male-gametophytic competitive ability. Correlated responses in the sporophyte show if component traits of the gametophytic competitive ability have the same genetic basis as sporophytic traits (genetic overlap).

The selection method was applied to a synthetic population (Long Ear Synthetic: BSLE). In the first experiment (Ottaviano et al., 1982) open-pollinated ears were used in the first generation, while the subsequent experiments were strictly based on selfing and

within-plant selection. Although the procedure used in the first experiment does not allow testing for haploid gene expression, interesting results were obtained. Gametophytic competitive ability was clearly affected by selection and positively correlated responses were observed for sporophytic traits. All these characters (seedling dry weight, root length and kernel weight) are expressions of growth, which may indicate that they are affected by the same basic physiological processes controlling pollen-tube growth.

The experiments in course are strictly based on within-plant gametophytic selection. One is carried out by means of selfing and the other is based on gametophytic selection according to a recurrent selection scheme. At present, we have only the results concerning the gametophytic traits of the experiment based on selfing. Altogether 30 S_3 lines (15 per group) have been tested for gametophytic competitive ability measured as proportion of fertilization in mixed pollinations. Base lines were more competitive than the apex ones (40% against 30%) This shows that response to selection is due to genes transcribed in the gametophytic phase.

The second set of data concerns gene expression studied by means of electrophoretic analysis of polymeric enzymes. The simplest situation is found in dimeric enzymes: when the enzyme is found in the pollen and it is under haploid control, the electrophoretic pattern of the enzyme produced in the pollen of a heterozygous plant reveals only the two parental bands, i.e., only homodimers can be formed because the gene is transcribed in the phase in which only one allele is present. However if control is sporophytic, the electrophoretic pattern is the same as that revealed by sporophytic tissues. We have also developed a cytogenetic method based on partially diploid pollen which allows it to be ascertained when the absence of heterodimers in the pollen is not due either to specific physiological conditions of the gametophytic cells or to specific systems of control of gene expression: the presence of the two alleles in the pollen should produce heterodimers also in the gametophyte (Frova et al., 1983).

By means of this technique we are studying several enzymic systems. The results obtained to date reveal a complex and interesting situation. There are genes (*Got-1, Adh-1 Phi*) showing haplo-diploid transcription, others such as *β-glu* that are pollen specific, i.e. the enzymic activity in the two phases is controlled by different genes.

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Female gametophyte - a source of modified plants

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Summary

Compared to our knowledge about the development and structure of the male gametophyte and culture in vitro of anthers or pollen, much less is known about the female gametophytes. More studies should be carried out to analyse the formation of the female gametophyte in vivo, under various environmental conditions, and its functioning after the pollen have germinated on the stigma. It also should be tried to find ways to modify the process of megasporogenesis, to obtain deviating types of gametophyte, for instance by means of culture in vitro of female gametophytes in various stages of development.

Descriptors: gametophyte, megasporogenesis, microsporogenesis, fertilization, culture in vitro, abnormal development

We possess a lot of information about the process of microsporogenesis, the structure of pollen grains, germination of pollen on the stigma and the growth of pollen tubes through the style. Thanks to many investigations, it has been shown that male gametophytes, especially the immature ones, are potentially able to develop into embryos and whole plants. This capability of the male gametophyte in culture in vitro and the expression of its genes into the wholly developed plants have been demonstrated among species of various taxa. Much less work has been done on the formation of the female gametophytes, especially with the intention of modifying the process of megasporogenesis in order to obtain gametophytes deviating from the normal type of development. When analysing the processes of megasporogenesis and microsporogenesis in vivo, we must not forget that in many, perhaps all plants, some abnormalities occur, which result in the formation of female and male gametophytes of different ploidy level. These events have been thoroughly analysed, for instance in *Solanum tuberosum*. In some microspores of *Secale cereale* and *S. montanum* we found 14 chromosomes, being equal to the somatic number. Additionally, a detailed analysis of 2-celled and 3-celled pollen grains revealed that some pollen grains deviate from the normal type of development (e.g. 2 equal nuclei instead of the generative and vegetative cells). Can we say that similar or other abnormalities occur also during megasporogenesis? The lack of data makes it impossible to give the answer.

It is a common opinion that the process of megasporogenesis proceeds more stably than the process of microsporogenesis. We do not know if through culture in vitro (by applying various chemical as well as physical factors) the former process can be induced

to deviate from normal development. It can be assumed that the development of the female gametophyte would proceed in a quite different manner during the culture of ovules or ovaries in vitro. If so, then the possibility of the formation of various female gametophytes, especially of different ploidy levels, could be of great value in some plant breeding programs.

Pollen selection can modify the sporophytic generation. It cannot be overlooked, however, that the female gametophyte plays the same role, especially the egg cell. This cell, due to the rich population of plastids and mitochondria, plays a significant role in the expression of genes in the sporophyte. It was clearly shown by Mulcahy (1984) that the pollination of stigmas with many pollen grains has some effect on various morphological features of plants. We do not possess sufficient data to explain this phenomenon. Very little is known about germination of pollen on stigmas and the functioning of the female gametophyte at that moment, especially the role of synergids. One should remember the interesting observations made on *Gossypium* by Jensen and his collaborators at the University of Berkeley in California concerning the ultrastructural reorientation of organelles of only one synergid immediately after pollen grains touched the stigma. What factor(s) evoke such enormous transformation of only one synergid (that one into which the pollen tube will enter) long before the tube reaches this cell? We still cannot answer this question. We do not know what kind of chemical compounds, important for the development of the zygote and the embryo, are brought by the pollen tube, which bursts inside the embryo sac. No information is available about test-tube pollination of ovules. Here, the direct pollination of ovules – in some instances – allows pollen-tube growth, which is the reverse of the situation for pollination in vivo.

The technique in vitro should be applied more intensively to the culture of female gametophytes in various stages of development. More efforts should also be undertaken to analyse development of female gametophytes in vivo under various environmental conditions. Only then it will be possible to obtain modified plants, not only through pollen grains, but also through the female gametophyte.

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In vitro techniques for germplasm storage

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Summary

In vitro methods for the storage of plant germplasm have a number of potential applications. As well as storing valuable laboratory stock cultures, they may resolve some persistent problems in plant genetic conservation. Cultures may be stored by growth limitation or cryopreservation. The former permits storage for ca. one year without transfer; the latter can, theoretically offer storage for indefinite periods. At the present state of development of methodology, cryopreservation can be recommended strongly for the storage of cell cultures and, on an empirical basis, for all other types of culture. Growth limitation can only be recommended for shoot cultures. In many cases, recovery after storage, including by cryopreservation occurs rapidly. The origin of recovery growth in shoot and other organised cultures subjected to cryopreservation gives some cause for concern since it may not always involve continuation of normal meristem growth and callusing may be prevalent. However, in studies where the phenotypic and genotypic stability of stored cultures have been tested, results are reassuring. There is a little evidence for genotypic variation in response to storage by growth limitation. However, adequately stringent tests have yet to be carried out here or with cryopreservation. Storage techniques are in use at some International Agricultural Research Centres and in tissue culture laboratories. Further implementation is to be encouraged. Information projects currently under way should help promote the methodology.

Descriptors: cold storage, conservation, cryopreservation, freeze-preservation, gene bank, genetic resources, germplasm, storage in vitro, tissue culture

Introduction – germplasm storage needs

In vitro methods are assuming considerable importance in several aspects of plant breeding and propagation. In consequence of this increased activity, attention is now turning to the need for good management of the cultures involved. They must be maintained economically, securely and under conditions conducive to genetic stability. Further, the maintenance of the cultures should be compatible with ongoing experimental work as well as medium and long-term storage needs.

Two other areas can be identified where storage in vitro is of relevance, namely biotechnology and genetic conservation. Whereas the former is of limited interest to the plant breeder, no-one would deny the need for the conservation of the broad genetic base of crop plants. Although it may be possible to generate new, valuable genotypes in culture to meet many agricultural requirements, it would be impossible to recreate the vast spectrum of genes existing in crops currently under cultivation and in their wild

relatives.

The importance of genetic conservation of crop plants has been recognised by the scientific community and in 1974, the International Board for Plant Genetic Resources (IBPGR) was created to encourage and initiate appropriate work. Much of the effort expended to date has been involved with the collection of germplasm and the establishment of seed gene banks. However, valuable as seed storage may be, it cannot meet all requirements. Two important classes of crop fall outside the scope of seed storage by conventional methods, namely those which are propagated vegetatively and those which produce short-lived ('recalcitrant') seeds (see Withers, 1982a; Withers & Williams, 1982).

The genetic resources of these problem crops are normally conserved in plantations which are geographically restricted, and open to environmental, pathological and political threats. Additionally, the distribution of germplasm from plantations poses far more problems than does the distribution of seeds. The consequent need for a new approach has come to the attention of several bodies including the IBPGR (see Withers & Williams, 1982), who, in 1982 convened an expert advisory group on culture in vitro.

Storage techniques

Variables Culture in vitro of plants involves a number of systems differing widely in their morphology, and their behaviour and requirements in culture. Consequently, it is impossible to make simple, categorical statements about appropriate storage techniques. Furthermore, the scientist will have differing demands depending upon his specific research activities, culture systems in use and available storage facilities. To accommodate these variables the following sections will deal firstly with general approaches to storage and then address each culture system in turn. (Details of methodology can be found in references: Withers, 1980a, 1980b, 1982b, 1983, 1984a, 1984b and the literature cited therein.)

Problems of maintenance under standard conditions For a number of years, scientists working with in vitro culture have maintained their experimental material in media and under environmental conditions conductive to relatively rapid growth. However, maintenance of large numbers of cultures not required for immediate use is wasteful both of time and material resources. Regular transfer of cultures increases the risk of loss by accident or microbial contamination.

More serious, however, than practical or economic constraints is the risk of unacceptable levels of genetic instability in cultures maintained in relatively rapid growth. Somaclonal variation is most marked in cultures which pass through an unorganised phase and least marked in systems involving non-adventitious regeneration from complex explants (Bright et al., 1982). If storage methods are to meet high standards of stability, this type of variation must be minimised.

Thus, some degree of control over growth is demanded. In the designing of storage methods, two approaches have been taken: either the reduction of the growth rate by various means or the suspension of growth by freezing (cryopreservation).

Growth limitation By reducing the temperature at which cultures are maintained, the rate of growth may be reduced considerably. A drop of 10 to 20 °C from the optimum

would appear to be generally feasible although there are exceptions wherein sensitivity to cold dictates a less severe reduction. Maintenance at a reduced temperature has only been explored for static cultures growing on semi-sólid medium. This restricts its application.

A second, potentially more widely applicable approach involves supplementing the culture medium with retardant chemicals. These include osmotically active compounds, natural hormonal inhibitors and synthetic inhibitors. Some less widely investigated approaches (see Withers, 1980b, 1982b) involving modification of the oxygen content of the atmosphere to which cultures are exposed, overlay with mineral oil, and dessication must await further development.

As a generalisation, growth limitation can extend the normal subculturing interval to one or even two years thus offering a real saving in costs, time and effort. However, indefinite periods of storage cannot be expected and it should not be overlooked that the maintenance of stable, carefully controlled conditions of, for example, cold storage may be technically demanding and relatively costly. Consequently, a means of completely suspending growth is very attractive.

Cryopreservation At the temperature of liquid nitrogen (−196 °C) all metabolic reactions cease, therefore indefinite storage is a real possibility, as long as the storage temperature is maintained. It should be emphasized that cultures are unlikely to survive rewarming to temperatures in the region of −80 °C for more than a brief period of time and uncontrolled warming, however brief, to the point of thawing of the intracellular contents (above ca. −40 °C) will almost certainly be lethal.

The cryopreservation procedure commences with the very important period of pregrowth before storage. In some cases, pregrowth handling may simply be a matter of choosing the most appropriate stage of growth, although in others, special subculturing routines and/or media are involved. Then follows treatment with cryoprotectant chemicals. The most common is dimethylsulphoxide (DMSO), used alone as for some organised systems, or, for disorganised systems (protoplasts, cells, callus), in combination with other compounds. Cryoprotectants have, conventionally, been chilled before slow addition to the specimen. Evidence is accumulating that this may not always be necessary (Finkle & Ulrich, 1982; Hauptmann & Widholm, 1982) but it is a wise precaution avoiding plasmolysis shock in sensitive cultures. Exposure to cryoprotectants for about 1 h is necessary to permit adequate uptake.

Transfer to the storage temperature should be carried out at a rate of cooling which minimises damage by ice crystallization or over-dehydration. In slow cooling, ice first forms extracellularly, creating a vapour pressure deficit which draws water from the cell. This reduces the amount of water remaining to form potentially damaging ice. However, if too much dehydration has occurred, the consequent shrinkage of the protoplast and its bounding membrane, the plasmalemma, will predispose the cell to injury later during thawing and rehydration.

Exceptionally, an alternative mechanism for avoiding freezing injury may be adopted. This involves freezing so rapidly that the ice crystals which form intracellularly are of a sufficiently small size to be inoccuous. In such cases it is essential that thawing also be carried out very rapidly to prevent ice recrystallization (reformation at a larger size). Slowly frozen material should not, in theory, always need to be thawed rapidly. How-

ever, in practice, it usually is beneficial to so do, indicating that even freeze-dehydrated cells may contain potentially damaging ice.

No special equipment is required to carry out ultra-rapid freezing, but for slow (or stepwise) freezing it is necessary to control the rate of cooling by the use of appropriate apparatus. This may be purpose-built and programmable or improvised (see Withers, 1984a; Withers & King, 1980). For storage, it is essential to use a purpose-built liquid nitrogen refrigerator.

After freezing and thawing the specimen is returned to culture. Washing is sometimes carried out, but there is evidence for injury as a result in cell cultures, for example (Withers & King, 1979). Generally, it does not appear to be essential. Recovery is usually carried out on semi-solid medium.

Storage procedures

Sources of recommendations The procedures which follow are based upon studies of the storage of the species and culture systems listed in Table 1. With the exception of cryopreservation of cell cultures, it is not possible to make very exact recommendations. However, sufficient information is available to give broad guidelines for the development of cryopreservation methods for all types of culture. These are given in Table 2 and supported by detail in the text, where general advice is also given on the application of growth limitation to shoot cultures.

Protoplasts It is not possible to maintain protoplasts in limited growth since wall regeneration and cell division transform their unique structure; they become cell or callus cultures. However, cryopreservation is a realistic possibility. Takeuchi et al. (1982) who have studied this in some detail have successfully preserved protoplasts of five species using the method given in Table 2. Wall regeneration, callus formation and (where appropriate) morphogenesis were demonstrated in recovering cultures.

Cell cultures Storage by growth limitation has not been developed successfully for cell cultures, probably due to neglect, although cryopreservation has been the subject of investigations for some ten years. During this time a routine, widely applicable, method has emerged (see Table 2; Withers & King, 1980). This has been successful with at least 19 species (see Table 1) which differ widely both in morphology and taxonomy.

For unresponsive species, a number of alternative treatments may be tried. These include: pregrowth in the presence of DMSO, proline or sorbitol; use of DMSO alone as cryoprotectant, exclusion of sucrose or proline from the cryoprotectant mixture; recovery in a liquid medium appropriate for growth of cells at low density and supplemented with the pregrowth additive; post-thaw washing with warm medium (see Cella et al., 1982; Finkle & Ulrich, 1982; Kartha et al., 1982a; Maddox et al., 1983; Withers & King, 1979, 1980).

Callus cultures The application of growth limitation to callus cultures has received some attention. Unfortunately, however, none of the studies have been extensive and no generally applicable method can be recommended.

A limited number of reports of cryopreservation indicate its general feasibility. The

Table 1. A list of the species which have been preserved successfully as cultures in vitro. Original references may be traced through Withers, 1980b, 1983, 1984b, Withers & Williams, 1982. Cell cultures marked with * are in storage at the Friedrich Miescher-Institut, Basel and were cryopreserved using the method of Withers & King, 1980. In all cases 'cryopreservation' refers to storage in liquid nitrogen, not at higher temperatures. (Key: P = protoplast, C = cell, Ca = callus, SE = somatic embryo, ZE = zygotic embryo, S = shoot culture or shoot-tip, PE = pollen embryo, A = anther).

Species	Storage method		Species	Storage method	
	growth limitation	cryopreservation		growth limitation	cryopreservation
Acer pseudoplatanus		C*	*Nicotiana tabacum*	Ca S	A PE C*
Arachis hypogaea	S		*Onobrychis viciifolia*		C*
Asparagus officinalis	S		*Oryza sativa*		C* Ca ZE
Atropa belladonna	C	A PE	*Panax ginseng*		C
Berberis dictyophylla		C*	*Pennisetum americanum*		C*
Beta vulgaris	S		*Petunia hybrida*		A
Brassica napus		C S	*Phleum* sp.	S	
Bromus inermis		P	*Phoenix dactylifera*		Ca
Capsicum annuum	Ca	C	*Pisum sativum*		S
Catharanthus roseus		C*	*Populus euramericana*		Ca
Cicer arietinum		S	*Populus* sp.		C
Corydalis sempervirens		C*	*Primula obconica*		A
Dactylis sp.	S		*Pseudotsuga menziesii*		C
Datura innoxia		C	*Pyrus* sp.		S
Datura stramonium		C	*Rhazya orientalis*		C*
Daucus carota	Ca SE	P C* SE	*Rhazya stricta*		C*
Dianthus caryophyllus		C S	*Ribes* sp.		S
Dioscorea deltoides		C	*Rosa* 'Paul's Scarlet'		C*
Elaeis guineensis		ZE	*Rubus* sp.		S
Festuca spp.		S	*Saccharum* spp.		C Ca
Fragaria spp.	S	S	*Sambucus racemosa*		C
Glaucium flavum		C*	*Solanum etuberosum*		S
Glycine max		P C*	*Solanum goniocalyx*		S
Gossypium spp.		A Ca	*Solanum melongena*		C*
Grossularia sp.		S	*Solanum stenotomum*	S	
Hordeum vulgare		ZE	*Solanum tuberosum*	S	S
Hyoscyamus muticus		C*	*Solanum* × *juzepczuki*	S	
Ipomoea batatas	S		*Solanum* × *curtibolum*	S	
Lactuca sativa		S	*Solanum* × chaucha	S	
Lolium multiflorum	S		*Sorghum bicolor*		C*
Lolium sp.		S	*Trifolium repens*	S	
Lycopersicon esculentum		S ZE	*Triticum aestivum*		ZE
Malus domestica	S	S	*Triticum monococcum*		C*
Manihot esculenta	S	S	*Vitis rupestris*	S	
Manihot utilissima		S	*Vitis* spp.	S	
Medicago sativa		C	*Zea mays*		C* Ca ZE
Nicotiana sylvestris		C			

Table 2. Recommended cryopreservation procedures.

Protoplast	Harvest cells in exponential growth. Isolate protoplasts. Cryoprotect with 5% DMSO + 10% glucose. Dispense into ampoules. Freeze at 1 to 2 °C min^{-1} to -35 °C. Transfer to liquid nitrogen. Thaw rapidly in warm water at +40 °C. Wash with liquid medium. Recover on semi-solid medium.
Cell	Pregrow on standard medium or medium supplemented with 6 % mannitol for 4 to 7 days. Harvest during active growth. Cryoprotect with 0.5 M DMSO + 0.5 M glycerol + 1 M sucrose or proline. Dispense into ampoules. Freeze at 1 °C min^{-1} to -35 °C. Hold at -35 °C for 40 min. Transfer to liquid nitrogen. Thaw rapidly in warm water at +40 °C. Recover on semi-solid medium. (No washing).
Callus	Select actively growing callus. Cryoprotect with 10 % DMSO + 10 % polyethylene glycol + 8 % glucose. Transfer to ampoule. Freeze at 1 °C min^{-1} to -40 °C. Transfer to liquid nitrogen. Thaw rapidly in warm water at +40 °C. Wash with liquid medium. Recover on semi-solid medium.
Embryo	Select small embryos. Cryoprotect with 10 to 15 % DMSO. Enclose in foil envelope or ampoule without surrounding cryoprotectant solution. Freeze at 1 °C min^{-1} to -40 °C. Transfer to liquid nitrogen. Thaw slowly in air. Recover on semi-solid medium. (No washing). (Methods recommended for cells and shoot-tips may also be suitable for zygotic embryos).
Shoot-tip	a. Dissect shoot apex including 2 to 4 leaf primordia. Pregrow for 2 days on a filter paper soaked in liquid medium (standard or containing 5 % DMSO). Cryoprotect with 10 to 15 % DMSO. Collect on the end of a hypodermic needle and plunge into liquid nitrogen. Thaw rapidly in liquid medium at +25 °C. Recover on semi-solid medium. b. Dissect, pregrow and cryoprotect as above. Transfer to ampoule. Freeze at 1 °C min^{-1} to -40 °C. Transfer to liquid nitrogen. Thaw rapidly in warm water at +40 °C. Wash in liquid medium. Recover on semi-solid medium.

most detailed study has been carried out by Finkle et al. (1979), Finkle & Ulrich (1979, 1982) and Tisserat et al. (1981) who have preserved callus of four species (see Table 1). The procedure used, summarised in Table 2, can be seen to have a similar basis to that recommended for cells and protoplasts. Further refinement might be brought about by cold-hardening the cultures, or applying the cryopreservation method recommended below for embryo cultures.

Embryos Under this heading are included zygotic embryos and secondary (asexual/somatic) embryos or embryoids. The former are of interest as inoculum material particularly in cereal work, and the latter as a route to mass propagation. The only report of growth limitation is that of Jones (1974) who demonstrated survival for a period of one year of an embryogenic culture of *Daucus carota* prepared by desiccation. The culture contained very early stage embryos which could be induced to undergo 'germination' by the addition of a sucrose solution. Callus treated similarly failed to survive.

Cryopreservation has been more successful in that embryoids of *Daucus carota* can be stored and recovered at a very high rate (see 'Survival levels' below). The slow freezing method successful with cell cultures of this species is, surprisingly, entirely unsuitable. However, a simple modification as detailed in Table 2 leads to success.

Preliminary findings (Withers, 1982c) with immature cereal embryos suggest consid-

erable flexibility in freezing requirements, although the rate of freezing used can influence the pattern of recovery growth. Relatively rapid freezing favours organized development by germination and relatively slow freezing favours callusing of the scutellum.

Bajaj (1977a) has used a slow freezing method to cryopreserve pollen embryos of *Atropa belladonna* and *Nicotiana tabacum*. Success was very limited, a decline in freeze tolerance with increase in embryo size and stage of development being very marked. Work by Bajaj, directed towards the cryopreservation of anthers of a number of species has shown some limited success but no single method has emerged for recommendation (see Withers, 1982b).

Shoot cultures and shoot-tips Cultured shoots are used widely in clonal propagation studies and are attractive to the conservationist since they are thought to be intrinsically more stable genetically than other types of culture.

Uniquely, shoot cultures have received sufficient attention and responded adequately clearly and consistently to permit the recommendation of storage by growth limitation. To date, some 22 species have been stored by culture at a reduced temperature, in the presence of growth inhibitors or by a combination of these two approaches (see Table 1). (These figures exclude several more reports recorded by Withers, 1982c and obtained by circulating a questionnaire to workers in laboratories world-wide – see 'Present application and future potential', below.)

The flexibility of approach which is possible here is illustrated by the different storage regimes which have been successful with potato: culture at 6 °C in the presence of 3% sucrose (Henshaw et al., 1980); culture at 10 °C in the presence of 50 mg l^{-1} *N*-dimethylaminosuccinamic acid and 2% sucrose (see Mix, 1984); culture at 10 °C in the presence of 1.25 mg l^{-1} abscisic acid and 6% sucrose, or at 25 °C in the presence of 4% mannitol and 0.5% sucrose (Schilde, personal communication); culture at 20 °C in the presence of mannitol at 0.25 or 0.5 mol l^{-1} (Withers & Marshall, unpublished observations).

Some general precautions are wise when adopting growth limitation. Although subculture may not be necessary for one year, much more frequent inspection (e.g. every four weeks) is essential to monitor for unexpected loss of viability, microbial contamination, depletion of culture medium, excessive callusing etc., and to ensure that the environment is stable. In the case of tropical species and others likely to be cold sensitive, the maximum tolerable temperature reduction should be determined. Preliminary work by Staritsky (personal communication) suggests that some of the symptoms of deterioration in storage may be alleviated by periodic transfer back to standard growth conditions.

Turning now to cryopreservation: some generalizations can be made. Most specimens require a period of pregrowth after dissection and before freezing, chemical cryoprotection (usually with DMSO) and are tolerant of post-thaw washing. In other respects procedures differ significantly. Some of the earlier successes (e.g. Grout & Henshaw, 1978) involved freezing shoot-tips very rapidly, employing the second of the injury avoidance mechanisms described earlier. However, as more reports have accumulated (e.g. Kartha et al., 1979), slow or step-wise freezing is emerging as a successful approach, offering a greater degree of reproducibility and control. In ultra-rapid freezing, direct contact with the coolant ensures a high rate of heat extraction but presents practical problems in storage and a risk of microbial contamination. Both approaches are detailed

in Table 2.

The fact that no single, consistently successful method is apparent for shoot-tips underlines the difficulty in working with organised material (see 'Embryos', above). Attempts to cryopreserve shoot-tips of cassava (*Manihot* spp.) further illustrate the problem. Two studies (Bajaj, 1977b; Henshaw et al., 1980) achieved some success by rapid freezing but recovery levels were very low (up to 13 and 8% respectively). In repeated attempts to consolidate these findings, or alternatively develop a slow freezing method, Kartha and colleagues (1982b; personal communication) failed. However, by modifying the slow freezing technique, they have achieved significant progress. The shoot-tips are cryoprotected and then placed in droplets on a piece of aluminium foil in a Petri dish. The dish is placed inside the freezing unit and cooling at 0.5 °C min^{-1} is applied. At a temperature of –25 °C the foil is transferred to liquid nitrogen. Thawing is achieved by immersion of the foil in warm (+37 °C) liquid medium. Further development is still required, as recovery rates range from less than 20 to over 80% overall and rates of plantlet regeneration from 0 to 20%.

Quantitative and qualitative aspects of recovery

Requirements For practical reasons, storage methods must permit survival at a high frequency. As will be shown below, this is not always easy to estimate consistently. Since the central aim of culture storage is the conservation of unique material, it is axiomatic that cultures should be recovered from storage unchanged in phenotype and genotype. Further, if use is to become widespread then there should be no significant genotypic variation in response to storage conditions.

Survival levels The ultimate, definitive test of survival is capacity to recover growth. However, in the development of storage methodology, particularly cryopreservation, it is invaluable to be able to test viability quickly and reproducibly in a large number of samples. For this purpose, the fluorescein diacetate, tetrazolium chloride reduction, and dye exclusion (e.g. Evans' Blue) tests are available (see Withers, 1980a, b). They are best applied some hours after recovery from storage and repeated at intervals.

Protoplast and cell cultures provide the most exact survival data. For the former, post-thaw results generally lie between 30 and 60%. For the latter, results have improved markedly over recent years and it is reasonable to expect up to 75% cell survival immediately after thawing, which stabilizes at this or a slightly lower level.

Callus and organised cultures are less amenable to viability testing, although the TTC test can indicate relative reactions to different storage temperatures, cryoprotectant treatments etc. (see Finkle & Ulrich, 1979). Most frequently, success in growth limitation is assessed by capacity to maintain a consistent, slow, but positive rate of growth, and in cryopreservation by capacity to produce new growing tissue.

The latter gives misleadingly high survival levels if directly compared with those for unorganised cultures, however. In studies of cryopreservation of embryos, for example, survival levels of up to 100% have been recorded, although areas of tissue death were evident in embryos capable of regeneration (Withers, 1979, 1982b). A similar situation holds in the recovery of shoot-tips, although survival levels range from under 10% to approaching 100%. Clearly the lower rates of success are unsatisfactory; whether the

relatively high ones are satisfactory in view of the occurrence of tissue damage, will depend upon the pattern of recovery and origin of new growth within the specimen (see below).

Patterns of recovery Little information is available on the course of recovery in cultures stored by growth limitation, although attempts have been made to assess rates of recovery after cryopreservation, and where appropriate, the anatomy of recovery.

Protoplast, cell and shoot cultures are capable of commencing recovery growth within ca. two days of thawing. Physiological investigations support the suggestion that there is a distinct period of 'convalescence' (Cella et al., 1982). Some early studies recorded longer periods intervening between thawing and recovery by relatively slow growth (e.g. Bajaj, 1976; Withers & Street, 1977). Electron microscopical examination reveals that under sub-optimal conditions of cryopreservation and recovery, cellular decline may continue long after thawing (Withers, 1980a), but that this is avoidable.

Inhibition of recovery can have much more serious consequences in the case of organised cultures. If genetic stability is to be assured, there should be minimal interference with normal process of development. Observations of shoot-tips of *Manihot esculenta* (Kartha et al., 1982b) for example show that callusing may be a common response after thawing. In various studies, the inclusion of hormones (e.g. gibberellin and NAA) or activated charcoal have been shown to promote organised development (see Withers, 1982b). However, a high percentage survival is probably the most important prerequisite for good recovery.

In a microscopical study of recovery in shoot-tips of *Pisum sativum,* Haskins & Kartha (1980) made the important observation that even when regeneration occurs relatively rapidly in a high proportion of the specimens, it may still be adventitious in nature, raising questions in relation to genetic stability.

Stability of phenotype and genotype Examples can be found of unorganised cultures retaining morphogenic potential after storage by cryopreservation (e.g. *Daucus carota:* Nag & Street, 1973; Withers, 1979; *Phoenix dactylifera:* Tisserat et al., 1981) or growth limitation (*Daucus carota:* Jones, 1974). These add to the many examples of organised cultures regenerating normally after preservation. Any observations such as an impairment of shoot regenerating capacity in frozen callus of *Saccharum* (Finkle & Ulrich, 1979) or tendency to callusing in shoot-tips (as above), can be attributed to structural damage rather than genetic modification. Nonetheless, careful examination of stability in morphogenic potential over different periods of time remains to be carried out.

Cell cultures perhaps provide a better system for testing stability since defined, biochemical mutants are available. King (see Table 1 and Withers, 1983a) has successfully cryopreserved a number of mutant cell lines and in a detailed study Hauptmann & Widholm (1982) have demonstrated retention of amino-acid analogue resistance in cell cultures of *Daucus carota* and *Nicotiana tabacum.* Further, Weber et al. (1983) have demonstrated the retention of the requirement for asparagine and glutamine in auxotrophic variants of *Glycine max* subjected to cryopreservation.

Genotypic variation in response to storage If in vitro storage is to become widely used, the methodology must be demonstrated to apply across a range of genotypes. In the

broadest sense, both cryopreservation and growth limitation meet this requirement being applied successfully to over 59 and 22 species respectively. Further, very few species have failed to respond to examination. Encouragingly, the list of successes includes cultures with widely different morphological features and environmental/cultural requirements. However, detailed quantitative studies of recovery in a range of genotypes of one species are lacking. That this must be carried out for reassurance is underlined by the observation by Henshaw et al. (1980) that in examining the tolerance of growth limitation of a range of potato genotypes, significant differences exist. Whereas some genotypes lose viability severely within six months, others can survive at a high level for eighteen months.

Additionally, recent observations by Barlass & Skene (1983) working with several *Vitis* species indicate differences in response to growth limitation both between species and between types of culture (proliferating shoots or single rooted shoots). Similar data on cryopreservation are completely lacking.

Present application and future potential

Methodology of germplasm storage in vitro has been accepted to a limited extent by scientists, including biotechnologists and genetic conservationists. However, it probably still suffers from a problem of credibility and lack of awareness of its potential utility. Less than a handfull of laboratories are using cryopreservation for the storage of stock cell cultures and none at all for other types of culture. This contrasts sharply with the level of use in microbiology, animal cell culture and animal husbandry (semen storage).

Perhaps surprisingly, interest in the use of storage in vitro for the conservation of genetic resources has overtaken the more immediately obvious applications in biotechnology. A survey carried out during 1980-1982 on behalf of the IBPGR (Withers, 1982a) revealed a growing interest and willingness to experiment with novel approaches to genetic conservation. Notable in this area is the work being carried out in the International Centres including CIP and CIAT (see Withers, 1982a). At the former, growth limitation is being used to store clonal collections of potato and at the latter in vitro methodology is being used to introduce cassava germplasm, free it from pathogens and carry out storage by growth limitation. Further, techniques have been developed to transport cultures world-wide from these centres. Studies are under way in association with the centres to develop cryopreservation methodology for both crops.

A crucial factor in the continued development of techniques and the eventual routine use of gene banks in vitro is the dissemination of information to potential users and other interested bodies. Two projects are under way at present which should help here. Firstly, Excerpta Medica is establishing a culture data bank ('MIRDAB') which includes plant material. Secondly, the IBPGR project which carried out the survey mentioned above is continuing to maintain a flow of information to and from scientists tackling problems in plant genetic conservation, and is computerizing relevant data.

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Preservation of potato germplasm in vitro

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Summary

Nodal segments of 220 potato varieties were used as starting material for long-term storage in vitro. The segments were cultured on a minimal liquid medium under normal conditions for three weeks. The plantlets were subcultured and maintained at 10 °C with an illuminance of 2 klx during 16 h per day. In the presence of growth retardants, cultures can survive for at least two years.

Descriptors: potato, *Solanum tuberosum*, germplasm, conservation, limited growth, growth retardants

After the review by Withers (1984) on current knowledge of techniques for germplasm storage in vitro, I can concentrate on the conservation of plant germplasm of vegetatively propagated crops, in particular potatoes.

Within the scope of the intent to preserve plant genetic resources at the Institute of Crop Science and Plant Breeding in Brunswick, a 'Living Collection' of potato varieties has been established.

Varieties used to be maintained by tuber propagation. The cost of maintaining the collection in clonal form is high and there is also a risk of losing material, particularly from diseases. Storage of clonal material as tissue culture is a useful alternative to traditional maintenance.

During the last two decades, remarkable progress has been made in plant tissue culture for storing germplasm of potato (Westcott et al., 1977; Westcott, 1981; Mix, 1981). Special techniques were developed since the constituent cells of shoot-tissue proved to be genetically stable. Plants regenerated from nodal segments in vitro should result in genetically identical progeny, which is prerequisite for maintenance of varieties. These attributes obviously make shoot tissue an ideal subject for long-term conservation of potato varieties, as long as cryopreservation for potato shows low survival. Loss of material cannot be tolerated.

Nodal segments from germinated tubers were taken as starting material. The surface-sterilized segments were placed in test-tubes on filter-paper bridges filled with a basic liquid medium of Murashige & Skoog (1962) with sucrose (concentration 20 g/l) and supplemented with the growth retardant *N*-dimethyl-aminosuccinamic acid (Alar or B-Nine) at 50 mg/l.

The segments develop small plantlets after about three weeks under normal condi-

tions. They then have to be cut again into nodal segments. After subculture for 3-4 weeks, they were again subcultured and maintained at 10 °C with an illuminance of 2 klx during 16 h per day. The cultures can survive at least two years without any loss of material.

To prevent any loss of varieties, each is maintained in 10 replicates, i.e. ten small plantlets of each variety. Also important is careful periodic checking of the collection.

Application of the growth retardant keeps the stems short. The selected storage conditions should additionally cause a reduction in the physiological activity, expressed as a reduction in growth rate of the stored plantlets.

Typical varietal differences in response to culture and to cultural conditions have been observed, but none of the 220 varieties has been found too difficult to maintain in vitro.

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Heredity of epigenetic-variant plants from culture in vitro

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Summary

Variations in plants regenerated from culture in vitro are not unfrequent, especially if the tissue was allowed to give callus. The heredity of some variant progenies was analysed. It proved not to be under direct control of genes but was controlled by interactions between genes and epigenetic factors, modified by culture in vitro. These interactions are kept through sexual reproduction under particular conditions. This knowledge could help us either to avoid unwanted variation for germplasm storage, or to use the variants for plant improvement.

Descriptors: variation, culture in vitro, epigenetic heredity, lettuce, *Lactuca sativa,* tomato, *Lycopersicon esculentum*

Culture of plants in vitro is becoming more widespread, either for propagation or for storage of germplasm. One of the main conditions for this technique is that it gives true-to-type products. Since several scientists have concluded that if they do not take special care, they will get unwanted variation in the regenerated material, it was necessary to analyse the genetic aspects of this polymorphism on suitable plant material.

The conditions for such material were to start with a diploid, autogamous species whose genetic stability could easily be observed. Moreover this model plant should be easy to grow in vitro, either under callusing conditions or in media allowing for regeneration, as desired. In a first set of tests lettuce (*Lactuca sativa*) was used, and in a second, to generalize, tomato (*Lycopersicon esculentum*), (Sibi, 1976; 1981; 1982).

In both groups of tests, the procedure was the same. Apparent homozygosis, i.e. absence of segregation in selfed progenies, was verified on the initial genotype that gave the material cultured in vitro. From this material, callusing strains were submitted to a certain number of transfers on a proliferating medium. Afterwards they were transferred to regenerating conditions and media to obtain plantlets. The regenerated plants were grown in a greenhouse and selfed. Their S_1 progenies were used for comparative trials with the selfed progenies from the initial standard genotype.

At this stage, variations occurred frequently in progenies from regenerated plants when the standard genotypes gave perfectly stable progenies. The variant progenies fell into two classes:

– A first class where variation was due to mutations or chromosomal rearrangements. This kind of family was easily detected, since it yielded Mendelian segregation or was

revealed through cytological observations.

- A second class where phenotype was not true to type, but showed fine within-progeny uniformity. These variant progenies were propagated by selfing without any segregation (for six generations in lettuce, for two in tomato). The frequency of these variants was rather high, and involved both qualitative characteristics (e.g. crushed leaves, branching, leaf colour) and quantitative traits (e.g. height, leaf size, growth parameters).

A certain number of such variants were carefully characterized. The heredity of the new phenotypes was subjected to diallele analysis, which was very convenient to provide evidence on:

- stability of transmission for a trait, which appears as significant variance for general combining ability (GCA),
- positive interaction when hybridizing two variants, which appears as significant variance for specific combining ability (SCA),
- asymmetric heredity, which may be observed as differences between progenies of a variant when used as female or as male parent. This is analysed by variance in row-column differences,
- special interactions of variants used as female progenitors when they are fertilized by some pollen types. This appears as a reciprocal significant variance.

The diallele-cross design included initial genotype and some selected well characterized variants. Each progeny was put in a comparative experiment (randomized block design). The data confirmed that such variation could be transmitted by selfing. When crossed to the standard, or to another variant family, the characteristics were transmitted following peculiar laws which cannot be related to Mendelian inheritance. Variances for GCA and SCA gave interesting facts about the genetical behaviour of this kind of variants. But much more revealing were the variances for asymmetric (female versus male) effects: in both lettuce and tomato, they were highly significant and show the evidence of epigenetic (maternal but in some cases markedly paternal) inheritance.

In this diallele analysis, the progenies from crosses between variants or from crosses between variants and standard may show transgression. These effects are not due to any heterozygosis: it has been shown that they remain after selfing. They are likely due to epigenetic interactions too. These results agree with other experiments on haploidization by culture in vitro of either anthers or ovules, which cultures, starting from homozygote genotypes, yielded variant progenies whose genetic behaviour was similar to our somatic variants (Kouadio, 1979; San Noeum & Ahmadi, 1982).

Moreover we studied also tomato to compare the rate of recombination between two markers in regenerated and in standard plants. The statistical analysis revealed a significant increase of the recombination in the material that had been treated in vitro.

All studies give a coherent set of data about variants. They show us what kind of mechanisms could be involved in such heredity: recent advances in genetics of higher plants, such as transposons, post-transcriptional events, linkats, genetic wavering in populations of cytoplasmic organelles could well account for what we call epigenetic factors which interact in the expression of genes. Such results may shed light either on the main precautions we have to take in order to avoid variations or accidents when storing germplasm and genetic material in vitro or what kind of use could be worthwhile for plant improvement with the transgressive variants.

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Selection for plant variation using cultured cells

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Summary

Plant cells in culture can be used to isolate simple biochemical mutants. Despite a shortage of good cell culture systems for the major crop plants, variation in several traits of agricultural interest has been obtained, either by applying a selection pressure to cultured cells or by screening regenerated plants. The tissue culture process appears to generate wide variation that is inherited by the progeny of regenerated plants. Gametophytic selection could be used to search for variation.

Descriptors: selection, plant, cell culture, mutant, haploids, breeding, resistance, variation, pollen

Introduction

The techniques of somatic cell culture are beginning to make valuable contributions to plant biochemical genetics. In particular the precise selection of specific mutations has begun to generate variation that was not hitherto known in plants, variation that is often not easily recognizable at the plant level and that as a consequence, is not easily selected for in the field or greenhouse. The existence of new biochemical traits in cell culture and the ability to manipulate them by cell fusion, by transfection and transformation and ultimately by plant regeneration has certainly added a new dimension to plant genetics. Since the early 1970s, there have been various attempts to sell this new somatic cell genetics to plant breeders or their employers as a revolutionary step towards higher yields. Judging by recent articles in the Economist or the Wall Street Journal, this crusade has not yet reached its climax. However, the whole campaign is both imprudent and ill timed. Plant breeders use genetics and they value variation very highly. They, therefore, have a continuous professional interest in new genetic systems and new sources of variation, and will not be slow to exploit any new opportunities that can be shown to be useful. On the other hand, whilst the potential of somatic cell culture undoubtedly exists and is worthy of research support, the immediate possibilities for manipulating crop plants in vitro are limited and, because breeding is a slow process, no direct, practical benefits of selection in vitro have yet been demonstrated.

My intention in this paper is not to sell anybody anything, nor is it to discuss such questions as the type of variation most urgently needed or the relevance of single gene versus polygenic traits. I will try simply to summarise for the information of plant

breeders: (1) the progress that has been made in the isolation in vitro of simple mutants of interest to geneticists, and (2) the extent to which variation of agronomic significance has been isolated using crop plant tissue cultures.

Biochemical mutants

One would expect to begin a project to isolate agronomic variation by using a system in which the basic procedures for doing selection and genetic analysis were well established. But it is only 10 years since it was first shown that traits selected in vitro could be expressed in regenerated plants and inherited by the progeny. These were reports by Maliga et al. (1973) of streptomycin resistance in *Nicotiana tabacum* and by Carlson (1973) of methionine sulfoxime resistance, also in *N. tabacum*. Almost all examples of variant isolation in which a simple biochemical trait was expressed in regenerated plants (and not just in the tissue cultures themselves) have been reported only within the last five years. Three such examples of plant somatic cell genetics are described briefly below.

Bourgin (1978) prepared mesophyll protoplasts from allodihaploid *N. tabacum* plants, irradiated them with UV and cultured them. A minimum of 2×10^5 protoplasts per 9 cm petri-dish survived the treatment and grew to colonies that were then transferred to a medium containing toxic levels of L-valine. In a typical experiment 9 resistant colonies were found out of 5×10^5 colonies tested. Plants regenerated from these colonies were also resistant and in crossing experiments two single gene mutations were identified, *Val^r-1* and *Val^r-2*, showing dominance and partial dominance, respectively. This is a simple, brief and reproducible method and one wonders why it took so long before someone did such a nice experiment.

Similar elegant experiments were carried out by Müller & Grafe (1978), who, as part of a programme to study the genetic control of nitrate assimilation, isolated mutants of *N. tabacum* lacking nitrate reductase (NR). Suspensions of cell aggregates, prepared by forcing tissue cultures through a sieve, were exposed to *N*-ethyl-*N*-nitrosourea. The cells were cultured for a short period to allow phenotypic expression, with amino acids in the medium instead of nitrate to support the growth of NR^- mutants. The mutants were then isolated by exposing the cells to chlorate. Because chlorate is reduced to toxic chlorite by the nitrate reductase enzyme, NR^- cells have higher chlorate tolerance. Chlorate resistance lines were isolated and shown to be nitrate reductase deficient as a result of mutations either in the structural genes of the enzyme (*nia*) or of a molybdenum-containing cofactor (*cnx*). Plants regenerated from the cultures are also NR^- and the *nia* mutants were shown to be homozygous for two recessive nuclear gene mutations (Müller & Mendel, 1982). Such mutants are of course conditionally lethal and one would expect difficulties in recovering anything but leaky types using whole plant selection (see Oostindier-Braaksma & Feenstra, 1973).

A further good example of a cell culture approach succeeding where attempts at the whole plant level have been disappointing is that of auxotroph isolation. Auxotrophs are without doubt very powerful tools for studying the fine structure and function of genetic systems. Over the past twenty years, isolation procedures using whole plants produced a collection of thiamine mutants in *Arabidopsis* but little else (reviewed by Redei, 1975), while in the last three years, the application of a cloning method using cultured haploid cells or protoplasts of three species has produced 12 different nutritionally deficient lines

(Savage et al., 1979; Gebhardt et al., 1981; Sidorov et al., 1981). For example, auxotrophs for histidine, tryptophan, leucine and nicotinamide as well as several nitrate reductase deficient and temperature sensitive lines of *Hyoscyamus muticus* were found by testing ca. 60 000 clones derived from MNNG-treated haploid mesophyll protoplasts. These traits, where tested, persist in regenerated plants (Gebhardt et al., 1981).

Thus cell culture techniques can be used to select precisely-defined single gene mutations of plants. The selection procedures for many such biochemical traits are clearly more easily applicable to cells in culture than to whole plants. Several traits have been repeatedly isolated in culture that were hitherto unknown in whole plants or were only recovered with difficultly as leaky mutants. The number of species in which biochemical mutants have been isolated in vitro is still very restricted. Most of the examples given above involved *N. tabacum.* Other species for which adequate cell/protoplast culture systems exist and from which mutants of this type have been isolated are *Datura innoxia* (Furner et al., 1978), *H. muticus* (Wernicke et al., 1979), *N. plumbaginifolia* (Bourgin et al., 1976; Nagy & Maliga, 1976), all of them members of the Solanaceae. One of the major factors at present limiting the isolation of agriculturally useful variation in vitro is the lack of adequate cell culture systems for most of the major crop plants, the most important property of a culture system being regeneration of plants from single (preferably haploid) cells or protoplasts. For example, there is as yet no established method for plant regeneration from single cell populations of any of the cereal species (see review by Thomas et al., 1979).

Mutants of agronomic interest

Selection for resistance is the most obvious use for cultured cells. Selection in the field for resistance, for example, to pesticides, pathogens or environmental stresses suffers from several well known technical problems: non-uniformity of soil conditions, irregular application of pathogen or pesticide, unpredictable and unreproducible climatic conditions, interactions between neighbouring plants (e.g. leaf overlap) etc. To some extent the problems are overcome by seedling tests in sand beds in the greenhouse using precise delivery equipment. However, it is difficult, for example, to mimic environmental stresses on a large scale and seedlings may not respond in the same way as mature plants in the field. The sowing of seedlings in a regular array is a laborious process and thus the number of plants screened is limited to hundreds or a few thousand. In contrast, toxic factors or stress conditions can be applied uniformly to millions of cells in a very small space and the results rapidly assessed. Because, however, several critical plant functions are usually missing from cell cultures, e.g. photosynthesis, translocation and transpiration, this approach may restrict the range of resistance mechanisms that might be selected. Furthermore, if seedlings in a greenhouse do not accurately represent the responses of mature plants growing in the field, then of course cell cultures are even further removed. There is also the danger of selecting for mutations in genes that are expressed in cell cultures but not expressed in any tissue of the whole plant. Consequently plants regenerated from resistant cultures may no longer be resistant. Despite these obvious objections there remains something intrinsically attractive about selecting for resistance cleanly and quickly among millions of cultured cells, and there are several examples where interesting traits have been selected in this way.

Pesticide resistance

Chaleff & Parsons (1978) plated suspension culture cells of diploid *N. tabacum* in medium containing the herbicide, picloram. Resistant colonies appeared 1-2 months later that were retested on selective medium. Plants regenerated from four resistant cell lines were also resistant and the trait was transmitted sexually as one semi-dominant and three dominant alleles of single nuclear genes. The four mutations were later found to define three separate linkage groups (Chaleff, 1980). The mechanism of resistance has not been determined; uptake of the herbicide was not impaired nor did the mutants detoxify the herbicide by formation of water soluble conjugates. The cells were not treated with mutagens and, so far as it is possible to calculate, the frequency of spontaneous mutants of this type in the cell cultures, including all of the possible genetic loci involved, was ca. 10^{-5} per cell.

There are other reports of herbicide resistant plants obtained in vitro: paraquat resistance in *N. tabacum* (Miller & Hughes, 1980); 2,4-D resistance in *Lotus corniculatus* (Swanson & Tomes, 1983) and asulam resistance in *Hordeum vulgare* (Gifford et al., 1982), and many reports of selected cell lines with greatly enhanced herbicide tolerance from which, however, no plants were regenerated. It would be useful to know the mechanisms involved but otherwise these latter reports are of little practical use (see Meredith & Carlson, 1982).

A further interesting example of selection at the somatic cell level is the scheme of Radin & Carlson (1978). The herbicides bentazon and phenmedipham do not inhibit growth of achlorophyllous *N. tabacum* cell cultures although both are toxic to whole plants. Thus instead of a direct approach using cell cultures, leaf cells of immature haploid leaves were γ-irradiated in situ and the plants allowed to grow further. The leaves were then sprayed with the herbicides. As the leaves developed to maturity, 'green islands' of healthly tissue could be distinguished on the otherwise yellow leaves. The green islands were then taken into culture in order to induce new adventitious shoots and regenerate plants. Plants resistant to each of the herbicides were recovered and crosses showed the resistance to be due mostly to recessive single gene mutations. Surprisingly, as found in the picloram selection, complementation tests indicated mutations in more than one gene, four genes for bentazon and two genes for phenmedipham.

Disease resistance

Selection for resistance to pathogens for which a toxin (preferably host specific) has been identified and isolated can in principle be carried out in vitro in a similar way to herbicide resistance selection (Brettel & Ingram, 1979; Ingram & Helgeson, 1980). The problems are immediately obvious: (1) in only a few cases have specific toxins been identified chemically; (2) a toxin may be active against a differentiated function not important for survival of cultured cells e.g. the action of tentoxin from *Alternaria tenuis* on chloroplasts (Steele et al., 1976); (3) the types of resistance mechanisms expressed in cell cultures will be limited e.g. it is difficult to imagine how a hypersensitive response would manifest itself in culture. Nevertheless, there still appear to be breeding situations where an attempt to find resistance in vitro is justified, given a toxin and a good culture system. The two examples below illustrate some of the possibilities.

The serious losses in the U.S. maize crop in 1970 caused by the susceptibility of male-sterile, T-cytoplasm maize to *Drechslera (Helminthosporium) maydis* race T are well known. By now equally famous are the attempts to isolate male-sterile lines resistant to race T by selection in vitro using the race-T toxin. Beginning with the demonstration by Gengenbach & Green (1975) that T toxin inhibited the growth of tissue cultures from cms-T but not cms-N maize, a programme of recurrent selection for T-toxin resistance was begun using immature embryo derived cultures of maize without prior mutagen treatment. Practically all plants regenerated from T-toxin resistant cultures proved to be highly resistant but had reverted to male fertility. The resistance was maternally inherited and it became clear that male sterility and toxin susceptibility were inseparable by this method of selection (Gengenbach et al., 1977; Brettel et al., 1980). Surprisingly, many of the plants regenerated from control cultures without toxin selection were also T-toxin resistant and male fertile. Mitochondria isolated from toxin-resistant cell lines were insensitive to the toxin. This fits both with original studies indicating the mitochondrion to be the site of T-toxin activity (Müller & Koeppe, 1971) and with the maternal inheritance of the trait. The linked acquisition of fertility and resistance was initially explained by sorting out of a mixture of cms-T and normal mitochondria during selection. However, it was later shown by restriction endonuclease digestion that the resistant plants still had a mitochondrial DNA pattern characteristic of cms-T cytoplasm (Kemble & Bedbrook, 1980; Gengenbach et al., 1981). It would seem, therefore, that T-toxin resistance was acquired in culture by a mutation or recombination event in the mitochondrial genome without prior mutagenesis and in the absence of the selective agent. In a plant breeding sense, of course, the T-toxin experiments failed; no resistant male-sterile lines were isolated. However, as a model for the application in vitro of a selection process difficult to carry out at the plant level the experiments are perfect.

More successful from a plant breeding point of view were the experiments of Behnke (1979, 1980), who used non-specific culture filtrates to select *Phytophthora infestans* resistant plants of *Solanum tuberosum*. Tissue cultures were initiated from six dihaploid clones of *S. tuberosum* known to produce cultures with good regeneration capacity. Small (ca. 1 mm diam.) culture pieces (some after X-irradiation) were subcultured onto media made up with culture filtrates of different *P. infestans* pathotypes. Colonies growing through this test were retested on toxic media. Thirty-six resistant lines were found among 41 040 non-irradiated inocula and 7 out of 1 400 irradiated cultures were resistant. Resistance was maintained during growth in non-selective conditions and resistant lines were cross-resistant to culture filtrates of all pathotypes used. Most important of all, plants regenerated from resistant lines were more resistant than controls in a test involving culture initiation from leaves placed on toxic media (Behnke, 1980). The average lesion size on leaves of resistant plants inoculated with a sporangial suspension was 25% less than on control plants. There was no difference in the sporulation of the parasite.

Although the few reported attempts to isolate disease resistance by selection in vitro have had little or no impact on plant breeding so far, a number of useful facts are emerging. First, toxin sensitivity is widely expressed in tissue culture, for example, using culture filtrates of *Alternaria solani* on potato and tomato (Handa et al., 1982), of *Phytophthora citrophthora* on *Citrus sinensis, Daucus carota, N. tabacum* and *N. sylvestris* (Breiman & Galun, 1981) or of *Claviceps fusiformis* on pearl millet (Bajaj et al.,

1980). Second, cells of host plants are more sensitive than non-host plants (Handa et al., 1982). Thirdly, resistant lines are more resistant in culture than susceptible lines (Helgeson et al., 1976; Ingram, 1976). It is true here again that progress is limited by the absence of suitable tissue culture systems.

Heavy metal tolerance

The toxicity of excess aluminium is a significant problem in many acid soils. Aluminium can be added easily and reproducibly to tissue culture media and cells are sensitive to aluminium. Furthermore, the mechanisms of tolerance proposed for whole plants: exclusion by alteration of a transport protein, or enhanced chelation with overproduced organic acids, should function also at the cultured cell level. Thus selection for tolerance of aluminium (or other toxic metals) would seem to be appropriate in tissue culture. Stable aluminium resistant cell lines of tomato were isolated by Meredith (1978). There was no difference in aluminium uptake between resistant lines and controls.

Ojima & Ohira (1982) isolated aluminium-resistant lines of carrot by subculturing cell suspension cultures for several months in presence of aluminium chloride. One resistant line examined in detail was shown to release higher amounts of organic acids, especially citrate, into the medium than wildtype cells. Aluminium toxicity on wildtype cells could be relieved by addition of the acidic fraction from medium conditioned by growth of resistant cells or by addition of citrate or malate. Plants regenerated from resistant cell lines set seed and aluminium toxicity tests were carried out on seedling progeny. Hypocotyl and root growth of seedlings from selected lines was greater than controls on high aluminium, and seedling roots showed a much reduced staining reaction to haematoxylin after exposure to aluminium, indicating reduced aluminium uptake. Problems exist with other soil metals and, for example, Koo (1982) has reported plant regeneration from haploid *N. tabacum* cell cultures resistant to Cu or Hg.

Salt tolerance

Several environmental stresses of interest to plant breeders are easily applied to cell cultures, in particular high salt levels and low temperatures. There are many reports of cell lines resistant to such stresses but there is increasing evidence that tolerance can appear in culture by gradual adaptation reactions. Two reports in which salt tolerant plants were regenerated are worthy of comment.

Normal cell lines of *N. tabacum* inhibited by 1.6 g l^{-1} NaCl were used to select for spontaneous or EMS-induced salt tolerance (Nabors et al., 1980). Lines were produced tolerating up to 8.8 g l^{-1} NaCl and this tolerance was maintained at least through to the F_2. Curiously, however, persistence of the phenotype in the F_1 appeared to be dependent upon the presence of salt during the regeneration process and F_2 plants were more tolerant if F_1 plants were continuously exposed to salt. There is to date no biochemical or genetical explanation for this salt requirement.

Preliminary data from Yano et al. (1982) point to salt tolerance in rice plants regenerated from immature embryo-derived cultures exposed to 37.5% seawater. Regenerated plants from tolerant lines grew and set seed when cultured in 17.5% seawater whereas control plants died. However, the tolerance of the progeny was not as marked.

Cold tolerance

Chilling resistant cell lines of *N. sylvestris* have been isolated by exposing plated cells for 21 days to –3 °C (Dix & Street, 1976). Resistant colonies were retested and several lines found that retained a significant level of chilling resistance. It is, however, quite certain in this case that the trait was not transmitted sexually in regenerated plants (Dix, 1977).

Poinsettia (*Euphorbia pulcherrima*) is a very popular greenhouse-cultivated ornamental that is propagated vegetatively, requiring a temperature of 25 °C for rooting and 20 °C for growth up to flower induction. Cultivation costs are therefore greatly inflated by heating costs. Progress towards the isolation of low temperature tolerant types is reported by Walther & Preil (1981), who have established a very efficient suspension culture/plant regeneration system for this species. Using X-irradiation and 170 days low temperature (12 °C) stress applied to plated cell cultures they have produced cold tolerant lines showing a small but significantly reduced leaf fall under cold stress compared to the original cultivar. Using a similar method they also isolated two chrysanthemum clones flowering earlier than the parent cultivar at low temperature (Preil et al., 1983). In these cases the question of sexual transmission is of minor importance so long as the character is stable through vegetative multiplication. In addition, the problem of induction of periclinal and sectorial chimeras seems to have been avoided in Poinsettia by plant regeneration from suspension cultures after selection (Preil & Engelhardt, 1982).

Improved quality

The proposal has been that selection can often be applied more efficiently to tissue culture than to whole plants. However, the characters so far mentioned, it can be argued, are primarily expressed in differentiated plant tissues and can actually be selected for in the field. Furthermore, some mechanisms of resistance to stresses may not be detected by application of selection in vitro. Crop plant tissue culture does, however, introduce some totally new ideas for selection, including procedures that are impossible to apply in planta or characters that are not seen by walking through a greenhouse or field. The best examples of these to date lie in the area of 'quality' traits, especially amino acid levels. The selection methods seek alterations in the regulation of specific biochemical activities. An obvious prerequisite for the design of the selection system is knowledge of the location of the activity and its regulation. Such knowledge in plants is all too frequently missing and tissue culture techniques in such cases have become a double-edged sword for attacking the problem: on the one hand seeking potential useful genetic variation, and on the other hand by this very search uncovering new information about the processes involved.

There have been many attempts to find mutations relieving feed-back inhibition of amino acid biosynthesis that cause accumulation of amino acids to a higher concentration than in wildtype. Hibberd & Green (1982) selected for resistance to growth inhibition by lysine plus threonine in immature-embryo derived cultures of *Zea mays*. A stably resistant line yielded plants whose progeny inherited the resistance as a single dominant nuclear trait. The free pool of threonine was increased 75-100 times in homozygous

mutant kernels, bringing an increase of 33-59% in the total threonine content of the kernels. Jacquemin & Dubois (1981) have also obtained resistance to lysine/threonine in wheat cell cultures that was inherited as a recessive character in the progeny plants.

Similar studies have been performed using toxic amino-acid analogues. Schaeffer & Sharpe (1981) isolated a line of *Oryza sativa* cells resistant to the lysine analogue, *S*-aminoethyl-L-cysteine, from which they regenerated plants. The levels of various amino acids were found to be increased both in the soluble pool and in the protein of seeds. The lysine content of seed protein hydrolysates was 10% higher in the selected line. Surprisingly, there was a 25-50% increase in total protein in selected seed, resulting in a large increase in total lysine per seed. There are as yet no field evaluation data for either the maize or the rice mutants.

Selected cell lines of *Medicago sativa* (alfalfa) resistant to the methionine analogue, ethionine, had higher levels of methionine, cysteine, cystathionine and glutathione than unselected controls (Reish et al., 1981). In one case, a tenfold increase in methionine together with a 43% increase in total free amino acids and a 40% increase in protein amino acids was recorded. Plants regenerated from the cultures were also ethionine resistant. No data are available for amino acid levels in regenerated plants.

Not exactly tissue culture, but a successful selection system all the same, is the application of the proline analogue, *trans*-4-hydroxyproline, to germinating embryos dissected from bulked M_2 seed of *Hordeum vulgare* (Kueh & Bright, 1982). Whole seeds were not used because of the masking effect of the endosperm on analogue toxicity. In this way, three resistant mutants were isolated that accumulated three times more soluble proline in leaves than normal plants. The resistance was inherited as a single semi-dominant allele. There was some suggestion of better ionic and osmotic adjustment in the one mutant line tested. After 7 days growth at constant concentrations of NaCl below 100 mmol/l or in gradually increasing concentrations up to 200 mmol/l the mutant grew better than the parent. The existence of such mutants offers a new approach to studying the relationship between osmotic stress and amino acid accumulation. A similar selection system was used by Cattoir-Reynaerts et al. (1981) to isolate lysine/threonine resistant M_2 plants of barley.

Improved efficiency

Many plants carry out two apparently wasteful oxidation processes yielding little or no ATP, namely cyanide-insensitive respiration and photorespiration. It has been proposed that crop plants can do without both processes and that their elimination would enhance yield.

Little progress has been made, however, in the isolation of alternate oxidase (cyanide-insensitive) mutants. A selection procedure seeking resistance to the fungicide carboxin, that had previously yielded mutants of *Ustilago maydis* blocked in the alternate oxidase, was applied to cell cultures of *N. tabacum* (Polacco & Polacco, 1977). Although resistance to carboxin was obtained that persisted in regenerated plants, there was no evidence of a change in cyanide-insensitive respiration.

Because of the observation that net photosynthetic CO_2 fixation is significantly increased in C_3 plants when photorespiration is inhibited at low oxygen concentrations (e.g. Zelitch, 1975) several schemes have been devised to select for mutants in the

glycolate biosynthesis pathway beginning with the enzyme RuP_2 oxygenase (Somerville & Ogren, 1979; 1980; Berlyn, 1980; Lawyer et al., 1980). This is an interesting example of apparently plausible selection schemes getting too far ahead of basic biochemical knowledge and it seems that mutant selection in this area will first clear up some of the biochemical questions before providing any useful traits for breeders. It is becoming clear that carbon entering the photorespiration cycle must be largely returned to the Calvin cycle if photosynthesis is to continue (Servaites & Ogren, 1977). This means that the only activity worth deleting is that of RuP_2 oxygenase, at the very beginning of the pathway.

Recent interesting work with *Arabidopsis* at the whole plant level has uncovered mutants in two other enzymes: phosphoglycolate phosphatase (Somerville & Ogren, 1979) and serine-glyoxalate amino transferase (Somerville & Ogren, 1980). The mutants were inviable and photosynthesised at greatly reduced rates under normal conditions promoting photorespiration. Further attempts, this time using cell cutures of *N. tabacum* aim at mutations blocking conversion of glycine to serine in the hope of favouring alternate pathways of glycine metabolism in which less CO_2 is lost. The strategy has been to look for mutants resistant to isonicotinic acid hydrazide (INH; Berlyn, 1980) and glycine hydroxomate (GH; Lawyer et al., 1980) both inhibitors of glycine to serine conversion. INH resistant *N. tabacum* lines were isolated from which resistant plants were regenerated. The resistance trait was dominant in crosses with sensitive lines. GH resistant plants were also found but inheritance of the trait was not reported. The biochemical basis for the resistance has not yet been described and there are no data on the photosynthetic performance of the mutants.

Unfortunately, these interesting genetic experiments could in the end prove to be misguided, as photorespiration may in fact function as a protective mechanism both for plants and for the environment in general. Photorespiration may protect against light and oxygen toxicity under normal CO_2-limited photosynthesis conditions by consuming ATP, $NADPH_2$ or reduced ferredoxin. C_4 plants lacking photorespiration have the photosynthetic capacity to remove all CO_2 from the air, an event that would destroy the temperature-controlling greenhouse effect. The balance between gross photosynthesis and photorespiration keeps changes in CO_2 within narrow limits (Tolbert, 1980).

Tissue culture variation

In all of the foregoing examples of selection for agronomic characters in tissue culture a selective pressure was applied directly to the cultures in the hope of eliminating all but cells, and thus plants, with a particular phenotype. In the course of such experiments over many years it has become clear, first, that deliberate treatment with mutagens is often unnecessary and, second, that variation also arises in many other characters than those of immediate interest. It seems that there is genetic variability in the tissues of the original explant and/or that passage through tissue culture itself causes mutations, rearrangements, deletions, enhanced somatic recombination, chromosome loss, or polyploidisation etc. (for reviews see Skirvin, 1978; Larkin & Scowcroft, 1981; Lörz, 1983). This has led to the idea that plants regenerated at random from tissue cultures should be examined in the field for useful characters or should be added to breeding populations to increase the genetic variance.

Tissue culture variation, popularised recently under the name of 'somaclonal variation', has been observed in many important crop plants: sugar cane (Heinz et al., 1977), maize (Edallo et al., 1981), oats (Cummings et al., 1976), sorghum (Smith et al., 1982), barley (Deambrogio & Dale, 1980), alfalfa (Reisch & Bingham, 1981), potato (Bidney & Shépard, 1981; Thomas et al., 1982). Although experiments have centered on vegetatively propagated crops like sugar cane and, more recently, potato, the phenomenon clearly also occurs in seed grown crops and is transmitted to sexual offspring. There seems to be no correlation between frequency of variation, the type of crop and the particular method of culture in vitro. Variation has been reported between plants regenerated from anther culture (Brown et al., 1983), adventitious buds on roots (Grout & Crisp, 1980), cell cultures (Reisch & Bingham, 1981) and protoplast cultures (Bidney & Shepard, 1981). On the other hand, there are some manipulations in vitro that do not seem to generate variation e.g. meristem tip culture, now an important means of propagation for many horticultural crops. The characters in which variation has been reported are many: e.g. nicotine content, pathogen resistance, sugar yield, leaf habit, plant height, maturity date, photoperiod requirement, chlorophyll content, leaf number, dry weight yield, tiller number, altered leaf waxes, flower form and pigmentation, perenniality. Some examples of quantitative studies of tissue culture variation are presented below.

Genetic changes in tobacco plants bearing marker loci have been examined after plant regeneration from tissue culture. Barbier & Dulieu (1980) regenerated plants in vitro from an intervarietal hybrid heterozygous at two loci involved in chloroplast differentiation. The initial plants had greenish-yellow leaves and it was easy to detect variants by examining the colour of plants regenerated in three different ways: directly from cotyledons, from cotyledon-derived tissue cultures after 3 passages, from protoplast-derived cell colonies. Crosses made between green regenerants and the homozygous parental lines showed that the variants were the results of single deletions or reversions or of double events (two reversions or one deletion and a reversion), the mutation frequency for each of the two loci being ca. 3.5×10^{-2}. The frequencies of green plants regenerated were: cotyledons 1.6%; cotyledon tissue cultures 6.8%; protoplast-derived colonies 6.6%. How much variation preexisted in the starting plant material is not clear from these data. However, Lörz & Scowcroft (1983) have presented an analysis of plants regenerated from *Su/su, Su/su* heterozygotes of *N. tabacum* via protoplast culture suggesting that 0.1–1.8% of the variants might be due to genetic differences already existing in the somatic cells of the starting plant. In both of the *N. tabacum* experiments described above several other phenotypic variants were observed among the tissue culture derived plants. A further, detailed analysis of first and second cycle dihaploid *N. tabacum* plants derived from anther culture and tested in agronomic performance traits showed that significant variation arose among lines from a single homozygous source in both the first and second cycles of anther culture (Brown et al., 1983).

The sexual progeny (R_1) of plants regenerated from tissue cultures (R_0) of a doubled *Oryza sativa* haploid were examined in the field by Oono (1982). Both dominant and recessive mutations were observed and mutant frequencies among the R_2 plants were very high. For example, the frequency of chlorophyll mutants (8.4%) was equivalent to that found in seed progeny following X- or γ-irradiation. Only 28% of R_1 progeny were normal for five characters examined and a further 28% carried two or more mutated characters. Several traits were maintained at least into the R_3 generation. An analysis of

150 R_2 lines revealed some agronomically useful characters including early heading, low plant height, long panicle length, and increased number of panicles and grains.

Zea mays plants (R_0) can be regenerated from a complex tissue culture arising on the scutellum of immature embryos. Edallo et al. (1981) scored for the presence of simply inherited mutations in R_2 plants; only segregations fitting a 3 : 1 ratio for endosperm and seedling mutants were considered. Both the endosperm and seedling mutants were phenotypically similar to spontaneous mutants previously described in maize but they occurred at much higher frequencies in the tissue culture derived plants. On average the R_0 plants carried one mutation each whereas control plants grown from seed were not visibly mutated. The mutation events were clearly random and continuous throughout the period in culture: plants regenerated from the same tissue culture were not genetically similar. It will be interesting to see in the next few years if plant regeneration from tissue culture proves to be a unique source of genetic variation of interest to plant breeders, particularly those working with crops that are highly polyploid, sterile or semi-sterile and vegetatively propagated.

Pollen selection

As stated in the introduction, the most important property of a cell culture system for selection is '... plant regeneration from single (preferably haploid) cells...'. I must now admit (as anyone with a passing knowledge of the plant life cycle will have realized already) that it is not really necessary to go to the trouble of producing sterile cultures to obtain such cells. Most plants produce them regularly in millions. I mean, of course, pollen grains. Although some properties of pollen are influenced by the sporophyte during microsporogenesis, expression of genes by pollen of higher plants has been described frequently (reviewed by Heslop-Harrison, 1979). Tanksley et al. (1981), for example, have compared isozyme profiles of pollen and sporophyte for nine enzymes in *Lycopersicon esculentum*. The structural genes of seven enzymes were transcribed and translated in the haploid gametophyte. Pollen is produced on a large scale: in *Z. mays* as many as 10^7 grains are shed per day per plant over a peak 7-day period, and it is possible to pollinate 50 or more ears with a single collection of pollen (300-500 pollinations a day), each ear yielding several hundred kernel progeny. In addition, many different pollen grain genotypes are produced by plants heterozygous for unlinked loci.

Mutants have been selected by applying a selective agent to mature pollen grains e.g. mutants of *Z. mays* lacking the major alcohol dehydrogenase isozyme (*Adh1-0*) were isolated by applying a volatile inhibitor, allyl alcohol, to grains just prior to pollination (Schwartz & Osterman, 1976; Freeling & Cheng, 1978). That selection for or against specific pollen functions leading to differential fertilization can occur has been clear for some time; it is often claimed as the cause of aberrant Mendelian ratios.

Using pollen mixtures from the cultivated tomato, *L. esculentum*, and a high altitude wild species, *L. hirsutum*, Zamir et al. (1981) showed that the *L. hirsutum* ecotype was more successful at achieving fertilization at low temperature. The *L. hirsutum* pollen is thus better adapted to chilling stress and, as has since been demonstrated (Zamir et al., 1982), the tolerance is determined by pollen genes. The critical question now is whether the tolerance of the gametophyte is reflected in progeny sporophytes and conditioned by the same gene(s). In other words, will selection of traits at the pollen grain level necessarily

lead to desired improvements of the whole plant.

Certainly, in the case of *Adh1-0* mutants of *Z. mays*, the condition selected for among pollen grains is expressed as a sensitivity to anaerobic conditions at all stages of sporophyte development. There are technical problems to be overcome in the application of chemical and environmental stresses to pollen, particularily when the pollen is short-lived or when a stress must be applied for a period of time in aqueous solution. However, it is interesting to see that pollination can also be achieved with partially germinated pollen (Raman et al., 1980; Walden & Raman, 1980) and that pollen shows differential sensitivity to pesticides (Startek & Walden, 1981).

Final word

The proposal is, therefore, that selection for agronomically useful traits at the single cell level (whether somatic or gametic) is possible and that this technique may make new, unique variation available to plant breeders.

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Selection of biochemical mutants in plants

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Summary

Recent advances in the field of somatic cell genetics have provided interesting results and new perspectives. Selection strategies developed in microbial genetics were successfully extrapolated to culture in vitro of cells of higher plants. Such selection has allowed the obtention of a broad array of mutant lines such as for auxotrophy and resistance. Their obtention and properties are considered, specially of those isolated by the use of positive selection techniques and concerning characteristics with an agronomic value such as overproduction of essential amino acids. Properties of mutants (characterised by a threonine or lysine overproduction) are presented both in model and crop plants.

Descriptors: mutant, selection, auxotrophic mutants, amino acid overproduction, protoplast culture, cell culture

The molecular basis of the genetic, biochemical and physiological processes in development of a crop plant is as yet poorly understood. However progress in plant breeding depends on the control and manipulation of such processes. A successful way in micro-organisms is to select and use mutants in which specific steps of the process are modified or blocked. The impressive progress in somatic-cell genetics in the isolation of mutants have been excellently reviewed by King (1984).

Before commenting on the prospects offered by culture of plant cells in vitro, I would recall two categories of biochemical mutants already obtained in higher plants by 'conventional' methods.

The first includes mutants in which the biosynthetic pathway affected is known. The following types have been reported by Nelson & Burr (1973): mutants affecting the photosynthetic apparatus, synthesis of storage proteins in cereals, carotenoid synthesis, imcompatibility reactions, synthesis of growth factors and synthesis of starch, essentially in maize.

The second type of mutants results from the emergence of zone electrophoresis associated with staining methods for proteins and enzymes. Numerous isoenzyme systems have been identified in plant populations and used to analyse the genetic and molecular basis of cellular regulatory mechanisms, as illustrated by alcohol dehydrogenase in maize (Schwarz, 1976; Freeling & Birchler, 1981). Such isoenzymes constitute ideal markers for somatic-cell genetics and are also useful for identifying cultivars of crop plants.

The main limitation, however, in obtaining mutants affecting a particular pathway or process resided in the lack of effective techniques for selection. A major contribution of cell culture in vitro has been to establish selection strategies for traits expressed at the cellular level.

Various relevant examples for auxotrophy or resistance are presented by King (1984). Most of the variant cell lines were obtained by applying a positive selection scheme, i.e. only potential mutants could grow. Resistance to antimetabolites, drugs, pesticides, toxins and mineral and environmental stresses are classical examples. Auxotrophic mutants, however, were obtained in *Hyoscyamus muticus* by non-selective screening of numerous protoplast-derived clones. Their selection implies a negative procedure since the desired mutant carries a metabolic defect that does not allow growth of the mutant cells on a normal medium. In our laboratory, I. Negrutiu and R. Dirks have adapted a bromodeoxyuridine (BrdU) treatment to *Nicotiana plumbaginifolia* protoplasts to enrich the amount of auxotrophes in surviving colonies. Auxotrophes for histidine, tryptophan, leucine, isoleucine, valine plus isoleucine and methionine were recovered. Ten such auxotrophes were detected among 15000 protoplast-derived colonies surviving the BrdU treatment (Negrutiu, 1983).

In such regenerated auxotrophic plants, we should be able to determine why previous attempts with entire plants have been unsuccessful, apart from the thiamine-less mutants of *Arabidopsis* (Redei, 1964; Feenstra, 1964; Jacobs, 1965) and tomato (Langridge & Brock, 1961).

Let me now come back to another aspect illustrated by King (1984), namely the possibility of improvement of nutritive value based on amino acid composition by means of somatic-cell culture.

A first approach in cereals has been to screen genotypes with an altered amino acid composition of endosperm proteins, such as the lysine-rich types, Opaque-2 in maize and Hiproly in barley. Another approach has been to look for mutations in the control mechanism of synthesis of essential amino acids like lysine, threonine and methionine. Such mutants should contain enzymes that are no longer feedback-regulated and so should overproduce the amino acid. The question is now how to design genetic changes in the metabolic pattern that ultimately increase the amount of specific amino acids. The rationale for selecting such overproducers is based on the growth inhibition exerted by the combination of amino acids like lysine plus threonine or lysine analogues and on assessment of feedback regulation for key enzymes of the aspartate pathway by which lysine, threonine and methionine are synthesized (Bright et al., 1978; Cattoir et al., 1980).

We have subjected various plant systems to such a selection scheme: barley embryos, *Arabidopsis* seeds, carrot embryoids, and leaf protoplasts of *Nicotiana sylvestris* and *N. plumbaginifolia*. In all systems, mutants with resistance to lysine + threonine were obtained. They are characterized by threonine overproduction in the pool of soluble amino acids associated with the insensitivity of the aspartokinase (AK) enzyme normally sensitive to feedback inhibition by lysine (Cattoir-Reynaerts et al., 1981; Cattoir-Reynaerts et al., 1983). However, until now, lysine overproducers were only obtained in *Nicotiana sylvestris* resistant to the action of a lysine analogue. They possess a desensitized dihydropicolinate synthase (DHPS), the first enzyme unique to lysine biosynthesis (Negrutiu et al., 1983a).

In some species as *Nicotiana sylvestris*, one mutation altering DHPS should be

sufficient to allow accumulation of lysine, the relative inhibition exerted by lysine on AK being not too stringent. In other species, such as barley, two mutations, one altering AK and the other DHPS are required, since lysine almost completely inhibits AK activity.

We have thus here a nice example in which theoretical expectations are met and where the selection procedure is also amenable to whole seed or embryo systems.

It provides also matter for discussion of the problem of expression of the selected cell trait at plant level. All altered phenotypes selected at protoplast level expressed the mutation in regenerated plants and progenies. The increased content of free lysine or threonine was traced to both leaves and fruits. In barley, the overproduction was still expressed in immature and mature endosperm, and in embryo of the seed.

More generally, it should be noticed that except for a tobacco line overproducing tryptophan (Widholm, 1978), mutations for amino acid overproduction induced and selected in cell culture were expressed in vegetative organs of mutant plants. Selection for feedback-insensitive mutants in amino acid synthesis could find a wide field of application certainly in forage crops. Such mutations inherited as monogenic, dominant or semidominant traits, represent thus valuable tools in plant breeding.

A last aspect of selection in cell cultures is the regeneration potential of different kinds of culture, namely callus and suspension cultures in contrast to protoplasts. We have surveyed 75 reports on selection of various types of biochemical variants in plant cells (Negrutiu et al., 1983b). All variants selected in mesophyll protoplast cultures have been regenerated into fertile plants, whereas only 17% of those selected from suspension of callus cultures produced fertile regenerants. So, protoplast cultures are valuable material in such mutation-selection experiments.

This discussion points also out that we have actually to be stricter in selection protocols with cultured cells. It is not enough to select for cell variants. We have to demonstrate by using the appropriate experimental system that the selected phenotype is relatively stable, expressed in regenerated plants and sexually transmissible. It is only in such cases that the induced variation can be fully exploited by the plant breeder.

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The potential of cell cultures for the production of salt tolerant cultivars

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Summary

The progress towards the production of salt tolerant plants through selection in cell cultures is briefly reviewed. The need for a fuller understanding of the mechanisms of salt tolerance in non-halophytes is emphasised and illustrated with reference to recent investigations on the role of proline. A clear protective effect, against salt stress, of exogenously applied proline has been demonstrated suggesting elevated levels of endogenous proline synthesis, for which there is a direct selection procedure, as a basis for improved salt tolerance.

Salt tolerance of proline-overproducing plants and cell cultures is currently under investigation in a *Nicotiana sylvestris* line with almost 100-fold increase in free proline.

Descriptors: selection, cell culture, salt tolerance, *Nicotiana sylvestris*, proline, hydroxyproline resistance

Introduction

For several reasons progress in the selection for salt tolerance in tissue and cell cultures seems a particularly appropriate topic to review within the context of this session. Firstly, improved salt tolerance is clearly a desirable agronomic trait. Secondly, it is one which has attracted considerable attention from a number of investigators over the last few years, with remarkably little success. It therefore serves to illustrate some of the difficulties already mentioned (King, 1984) in applying this conceptually simple approach to a practical problem. Finally, salt tolerance is an area in which recent physiological investigations, with both whole plants and cell cultures, have yielded insights which suggest new approaches to the selection of tolerant cultivars.

In the last ten years salt tolerant cell lines have been reported for:

- *Nicotiana sylvestris* (Zenk, 1974; Dix & Street, 1975)
- *Nicotiana tabacum* (Nabors et al., 1975; Hasegawa et al., 1980)
- *Capsicum annuum* (Dix & Street, 1975)
- *Medicago sativa* (Croughan et al., 1978)
- *Kickxia ramosissima* (Mathur et al., 1980)
- *Citrus sinensis* (Ben-Hayyim & Kochba, 1982)
- *Oryza sativa* (Yano et al., 1982)
- *Colocasia esculenta* (Nyman et al., 1983)

Only in the case of *N. tabacum* (Nabors et al., 1980) has sexual transmission of salt

tolerance, at the whole plant level, been satisfactorily demonstrated. This success may have provided encouragement and an added stimulus to other workers in the field. The problems with the other systems have been at the level of plant regeneration, transfer of plants to soil, expression of resistance in the whole plant, induction of flowering, fertility, or heritability. Sexual transmission of resistance in some lines may be currently under investigation. Both Yano et al. (1982) and Dix et al. (1983) report a reduced level of resistance in progeny compared to their parents, and in the latter case this is not found in intact seedlings, but only in callus derived from them.

There is a growing conviction that adaptation to salt stress may be a common feature in cell cultures, giving rise to epigenetic variants of sufficient stability to obscure the lower frequency occurrence of genuine genetic variants. This suggests that a careful reappraisal of the whole approach to the selection of salt resistant lines may be in order.

Cell cultures have also been used in investigations on the basis of salt stress damage and tolerance. Differences in salinity tolerance at the whole plant level are reflected by differences in the responses of callus (Tal et al., 1978; Orton, 1980) and protoplast (Rosen & Tal, 1981) cultures, and salt tolerance in halophytes appears to include a substantial cellular basis in some species (Hedenstrom & Breckle, 1974; Warren & Gould, 1982) but not in others (Smith & McComb, 1981).

Of particular interest here are results relating to a role of proline in relation to salt tolerance (Steward & Lee, 1974). Katz & Tal (1980) and Dix & Pearce (1981) have demonstrated elevated levels of proline synthesis in both salt sensitive and tolerant cell cultures in response to salt but exogenous proline was unable to ameliorate the effects of salt on a sensitive cell line of *N. sylvestris* (Dix et al., 1983). A protective effect of proline has, however, been observed in whole plants (Bar-Nun & Poljakoff-Mayber, 1977) and organised tissues (Mathur et al., 1980). These findings suggest a link between selection for salt tolerance and selection for amino acid overproduction via amino acid analogue resistance (Widholm, 1975). This link is supported by the recent results of Kueh & Bright (1982) who have demonstrated both proline accumulation and enhanced salt tolerance in hydroxyproline resistant barley plants.

We have been investigating the effect of exogenous proline on inhibition of *N. sylvestris* seedlings and callus by salt, and the characteristics of a hydroxyproline resistant line of *N. sylvestris*.

Materials and methods

Detailed conditions for the initiation and culture of *N. sylvestris* callus, and plant regeneration, have been described in earlier papers (Dix et al., 1977; Dix, 1981). Callus and seedling growth tests were performed as described by Dix et al. (1983).

The hydroxyproline resistant line, HPR105, was isolated from *N. sylvestris* (NS) callus culture on medium supplemented with 10 mM hydroxyproline. HPR105A is a callus line initiated from a regenerated plant and retaining its hydroxyproline resistance.

Amino acid extraction and analysis was performed as described previously (Dix & Pearce, 1981).

Results and discussion

The effects of proline on NS callus cultures were investigated at lower concentrations (proline at 0.1 and 0.5 mmol/l) than previously (Dix et al., 1983). Growth of NS callus is largely inhibited at 1% NaCl and completely at 1.5% NaCl. Neither of the proline concentrations tested gave any improvement of growth on saline medium and the higher concentration was itself partly inhibitory.

Using NS seedlings, however, a pronounced protective effect of 0.1 mmol/l proline was found at 1% NaCl (Table 1), as assessed by both shoot and root growth. The more severe stress caused by 1.5% NaCl could not be reversed, and at 0.5 mmol/l proline itself became strongly inhibitory.

These results suggest that exogenously applied proline can protect differentiated (seedlings) but not undifferentiated (callus) *N. sylvestris* against salt stress.

Amino acid analysis shows the hydroxyproline resistant (HPR105A) callus to contain 14.21 μmol proline g^{-1} fresh weight, as compared to 0.16 μmol g^{-1} in sensitive NS callus, representing almost 100× increase in free proline. In regenerating HPR105A tissue, consisting mostly of shoots, this falls again to 0.60 μmol g^{-1}, only four times higher than the sensitive control yet comparing favourably with the three times increase found by Kueh & Bright (1982) in the leaves of hydroxyproline resistant barley plants.

Preliminary investigations on the salt tolerance of HPR105A suggest a small increase in tolerance (compared to NS callus) of undifferentiated callus, and a more pronounced increase in the case of regenerating tissue, although in neither case does this compare with that found in undifferentiated lines selected directly for salt tolerance (Dix et al., 1983). Thus cell cultures obtained by this indirect selection procedure have a lower level of salt tolerance than those selected for growth in medium containing NaCl, but there may be a greater chance of the tolerance being expressed in the intact plant, and resulting from a stable genetic change.

Selection procedures of this kind based on careful consideration of physiological or biochemical mechanisms should also be applicable to other agronomic traits, including yield, and resistance to various stresses, such as those caused by temperature extremes, metal ions, herbicides and diseases.

Table 1. Effect of proline on salt tolerance of seedlings of *Nicotiana sylvestris*. Mean values of shoot fresh weight and adventitious root length of seedlings after 6 weeks on medium containing NaCl and/or proline.

Proline mmol/l	Shoot weight (g)		Adventitious root length (mm)	
	0% NaCl	1% NaCl	0% NaCl	1% NaCl
0	0.10	0.02	69	6
0.1	0.08	0.09	24	24

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Selection in vitro for resistances

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Summary

Attempts were made to select for disease resistance with pathotoxins in callus cultures and in microspore populations of potato and barley. Potato plants were regenerated from calluses grown in the presence of filtrates from liquid nutritional media of *Phytophthora* or *Fusarium*. Plants with *Phytophthora* resistance in vitro were screened in the field. Amongst microspore-derived barley and potato lines, resistant genotypes are overrepresented, probably as a result of spontaneous selection in the microspore population. Additionally, with fusaric acid, microspore calluses could be identified that were resistant to that pathotoxin.

Descriptors: potato, *Solanum tuberosum*, barley, *Hordeum vulgare*, selection in vitro, resistance, microspores, *Phytophthora infestans*, *Fusarium* spp.

Introduction

The actual contribution to resistance breeding of selection in tissue culture is not much more than demonstration of the feasible. Especially for applied aspects, the spectrum of reproducible results is rather small (Wenzel, 1983). In tests with barley and potato, two approaches were adopted: selection in microspore populations and in calluses.

Selection in callus populations

The most direct way of screening for disease resistance is opened up for those pathogens, which attack their host by toxins and where consequently such toxins can be added to the culture medium. In potato, this is true for *Phytophthora infestans*, *Fusarium oxysporum*, *F. coeruleum* and *F. sulphureum*, but only for *F. oxysporum* has the toxin been identified, namely fusaric acid. For the other pathogens, one has to work with a fungal growth medium (filter-sterilized and diluted or concentrated) containing the toxins.

Selection in vitro for *Phytophthora* resistance was started by Behnke (1979) with five dihaploid potato clones, from which morphogenesis – a prerequisite for such experiments – is easy to achieve (Behnke, 1975; Wenzel et al., 1980). Callus pieces 3 mm across were plated on media containing up to three times the concentration of the fungal nutritional medium, after three weeks of fungus growth. This medium was used either directly for preparation of the regeneration medium, or after additional extraction with

organic solvents. For *Phytophthora,* the effect of toxin can easily be measured by the formation of secondary callus from leaves plated on toxin-containing media or by growth of shoot-tip cultures. After threefold concentration, a killing rate of 95% was achieved for calluses.

Surviving calluses were transferred at least five times before regeneration was induced. First tests for resistance were performed on greenhouse-grown selected clones. It could be demonstrated that diameter of local lesions after mechanical inoculation with spores was significantly reduced from that for unselected control plants. The number of sporangia formed later did not differ which means that only infection efficiency was reduced and not the growth rate of the fungus or the sporulation rate. From 42440 calluses, 173 (0.41%) proved resistant; of these, 36 plants showed resistance in the greenhouse test, 34 of which were screened in the field. As the starting material was only selected for regeneration capacity but not for virus or any other resistance, most of the clones became infected in 1981 with virus and in 1982 with *Erwinia.* In the first year, only five and in the second year seven clones stayed healthy and free of *Phytophthora.* Fortunately the seven of the second year included the five of 1981. In order to get some idea of what might have happened during or before selection, we tested the protein patterns of selected and susceptible clones and compared them with the starting material. The electropherograms on polyacrylamide gels showed only slight differences. It was not possible to correlate one specific alteration to a specific behaviour during selection (Wenzel et al., 1983). The resistance has been maintained, however, over several years of vegetative propagation, which is strong argument for a stable mutation.

Selection in microspore populations

Selection amongst microspores would be simplified, when their culture in isolation from the anther can be obtained. In potato, culture of an isolated microspore is not easy as the exine is rather thick and microspore development cannot be monitored without fixation. Cereal microspores offer a better system. From microspores of the spring barley variety Dissa, several thousand calluses and first green plants were regenerated (Köhler & Wenzel, 1983). In this system, we applied fusaric acid as selecting agent at the microcallus stage, at a concentration of 0.1 mmol/l. From 1003 calluses four were screened as resistant, a range close in success of selection to that with the *Phytophthora* system. Attempts to regenerate surviving structures are in progress.

Besides this system with a defined compound, spontaneous selection can be observed also in microspore populations. It is always a central problem in microspore culture to regenerate a sufficient number of plants from an interesting genotype for further breeding work; the numbers are never large enough to guarantee the statistical presence of the better genotype. But all androgenetic lines, which showed variety characteristics, have been selected from rather small populations (Foroughi-Wehr et al., 1982). Probably spontaneous selection has taken place in vitro. This, however, raises the question whether only specific classes of microspores possessing, for instance, regeneration capacity will survive, or whether selection is random.

Cell regeneration is surely linked to internal concentrations of hormones. Genotypes that do not require too specific a hormone constellation or that contain a flexible regulation system respond more often under the imprecise external hormone concentra-

tions provided in the culture media. It looks as if these responsive genotypes are at the same time the most vigorous ones. Vigorous types are also of practical interest, which means that selection in vitro and commercial breeding share a common aim. However representative growth might also be desired; we have to admit that this cannot be guaranteed with the available microspore culture techniques.

Regardless of the previous statement, we detected the resistance we were looking for in our microspore-derived lines of potato and barley. In barley, this was resistance to mildew and to barley yellow mosaic virus. We have screened more than 300 androgenetic lines and found about half of them to be resistant to the virus. This means that there is either a positive linkage between resistance and regeneration, or a rather simple inheritance (Friedt et al., 1983). For mildew resistance, the number of regenerated resistant lines was rather high, which means that the desired genotypes were detected regardless of any spontaneous selection. These two resistances are probably not polygenically inherited; this is also true for the extreme virus resistance of potato. But even for polygenically inherited resistances, surprisingly high proportions of resistant clones could be identified in potato after microspore regeneration.

Conclusion

In microspore selection, the major advantage is probably that quantitative characters can be combined in a genotype in the homozygous condition through a haploid step; this character can then be transferred to the next generation like a qualitative trait. For callus selection, the question is still open of where this new resistance is coming from. Regardless of whether it is a culture-derived somaclone or an induced or spontaneous mutation, such variation does not advance plant breeding as such. The variability can be used in applied programs only if it is combined with a powerful selection system. In all cases, we have to be aware that resistance is a complex trait, separable into an infection, penetration, growth and propagation phase. For each stage, different mechanisms of the host are acting with the pathogen. Selection in vitro can work only at some of these levels, and as long as, for instance, even an infection process is not understood, such selections follow rather empirical routes.

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Manipulation of protoplasts, organelles and genes: applicability to plant breeding

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Summary

Recent research achievements in plant-cell culture and protoplast manipulations and in molecular genetics provide means to augment conventional plant breeding by novel techniques. The main available tools are androgenesis, protoplast culture, protoplast fusion and molecular procedures for the identification of nuclear genomes, plastomes and chondriomes.

Focusing on organelles, which harbour important breeding traits and which cannot be handled by conventional means, we developed a general procedure for unidirectional transfer of chloroplasts and mitochondria between plants. This procedure is based on 'donor-recipient' protoplast fusion, in which the nuclear division of the 'donor' protoplasts is arrested and the organelles of the fusion-progeny plants are identified by their phenotypic expression (e.g. chlorophyll deficiency, resistance to chemical agents, male sterility) as well as by the restriction pattern of DNA (deoxyribonucleic acid) in the respective organelle. Experimental results indicated that in some 'donor-recipient' combinations both chloroplasts and mitochondria could be transferred independently between species and that a rather early sorting-out of the two types of organelles took place. In other cases sorting-out (e.g. of chloroplasts) was delayed beyond sexual reproduction and there were indications that in certain 'donor-recipient' combinations only one of the two types of organelles (e.g. chloroplasts) could be transferred.

A hypothetical scheme is presented in which a given crop (*Cucumis melo* L.) is handled by protoplast manipulations, androgenesis and conventional breeding in order to achieve breeding goals such as herbicide resistance, male sterility and good fruit quality.

Descriptors: protoplast, organelle, chloroplast, mitochondrion, plasmone, chondriome, plastome, restriction pattern, cytoplasmic male-sterility

Introduction

During the 1970s, we have witnessed several research achievements which are having considerable impact on crop improvement. Among these, the most relevant to our discussion are the following: haploidization by androgenesis and other means; isolation of protoplasts and their culture up to regeneration of functional plants; cloning of plants in vitro; regeneration of somatic hybrid plants resulting from protoplast fusion; development of gene vehicles which may allow insertion of alien genes into a given plant genome; methods for isolation of mutants in vitro.

Each of these research achievements would require a lengthy review for adequate description, including 'state of the art' and applicability to plant improvement. Cloning of plants is outside our scope. The paper of King (1984) covers the utilization of plant

protoplasts for the induction and isolation of biochemical mutants and that of Schilperoort (1984) deals with the tools for transferring genes into plant genomes. The aim of this paper is to indicate how conventional breeding can be augmented by novel approaches. We shall indicate the novel tools available for crop improvement, providing references where details may be found. We shall then focus on one type of plant-cell manipulation, in which our laboratory is presently engaged, and finally take a hypothetical test-case as example of how breeding aims in a specific crop can be approached by combining sexual crosses with culture in vitro and protoplast manipulations.

Tools

Modern crop improvement may complement conventional breeding with novel tools available for several cultivated species: haploidization, mutagenesis and mutant selection at the cellular level, protoplast-to-plant systems and somatic hybridization. In addition, recombinant DNA techniques may become available for crop improvement (e.g. Cocking et al., 1981).

Haploidization This 'tool' can serve plant breeding by revealing the phenotypic expression of recessive traits and by facilitating homozygosis. It can be achieved by two main methods: gynogenesis and androgenesis (review: Chu, 1982); the latter method (review: Maheshwari et al., 1982) is applicable to a much wider range of crop plants, is highly reproducible in some crops (e.g. tobacco and other Solanaceae), fairly reproducible in several major crops (e.g. wheat) and possible, but with low yields, in other crops (e.g. *Citrus*). Androgenesis is not yet possible in some major crops (e.g. cotton). Neither is this method applicable to all cultivars of species (e.g. *Triticum aestivum*), in which some cultivars readily undergo androgenesis.

Mutagenesis and mutant isolation at the cellular level Induction and selection of mutants with agronomically useful traits may be initiated at the cellular level (reviews: Kitto, 1981; Maliga et al., 1982; Chaleff, 1983). This approach has several obvious advantages: a vast number of cells can be mutagenized and screened; the genetic variability of cultured cells can be exploited and chimeras may be avoided. On the other hand, this 'tool' has limitations: its application is limited by regeneration capacity, and screening is restricted to traits that can be revealed in vitro.

Protoplast-to-plant systems The ability of cultured cells to regenerate functional plants is prerequisite for the application of cell manipulations in breeding. Protoplasts provide the most reliable source of individual cells and are the only current means of obtaining somatic hybrid plants. Protoplasts will also be the material of choice for the integration of alien cloned genes into target plants. Thus, the availability of a protoplast-to-plant system in a given crop is a key consideration for application of cell manipulation in that crop's improvement. The progress in this field of endeavour, during the last twelve years, is impressive. The first (Takebe et al., 1971) and subsequent protoplast-to-plant systems were developed in 'model plants' such as *Nicotiana* spp., *Petunia* spp., *Hyocyamus muticus* (reviews: Vasil & Vasil, 1980; Binding et al., 1981) and in carrot (Grambow et al., 1972). Subsequently such systems became available in Gramineae (Vasil, 1982) and even

Table 1. Recent reports on protoplast-to-plant systems in cultivated plants.

Family	Species	Common name	Authors
Compositae	*Cichorium intybus*	chicory	Crepy et al., 1982
	Lactuca sativa	lettuce	Engler & Grogan, 1983
	Senecio vulgaris	–	Binding & Nehls, 1980
Cruciferae	*Brassica oleracea*	cabbage, cauliflower	Lu et al., 1982; Xu et al., 1982a; Vatsya & Bhaskaran, 1982
	Brassica napus	rape	Xu et al., 1982a; Li & Kohlenbach, 1982
Leguminosae	*Medicago* spp.	lucerne	Kao & Michayluk, 1980; Johnson et al., 1981; Arcioni et al., 1982; Xu et al., 1982b
	Trigonella corniculata	–	Xu et al., 1982b
	Trifolium repens	clover	Gresshoff, 1980
Rutaceae	*Citrus aurantium*	sour orange	Vardi et al., 1982
	Citrus limon	lemon	Vardi et al., 1982
	Citrus paradisi	grapefruit	Vardi et al., 1982
	Citrus reticulata	mandarine	Vardi et al., 1982
Scrophulariaceae	*Digitalis lanata*	–	Li, 1981
Solanaceae	*Capsicum annuum*	sweet pepper	Saxena et al., 1981b;
	Lycopersicon esculentum & *L. peruvianum*	tomato	Mülbach, 1981; Zapata & Sink, 1981; Morgan & Cocking, 1982; Köblitz & Köblitz, 1982
	Solanum melongena	egg-plant	Bhatt & Fassuliotis, 1981; Jia & Potrykus, 1981; Saxena et al., 1981a
	Solanum tuberosum	potato	Shepard et al., 1980; Thomas, 1981; Karp et al., 1982

in an orchard crop (Vardi et al., 1975; Galun et al., 1977). Protoplast-to-plant systems are now available for several economic crops (Table 1) and the list will probably extend.

Somatic hybridization The potential advantages of establishing somatic hybrid plants by fusion of two protoplasts having different nuclear genomes has been thoroughly discussed in several reviews (e.g. Schieder & Vasil, 1980; Schieder, 1982; Shepard et al., 1983). These authors also listed the somatic hybrids obtained by this technique. Unless fusion is undertaken between phylogenetically distant species, the chromosome number of the resulting regenerated hybrid plants will be the sum of the parental-plant chromosomes. Thus a haploidization step should precede protoplast fusion or the resulting somatic hybrids should be haploidized in order to restore normal chromosome number. Success in somatic hybridization was achieved mainly with model plants and ornamentals, but hybrids between two crops like tomato and potato have also been reported (Melchers et al., 1978; Schiller et al., 1982; Shepard et al., 1983). These hybrids do not reproduce sexually but may become useful by vegetative propagation.

One aspect of somatic hybridization, which will be detailed below, is to establish a

fusion product having only one functional nucleus but organelles from both fusion partners (Galun & Aviv, 1978). A general procedure to obtain such fusion products and to secure the respective cybrid plants was developed in our laboratory and termed the 'donor-recipient' technique (Zelcer et al., 1978. Reviews: Galun, 1982; Galun & Aviv, 1983). This procedure may thus lead to unidirectional transfer of organelles, either intraspecifically, between cultivars and mutants, or interspecifically, between cultivars and their wild relatives.

Organelle transfer by protoplast fusion

Genetic information encoded in angiosperm organelles Chloroplasts and mitochondria harbour in their respective genomes (i.e. the plastome and the chondriome) a multitude of genes; several of these genes were shown to have breeding value and additional important breeding traits are assumed to be coded by the organelle genomes since these traits are 'cytoplasmically' inherited in crops whose organelles are transmitted maternally (reviews: Bohnert et al., 1982; Leaver & Gray, 1982; Galun, 1982; Galun & Aviv, 1983).

To list all the known genes coded by the angiosperm plastome is beyond our scope but let it be noted that, in addition to being autonomous for rRNAs and tRNAs, the plastome encodes for several polypeptide subunits of enzymes involved in photosynthesis, such as the large subunit of ribulose-1,5-biphosphate carboxylase/oxygenase, subunits of the ATP synthetase complex and other polypeptides of the photosystems. Probably each of these proteins has subunits coded by the nuclear genome, translated on cytoplasmic ribosomes, transferred into the chloroplasts and finally complexed there with plastome-coded polypeptides. Thus a concerted synthesis and subunit complementation seems to be required (Bohnert et al., 1982). There is good genetic evidence that traits like streptomycin (Maliga et al., 1975), lincomycin (Cseplo & Maliga, 1982), and tentoxin (Durbin & Uchytil, 1977) resistances, as well as resistance to the herbicide atrazine (Gasquez & Barralis, 1979) are controlled by the plastome.

The genetic information encoded in the mitochondrial genome (the chondriome) of angiosperms is only beginning to appear (Leaver & Gray, 1982) but there is fair evidence from several crop plants that resistance to specific toxins and alloplasmic (i.e. 'cytoplasmic') male-sterility are chondriome-controlled. According to information from mammalian and fungal mitochondria, the chondriome encodes the organelles tRNAs and rRNAs. Several enzymes in the mitochondrion seem to have subunits coded by the chondriome. Thus, as in the chloroplast, a concerted syntheses of nuclear and chondriome-coded polypeptides seems to be required for the normal function of mitochondria.

Transfer of organelles by protoplasts manipulation In almost all crop plants, chloroplasts are transmitted maternally (Sears, 1980), and probably so too are mitochondria. Thus organelle-controlled characters are rather difficult to handle by conventional breeding. Moreover, since chloroplasts and mitochondria are transmitted together, sexual hybridization cannot cause the transfer of only one of these organelles from a donor source into a target cultivar.

We have recently reviewed the studies on somatic hybridization in angiosperms in which the organelle composition of the hybrid progeny was followed (Galun, 1982;

Galun et al., 1982b; Galun & Aviv, 1983). Briefly, the fate of chloroplasts was followed in most of the reported interspecific somatic hybrids of *Nicotiana* as well as in the tomato-potato hybrid. In earlier studies (e.g. *N. glauca* + *N. langsdorffii, Lycopersicum esculentum* + *Solanum tuberosum, N. tabacum* + *N. debneyi*), the chloroplasts were identified by isoelectric focusing of the large subunit of their respective ribulose-1,5-bi-phosphate carboxylases. In later studies the responses to streptomycin and tentoxin, and the restriction pattern of the cpDNA were used to characterize the chloroplast compositions of somatic hybrids and cybrids. More recently a rather simple procedure was developed in our department, based on hybridizing, by the Southern blotting technique, radioactive cpDNA probes to blots of gels after electrophoresis of total plant DNA digested with specific endonucleases (Fluhr et al., 1983b). By this procedure only small amounts of tissue (200 mg or less) are required and ten or more samples can be handled simultaneously.

The fate of mitochondria in somatic hybrids and cybrids, resulting from protoplast fusion was followed in only a few cases (Galun, 1982; Galun et al., 1982b; Galun & Aviv, 1983). Typically (contrary to chloroplasts), the restriction patterns of the mtDNA of hybrids (and cybrids) are similar but not identical to either of the fusion partners (e.g. Belliard et al., 1979; Nagy et al., 1981; Galun et al., 1982a; Aviv et al., 1983). There are indications (Nagy et al., 1983) that coexistence of different chondriomes in one fusion cell is a necessary condition for mtDNA rearrangement in *Nicotiana*. Nevertheless there is a 'phenotypic' sorting-out of characters (e.g. male sterility), which are assumed to be controlled by the chondriome. Hence, for practical breeding, mitochondria may be considered to sort out after protoplast fusion, in a manner similar to the sorting out of chloroplasts.

An efficient way to transfer either or both chloroplast and mitochondria unidirectionally from a 'donor' plant to a target ('recipient') plant was developed in our laboratory. We called it the 'donor-recipient' technique. By this technique, the nuclear division of 'donor' protoplasts is arrested with X-rays or gamma-rays, before fusion with 'recipient' protoplasts. With suitable selection procedures, only colonies from heterofusions are retained. These colonies should result from fused protoplasts containing functional nuclei from the 'recipient' only but organelles from both fusion partners. This procedure was improved by P. Maliga and associates by treating 'recipient' protoplasts with iodoacetate, which causes a transient metabolic inhibition. The inhibition is removed when the 'recipient' protoplasts are fused with the irradiated 'donor' protoplasts (e.g. Sidorov et al., 1981). The 'donor-recipient' technique, with or without the modification is a much more efficient tool for organelle transfer than regular somatic hybridization, by which the progeny is composed of hybrid nuclei, which are commonly tetraploid. Such nuclei are often troublesome when the hybrids are wanted for breeding purposes.

The results of recent studies on organelle transfer by protoplast fusion and somatic hybridization (including cybrid formation) are summarized in the following paragraphs.

When chloroplasts from two sources are introduced into one heteroplastomic fusion cell and the cell is cultured to result in a hybrid (or cybrid) plant, it is common (albeit with notable exceptions, Fluhr et al., 1983a; 1983b) that sorting-out of chloroplasts follows, resulting in fusion plants with homoplastomic cells identical in either parental cpDNA. However despite a sorting out of mitochondrial characters, the resulting cybrids' mtDNAs are similar but rarely identical to those of either fusion partner.

There are cases (e.g. Zelcer et al., 1978; Aviv & Galun, 1980; Aviv et al., 1980) in which chloroplasts and mitochondrion-controlled characters (e.g. male sterility) sort out independently. However either the 'donor' chloroplasts or its mitochondria may not be compatible with the 'recipient' nuclei, thus resulting in transfer from the 'donor' of only one of the two kinds of organelles. We do not have much information on interspecific organelle-nuclear compatibilities but at this stage we should keep in mind that there could be differences between plastome-nuclear and chondriome-nuclear compatibilities (Aviv et al., 1983).

Detailed information on chloroplast and mitochondria transfers is as yet mostly restricted to model plants of the genus *Nicotiana*. Though the same basic mechanisms probably operate in other genera, verification is essential before the techniques developed in *Nicotiana* be employed in breeding of crop plants.

Combining protoplast manipulation and androgenesis with conventional breeding: a demonstrative example

With the aim to achieve breeding goals such as herbicide resistance, male sterility and good fruit quality in *Cucumis melo* (melon) practical combination of protoplast manipulations and induced haploidization with conventional breeding will be demonstrated by a hypothetical example (Fig. 1). As yet, there is no protoplast-to-plant system in *C. melo*, but melon protoplasts can be isolated and cultured up to the maintenance of callus culture (G. Wackerle & E. Galun – unpublished). Regeneration of plants from the latter calli is thus feasible. Likewise we have no information on chloroplast-controlled herbicide resistance in melon cultivars or in their wild relatives and chloroplast-controlled herbicide resistance has not yet been transferred by protoplast fusion from a resistant wild species into a cultivated crop, though attempts along this line have been reported

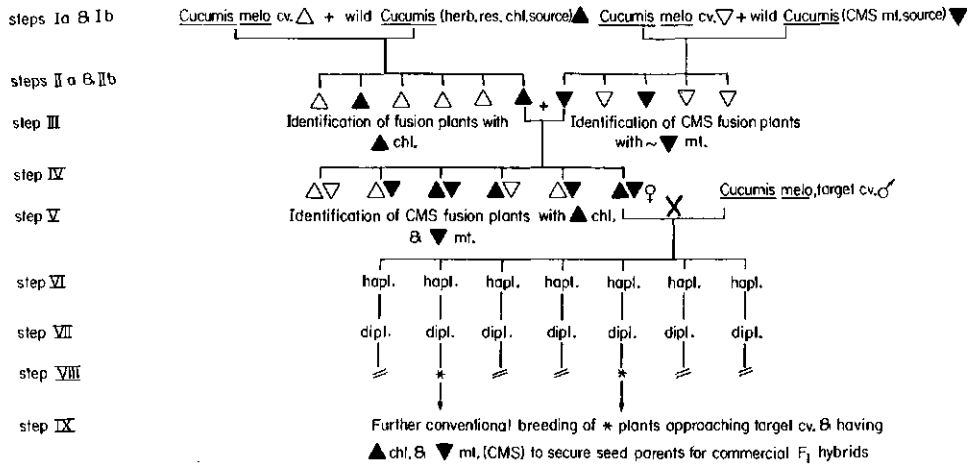

Fig. 1. Hypothetical scheme for the utilization of protoplast manipulations, androgenesis and conventional breeding to produce a herbicide-resistant and male-sterile breeding line, which may be further used as seed parent in hybrid seed production. See text for explanations.

(Binding et al., 1982). Neither do we know whether mitochondria from alien *Cucumis* species will cause alloplasmic male-sterility when transferred into melon cultivars, but this possibility is in the line of previous information. Finally, androgenesis has not yet been reported in melon but it has proved possible in the related cucumber (Lazarte & Sasser, 1982). Thus, although the scheme presented in Fig. 1 is hypothetical in several respects, it is not too far from reality. This example should be useful to demonstrate the general procedure.

In the following, the scheme will be detailed step-wise.

- Step Ia. Protoplasts will be isolated from a cultivar, these protoplasts will serve as 'recipients'. In parallel, protoplasts will be isolated from a *Cucumis* source with chloroplast-controlled herbicide resistance and will serve as chloroplast 'donors'. 'Recipient' and 'donor' protoplasts will be treated by techniques described in the previous chapter, fused and cultured up to the stage of plantlets.
- Step Ib. This step will be undertaken in parallel to Step Ia, but 'donor' protoplasts will be obtained from a wild *Cucumis* species, whose choice may be based on previous information, indicating that the combination of its 'cytoplasm' induces male sterility when combined with *Cucumis melo*. For instance male sterility could be obtained by crossing this wild species with pollen of *C. melo*, followed by repeated backcrossing to *C. melo*.
- Step IIa. The cybrids resulting from Step Ia will be screened for their chloroplast compositions and those having herbicide-resistant chloroplasts will be retained.
- Step IIb. The cybrids resulting from Step Ib will be screened at the plantlet stage for mitochondrial compositions and those having wild *Cucumis* mitochondria will be retained, or the plantlets will be kept up to flowering and those showing male sterility will be retained.
- Step III. Protoplasts from plants retained from Step IIa will be fused with protoplasts of plants retained from Step IIb. A 'donor-recipient' fusion procedure will be employed leading to cybrids. Protoplasts of either Step IIa or Step IIb may serve as 'donors' and, respectively, protoplasts of either Step IIb or Step IIa may serve as 'recipients'. The fusion products will be cultured up to the plantlet stage.
- Step IV. The plantlets obtained in the previous step will be screened for both chloroplast and mitochondria. Those containing herbicide-resistant chloroplasts and mitochondria from the wild *Cucumis* will be retained. Rather than testing for mitochondria, the screening may be delayed up to flowering and male-sterile plants with the required chloroplasts will be retained.
- Step V. Plants retained from Step IV will be crossed with pollen from a target *C. melo* cultivar and as many plants as possible will be raised to flowering. These plants should be male-sterile.
- Step VI. Anthers from Step V plants will be cultured to induce androgenesis. From experience with tobacco, tomato and other plants, male sterility should be manifested beyond the first microspore mitosis – thus there is a high probability that male sterility will not interfere in androgenesis. The haploids obtained from anther culture will be cultured to the plantlet stage.
- Step VII. If diploidization does not occur spontaneously, an induced diploidization will be performed.
- Step VIII. The diploid plants obtained by the previous step will be screened for

horticultural characters (e.g. yield, fruit quality) and those closest to the target cultivar will be retained.

- Step IX. The latter plants may be propagated by repeated backcrossing with the target cultivar and will serve as breeding material, containing the required organelle composition, in order to establish potential seed-parents for commercial F_1 hybrid melon varieties.

The male-sterile breeding lines established in Step VIII will serve in conventional breeding procedures (Frankel & Galun, 1977) to find the best hybrid combination between these lines and normal breeding lines which will serve as pollen parents.

The steps may be amended in several ways. For example if a plant-to-protoplast system be developed in a target cultivar, Step V and possibly even Steps VII and VIII can be eliminated. It may also turn out that Step V will be required but rather than haploidizing the F_1 at this step the plants should be backcrossed several times to target cultivars.

Conclusion

The main message of this presentation is to indicate how protoplast-culture and fusion techniques currently available, with the possible addition of haploidization, can be combined with established procedures of plant breeding (Borlaug, 1983) in order to improve crops in the immediate future. Other techniques, like direct integration of useful genes into the plant genome (Cocking et al., 1981) or the use of recombinant DNA in conjunction with appropriate gene-vehicles (as described by Schilperoort, 1984) are still in the experimental stage but may become useful within the next decade.

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Genetic manipulation of cytoplasmic plant genes

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Summary

We are developing genetic manipulation methods for genes of plant cell organelles. Our attention is focused on determining the molecular basis of cytoplasmic male-sterility in *Petunia hybrida* and on transfer of this property by somatic cell cybridization. We are also using autonomously replicating sequences (ARSs) from *P. hybrida* chloroplast DNA to construct vectors for transfer of specific organelle genes.

Descriptors: cytoplasmic male-sterility, chloroplast, mitochondrion, autonomously replicating sequences (ARSs), cell organelle vectors

Introduction

Essentially all efforts directed towards the development of genetic manipulation systems for plants are being focused on nuclear genes. However a number of important plant genes are located in the cytoplasmic cell organelles. The DNA of chloroplasts and mitochondria codes for polypeptides involved in essential processes like respiration, photosynthetic CO_2 fixation and ATP synthesis. Moreover, other commercially interesting properties like cytoplasmic male-sterility and resistance to certain plant pathogens and herbicides are also located on cell organelle DNA. The aim of our research is to develop manipulation methods for the cytoplasmic genetic information of cultivated plants. Our attention is focused on determining the molecular basis of cytoplasmic male-sterility in *Petunia hybrida* and on the transfer of this property to other, economically important species of the family Solanaceae by somatice cell cybridization. These techniques permit the transfer of entire cytoplasms, including organelles, between plant species. A more direct approach for the transfer of desired cytoplasmic properties would be the transfer of specific organelle genes. For the introduction of such genes, we have isolated the replication origins of organelle DNA by molecular cloning and we use these for the construction of cloning vectors that can replicate autonomously in plant organelles.

Molecular basis and transfer of cytoplasmic male-sterility

Cytoplasmic male-sterility (CMS) is a maternally inherited trait, which is of great economic importance for the commercial production of hybrid seed because it prevents normal pollen development and thus eliminates self-fertilization of the seed parent plant. The aim of our research is to broaden the applicability of this property, which is as yet limited because it is found only in a few plant species, including *Petunia hybrida.*

Molecular basis of cytoplasmic male-sterility in Petunia hybrida To elucidate the molecular basis of CMS in *Petunia hybrida,* DNA of chloroplasts and mitochondria of fertile (F) and CMS plants were characterized with restriction endonucleases. Mitochondrial DNA of F and CMS plants showed various differences when digested with a number of restriction endonucleases (Fig. 1A). Such differences were not observed between the restriction fragment patterns of chloroplast DNA of these plants.

Differences between F and CMS plants were also observed at the level of polypeptide synthesis in isolated mitochondria. In mitochondria of fertile plants, a polypeptide of molecular weight 38000 daltons was synthesized that was not observed in CMS mitochondria (Fig. 1B). The absence of this polypeptide in CMS mitochondria was associat-

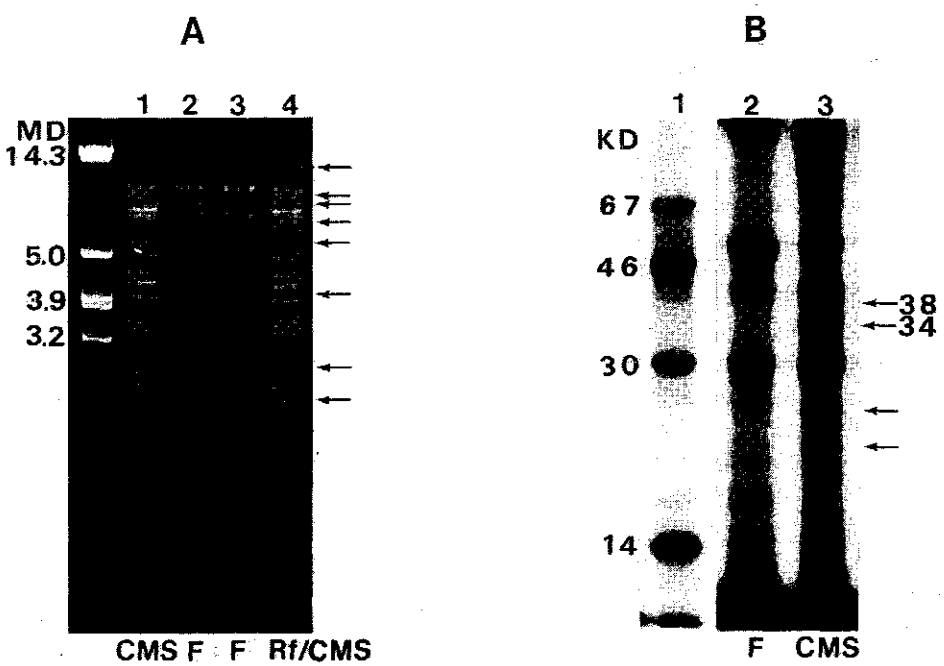

Fig. 1. A. Electrophoretic patterns on agarose gel of BamHI digested mitochondrial DNA from two cultivars of fertile *Petunia hybrida* plants (Lane 2 and 3), a cytoplasmic male-sterile plant (Lane 1) and a plant with restored fertility (nuclear *Rf* gene) with CMS cytoplasm (Lane 4).
B. Autoradiograph of electrophoretic patterns on polyacrylamide gels of [^{35}S] polypeptides, synthesized by isolated mitochondria from fertile (Lane 1) and CMS (Lane 2) *P. hybrida* plants.

ed with the presence of a polypeptide of 34000 daltons that is not synthesized in F mitochondria.

Differences in polypeptide synthesis between F and CMS plants were not observed in chloroplasts. The differences between F and CMS plants so far observed all concern the mitochondria. Therefore these results support the hypothesis that mitochondrial DNA is responsible for the cytoplasmic male-sterility in *Petunia hybrida.*

Transfer of cytoplasmic male-sterility from P. hybrida to other species We are now trying to transfer CMS from *P. hybrida* to other plants of the family Solanaceae by cybridization. This technique, which involves the use of a fusion partner with nuclei inactivated by X-rays (Aviv & Galun, 1980) can be combined with the use of recipient protoplasts treated with iodoacetate, which causes a transient inhibition of cell metabolism, that is restored by fusion (Siderov et al., 1981). Essential technical procedures for such an approach as protoplast isolation and plant regeneration have been accomplished recently in our laboratory for cultivars of *Lycopersicon esculentum* such as 'Bellina' and 'Money Maker'. The paper of Galun & Aviv (1984) deals in detail with the cybridization technique and the fate of organelles and their DNA in the resulting heteroplasmic state in the fusion products.

Construction of vectors for organelle genes: cloning of autonomously replicating sequences (ARSs) of P. hybrida chloroplast DNA

Vectors for organelle genes must meet two requirements: they must contain marker genes that permit selection or detection of cells harbouring organelles with the vector, and properties must be present for stable maintenance of the vector in the organelle.

For this purpose chloroplast DNA sequences of *P. hybrida* which are capable of autonomous replication were isolated in yeast by the method of Stinchcomb et al. (1980). This method has been employed successfully for the isolation of various autonomously replicating sequences (ARSs). The essence of the method is the use of a vector, YIp5 (Struhl et al., 1979) composed of the *E. coli* pBR322 vector and the yeast *S. cerevisiae ura*-3 gene, but lacking an origin of replication suited for the yeast host. So, this vector allows cloning of DNA sequences that can function as an origin of replication in yeast.

To isolate *P. hybrida* chloroplast DNA fragments with ARSs, restriction enzyme digests of purified chloroplast DNA were prepared with the enzymes EcoRI and BamHI and ligated into the linearized vector YIp5. After transformation of *S. cerevisiae* strain DL1 with the ligated preparation, several ura^+ transformants were obtained. Analysis of the transformed yeast cells showed a typical ARS phenotype (Stinchcomb et al., 1980). For more detailed analysis of the plasmids present in the yeast cells, DNA preparations were isolated from three different yeast strains and transformed into *E. coli* strain JA221. Restriction enzyme analysis of DNA preparations isolated from the transformants showed that plasmid pPCY3 had a BamHI insert of about 55 base pairs and that plasmids from the other two transformants (pPCY20 and pPCY28) both contained a 5.4 kb EcoRI fragment (with 5400 base pairs) but in reversed orientation. The chloroplast DNA character of the inserts was confirmed by specific hybridization of the inserted fragments with [^{32}P] DNA from chloroplasts (Fig. 2).

As the 55 bp BamHI insert had no homology with the 5.4 kb EcoRI insert (results not

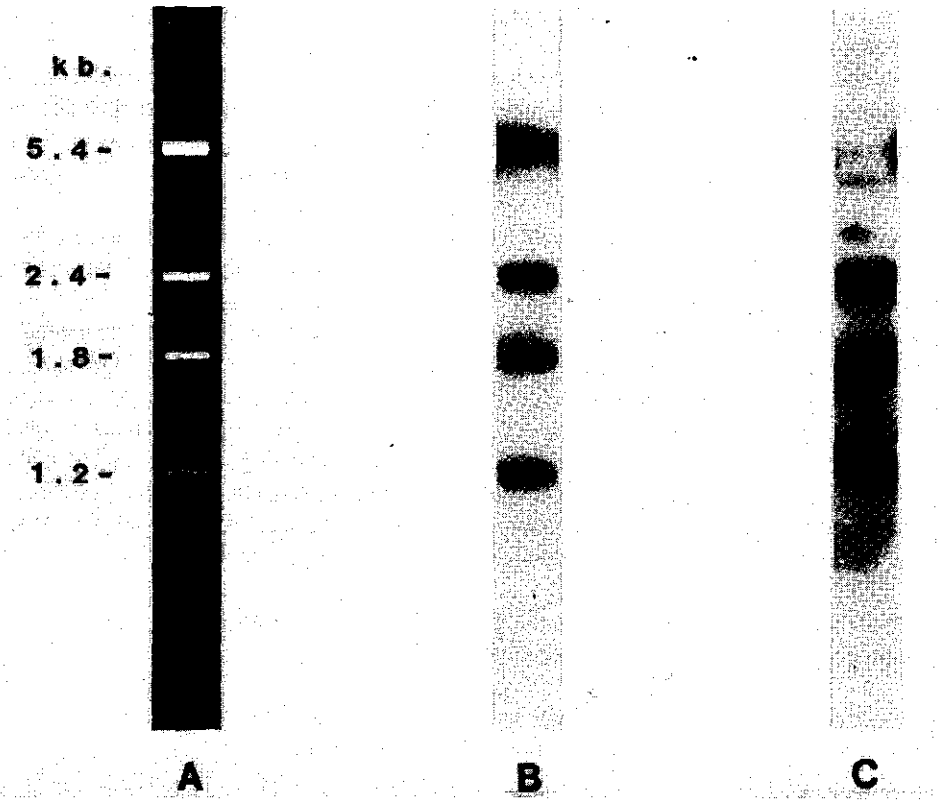

Fig. 2. Identification of the chloroplast nature of the ARS (autonomously replicating sequence) insert into plasmid pPCY20. Electrophoresis on agarose gels of the EcoRI/HindIII double-digest of pPCY20 DNA (Lane A) shows the vector fragment (5400 base pairs) and three other fragments (2400, 1800 and 1200 base pairs) originating from the cloned insert. Hybridization of these fragments after transfer onto nitrocellulose paper showed that all four bands hybridized with [^{32}P] pPCY20 DNA (Lane B) and that [^{32}P] chloroplast DNA hybridized specifically with three inserted fragments (Lane C).

shown), the *P. hybrida* chloroplast genome must contain at least two different sequences capable of autonomous replication in yeast. Experiments are in progress to determine whether the same autonomously replicating sequences can be isolated with other restriction enzymes or that still other sequences exist, and to map the ARSs on the chloroplast genome. Studies of these plasmids in a replication system in vitro prepared from isolated chloroplasts (Overbeeke et al., 1983) provide further evidence whether these sequences function in chloroplasts as an origin of replication.

Insertion of such ARSs in plasmids with an appropriate organelle gene that can function as selection marker (Galun & Aviv, 1984) should result in a vector for plant organelles. The availability of such vectors and of techniques like cybridization opens the way for introduction into plants of organelle genes involved in economically important properties such as photosynthesis, cytoplasmic male-sterility and resistance to herbicides and plant pathogens.

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Cytoplasmic hybridization in higher plants

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Summary

Rapeseed plants regenerated after protoplast fusion, combining desirable cytoplasmic traits of *Brassica napus* and *Raphanus sativus* or *Brassica campestris* and *Raphanus sativus* underline the usefulness of cytoplasmic genetics already described on a model species, *Nicotiana tabacum*.

Descriptors: protoplast, organelle, chloroplast, mitochondrion, cytoplasmic male-sterility, atrazine resistance

Introduction

By gathering in the same cell different cytoplasmic genomes (normally isolated by sexual reproduction), protoplast fusion offered new experimental means to test out possibilities of exchange and recombination. With this view, clear cut experimental systems are available in the form of cytoplasms belonging to distinct species which are easily distinguished genetically and biochemically.

We describe briefly here results we have obtained in *Nicotiana* and *Brassica*.

The case of Nicotiana species

Two varieties of *N. tabacum* were used, differing by nuclear markers and also by the cytoplasm (*N. tabacum* for the first one which was fertile and *N. debneyi* for the second one which was male-sterile with strongly feminized flowers). After protoplast fusion, two kinds of new combinations were obtained among regenerated plants without selection at the tissue culture level: nuclear hybrids (about 3%) and about 20% so-called cybrids because they possessed a nucleus of one or the other parents but new flower morphologies (Belliard et al., 1977). These new types of flower found also in the great majority of hybrids were stably maternally inherited. This trait can be defined as follows: petals (P) are more or less coalesced; the length of stamen filament (F) is variable. Although these traits depended on environmental conditions, it was possible to define each cybrid by its progeny in field trials in 1977 and 1978. Figure 1 shows the distribution of progenies of some cybrids according to P and F traits (each number corresponds to a given cybrid). The main conclusions were as follows:

- The cybrids are distributed, roughly between the parents, but sometimes with reverse

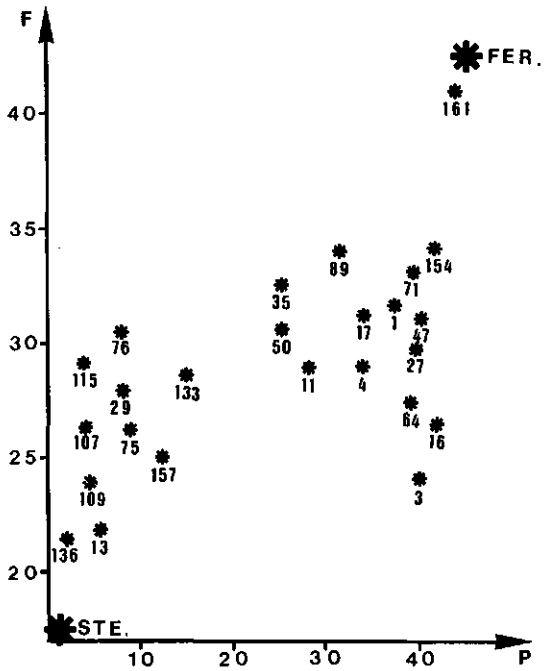

Fig. 1. Distribution of a sample of cybrids of *Nicotiana tabacum* regarding two traits of flower morphology. Each number corresponds to the progeny (75 plants) of a given cybrid. Differences are significant between cybrids. Each type of flower was stable through further generations. FER: fertile parent. STE: sterile parent. P: lenght over which petals were coalesced (mm). F: length of filament (mm).

combination of the two traits.

- It is impossible to make discrete classes of cybrids.
- The number of different flower morphologies approaches the number of cybrids obtained.
- Moreover the same is true for progenies of hybrids after several backcrosses with normal *N. tabacum* to go towards the diploid chromosome number.

Apart from these 'stable' cybrids, about 10% show 'unstable' phenotype, which can be fixed after one or two backcrosses and may correspond to somatic sorting out of cytoplasmic determinants.

At the molecular level, several plants were tested by DNA restriction pattern. CpDNA analyses show that flower morphology had nothing to do with plastid type (*N. tabacum* and *N. debneyi*) (Belliard et al., 1978). At the outset, each cybrid and its progeny, studied in some cases until the 6th generation, possess their own mtDNA restriction pattern. A cybrid possesses some more or less specific fragments of each parent and new bands in almost all cases. The more it possesses specific bands of the fertile parent, the more its flower morphology approaches normal (and inversely). This appears true also when using another enzyme (Belliard & Vedel, unpublished results).

In view of these new phenotypes, the most appropriate hypothesis is DNA recombina-

tion between parental mitochondrial DNA (Belliard et al., 1979). This hypothesis is based on the newness of the phenotypes, their mode of sexual transmission, their stability during vegetative propagation as well as in successive sexual generations, the absence of such variation in controls without protoplast fusion and the biochemical characterization of organelle DNA.

Alternative hypotheses can now be tested with experimental data. That a mixture of parental mitochondria explain the new flower types is ruled out both by DNA patterns which are never the sum of parental patterns, and by the genetic analysis of progenies: it is very difficult to imagine a continuous variation in morphology between progenies of cybrids without variation inside the progeny when cybrid types are so numerous. Another hypothesis would be that mtDNA of *N. tabacum* is composed of several distinct molecules. Cybrids would then result from 'interchromosomal' recombination between parental mitochondrial chromosomes. This hypothesis alone cannot explain either the distribution of phenotypes (one would expect discrete classes of phenotype depending on the combined chromosomes) or the DNA patterns obtained (the number of chromosomes being as high in this hypothesis as the number of specific fragments!).

Another hypothesis refers to DNA rearrangements without exchange between parental mtDNA. Such rearrangements have been found in plants regenerated after a long period of tissue culture in male-sterile maize giving male-fertile plants (Kemble et al., 1982). This hypothesis does not easily explain either the patterns obtained (occurrence of a mixture of some specific parental fragments, being highly improbable reassociations, considering their number) or – and this is a strong argument – the fact that such changes do not occur in controls without fusion. (It cannot be ruled out that some modifications are superimposed by the tissue-culture techniques, but controls show that this practice does not affect the cytoplasmic male-sterility nor flower morphology in this experimental system.)

The case of Brassica species

Starting from a sexual cross between cytoplasmically male-sterile *Raphanus sativus* (Ogura, 1968) as female and *Brassica oleracea* as male, the *Brassica* genome was introduced by repeated backcrosses into radish cytoplasm (Bannerot et al., 1974). *B. napus* and *B. campestris* alloplasmic lines have been derived from the same cytoplasm by crosses and backcrosses with these species. In all alloplasmic combinations, resulting plants exhibited yellowing at low temperature (below 15 °C) and although green at higher temperature, they always maintained a low level of chlorophyll (Rousselle, 1981). In all cases, the plants are cytoplasmically male-sterile (cms).

In addition, the flowers of these plants have less developed nectaries and a reduced production of nectar. This may be an important defect in hybrid seed production, since the cms plants are less attractive for honey bees, the most important agents in cross-pollination (Mesquida & Renard, 1978).

Another type of alloplasmic situation is of interest for *Brassica* crops. An atrazine-resistant *B. campestris* biotype has been discovered (Maltais & Bouchard, 1978; Souza-Machado et al., 1978). This resistance has been proved to be maternally inherited and associated with the reducing side of Photosystem II (suggesting that this trait is encoded in plastid DNA). Indeed alloplasmic *B. napus* lines with *B. campestris* atrazine-resistant

cytoplasm have been shown to be fully resistant to this herbicide (Beversdorf et al., 1980). Atrazine resistance could be useful in weed control. An attempt to transfer atrazine resistance by protoplast fusion from *Solanum nigrum* to *Solanum tuberosum* has been published (Binding et al., 1982).

By fusing rapeseed protoplasts, the one bearing *Brassica napus* cytoplasm, the other *Rhaphanus* cytoplasm, we obtained plants with a new hybrid cytoplasm: they possess rapeseed chloroplasts ensuring a normal chlorophyll content of the plant and mitochondrial DNA sequences of *Raphanus*, inducing male sterility. In a second experiment, the same rapeseed line with *Raphanus* cytoplasm was fused with a protoplast of a rapeseed line possessing atrazine resistant cytoplasm of the Canadian biotype of *Brassica campestris*, resulting in cybrids, that combined the cms trait (*Raphanus* mitochondria) and atrazine resistance (*B. campestris* chloroplast) associated with the rape (*B. napus*) nucleus. Such plants cannot be obtained by conventional means and could be useful in *Brassica* crop improvement (commercial production of F_1 hybrid seeds) as well as in fundamental research on cytoplasmic male-sterility.

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New procedures for selection and cultivation of somatic hybrids and cybrids

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Summary

A new technique is described for the production of somatic hybrids and cybrids. The method is based on the use of microcapillary pipettes with which fusion bodies can be picked out. Culture of the fusion bodies is undertaken in protoplast suspensions derived from an auxotrophic mutant. Selection of the somatic hybrids or cybrids follows by the removal of the supplement essential for survival of the auxotrophic cell colonies.

Descriptors: tobacco, *Nicotiana,* fusion bodies, nurse culture, auxotrophic mutants, somatic hybrid, cybrid

Galun & Aviv (1984) propose a breeding scheme for *Cucumis melo* combining protoplast fusion, androgenesis and conventional breeding. However they have not mentioned how they will select the cybrids after fusion of the protoplasts. Selection of the few somatic hybrids or cybrids from a mixed population of regenerating protoplasts is a key step in successful hybridization experiments (Schieder, 1982). This paper presents a selection procedure for somatic hybrids which also could be used for the production of cybrids.

The method is based on the use of microcapillary pipettes with which somatic hybrid fusion bodies can be picked out and transferred to a protoplast suspension derived from auxotrophic mutants (Hein et al., 1983). Selection of somatic hybrids or cybrids follows after visible colonies have developed by removal of the supplements necessary for survival of the auxotrophic cells. The following experiments demonstrate the potential of this method. Protoplasts derived from cell suspensions of *Nicotiana tabacum* were mixed with mesophyll protoplasts of *N. paniculata, N. sylvestris,* or *Petunia* 'Mitchell' (Mitchell et al., 1980) and treated with polyethylene glycol (PEG) (volume fraction 0.25) dissolved in a mixture of mannitol solution (0.45 mol/l) and $Ca(NO_3)_2$ solution (0.1 mol/l). After washing with $Ca(NO_3)_2$ solution (0.275 mol/l), numerous fusion bodies were observed and were picked out with micropipettes by means of a micromanipulator. They were transferred into a protoplast suspension derived from the nitrate reductase deficient cell line CNX-68 of tobacco, originally isolated by Müller & Grafe (1978). This mutant cannot metabolize NO_3^- as sole source of nitrogen. However in media supplemented with amino acids, growth is optimum. The mutant and the heterokaryons were cultivated together in a regenerative medium supplemented with amino acids, in which

they all developed to visible colonies. After transfer of the cell colonies onto agar medium with NO_3^- as sole nitrogen source, only the somatic hybrid colonies survived and grew further.

Numerous hybrid colonies between *N. tabacum* and *N. paniculata* or *N. sylvestris* were obtained by this selection procedure (Hein et al., 1983). A few presumed hybrid colonies between *N. tabacum* and *Petunia* 'Mitchell' have also been observed (Hein & Schieder, unpublished). Somatic hybrid character of the cell colonies was confirmed by isoenzyme investigations and for a few colonies by gas chromatography by the method of Ninnemann & Jüttner (1981). We believe that this method will also be useful for other plant species. For example, divisions of fusion bodies between the two potato species *Solanum tuberosum* and *S. berthaultii* have been observed after transferring them into a protoplast suspension derived from the tobacco mutant CNX-68 (Hein, unpublished). This observation demonstrates that it is not obligatory that the mutants used as a 'nursery' are derived from one of the fusion partners.

This selection and cultivation procedure for somatic hybrid fusion bodies should be generally applicable and could also be used for production of cybrids with the donor-recipient technique of Zelcer et al. (1978). Figure 1 is a selection scheme for the transfer of male-sterility inducing cytoplasm (CMS) from one species to another with the help of microcapillaries and using a protoplast suspension derived from auxotrophic mutants as

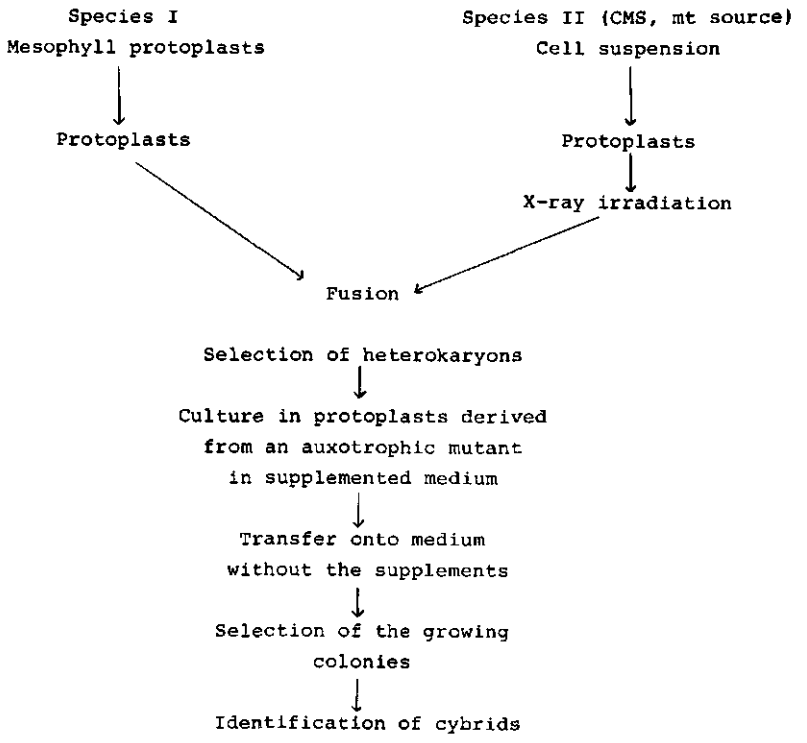

Fig. 1. Selection and cultivation scheme for fusion products in cybridization experiments.

'nursery'. Auxotrophic mutants from several other plant species, for instance of *Datura innoxia, Hyoscyamus niger, Petunia* 'Mitchell' line,. *Nicotiana plumbaginifolia* and soybean (King et al., 1980; Gebhardt et al., 1981; Steffen & Schieder, 1983; Márton et al., 1982; Roth & Lark, 1982) exist which also could be utilized as a nurse culture and it is hoped that in the near future such mutants can be obtained from many other plant species as well.

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Potential of cells and genes of higher plants

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Summary

In the past thirty years, molecular biological research has given rise to the development of molecular genetics. This new science has furnished understanding of the structure and organization of genes and of the regulation of their expression leading to a certain phenotype. Although most research in molecular biology and molecular genetics was originally focused mainly on the bacterium *Escherichia coli,* it has now been extended to various eukaryotes including plants. Eukaryotes have a much more complex genome structure than bacteria. Study on this structure in eukaryotes became feasible only by the development of recombinant-DNA technology about ten years ago. It is now well established that individual genes can be isolated and functionally reintroduced either into the same organism or into completely different organisms by this technology, i.e. genes can be transferred functionally from prokaryotes to eukaryotes and vice versa. Recent developments in molecular biology of plants have shown that by the Ti (Tumor-inducing) plasmid of *Agrobacterium tumefaciens* one – or a number of – foreign genes can be introduced and expressed in plants and that introduced genes can be stably transmitted into progeny.

An important feature of the application of recombinant DNA in the genetic manipulation of plants is that not only plant genes can be used but, if required, also genes from other eukaryotes including animals, or from bacteria. Moreover genes can be added to an otherwise unchanged genome with this technology. An essential contribution has been the development of methods for the isolation, culture and regeneration into intact plants of naked plant cells, protoplasts, from organs of various plant species. Protoplasts can also be fused, i.e. somatic-cell hybridization, for asexual mixing of genomes and cytoplasms derived from related and less related plants. Both types of approach for genetic manipulation of plants, i.e. the introduction of defined genes by recombinant DNA and somatic-cell hybridization could bypass barriers that are inherent in conventional methods for transfer of genes. Therefore genetic manipulation of plants could have far-reaching prospects in plant breeding, when properly combined with conventional methods, and in industrial production of valuable plant products in plant-cell biotechnology. However, many complex problems have still to be solved before we learn how to make optimum use of the potential of cells and genes of higher plants in the next decades.

Descriptors: molecular genetics, gene structure, gene organization, recombinant DNA, genetic manipulation, somatic hybridization, gene transfer, Ti plasmid, plant cell biotechnology, binary-vector system

Introduction

Mankind has explored the potentials of plants for more than 10000 years. At first, plants were noticed that occasionally developed more beneficial features on their own. Without any knowledge of heredity, man learned to use the better plants to obtain cultivated plants with higher productivity. Well devised improvement of plants by sexual crosses and selection started at the beginning of this century when Mendel's laws became known. However, the lack of knowledge on the nature of genes and their mode of action was still a limitation for optimal exploitation of the potentials of genes of higher plants.

The past thirty years of molecular biologic research have changed this situation by the discovery of DNA as the chemical substance carrying the blueprint of life and by the unravelling of the genetic code and the functional structure of DNA molecules. A new science called molecular genetics was born. It furnished understanding of the structure and organization of genes and of the regulation of their expression. Although most research in molecular biology and molecular genetics was originally focused on the bacterium *Escherichia coli,* it has today been extended to various eukaryotes including plants more recently. Eukaryotes do have a much more complex genome structure than bacteria. Molecular studies on their genome became feasible only by the development of recombinant-DNA technology about ten years ago. It is now well established that individual genes can be isolated and functionally reintroduced either in the same organism or in completely different organisms, i.e. genes can be transferred functionally from prokaryotes into eukaryotes and vice versa. Recent developments in molecular biology of plants have shown that one or more foreign genes can be introduced in plants via the Ti (Tumor-inducing) plasmid of *Agrobacterium tumefaciens.* Such introduced genes can show expression and can also be stably transmitted into progeny. A valuable feature of the application of recombinant DNA in the genetic manipulation of plants is that not only plant genes can be used, but, if desired, also genes from other eukaryotes like animals or from bacteria. Moreover, with this technology, genes can be added to an otherwise unchanged genome. An important contribution to genetic manipulation of plants has been the development during the past ten years of methods for the isolation, culture and regeneration into intact plants of millions of naked single cells, called protoplasts. Protoplasts can be fused: somatic-cell hybridization can be performed for asexual mixing of genomes and cytoplasms originating from genetically related and less related plants. Each of the two types of approach for genetic manipulation of plants, i.e. the introduction of defined genes via recombinant DNA, and somatic-cell hybridization, has its own potentials to bypass certain barriers that are inherent in conventional methods of transferring genes. Genetic manipulation might not only have wide prospects in plant breeding, when properly combined with conventional methods, but will also have its impact on the industrial production of valuable chemicals by plant-cell biotechnology. However, many complex problems have to be solved before we know how to make optimum use of the potentials of cells and genes of higher plants in the next decades. The aim of this contribution is to assess, in broad lines, problems and prospects for the new genetic technologies in somatic-cell genetics, with special reference to the principles of the recombinant-DNA technology and the most advanced gene vector for the introduction of new genes into plants, i.e. the Ti plasmid. Studies on this plasmid also tell us about genes that control morphogenesis in plants.

Objectives of genetic engineering with plants viewed as production units

Whether any one or a combination of procedures will be used at a given time depends on which procedure will lead to a desired product for the lowest cost price. In such a capitalistic philosophy, one can look at plants as production units (Figure 1). A product has a use, which fits with market demands, and certain conditions have to be met for its production. Commercial success is stimulated by improving the product for quality and yield, both of which are strongly influenced by energy supply and favourable environmental conditions.

In nature, however, conditions are often far from favourable and rather unpredictable. Diseases, insects, cold and heat, drought, salinity, weeds and too much rain are just a few of the problems encountered. They can seriously diminish quality and yield. Losses of 30% or more are not rare. There are two ways of escaping these problems:
- isolation of plants that are less susceptible to environmental conditions
- culture of plants under controlled artificial conditions.

The choice depends on feasibility and cost price.

The first way is the most common nowadays. Much effort in plant breeding is directed towards this objective. Crop improvement, however, is a slow and tedious process involving operations that take a lot of time, such as backcrossing and testing, or are difficult to achieve, for example tapping into wild gene pools or inbreeding naturally outcrossing crops. And although often neglected in discussions on plant breeding versus genetic engineering, conventional plant breeding is actually an expensive process, and its

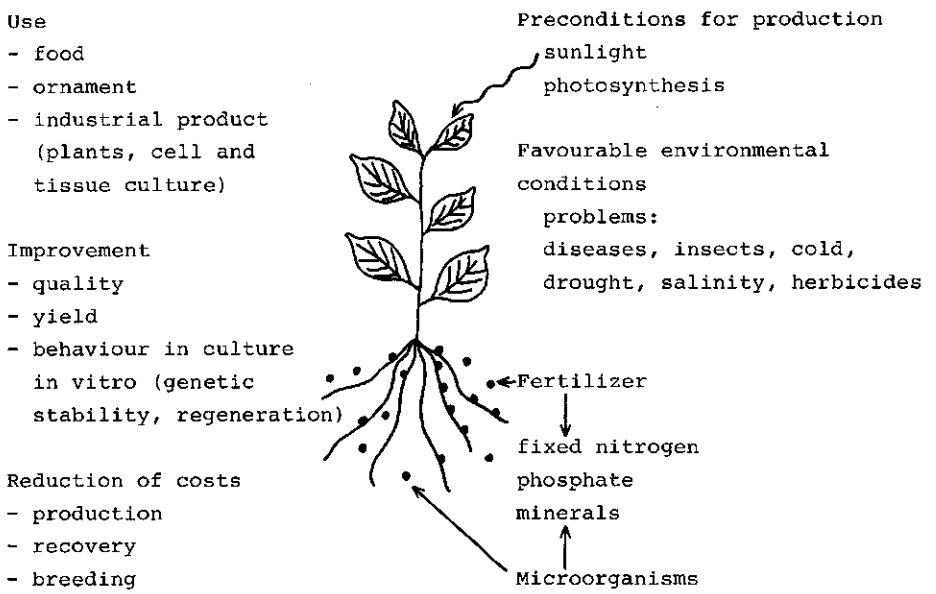

Fig. 1. The plant viewed as a production unit. Various biotic and abiotic factors affect quality and yield. In future, a strategy based on integrated use of conventional and unconventional methods in plant breeding might improve plants for better use at lower cost price.

costs increase the cost price of a product, whether or not it is performed by public or private research.

It is expected that genetic engineering will offer facilities to shorten the time frame of breeding processes by the introduction of more straightforward procedures. However, the genetics, for instance, of resistance to biotic and abiotic factors is mostly too complex to be handled by genetic engineering either. At the moment procedures are being developed for introduction of single bacterial genes into crops, which will give rise to resistance to insects and herbicides. A few commercial successes on these objectives are the first to be expected in the coming five years.

The culture of plants under artificial conditions is already well known in horticulture by means of greenhouses. Although a high degree of perfection is obtained with greenhouses, by which, for instance, various kinds of vegetables cultured in this way compete well with those produced under field conditions, the steep increase in cost of fuels in recent years tends to set a limit to their commercial use. However, a remarkable industrial development by Schulte & Lestraden Beheer BV (Sassenheim, the Netherlands) opens up ways even of extending the large scale production of horticultural crops and possibly also of valuable agricultural crops under artificial conditions, with a minimum amount of energy. This system allows plants to grow that are not sufficiently hardy to be grown in the field. In this way the new industrial technology joins genetic engineering in filling the existing gap between culture in vitro and the growth of mature plants in the field (Figure 2).

With regard to energy supply in nature, it is obvious that sunlight is an inexpensive but sometimes also destructive source of energy for plant growth. The energy of light is converted into chemical energy during photosynthesis, which allows CO_2 fixation in chloroplasts. The enzyme for this process is ribulose-1,5-biphosphate carboxylase (RuBP), which consists of a small and a large subunit. A nuclear gene encodes the small subunit and a gene in chloroplast DNA carries the genetic information for the large subunit. After the synthesis on ribosomes in the cytoplasm, the small subunit is transported into the chloroplast. To obtain a higher yield, it is thought to be of interest to explore ways of increasing the photosynthetic capacity of crops, having a C_3 pathway of photosynthesis, to the level present in C_4 plants. Typical examples of C_4 plants are tropical grasses such as maize and sugar cane. They do not lose much energy by photorespiration, like in C_3 plants. Photorespiration is due to the fact that RuBP has both carboxylase and oxygenase activity. The oxygenase activity of this enzyme in C_4 plants is inhibited by a high concentration of CO_2. By an ingeneous organization of mesophyll and bundle-sheath cells, the CO_2 accumulates in the bundle-sheath cells where CO_2 fixation takes place. The morphological structure needed for the C_4 pathway is not present in C_3 plants. Nevertheless, one is looking to what extent certain principles of the C_4 system can be introduced into C_3 plants by the new genetic technologies. It would probably be sufficient to eliminate the oxygenase activity of RuBP carboxylase. This might be achieved, in principle, by genetic manipulation of the genes involved. These genes have been isolated and their base sequence determined (McIntosh et al., 1980; Berry-Lowe et al., 1982).

Besides inexpensive energy from sunlight, plants require a much more expensive product, in terms of energy, namely nitrogen fertilizer. Plants have to rely on a sufficient supply of fixed nitrogen for their protein biosynthesis. It is taken up by their roots and relatively small amounts of fixed nitrogen, if properly applied to the rhizosphere, would

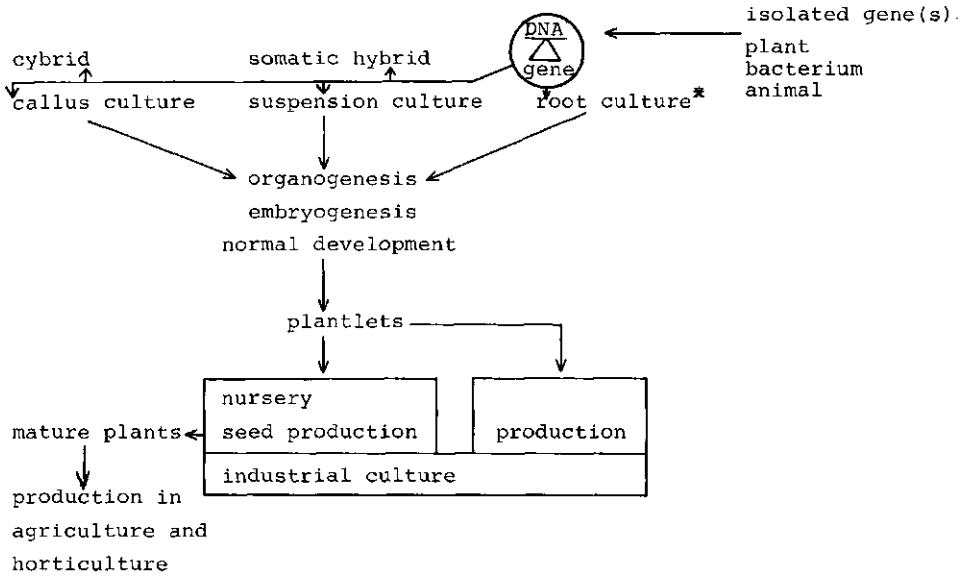

Fig. 2. Capabilities of cells and genes of higher plants in plant biotechnology, i.e. as aids and appliances in plant breeding. If normal plants can be regenerated from genetically manipulated cells, their first production or testing might be accomplished by industrial culture in order to overcome any weakness for direct growth in nature. Complete artificial production can also be considered in certain cases. Cybrids have a new combination of nucleus and cytoplasm, and somatic hybrids carry a new combination of chromosomes. Using the recombinant-DNA technology any desired gene or genes from plants, but also from other organisms, can be introduced and expressed. Target-oriented modification or replacement of existing genes might become feasible too.

actually be enough. However, nitrogen fertilizer cannot, for various reasons, be applied very precisely to plants at the proper place, so that in general an overdose is given in agriculture. Nitrogen fertilizer is becoming increasingly expensive, since its synthesis requires a large energy input and consequently its price is related to that of oil, which has risen considerably in the past years. Especially developing countries can hardly afford the use of nitrogen fertilizer on a large scale. Moreover, the extensive use of large amounts of nitrogen fertilizer tends to lead to serious water pollution in some Western countries. So all over the world, scientists are studying whether more efficient use of biological nitrogen fixation might be possible in order to decrease the use of nitrogen fertilizer.

In nature, various free-living and symbiotic micro-organisms furnish plants with fixed nitrogen in the form of ammonia by reduction of free nitrogen. Especially biological nitrogen fixation by *Rhizobium* spp., in their symbiotic interaction with legumes, contributes considerably to the natural production of fixed nitrogen. Genes for various steps in symbiotic nitrogen fixation by fast-growing rhizobia have recently been traced in the bacteria to a large circular extrachromosomal DNA, called Sym plasmid (Hooykaas et al., 1981). This finding makes these genes more accessible to genetic manipulation and

molecular genetic studies, which will elucidate the molecular mechanism of the symbiotic association.

In the search for ways to extend the use of biological fixation of nitrogen in agriculture, two approaches are being followed:

- Genetic manipulation of bacteria, either to improve the efficiency of their natural nitrogen-fixing capacity, or to create new associations with plants. In the latter case, one can try to extend the host range of existing nitrogen fixers or to introduce the capacity of nitrogen fixation into bacteria that either associate with plants or live in the rhizosphere.
- Genetic manipulation of plants, i.e. the introduction of bacterial *nif* genes in order to obtain plants that fix nitrogen by themselves.

Considering these approaches, we should bear in mind that biological fixation of nitrogen is also an energy-consuming process. In symbiotic associations, energy is tapped from the plant in the form of photosynthetic products that are delivered to the bacteria. Plants should not be forced to contribute too much energy, since this would affect their growth, leading to poor quality and lower yields. How serious this energy problem will be in the first approach cannot yet be easily assessed. The major advantage of the use of indigenous bacteria, which have an established association with plants or otherwise accumulate in the rhizosphere, is that the fixed nitrogen is offered to plants at the proper place for direct use. The use of indigenous strains is important since natural populations of micro-organisms in the uncontrolled environment of the open field are in general successful competitors with foreign strains.

The first approach looks the more promising, especially if bacteria are used which do not depend on the plant for their energy requirement, but can use nutrients from the soil. It is, moreover, much easier to manipulate bacteria genetically, since the technology is well known. For the second approach, if the complex set of *nif* genes can ever be expressed properly in plant cells, all energy needed for nitrogen fixation has to be furnished by the plant, which can be expected to interfere with plant development and growth. In addition, the nitrogenase, which is the enzyme for the reduction of free nitrogen to ammonia, is rapidly inactivated by oxygen and should somehow be protected in the plant cells. Because of the genetic and physiological complexity of the system, the second approach is unlikely to result in a commercial success in the near future, if ever.

For agricultural production, fertilizers containing phosphorus compounds and minerals are needed too. Especially the phosphorus compounds that dissolve well could become a limiting factor. The application of this type of fertilizer is important because phosphorus compounds, if present in the soil, are often in a form not accessible to plants. In nature, however, mycorrhizal soil fungi are helpful to many plants: by colonizing the plant roots, a sort of extension of the root system is brought about. The fungi make phosphate available to plants by converting the phosphate into a soluble form and transporting it to the roots of the plant. They also transport water to the plant, collected beyond the reach of the plant's root system. Plants grown on land reclaimed from strip-mining have been inoculated with success with a strain of mycorrhizal fungi (Brill, 1981). Other strains are thought to attain soon a considerable economic importance in forestry because they stimulate the growth of tree seedlings. So far, however, relatively little work was done to match specific strains of mycorrhizal fungi to specific plants and growing conditions.

Plants can benefit from many other associations with micro-organisms that are just

beginning to be understood. Some *Pseudomonas* strains, for instance, have been shown to detain iron in the soil. By this means, the iron near the roots of the plant is unavailable to potentially harmful fungi and bacteria, which need iron for growth, though iron is still available to the plant. This sort of microbial protection, which has been claimed to work by aiding the bacteria to sugar-beet seeds or to potatoes, increases the yield of the plants (Brill, 1981).

The exploitation of micro-organisms in the soil is hardly new in agriculture. As early as Roman times, legumes were used to enhance the fertility of the soil. The agricultural community has only recently begun to recognize the wider potential of the design of micro-organisms important to agriculture. Recombinant-DNA technologies should stimulate this development not only in designing appropriate micro-organisms but also in improving plants for an effective interaction with such micro-organisms. Much, however, has first to be learnt on the processes involved in recognition and colonization of plants by micro-organisms.

Plant-cell biotechnology

The development of advanced techniques in cell and tissue culture, and the possibility of genetic manipulation of plant cells, open up prospects for rapid progress in plant-cell biotechnology. It could raise plant-cell biotechnology to a level of commercial interest.

The importance of plant-cell cultures for production of high-priced chemicals is based on numerous observations that in undifferentiated cells a wide variety of important secondary plant constituents may be formed and that the selection of high-producing cell strains is possible (Zenk, 1978). Further interest stems from investigations on biotransformations. In such reactions, a precursor of a certain compound is fed to cells, which can then transform the precursor into the desired compound. Some of these reactions such as position-specific hydroxylation or glycosylation of a multifunctional substrate may be of practical value, because such reactions cannot be performed by chemical routes nor with microbial cultures. In general, plant cells possess many biochemical capabilities to transform a great variety of substrates.

Plant chemicals are used to produce many industrial products, such as pharmaceuticals, contraceptive agents, anti-neoplastic agents, anti-microbial agents, virus inhibitors, insecticides, emulsifying agents, valuable fats and oils, natural pigments, flavouring agents, aromas, sweeteners and enzymes. Virtually all the valuable plant compounds are secondary metabolites, since their production is considered not to be essential for the processes keeping the plant cells alive. The vast range of chemical structures involved are intermediates or end-products of biosynthetic pathways, in which each conversion is catalysed by a highly specific enzyme. Cell cultures quite often differ in their qualitative and quantitative spectrum of secondary constituents from the parent plant and its organs and from corresponding callus tissues. Secondary products have often been isolated from cell cultures, though they were absent from the intact plants. Through the observed shift in the pattern of secondary constituents, plant-cell cultures could be a source of useful novel plant products. With suitably devised assays, novel products might be identified with interesting biological and pharmacological properties. Because of strict laws, new pharmaceutical products must pass a costly, long line of trials before they can be put on the market. Therefore, at this early stage of plant-cell biotechnology, the key to

commercial success will be to focus on expensive chemicals that are already in use.

Large-scale fermentation of plant cells can provide a source of valuable plant products that is not subject to variation of crop yields or to the uncertainties of international trade. But it is not realistic to think that plant-cell fermentation will ever yield bulk commodity chemicals. A good chance for commercial production by plant-cell biotechnology in the next ten years is possible for chemicals like anticancer alkaloids, steroids such as the cortisones, oestrogens used in birth-control pills, anticlotting agents such as heparin, insecticides, flavouring agents and sweeteners. Many of these products are obtained from plants now, but some of the plants are costly or difficult to obtain. For example, the sex hormones used in birth-control agents are still derived from the tuberous roots of *Dioscorea*, which must be laboriously harvested from the Central American rain forests. Yet, even if plant cells can be successfully cultured for these plant species, the overall process may be more expensive than extracting the drugs or chemicals from whole plants that are in plentiful supply.

Promising progress has been made in mass cultivation of plant-cell suspension cultures in large volumes (up to 20000 l). Long fermentation, up to several weeks, together with the various problems of sterility, and of rheology and viscosity at high density of cells, as well as of aeration and foaming can be controlled. Improved airlift fermentors, which are being developed, should speed up the biotechnological application of plant-cell cultures. Through the decisive role of economic factors in industrial production, suspension cultures will only compete successfully with the high standard of production processes based on bacteria or fungi if efforts are directed towards plant-specific reactions. The more expensive growth media and the longer fermentation periods required for plant cells are just two of the factors hindering commercial application. This, however, might be partly compensated by immobilization of plant cells, for instance in alginate. From studies so far, remarkable results have emerged (Brodelius & Mosbach, 1982). In immobilized condition, the cells do not divide and possibly as consequence of this can produce secondary metabolites known from plants but not detected in suspension cultures. The compounds are excreted from the cells after accumulation in their vacuoles. The immobilized cells, packed in a column, can be eluted with various suitable solvents and still continue synthesis of the compounds for a period as long as 40 days or more. It is not known yet whether this observation represents a general principle for many plant species and secondary metabolites.

Nevertheless, immobilized plant cells together with the introduction by recombinant-DNA techniques of plant genes for important key enzymes, could allow the production of large amounts of certain secondary metabolites and could make plant-cell biotechnology a commercial reality within ten years. But first, scientists have a long way to go before they understand the genetic and environmental factors that influence the kind and yield of products made by plant cells. In spite of striking differences in the pattern of accumulation of secondary metabolites, the genome of every cell contains all the information necessary to generate the full secondary metabolic capability of a certain plant species. In order to understand the regulatory systems in secondary metabolism, the enzymes involved in the relevant pathways as well as the genes that code for them have to be identified and characterized in detail.

Plant-cell cultures not only have to compete with plants as a source of starting material, but also with production processes which effectively can be accomplished

either with bacteria and fungi or with enzymes obtained in large amounts for instance from genetically manipulated micro-organisms. Especially, if a chemical route is not available or too expensive to convert a cheap substrate into an expensive chemical in one step, micro-organisms and enzymes will be the obvious choice. The micro-organisms can be provided with a relevant plant gene if this gene has been identified and isolated. However when a plant product can only be synthesized by a multi-step pathway, the application of plant-cell cultures stands a good chance. In such a case, the transfer and proper expression of all the relevant plant genes in micro-organisms is surely not easy to achieve. In this respect it is also important to note that many secondary metabolites of plants are toxic to microbes. The recent development of procedures for application of recombinant-DNA technology to plant cells has made plant-cell cultures an interesting alternative to microbial cultures. The various routes genetically manipulated cells or plant genes can follow for their exploitation in plant-cell biotechnology is indicated in Figure 3.

The combination of genetic engineering with new biotechnological processes for the

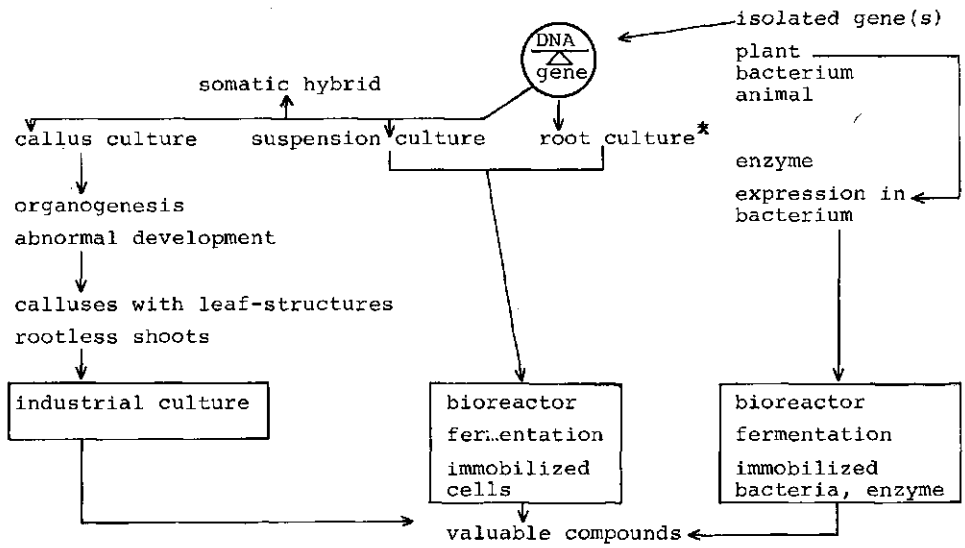

Fig. 3. Capabilities of cells and genes of higher plants in plant-cell biotechnology, i.e. for the industrial production of expensive plant chemicals by bulk fermentation of (immobilized) cells, roots or micro-organisms and with (immobilized) enzymes. Known secondary metabolites are often produced specifically in differentiated plant tissues. So root cultures and calluses both have a high capacity to develop abnormal leaf-like structures or rootless shoots, and might be a major source of products, if the level of production is comparable to or higher than in plants. To save energy, sprouting calluses might be grown in a closed-energy system for artificial industrial culture. Genetic manipulation with somatic-cell hybridization or recombinant-DNA technique can be applied to increase the cellular level of product. Alternatively, a unique plant gene can be introduced and expressed in micro-organisms for production of the corresponding valuable enzyme. Either the micro-organism or the isolated enzyme can be used for industrial synthesis of the product.

exploitation of the capabilities of cells and genes of higher plants, might in due course, replace plants as production units for certain purposes. The ultimate impact of this development is still speculative and depends on social and economic factors.

Approaches and problems in genetic manipulation of plant cells

The genetic manipulation of plant cells can be performed in basically two different ways which are often called the cellular and molecular approach.

In the cellular approach, plant protoplasts are fused by somatic-cell hybridization. Intake by protoplasts of isolated nuclei, chloroplasts, mitochondria or even isolated chromosomes can also be considered part of the cellular approach. The objective is to mix in an asexual manner more or less intact genophores, to obtain either a new combination of nucleus and cytoplasm in the form of cybrids or a new combination of chromosomes in somatic hybrids. Various procedures, which lead to the isolation of cybrids and somatic hybrids (Cocking, 1979), are summarized in Figures 4 and 5, respectively.

The molecular approach is primarily focused on DNA surgery, i.e. the introduction and expression of new genes and, if possible, the modification or replacement of existing genes. Here also the objective can be to change nuclear or cytoplasmic characteristics. The approach is based on recombinant-DNA technology and will be dealt with in more detail in the following sections. Figure 6 lists the various procedures that are used or are being tried for the introduction of DNA into plant cells.

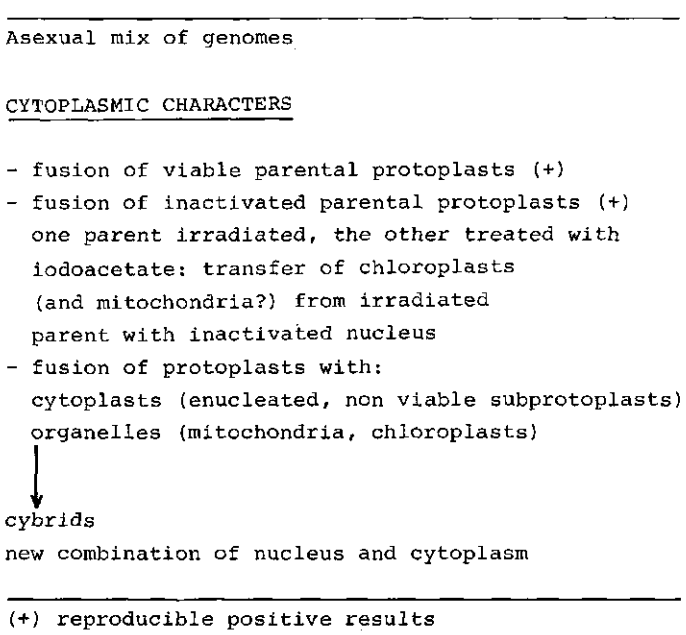

Fig. 4. To alter cytoplasmic characteristics of a cell, cybrids can be obtained by asexual mix of genomes with various approaches. Only a few have resulted in reproducible positive results.

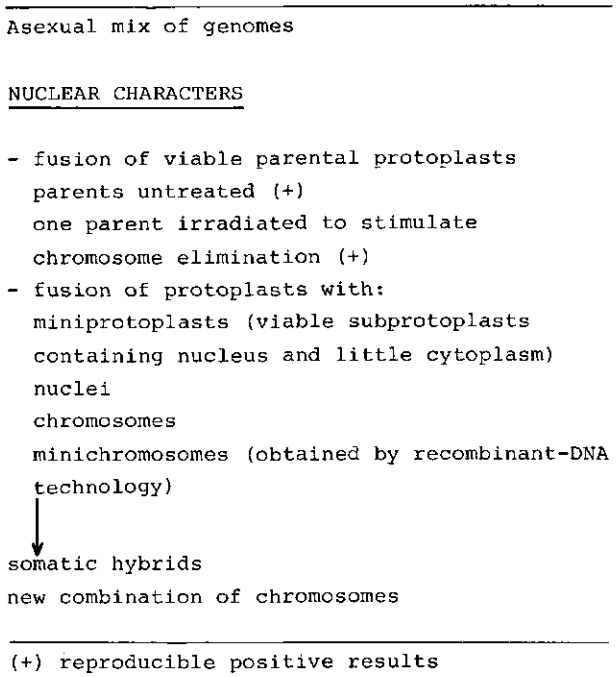

Fig. 5. To alter nuclear characteristics of cells, somatic hybrids can be obtained by asexual mix of genomes with various approaches. Only a few have resulted in reproducible positive results.

In assessing the prospects of genetic manipulation of plants, one must recognize those problems that can not easily be solved because of their tight linkage to biological principles or rules that cannot be circumvented. Plants, like other higher organisms, have the complexity of a differentiated multicellular structure, as well as the eukaryotic genetic complexity, which is orders of magnitude greater than in bacteria (Flavell, 1982; Messing et al., 1983). In contrast to bacteria, which have a relatively simple haploid genome spread through the interior of the cell, plant cells are diploid or even polyploid, with their DNA packed as separated entities or chromosomes in a nucleus, which is separated by a membrane from the cytoplasm. In bacteria, the complete process of transfer of genetic information from DNA to RNA to protein all takes place at the surface of the genome, whereas in eukaryotic cells, most of the RNA is synthesized and processed in the nucleus and has then to be transported through the membrane before it is translated into protein on cytoplasmic ribosomes. Looking at this route of genome expression only, it is obvious that regulation of gene expression in eukaryotes would be, and indeed is, quite different from that in prokaryotic bacteria. The regulatory sequences before and after the protein-coding region in genes of prokaryotes and eukaryotes are so different that the natural genes in general are not properly expressed if they are exchanged. Moreover, plant cells have three completely different genophores, i.e. nucleus, chloroplasts and mitochondria, which interact in a complex way regulatory

signals, gene products and possibly even genes by which number, ratio and function of chloroplasts and mitochondria are controlled (Leaver-et al., 1984; Walbot et al., 1980).

The amount of DNA in organelles is relatively small and the structure, organization and expression of the genes in chloroplasts are similar to those in prokaryotic genes. However the amount of nuclear DNA in plants far exceeds the amount needed to hold the genetic information. Many of the genes in eukaryotes are larger than in prokaryotes, because of the presence of intervening sequences or introns, which do not code for protein and are absent in bacterial genes. In addition, some eukaryotic genes are organized into multigene families, i.e. many more or less identical copies of a gene are

Introduction of well defined genes (recombinant-DNA)

NUCLEAR CHARACTERS

Transformation of protoplasts
- naked DNA (+)
- encapsulated DNA
  liposomes (±)
  protein coat
  bacterial spheroplasts (±)
- microinjection
- cocultivation with
  Agrobacterium tumefaciens (+)
  (dicotyledons only)

Transformation of egg cells
- fertilization with transformed
  germinated pollen
- microinjection of fertilized
  egg cells through pollen tube
  entrance of ovule (micropyle)

CYTOPLASMIC CHARACTERS

Transformation of
- chloroplasts
- mitochondria

(+) reproducible positive results
(±) reproducibility not well established

Fig. 6. Recombinant-DNA technology can be used in trying to change either nuclear or cytoplasmic characteristics of a cell. Transformation of cells with DNA is prerequisite. Various approaches are followed, but only a few of these have given reproducible stable transformation of plant cells for nuclear characteristics. No proof is yet available of transformation of chloroplasts or mitochondria.

present, which is also found for plant genes. Nevertheless, 80% or more of the DNA in plants does not code for sequence specific (regulatory) functions or proteins. In spite of much speculation, little is known about the molecular significance of excess DNA. However some biological phenomena are known to be correlated with the amount of nuclear DNA in a remarkable fashion (Bennett, 1982).

The C-value and amount of DNA appear to play a major role in determining the maximum rate of development in angiosperms, and hence where they survive and the range of lifecycle types they can display in a particular environment. In other words, the C-value of the DNA sets a limit to the present range of phenotypes that can be achieved by genetic control. Nuclear DNA can thus affect the plant phenotype by its mass or size, independent of the information it encodes. A 33% difference in C-value between wheat and rye, which determines differences in the rates of development of these species, is directly responsible for nuclear instability and for abortion during endosperm development in seeds of triticale, the hybrid between wheat and rye. The possible consequences of changing the C-value and amount of nuclear DNA to a large extent by genetic manipulation should, therefore, be considered in assessing the feasibility of a certain objective in plant improvement. The same warning holds for objectives aiming to gain new combinations of chromosomes, especially if chromosomes of genetically remote plants are mixed.

The spatial disposition of chromosomes appears highly ordered with specific associations of pairs of long arms or pairs of short arms for the haploid complement as a basic unit in diploids (Bennett, 1982). Consequently, arm length determines chromosome position, and presumably genes exist that control the relative length of any component of the genome. Because of rules that seem to govern the spatial ordering of chromosomes, the appearance of a karyotype will often tend to be conserved. At first glance, we may even wonder how much we are allowed to change in chromosome composition by asexual mixing of genomes in order to get a new combination of chromosomes with a still stable karyotype, carrying the genetic information planned to be present and properly expressed in the newly designed plant. With regard to proper expression, it is reasonable to assume that the ordered disposition of chromosomes, and consequently the spatial association of genes on heterologues, will influence regulation of gene expression. Rules on ordering of chromosomes, determining chromosomal behaviour and efficiency of gene action, could be considered as the basis of one sort of somatic incongruity at the nuclear level, which might affect the prospects of somatic-cell hybridization or the functional uptake of isolated chromosomes in its application for plant breeding. At the cytoplasmic level another sort of somatic incongruity can occur through the precise interaction between nucleus and organelles, as well as between mitochondria and chloroplasts. It can be expected that this is expressed more strongly if genophores of genetically remote plants are mixed. The various forms of somatic incongruity are only just beginning to be recognized, and will seriously affect the timing and synchronization of regulatory signals required for regular and functional expression of genes, in particular in morphogenesis (Hogenboom, 1984).

Since biological rules exist, it is still much too optimistic to believe that, to obtain new plant varieties, somatic-cell hybridization and other approaches in vitro can easily bypass natural barriers set by sexual incompatibility and incongruity. Where regeneration of plants is not required, as in plant-cell biotechnology, the cellular approach might

find more ready application.

Although the cellular approaches in somatic-cell genetics may prove of limited value in plant breeding, they are undoubtedly of great scientific value in understanding processes of somatic incongruity and other fundamental questions such as gene expression, chromosome behaviour and the position of genes, with a biochemically defined function, on chromosomes. This belief stems from the considerable amount of information gained by somatic hybridization of mammalian cells from genetically remote parents.

In general, the goal of plant breeding is to introduce only a few characteristics or genes in an otherwise unchanged genetic background. Both asexual and sexual crosses need a lot of backcrosses to achieve this goal. If we could perform precise gene surgery we might eliminate some of the steps in this slow and tedious process. In essence, the capabilities of the recombinant-DNA technique do offer the means for DNA surgery. Its target-oriented principle will avoid many of the problems related to somatic incongruity, which are inherent in the cellular approach.

It has often been argued that the same optimism used to hold about the prospects of inducing mutations by irradiation or with chemical agents, though their practical value proved to be small. However the applied procedures for induction of mutations can be likened to the use of a rifle filled with hailstones. One might reach its desired target, whereas many do but cause unpredictable and unknown damage to the genome. If the recombinant-DNA technique can be applied as accurately to plant cells as to bacteria and yeast, it will provide target-oriented methods without accompanying unpredictable side-effects. At this early stage of development of a recombinant-DNA technology for plant cells, many prerequisites still have to be fulfilled (Figure 7).

The target for the introduction of well defined genes by the molecular approach is not only the nucleus but also the chloroplasts or mitochondria in order to modify cytoplasmic characters (see also Figure 6). Promising results have been achieved for the chromosomal integration and expression of foreign genes. However no success has yet been reported with stable transformation of organelles.

The interest in DNA transformation of organelles stems not only from the fact that chloroplasts are centres of photosynthesis and mitochondria the energy power plants in the cell. Both organelles also play a role in certain disease resistances. Mitochondria play a role in cytoplasmic male-sterility (CMS) (Leaver et al., 1984). Chloroplasts synthesize compounds that participate in certain biosynthetic pathways involved in the production of secondary metabolites. In particular as centres where new proteins are synthesized, chloroplasts or plastids could be interesting objects of study in the molecular approach. This idea is based mainly on the large number of copies per cell and their bacterium-like genome organization and gene expression, which are being well characterized.

Chloroplasts can now be isolated such that they remain synthetically active in vitro for many hours. Whether they can take up DNA and can be stably transformed in vitro or in vivo is not known. Transformation is a rare event in general and it is not likely that selection in vitro of any transformants among the many untransformed chloroplasts can ever be achieved. Millions of chloroplasts would have to be stimulated to divide well in vitro and would have to be plated like bacteria with a high plating efficiency. So DNA-treated chloroplasts in some way always have to be reintroduced into cells in order to see whether transformants are present. However that is not readily achieved. Attempts

GENERAL

Shuttle gene vectors
- autonomous replication
- integration

Gene characterization and isolation

Gene expression
- continuous
- regulated

New proteins
- stability
- disposition
- activity

Genetic stability
- genetic background of host
- introduced genes

PLANT BIOTECHNOLOGY

Regeneration
Stable vegetative propagation
Stable sexual transmission

PLANT-CELL BIOTECHNOLOGY

Large-scale fermentation
Immobilization of cells
Product recovery

Fig. 7. Conditions to be fulfilled before genetic manipulation of plant cells with recombinant-DNA technology can have commercial success in plant biotechnology and plant-cell biotechnology.

to introduce chloroplasts into protoplasts by a fusion procedure have not been very promising, since many chloroplasts disintegrate because of the fusion of chloroplast membrane and protoplast membrane. Neither is microinjection of DNA-treated chloroplasts into protoplasts likely to result in easy detection of cells with stably transformed chloroplasts. So the only reasonable way of studying transformation of organelles is to introduce the DNA directly into cells. The DNA should then be provided with both sequences for integration or replication in chloroplasts and a marker gene for selection of cells specifically carrying transformed chloroplasts. Since for instance biosynthesis of protein in chloroplasts is similar to that in bacteria and different from such biosynthesis in the cytoplasm, resistance to the antibiotic streptomycin can be used in selection.

Various methods that can be used for transformation of protoplasts are summarized

in Figure 6. Only a few have been successfully applied for the stable transformation of nuclear characteristics. For dicotyledons but not for monocotyledons, *Agrobacterium tumefaciens* has been shown to be the most efficient micro-injector for the introduction of DNA into plant cells.

One of the major problems in genetic engineering of plant cells lies in the need to use cells that are cultured in vitro. In the cellular approach, but also in various procedures of the molecular approach, protoplasts are used which, after having been manipulated, have to be cultured and subcultured as callus tissue before one can try to regenerate plants. Maintenance of plant cells in tissue culture causes, as was called by P.J. King (pers. comm.) a kind of genetic earthquake.

Cell and tissue cultures from most plant species show considerable genetic instability, i.e. polyploidy, aneuploidy, chromosomal breakage, chromosomal rearrangements and various kinds of small mutations. Cells in tissue culture, that spontaneously or after stimulation with hormones are capable of regenerating tend to loose their regenerative or morphogenic capacity after being subcultured for a long time. Tissue cultures derived from many crops, unfortunately, cannot be stimulated to regenerate plants in tissue culture either at all or in a reproducible way. Anyhow, in procedures for genetic manipulation, tissue culture should be kept as short as possible or preferably be avoided. For effective application of genetic manipulation in plant breeding more must be understood about the molecular processes that control cellular differentiation and regeneration.

In the molecular approach, there might be an escape from the tissue culture problems. For manipulation of nuclear characteristics, recombinant DNA could, for instance, be injected into fertilized egg cells (Figure 6) as has been successfully accomplished with mice (Palmiter et al., 1982) and *Drosophila* (Rubin & Spradling, 1982), or one can try to use transformed pollen. Another alternative could be to use root cultures in which embryogenesis can take place (Figure 2). Root cultures derived from various dicotyledons are obtained quite easily from plants infected with *Agrobacterium rhizogenes*. This plant pathogen causes the hairy root disease. From wounds infected with this organism, numerous adventitious roots develop. These roots are composed of transformed cells that carry foreign DNA originating from a large extrachromosomal DNA molecule or plasmid from the inciting bacterium (Chilton et al., 1982). This Ri plasmid, like the Ti plasmid of *A. tumefaciens*, is used as a gene vector for plant-genetic manipulation.

Recombinant-DNA technique

Recombinant-DNA techniques make it possible to transfer segments of DNA from one organism to another (Cherfas, 1982). The procedure requires five elements depicted in Figure 8.

A method is needed for breaking and joining DNA molecules. DNA molecules can be cleaved at specific positions by special enzymes called restriction enzymes, which have been extracted and purified from various micro-organisms. Each of these enzymes has its own specificity in recognizing a particular sequence of four or six nucleotides in the DNA molecule. Within such sequences some enzymes make a clean cut straight across the DNA molecule, leaving behind flush-ended DNA fragments. Others make a staggered break, which leaves each fragment with a protruding single strand end (Figure 8).

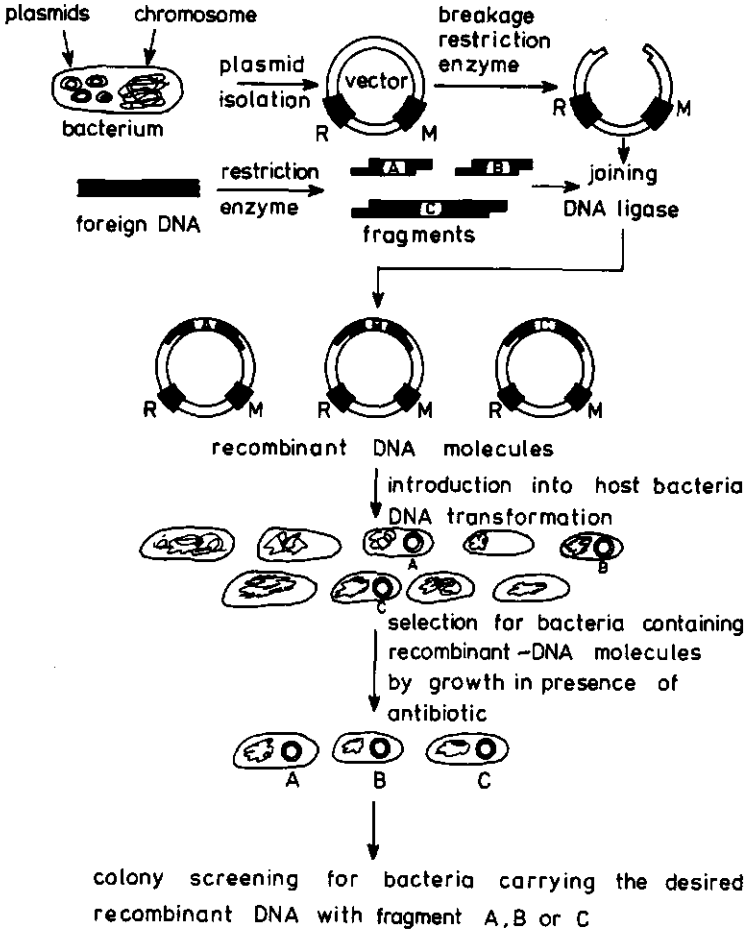

Fig. 8. Strategy for cloning DNA in a plasmid consists of five steps: specific DNA cleavage, DNA joining, DNA transformation, selection of bacteria with recombinant DNA, colony screening for target gene or particular DNA fragment. R, origin of replication (replicator) and M, antibiotic marker gene for selection, on vector plasmid.
A, B and C, DNA fragments with protruding ends complementary to the protruding ends of the vector plasmid cleaved with the same restriction enzyme. After transformation and selection, three sorts of bacterial colonies are obtained, whose cells carry vector plasmids with A, B or C.

Enzymes that make a staggered cut are especially important, because the single strands that they leave protruding are complementary in base sequence. Under the right conditions, the complementary bases will pair up again. So, if two different sorts of DNA both are split into pieces with one of these enzymes and subsequently all the fragments are mixed, the chances are that fragments from the two sorts of DNA will come together in a new hybrid molecule. A new combination of genes i.e. recombinant DNA, will be made when the fragments are covalently joined by an enzyme called DNA ligase. If a restriction enzyme leaves DNA fragments with blunt ends, other types of enzymes can be used for adding complementary single-stranded tails, containing only one type of bases, to the

flush-ended DNA fragments from the two sorts of DNA. Synthetic linkers are used too. These linkers are short double-stranded pieces of DNA synthesized to contain a specific restriction-enzyme-recognition site. The recognition site and enzyme are so chosen that staggered-ended fragments are obtained. Linkers are ligated to the flush-ended DNA fragments, after which the described procedure for staggered-ended fragments is followed. By making a wise choice from the catalogue of about 300 known restriction enzymes the genetic engineer can find one to perform practically any task. Almost any sequence of bases can be traced and cut at will.

A DNA molecule will only replicate if it carries a special DNA sequence, called a replicator. Since most DNA fragments will not harbour a replicator, they have to be attached to a DNA molecule carrying such a structure in order to multiply and to be maintained stably in a host. Such a DNA molecule with a replicator is called a gene vector (carrier). Viruses, phages (similar to viruses, but attacking bacteria) and plasmids are common vectors.

Plasmids are small lengths of DNA, usually with ends linked to form a circular molecule. They are found outside the chromosomes of bacteria in the cell fluid. Plasmids present in many copies are especially important. While the bacterial chromosome consists of about 4 million base-pairs, circular plasmids have several thousand base-pairs. Many have been found or are artificially constructed in such a way that they carry unique recognition sites for various restriction enzymes. At these unique sites, the plasmids can be cleaved for insertion of foreign DNA. They can then be joined to a foreign gene and relinked to form a loop, while still retaining their ability to replicate in a host cell.

Having linked a DNA fragment into a vector, the recombinant DNA should in some way be made to enter cells of the host organism. Several procedures have become available to transform bacteria, yeast, mammalian cells and also plant cells (Figure 6).

Transformation of cells is, however, a rare event. So a method is required to separate cells that have taken up new genes from those many cells that have not. Commonly used plasmids are those that carry a trait of antibiotic resistance. When such a plasmid is used as a vector, it carries not only the gene into the host cell, but also the ability to resist an antibiotic. By treating all cells exposed to the vectors with the antibiotic, one can suppress those cells that have not acquired the gene for antibiotic resistance and allow only those that have taken up the plasmids to grow and reproduce themselves.

The fifth element of recombinant-DNA techniques is required for cloning many different fragments at the same time, e.g. for making a genome clone bank or library. One must be able to recognize those bacteria that contain the cloned piece of DNA or gene in which one is interested. Various procedures have been developed for screening of bacterial clones on the presence of bacteria harbouring the desired DNA clone (see also next section). Nowadays, the word clone is being used in different senses. It is derived from the Greek *klon*, a slip or cutting to propagate a plant. Apart from plant cloning, we have to distinguish cell cloning and molecular or gene cloning. Cell cloning means the formation of a group of genetically identical cells, all arising from a single cell, such as a plant protoplast. Molecular or gene cloning is the formation of many identical gene copies replicated from a single gene introduced into a host cell. For a DNA fragment, which might carry more than one gene, we speak about a DNA clone.

Successful completion of all the five steps permits the investigator to clone the

transferred gene. That is, the host cell is allowed to multiply and the plasmids or other carriers replicate themselves, giving a large number of identical transplanted genes scattered among the host cells. These modified host cells may then carry out the function governed by the foreign genes they now contain: an ability they did not have before. Essential in this, however, is that the appropriate regulatory sequences for the expression of the gene in its new host are attached to the protein-coding region of the gene. The regulation of gene expression in eukaryotes is not yet well understood. The rationale for the expression of recombined genes in eukaryotes is still incomplete.

Isolation of genes

Although quite a few genes have been isolated directly from fragments of viral and bacterial chromosomes, the isolation of specific genes from fragmented eukaryotic chromosomes is still difficult and time-consuming. One of the problems is that although various genes do exist in multiple copies or multiple gene families, as we now know, a good many others do not and are a minute fraction of the large amount of DNA in a plant cell. Moreover, many complex traits that are of agricultural importance, like yield and resistance to biotic and abiotic factors, are polygenic traits, the genes of which are neither well identified nor biochemically characterized. Strategies for the isolation of plant genes are therefore based on two general states of the art: (a) a trait is identified for which the mRNA or protein can well be purified; (b) no extensive knowledge of the specific gene product(s) exists.

In both instances it is useful first to construct a gene library or clone bank. The entire genome of a plant is chopped at random into little bits. Every bit is inserted into a cloning vehicle and used to transform a bacterium, commonly *Escherichia coli.* The multitude of host cells, each carrying some random fragment of the genome, represents that genome carved up into manageable pieces. The collection of colonies derived from these cells form a library that can be consulted for any genetic information thought to be in the genome. All that remains, is to find which of those bits contains the target. The collection is maintained as a living library of the genome that can be used each time a gene from that species is wanted.

If the protein responsible for a trait is available in purified form, various procedures can be followed to find the target gene in the library. A portion of the protein can be sequenced to predict a portion of the mRNA sequence. The mRNA is used to synthesize the corresponding DNA, which is used to screen the library for the target gene by using the nucleic acid hybridization technique. Alternatively one can make antibodies to the protein and use them to isolate polyribosomes or polysomes synthesizing the protein of interest. First a polysome preparation is made from lysed cells. Among the many polyribosomes in the population, there will be some synthesizing the particular protein from its mRNA template. The specific antibody-polyribosome complex will be precipitated and thus separated from the mixture of polyribosomes. The specific mRNA, from which the protein was made by means of the ribosomes attached to the mRNA, can then be extracted from the antibody-precipitated polyribosomes. It can be isolated in nearly pure form, free of other mRNAs, by chromatography. Subsequently the specific mRNA is used as the template for enzymatic synthesis of the complementary DNA (cDNA) with the enzyme reverse transcriptase. The single-strand cDNA is now replicated by DNA

polymerase I to yield double-stranded DNA (ds cDNA) specific for the protein whose gene is being sought. This DNA can be used as a probe to find the target gene in a library. Generally the ds cDNA is first cloned in *E. coli* to produce sufficient DNA. Since the synthetic cDNA can specify the amino acid sequence of a given protein, one can also try to use it directly for genetic manipulation of plants. Note, however, that this synthetic cDNA isolated from a eukaryotic mRNA is not identical with the natural gene for this protein, because it does not contain the introns nor the regulatory sequences required for expression. The regulatory sequences can be attached to it. It is not yet known whether the introns always have to be added again too for expression. In a number of cases this seems not to be essential, but if required, synthetic introns can be added.

If neither DNA or RNA probes are available but the protein one is looking for is known, 'hybrid arrested translation' (HART) can be used. It combines the sensitivity of immunological methods with the ability of a nucleic acid strand to recognize its complement, to form one of the most powerful methods for finding a cloned gene in a million. The method is especially useful if it is difficult to isolate mRNA because of the few copies in the cell. According to the sort of gene, mRNA can be present as rare, moderately abundant or abundant mRNA. It is extremely difficult to isolate mRNA from low abundance classes. In the HART procedure, each clone's DNA is extracted and purified and the two strands are separated. The strands are mixed with the raw RNA from the donor cells, whose DNA was recombined with the vector. Each bit of donor mRNA binds to its complementary DNA if that DNA is present. The whole is then put into a cell-free system in which everything needed to synthesize proteins from mRNA is present. Only those mRNAs in the mixture that are not bound to DNA are still free to direct the synthesis of protein. If the protein one is looking for is detectable in the mixture, it is likely that the corresponding gene is not present in the clone. To detect the protein, radioactively labelled antibody is used. If the protein is being made by the cell-free system, then it will bind to the antibody and the radioactivity will reveal it. But if the purified recombinant DNA from the clone contains the gene one is interested in, the mRNA will have bound to the gene and none of that protein will have been synthesized. To check whether this is indeed the reason for the absence of the protein, one can separate the mRNA and DNA by gentle heating and see if the protein is then synthesized.

If little or no knowledge of gene products is available one can use 'subtractive' methods and 'shotgun' methods.

The subtractive method is based on the use of two really isogenic plants, except that the gene of interest is present in one of the two plants (A) but not in the other (B). The genes from Plant B, which does not carry the trait, are used to 'filter out' all of the common genes. Only the genes unique to Plant A will then be isolated. In outline, the filtration is performed by using nucleic acid hybridization procedures and cloned cDNA derived from the entire mRNA populations. The technique seems applicable to a wide array of genes. However, it requires closely related plants for the subtraction step. It may not be usable for extremely minor gene products, because there is no guarantee that virtually all rare mRNAs are represented in the cDNA clone bank.

Shotgun methods make use of a phenomenon called 'complementation'. Host strains that lack a specific gene cannot grow because they need the enzyme coded for by that gene, e.g. the enzyme to synthesize an amino acid or a vitamin. Such strains are called auxotrophic mutants. They will only grow if their requirement is added to the culture

medium. They can also grow in the absence of their requirement, if the cells have acquired a vector that contains the missing gene. This gene, if properly expressed, will supply or complement the missing enzyme. Also plant genes in a vector can be identified by complementation of auxotrophic traits. In a shotgun method, random DNA fragments obtained by restriction enzyme cleavage can be cloned into yeast or bacterial 'expression' plasmids or can be 'shotgunned' with a plant vector into recipient auxotrophic plant cells. The binary plant vector system (see later) might in particular be useful for such experiments. A selective screen to transformed cells will result in survivors that have the DNA fragment carrying the gene of interest. Shotgun methods are suitable for extremely minor gene products. However they are applicable to a relatively few types of genes, namely those selectable in culture. Furthermore, substantial technological development is still necessary.

Another way of identifying and isolating genes that correspond with a certain phenotype is offered by mobile genetic elements like transposons (see next section).

In future, all the laborious and tedious work of identifying and isolating natural genes may not always be necessary. The rapid progress that is made in automated procedures for chemical synthesis of DNA may lead to designer genes. With knowledge of protein structure and function, one might actually design a gene to do a specific job. Although recombinant-DNA technology has advanced rapidly in the last few years for plants too, complex polygenic traits, like yield, cannot yet be tackled.

Mobile genetic elements

Both genetic and biochemical evidence has shown that a gene or set of genes of a prokaryotic or eukaryotic chromosome sometimes leaves its original position and undergoes transposition to some other site in the genome (Shapiro, 1983). Such mobile genetic elements are called transposable elements or transposons. The capacity of transposons to be inserted at different sites in DNA is conferred by short extensions or repeats, which contain inverted base sequences, at each of their ends, called insertion sequences. The flanking sequences on the recipient are uninverted repetitions of a short sequence (typically 5-9 base pairs), that was present once before insertion of the transposon, as a target site. The target site on the recipient DNA is duplicated during transposition. The important point is that a transposon need not to be homologous to the recipient DNA because the specificity of insertion is determined primarily by protein-DNA interactions rather than by base-pairing. The insertion process, therefore, is quite different from general genetic recombination or homologous recombination, which needs base-pairing between a recipient DNA strand and a DNA strand of the incoming DNA, that will recombine. In *E. coli*, the genes that control homologous recombination, i.e. the *rec* genes, do not participate in the insertion process of a mobile genetic element. Their insertion process is also said to be an illegitimate recombination. The smallest mobile genetic elements are the insertion sequences (IS) which have a length of about 1000 bases. In contrast to transposons, IS elements do not carry a gene. The terminal sequences of some transposons are identical to one of a number of insertion sequences that also can act independently. It seems likely that a transposon is formed when one or more genes become bounded by a pair of insertion sequences. Certain genes for antibiotic resistance are on a transposon and by this can jump to any DNA (phage, chromosome

or plasmid) present in a bacterium. Transposition leads to random mutagenesis of genomes if insertion occurs in a gene. The gene is then interrupted and inactivated. However IS elements and the insertion sequences at the terminal ends of transposons can also markedly affect the expression of nearby genes. Insertion sequences usually block the transcription of distal genes in a transcription unit, but also can act as new promotors. Furthermore, insertion sequences enhance chromosomal rearrangements such as deletions and inversions.

Transposon mutagenesis has been exploited as a technique for identification of genes by associating mutations at particular sites with changes in particular functions (phenotype). Transposons carrying a gene for antibiotic resistance are regularly used in this way. The transposon identifies itself, because the bacterium is rendered resistant to an antibiotic. It also flags its position, because the pattern of restriction endonuclease fragments is changed by the insertion of new DNA into an old fragment, giving rise to new ones. Using the technique of transposon mutagenesis a genetic map of the *Agrobacterium tumefaciens* octopine Ti plasmid has been obtained (see later).

Mobile genetic elements are far from unique for prokaryotes and were actually first detected and characterized in considerable detail for maize by Barbara McClintock (1951). Nearly 20 years later, similar elements were studied at the DNA level first in bacteria and later in a variety of eukaryotic organisms, notably *Drosophila melanogaster* and the yeast *Saccharomyces cerevisiae*. The structure of these latter elements has been elucidated rapidly, including their complete DNA sequences. Much progress at the DNA level has also been made for certain transposable elements responsible for mutations at the shrunken (*Sh*) locus in maize (Federoff et al., 1983). The *Sh* locus codes for endosperm sucrose synthase. These transposable elements are known as the activator (Ac)-dissociation (*Ds*) family of controlling elements in maize capable of causing unstable insertion mutations, as well as breakage and rearrangement of chromosomes. Determination of the DNA sequences of a few isolated *Ds* elements has indicated that they resemble other transposons in having inverted DNA repeats at the ends and in creating short direct DNA duplications at the site of insertion on the recipient DNA.

DNA probes derived from the transposable elements could be used to isolate genes, previously tagged by the insertion of the element, from mutants that have occurred spontaneously. However, a *Ds* element appears to be present throughout the maize genome in multiple copies. This will make the identification of particular genes mutated by the insertion of *Ds* difficult, but not impossible. Experiments along these lines are in progress in various laboratories.

Gene vectors for plants

Since many of the recombinant-DNA manipulations are performed with *E. coli*, a cloning vehicle for plants should preferably be a 'shuttle gene vector' (Figure 9). This means that it must contain DNA sequences for replication and selection in bacteria as well as in plant cells. For stable maintenance of introduced genes during cell division and sexual transmission to progeny, a vector containing DNA sequences that can force the integration of foreign DNA into the genome is preferred over vectors showing autonomous replication. Furthermore the gene vector should contain several unique restriction sites for stable insertion of genes into the vector.

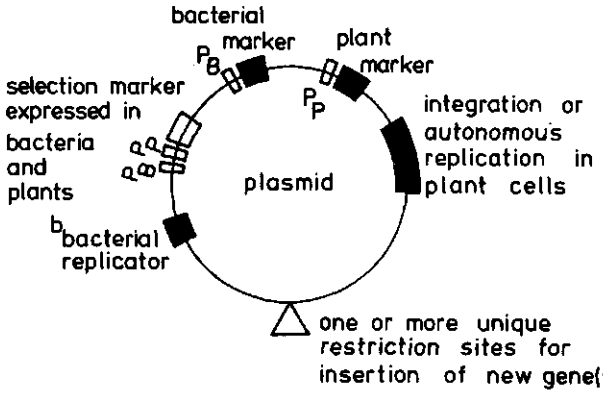

Fig. 9. A shuttle gene vector for plants needs DNA sequences for maintenance in bacteria and plants as well as for selection of transformants. For expression through RNA, particular regulatory sequences called promotors have to be present. Bacterial and plant promotors are indicated P_B and P_P, respectively. A marker gene for selection in both bacteria and plant cells is obtained if the protein-coding region is linked to P_B and P_P. Unique sites for various restriction enzymes are preferred for insertion of one or more genes.

There are various ways of achieving autonomous replication of a vector for plant genes. Minichromosomes can be constructed with the recombinant-DNA technique that still carry a centromere and autonomously replicating (AR) sequences. Such AR sequences are present in eukaryotic chromosomes at regular distances in the DNA. Minichromosomes as well as AR sequences inserted into a plasmid have been successfully used in yeast (Clarke & Carbon, 1980; Stinchcomb et al., 1980).

Plant viruses can be used too for the development of autonomously replicating vectors. Plant viruses containing RNA as genetic material might be used via cDNA for construction of vectors. But not much has yet been achieved with these viruses. Currently most attention is paid to cauliflower mosaic virus (CaMV), a DNA-containing plant virus. Attempts are being made to insert foreign DNA into the CaMV genome and to introduce this DNA into plant cells by infection of susceptible host plants. CaMV has a limited host range. Progress has been made with the encapsidation and propagation of inserted DNA fragments (Howell et al., 1980). However stability, size limit and expression of foreign DNA inserted into the CaMV genome need further study to establish whether this system can be used for plant transformation.

No plant viruses have been found that can integrate their genetic information into the host genome. But it has been well established now that the plant pathogens *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* can introduce a particular part of their DNA into plant cells. *A. tumefaciens* causes crown galls, and *A. rhizogenes* causes hairy root disease in dicotyledons. Monocotyledons are not susceptible to these agents. Large plasmids in these agrobacteria, the Tumor-inducing (Ti) plasmid and Root-inducing (Ri) plasmid, confer the disorder on the host. Both sorts of disorder result from transfer, and functional integration into plant chromosomes of a particular part of the Ti or Ri plasmid. The bacteria always reside outside the cells and do not penetrate the cells that are being transformed. They firmly attach to the walls of cells in an infected area of a wound in order to transfer DNA.

Since transposable elements are present in plants, these elements might ultimately be used in a plant gene vector for the integration of foreign genes into chromosomes as was demonstrated first in bacteria and recently in *Drosophila* (Rubin & Spradling, 1982). Transposable elements like those in maize might be exploited in gene transfer experiments on monocotyledons, for which Ti-plasmid derived gene vectors perhaps cannot be used.

The Ti plasmid

The crown gall disease is characterized by unlimited plant cell proliferation (gall formation). Crown gall tumor tissues differ from untransformed tissues by their ability to grow autonomously on synthetic media in the absence of hormones, i.e. auxins and cytokinins. Moreover, tumor-specific compounds called opines are produced by them as a direct result of genetic transformation by the bacterium. Opines are unusual amino acid derivatives that accumulate in the plant cells. They have no known useful function in the plant cells. Neither are they responsible for the tumorous state of the cells. However opines can be useful to agrobacteria, since they serve as a source of carbon and nitrogen, and some of the opines also induce conjugation between the bacteria. The Ti plasmid of *A. tumefaciens* carries the genetic information for tumor formation, synthesis of opines in crown gall cells, catabolism of these compounds by the bacterium and conjugation between bacteria. The type of opine produced in the tumors defines the Ti plasmids as octopine, nopaline or leucinopine Ti plasmid.

Crown gall disease is a clear example of genetic manipulation by recombinant DNA in nature (Figure 10). By a still unknown mechanism, *Agrobacterium* introduces part of its Ti plasmid, called T-region, into plant cells. The bacterial DNA recombines with the chromosomal DNA in the plant nucleus. As part of the plant genome, this piece of foreign DNA is called transferred DNA (T-DNA). The mass of T-DNA ranges from 8 to 16 megadaltons. The T-DNA is expressed in various RNA transcripts carrying a poly-adenine tail, which is characteristic for eukaryotic mRNA. The enzymes for opine synthesis have been shown to be one of the products of T-DNA transcripts. Other T-DNA genes, namely the oncogenes (*onc* genes), are responsible for the tumerous or oncogenic state of crown gall cells. Through the activity of *onc* genes the plant cells are stimulated to divide continuously and so provide the bacteria with a 'niche' with sufficient food in the form of opines. Knowledge of how agrobacteria genetically manipulate plant cells for their own benefit can be used for our own objectives. We need to know more about those genes and sequences of the Ti plasmid that are essential for transfer, integration and expression of T-DNA in plant cells. Here, we will deal with the knowledge gained for the octopine Ti plasmid only (Figure 11). For detailed information, the reader is referred to recent reviews (Hooykaas & Schilperoort, 1984; Chilton, 1983).

Molecular genetic studies have demonstrated that about half of the plasmid does not carry any genes involved in tumor induction. It harbours mainly catabolic functions for utilization of octopine (*occ*) and arginine (*arc*), while also genes for conjugation (*tra*) and exclusion of a phage (*ape*) are located on this catabolic or degradation part of the Ti plasmid. All genes responsible for tumor induction are in the other half in two regions called the virulence (Vir) region and the T-region.

The T-region carries the *onc* genes, which are active in the plant cells as part of

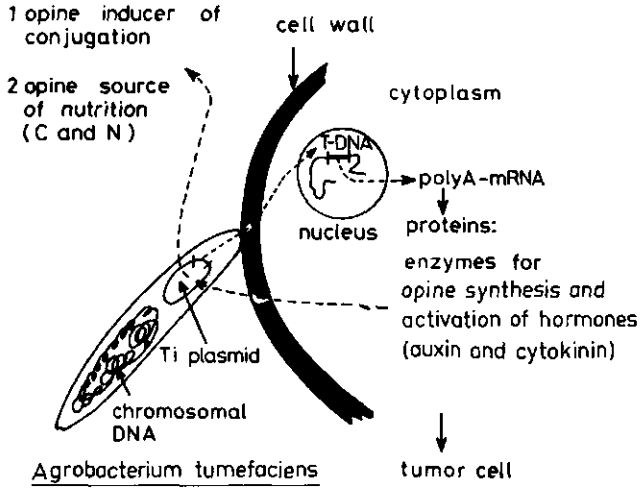

Fig. 10. In nature, *Agrobacterium tumefaciens* genetically manipulates plant cells for its own benefit. The T-region, of a tumor-inducing (Ti) plasmid is transferred and integrated as T-DNA in chromosomal DNA present in the host nucleus. Expression of T-DNA genes leads to synthesis of opines and the activity of the hormones auxin and cytokinin; the hormones result in tumor development. The opines are useful only for the bacteria as source of nutrition and as inducers of bacterial conjugation.

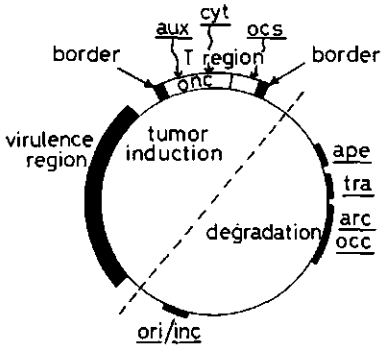

Fig. 11. The octopine Ti plasmid can be separated into two halves, of which only one is required for tumor induction. The other half carries genes for catabolism of octopine (*occ*) and arginine (*arc*), as well as genes for conjugation (*tra*), exclusion of a phage (*ape*), incompatibility (*inc*) and the origin of replication (*ori*). The T-region carries genes for auxin (*aux*) and cytokinin (*cyt*) activity and a gene for octopine synthesis (*ocs*) in tumor cells. It is flanked by direct repeats of 23 base pairs, called border sequences. The virulence region carries genes involved in some mechanism by which the T-region is transferred and integrated as T-DNA in plant cells.

T-DNA. Mutagenesis of the T-region has revealed that it consists of several loci, and that it is bordered by particular base sequences or repeats called border sequences. Besides a gene for octopine synthase, the enzyme that catalyses synthesis of octopine in tumor cells, at least two loci have been identified on T-DNA that control cellular growth and differentiation in the plants. Inactivation of one locus triggers shoot formation (shooter mutants), while disruption of the other results in root formation (rooter mutants) from relatively small tumors when the mutants are tested on *Kalanchoe* or tobacco plants. Infection with a mixture of shooter and rooter mutants gives rise to normal tumors. The same result is obtained when a shooter mutant is supplemented with auxin or a rooter mutant with cytokinin. This indicates that the shooter mutant is affected in a locus for some auxin-like activity (auxin locus), while the rooter mutant is affected in a locus for cytokinin-like activity (cytokinin locus) (Ooms et al., 1981). The combined activity of the two loci leads to unorganized tumors. They are probably directly responsible for the autotrophic growth of the tumor tissue. Study on the function of these T-DNA loci and on the targets of their products in plant cells might shed light upon molecular processes that control regeneration of plant cells.

The Vir region of the Ti plasmid has been mapped, and there is only a small distance between this region and the T-region. In contrast to mutations in the T-region – which give rise to tumors with altered morphology – mutations in *vir* genes abolish or attenuate tumor formation. As the virulence region of the Ti plasmid has never been detected in transformed plant cells and mutations in genes in this region can be complemented in trans in *Agrobacterium* strains, *vir* genes are probably only expressed in the bacterium where they regulate the virulence (Hille et al., 1982). Some of them could play a role in processing the DNA of the Ti plasmid into T-DNA as found in transformed cells, by recognition of the border sequences of the T-region. Other *vir* genes are supposed to be important in establishing a mechanism for transfer of the DNA of Ti plasmids into plant cells.

Ti plasmids as plant-gene vectors

The rapid progress in our knowledge of the molecular basis of crown gall formation has led to the recognition of Ti plasmids as possible vectors for foreign DNA. Two methods have been successfully applied for the transformation in vitro of single plant cells. These are based on DNA transformation, using naked Ti-plasmid DNA, or *A. tumefaciens* itself as a micro-injector for the introduction of genes in plant cells. Both techniques use the ability of crown gall cells to grow in the absence of hormones for selection of transformed cells from a large population of normal cells. The DNA transformation procedure involves treatment of tobacco protoplasts with polyethylene glycol and Ca^{2+} in the presence of octopine Ti-plasmid DNA and carrier DNA (Krens et al., 1982). By this technique we can isolate hormone-independent calli at a frequency of about 0.01% of the original number of protoplasts.

A. tumefaciens is used as micro-injector in the cocultivation procedure (Marton et al., 1979). The agrobacteria are added to tobacco leaf protoplasts, which have formed a new cell wall, but have not yet divided. After about 30 h of cocultivation, the protoplasts are washed and cultured in the presence of antibiotic in order to eliminate the bacteria. Agrobacteria are very effective in transforming plant cells. With the cocultivation

procedure, a few per cent of the initial population of tobacco protoplasts are transformed. A striking difference has been observed in the structure of T-DNA integrated into the tobacco genome by the two methods. When *A. tumefaciens* is used in vitro the T-DNA is generally present as the well known piece of DNA also found in tumor cells induced on tobacco plants. However if Ti-plasmid DNA is used to obtain transformants, the size of T-DNA is often not limited by its known borders, i.e. the size of T-DNA is variable. Smaller as well as larger stretches of T-DNA are found. In addition, the integrated DNA in some transformants seems to be more scrambled and various rearrangements might have occurred (Krens et al., 1983). Therefore, the use of *Agrobacterium* itself as a micro-injector instead of naked DNA has the advantage of both a higher frequency of transformation and a more predictable distinct size of the transferred DNA. On the other hand, DNA transformation is nowadays presumably the only method for monocotyledons, since these are not susceptible to *Agrobacterium*. Since *Agrobacterium* is so efficient in the transformation of plant cells, much work has focused on the construction of vehicles derived from Ti plasmids to be used in *Agrobacterium* for the introduction of foreign genes into plant cells. Obviously, genes that are to be introduced into the plant genome have to be inserted into the T-region of the Ti plasmid first. Because of the large size of the Ti plasmid and the various functions needed for virulence, it seemed impossible until recently to develop a simple cloning vector derived from the T-region. Various combinations of in vitro and in vivo methods have been used for the insertion of DNA segments into the T-region. The principle of these methods is mutagenesis (insertion of a foreign fragment of DNA) of a T-region clone propagated in *E. coli*, followed by subcloning of the mutated clone in a wide-host-range vector, and transfer of the constructed plasmid into an *Agrobacterium* strain containing a Ti plasmid. The use of a wide-host-range vector is required, because of the inability of the usual *E. coli* vectors to replicate in *Agrobacterium*. In *Agrobacterium*, the foreign DNA is then inserted in the T-region of the Ti plasmid by homologous recombination (double crossing-over) between identical parts of the T-region present on both the Ti plasmid and the wide-host-range plasmid. The wide-host-range plasmid is subsequently eliminated (cured) from the agrobacteria. The final step, involving double crossing-over, is performed in *Agrobacterium* as a host, because the host range of the Ti plasmid is confined to Rhizobiaceae, i.e. the Ti plasmid does not replicate in *E. coli*. To overcome this practical disadvantage, we have extended the Ti plasmid's host range to *E. coli* through the isolation of a stable Ti cointegrate (two plasmids linked together to form one plasmid) containing the Ti plasmid and the wide-host-range plasmid R772. This particular cointegrate plasmid is perfectly stable and replicates in *A. tumefaciens* as well as in *E. coli*. *Agrobacterium* strains carrying this cointegrate have the normal capacity to induce tumors in plants.

In order to use the cointegrate plasmid in *E. coli* for insertion of foreign DNA into the T-region (site-directed mutagenesis), the following steps are performed (Figure 12). In a T-region fragment, cloned on an *E. coli* vector plasmid (e.g. pBR), a DNA fragment carrying a gene for antibiotic resistance is inserted into a suitable site, leaving sequence homology to the T-region at both sides of the insert. Subsequently, the R772::pTi cointegrate plasmid and the *E. coli* vector, carrying the mutated T-region, are put together in *E. coli* cells. To obtain a cointegrate plasmid, in which by double crossing-over the foreign DNA insert from the *E. coli* vector is introduced into the T-region of the

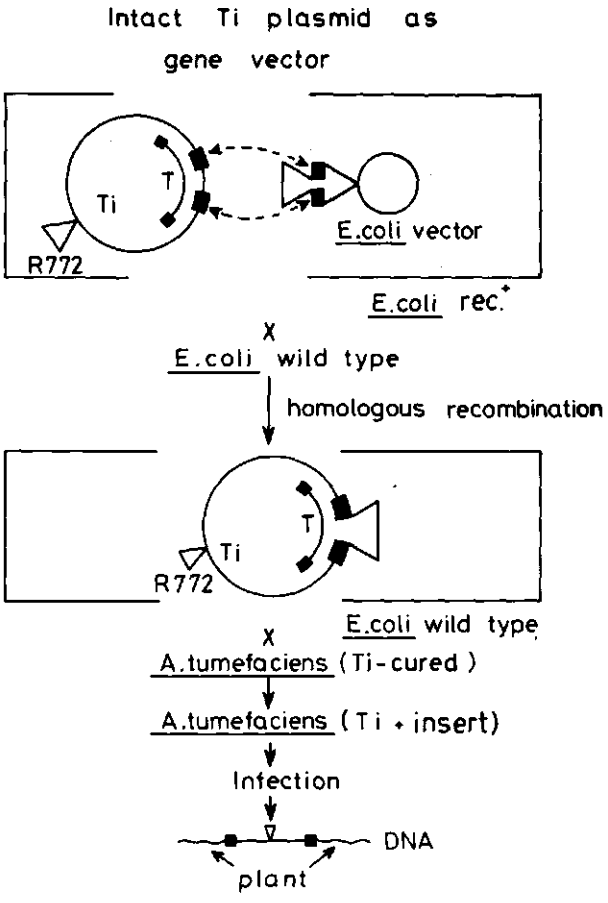

Fig. 12. Site-directed mutagenesis of the T-region by insertion of a DNA fragment. The whole procedure can be accomplished in *E. coli* with a stable cointegrate plasmid (R772::Ti) consisting of a wide-host-range R plasmid (▽R772) and a Ti plasmid. T stands for T-region flanked by border sequences (■). The DNA fragment that has to be integrated in the plant genome via the T-region is indicated by ▽. First it is inserted in a segment of the T-region cloned in an *E. coli* vector. The DNA fragment is transferred and inserted into the T-region of the R772::Ti cointegrate via homologous recombination (double crossing-over) between identical T-region parts, by combining the cointegrate plasmid and the mutagenized T-region clone in one *E. coli* cell. Through subsequent conjugation with a wild-type *E. coli* strain and proper selection, cells with only the cointegrate plasmid, carrying the foreign DNA fragment as an insert in the T-region, are obtained. The cointegrate plasmid, with a genetically manipulated T-region, is transferred into Ti plasmid cured agrobacteria, which thereafter can take care for transfer and chromosomal integration of the foreign DNA into plant cells.

cointegrate plasmid, *E. coli* cells with both types of plasmids are conjugated or crossed with cells of another *E. coli* strain. Cells that have only acquired the cointegrate plasmid with the insert in its region are selected for the resistance marker present on the insert and contraselected for a marker on the pBR vector. The R772::pTi plasmid, thus genetically

manipulated in *E. coli*, is then transferred to *A. tumefaciens* cells that do not contain a Ti plasmid themselves. *A. tumefaciens*, carrying the R772::pTi plasmid, is then used to transform plant cells either in vivo or in vitro. With this approach of site-directed mutagenesis, many mutations, both insertions and deletions, have been introduced in all parts of the octopine T-region (Hille et al., 1983). A major conclusion from such experiments is that *onc* genes of the T-region need not be active nor present for proper transfer, integration and expression of remaining T-region genes such as those encoding the enzymes for opine synthesis.

With comparable systems, the intact Ti plasmid has been used as a vector for introduction, integration and expression of various foreign genes. Foreign genes introduced into plant cells are not expressed if they lack the eukaryotic regulatory sequences for expression. To overcome the potential barrier to the expression of foreign genes in plants, chimaeric genes have been constructed, consisting of the regulatory signals from opine genes and the protein-coding sequences derived from foreign genes. In this way various bacterial genes for antibiotic resistance (Herrera-Estrella et al., 1983; Fraley et al., 1983) and some unrelated plant genes, e.g. for the storage protein of beans, called phaseolin, were shown to be expressed in plant cells. An interesting feature of the regulatory sequences that act as triggers (promotor sequences) for transcription of opine genes is that they give constitutive expression of these genes in callus as well as in all tissues of plants, including their F_1 progeny, regenerated from T-DNA-transformed cells (Memelink et al., 1983; Otten et al., 1981).

Although successful applications have been achieved with systems based on homologous recombination in order to insert foreign genes in the T-region they all have serious drawbacks. First, one has to link a selectable bacterial gene to the DNA that has to be introduced into plant cells in order to monitor whether a successful cross-over has been achieved in the bacterium. In other words, a useful but not selectable foreign gene has always to be linked to a resistance gene of the bacterium, if one wants to isolate bacteria carrying the insert in the T-region. Second, relatively large pieces of the T-region have to be present for homologous recombination. Third, the procedures are rather laborious because of the need to use the large intact Ti plasmid. Ideally one only wants to introduce useful genes, avoiding the presence of large remnants of T-DNA, by using a small plasmid as is done for recombinant-DNA work with *E. coli*. Therefore, in order to construct a more advanced plant-gene vector derived from the Ti plasmid, we tried to disconnect the T-region from the Vir region without loss of their function. We constructed two compatible plasmids, one carrying the T-region, the other the Vir region, of the octopine Ti plasmid (Hoekema et al., 1983). The results obtained with such plasmids are summarized in Figure 13. *Agrobacterium* strains carrying only one plasmid with either the T-region or the Vir region are not able to induce tumors. However when both sorts of plasmid were combined within one cell, an *Agrobacterium* strain was obtained with normal tumor-inducing capacity and octopine was detected in the tumors. This result means that the T-region and Vir region can be separated without loss of oncogenicity. The possibility that the tumor-inducing capacity of the bacteria in the binary-vector system is due to formation of a cointegrate plasmid consisting of both types of plasmids is very unlikely since the two plasmids do not share any DNA homology and illegitimate recombination between the two types of plasmids, as a result of jumping transposable elements, was not detected. In other words, if it did occur, the relative frequency must

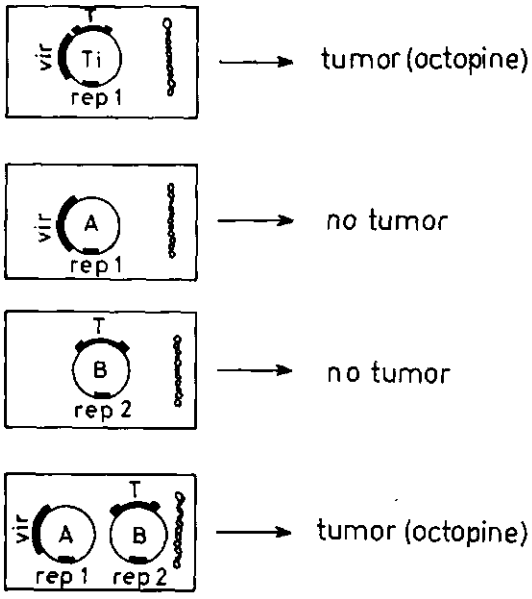

Fig. 13. The binary-vector system is based on functional separation of T-region and Vir region on different plasmids. The T-region plasmid (B) and the Vir region plasmid (A) alone do not confer tumorigenicity. Agrobacteria acquire a normal tumor-inducing capacity when both compatible plasmids A and B are present within one cell. The origin of replication or replicator of these plasmids is indicated by rep 1 and rep 2. The chromosomal DNA, carrying other genes involved in virulence, such as those for attachment to plant cell-walls, is indicated by a condensed DNA structure on the right of the cells.

have been less than 10^{-4}. If only agrobacteria with a cointegrate plasmid were virulent, a few of them, among tenthousands of avirulent agrobacteria, would have had the capacity to induce tumors of a size otherwise found when large numbers of only virulent agrobacteria were inoculated. Such a situation however, is not possible, since it is known that avirulent agrobacteria compete with virulent agrobacteria for attachment sites on the plant cell wall. A control experiment verified this fact.

Inserts in T-region vectors can now be constructed in *E. coli* without the necessity to transfer the insert afterwards into an intact Ti plasmid by homologous recombination. Vectors carrying a genetically manipulated T-region can be directly introduced into an *Agrobacterium* strain that already has a Vir plasmid. Without any further manipulations, the agrobacteria can subsequently be used for infection and transformation of plant cells. However, it would still be desirable not to have segments of the T-region present as part of the foreign DNA that becomes integrated in the plant genome. The *onc* genes can be eliminated without affecting transfer and integration of T-DNA in plant cells. This indicates that a construction, carrying only the 'essentials' of the T-region for its transfer, like the border sequences, could perhaps be transferred in the presence of a Vir plasmid. To test this we constructed plasmids derived from the T-region of the octopine Ti plasmid and containing only the octopine synthase gene (as a marker to identify transformed cells) flanked by fragments containing border sequences. These

plasmids were – by stable cointegrate formation with R772 – transferred from *E. coli* into a recombination-deficient *A. tumefaciens* strain, containing a resident nopaline Ti plasmid. The resulting *Agrobacterium* strains were then used to infect tomato plants. And indeed, both nopaline and octopine synthase activity were detected in the tumors. So in the presence of an intact Ti plasmid a plant-gene vector, harboring foreign DNA between fragments with border sequences of the T-region, readily effectuate transfer and integration of genetic material into plant cells. The use of such small vehicles to transfer foreign DNA into plant cells in the presence of the Vir region plasmid is currently under study. Promising results have already been obtained. In Figure 14, the strategy for the construction of advanced plant-gene vectors is depicted. The development of these vectors is still in its infancy, but it is quite likely that besides the border sequences of about 23 bases no other DNA needs to be present than those useful genes that have to be

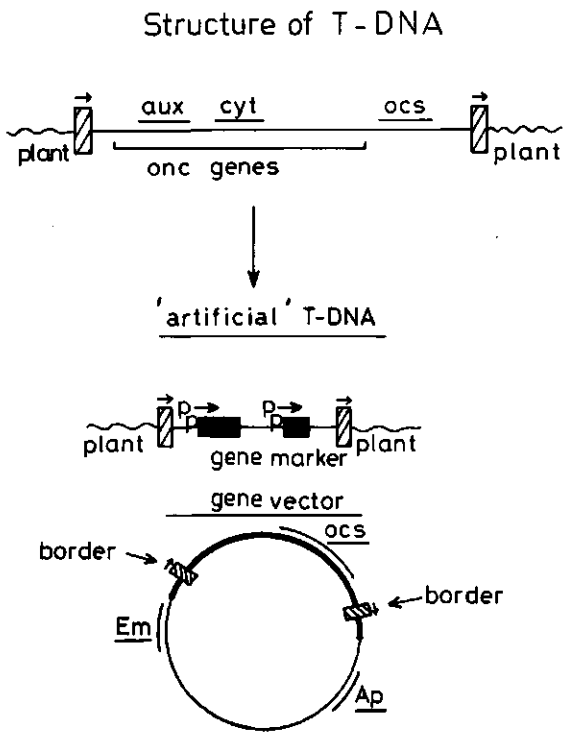

Fig. 14. 'Artificial' T-DNA is constructed by placing the gene to be introduced into plant cells, together with a marker gene for selection or identification of transformed cells, between fragments containing the T-region border sequences (▨). *Onc* genes in the normal T-DNA structure are not required for its transfer and integration into plant chromosomes. *Aux* and *cyt* stand for auxin and cytokin loci, respectively. Promotor sequences for expression in plant cells are indicated by P_P. In the construction of an advanced plant-gene vector, that can be used in the binary-vector system, the octopine synthase gene (*ocs*) has been used as marker gene. The plant-gene vector is introduced into *Agrobacterium* by mobilization and cointegrate formation with R772, screening for integration of R772 into one of the two antibiotic-resistance markers (*Em* and *Ap*) outside the artificial T-region. *Em* gives resistance to erythromycin and *Ap* to ampicillin.

integrated in the plant. This is the ideal situation, unless marker genes have to be added for selection of transformants. It can be envisaged that it is not in all instances desirable to introduce a selectable marker such as a gene for antibiotic resistance into a crop. The use of *A. tumefaciens* as micro-injector of DNA has given high percentages of transformants. Selectable markers are then not required, since transformants can be identified by screening for the presence of foreign DNA. So far, however, *A. tumefaciens* can be used only for dicotyledons. It also should be noted that the foreign genes do not a priori have to be expressed, even if they carry the proper promotor sequences. Other regulatory sequences are sometimes needed and also the chromosomal position can affect gene activity. Much more information is needed on these aspects and on differential gene expression in plants in general.

The application of the recombinant-DNA technology to plants would further be improved if procedures were found for site-directed integration of genes. This would be important indeed, if chromosomal position seriously influences proper expression of the introduced genes. Such procedures, moreover, are essential if one wants to modify or replace existing genes. They would offer approaches for accurate and target-oriented gene-surgery. So far, T-DNA has been integrated into the plant genome at random. The mechanism of integration is not yet understood nor is it known whether the T-region border sequences play a role in this process. Nevertheless, the binary-vector system can be used to study whether site-directed integration can be forced by homologous recombination. The plant-gene vector could be provided with specific fragments of plant DNA.

The binary-vector system can also be used for efficient introduction with *A. tumefaciens* of plant-gene vectors, carrying AR sequences or a centromere, if autonomous replication of foreign genes is required. However we do not know whether T-region border sequences act against autonomous replication. Neither do we have proof whether AR sequences, although they function in yeast, lead to autonomous replication in higher eukaryotes.

The results obtained so far, including those showing that T-DNA is stably transmitted to progeny by sexual crosses, indicate that we have made the first steps in applying recombinant-DNA techniques to genetic manipulation of plant cells. Further progress awaits identification and isolation of genes of importance in agriculture or plant-cell biotechnology.

Genetic engineering versus plant breeding

Unconventional methods for transferring genetic information could well be the final step by which mankind fully exploits the potentials of cells and genes of higher plants. It is quite clear that the new genetic technologies are still in their infancy. Many problems await solution, and new problems will certainly arise. We do not know yet how long it will take to solve the existing problems. So we can only speculate about the range of these technologies. Luckily we can look to the progress made with various micro-organisms and animal systems for spectacular achievements.

The rapid progress made with these living systems, even with those that have a complex cellular organization, were only possible after several decades of extensive research in molecular biology, genetics, cell biology and physiology. The results from these studies clarified the nature and action of genes and increased our understanding of

how a genotype is translated into a certain phenotype. Also a rapid increase in our understanding of the molecular basis of cell differentiation and morphogenesis, which is the big challenge of molecular biology nowadays, can be observed.

It is painfully clear to anyone who is attempting to carry out plant breeding with genetic manipulation that there are serious gaps in fundamental knowledge of plant genes and biochemical mechanisms in relation to plant productivity and resistance to biotic and abiotic factors. If sufficient priority is given to fundamental research on plants, genetic manipulation may become a practical tool in plant breeding and in plant-cell biotechnology.

Molecular biology of plants is perhaps a decade old. Although information and technology is expanding explosively, it will doubtless take another decade before we know how to exploit cells and genes of higher plants to the full. We may remember that it took quite a time before the significance of Mendel's laws were recognized and applied in plant genetics and plant breeding. Thus the impact of molecular biology on plant breeding and plant-cell biotechnology needs time.

In my opinion, genetic manipulation is worth the effort. We might expect a resurgence of plant genetics rather than a revolution in plant breeding. The plant breeding component of increased yields is expected to maintain a major impact and therefore no levelling off of the plant breeding contribution is envisaged. On the other hand the prognosis for success in meeting the world's ever-expanding food needs depends, in part on basic research in agriculture and enhanced capabilities for genetic manipulation. We cannot afford not to pursue every available avenue to improve food production. So plant breeders maintain a role as the key strategists in crop improvement, by becoming intimate with these technologies. Molecular biologists and plant breeders should be getting together and determine how they can best capitalize their joint knowledge. Both must try to appreciate the strengths and weaknesses of conventional and unconventional methods in creating successful new cultivars. Many of the cellular and molecular genetic techniques will have their greatest impact embedded within a breeding strategy, at various specific steps, assisting the breeder in making the difficult or 'impossible' more readily achievable and compressing the time scale for achievement. Where this type of interaction occurs genetic manipulation in agriculture will advance first.

It is my personal feeling, that for healthy development of the new genetic technologies, we must not expect significant practical results soon, despite the optimistic sounds from the media, which some molecular biologists tend to believe. In the next five years, we might expect the first simple agricultural applications. It will take another 20 years before the new genetic technologies are in use more regularly in plant breeding. Genetic manipulation of plants must first be accepted as a powerful tool to increase our knowledge of the biology and genetics of crop plants, including tropical plants. In 10 years, recombinant-DNA technology could have more impact on commercial plant-cell biotechnology.

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Abstracts of posters

Factors in development of multiple shoots from apical buds of Phaseolus vulgaris L.

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To test the effect of genetic and environmental factors on the ability to regenerate multiple buds from apical buds of bean (*Phaseolus vulgaris* L.), four genotypes were used.

Apical buds of 10-day-old plants, aseptically grown, were cultured in test tubes, 10 cm long and 1.5 cm diam., charged with 4 ml MS (Murashige & Skoog) basal medium supplemented with 6-benzylamino purine (BA) (5, 10 and 20 μmol/l) and naphtaleneacetic acid (NAA) (0, 2 and 10 μmol/l) in a factorial combination. The pH was adjusted to 5.6. Culture room was maintained at 22-23 °C with light-dark cycle of 16 to 8 h. Light was supplied with Gro-lux lamps providing 2000 lx at culture level.

The best result was obtained from genotypes Bico de Oura (B.d.O.) and P1136 which produced multiple buds from at least 50 % of the explants (average of all media). The highest proportion of explants with multiple buds was achieved with genotype P1136 (87.5%) on the medium added with 5 μmol/l BA and with the genotype B.d.O. (90%) on the medium with 10 μmol/l BA. The highest production of multiple buds per explant has been obtained from genotypes P1136 and B.d.O. cultured on media containing BA at 10 and 20 μmol/l, respectively. Genotypes Lisa and 63227 produced multiple buds from 16 and 19% of the explants (average of all media), respectively. Medium with 10 μmol/l BA gave the highest percentage of explant with multiple buds (30% for both genotypes). The number of multiple buds per explant was very low in all media and most of them became vitreous rapidly.

In a subsequent trial, genotypes P1136 and B.d.O. were cultured on media containing BA at 10 or 20 μmol/l alone or with glutamine (400 mg/l) or adenine (100 mg/l). Both compounds increased the number of healthy cultures, though not significantly.

Genotype P1136 tested on these six media behaved slightly better in number of healthy explants, if cultured in the test tubes (77.4%) than in 120-ml flasks (71.3%).

Elongation of multiple buds was easier with genotype B.d.O. In the same genotype, buds from axillae of primary leaves behaved better than apical buds.

The technique offers possibilities of inducing genetic variation.

Descriptors: bean, *Phaseolus vulgaris*, apical buds, propagation in vitro

Selection for yield through selection for rythm of development in spring barley

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The aim of this investigation was to determine whether an optimal rythm of development with respect to kernel yield exists, and if so, how this could be used to improve the efficiency of selection in a breeding program. Five populations were extensively investigated: in 1981 pop. 6454 (Breuns D9 × Delisa) and in 1982 pop. 76023 (Valeta × Effendi), 76024 (Valeta × Zephyr), 76025 (Camila × Effendi) and 76026 (Camila × Zephyr). About 100 lines of each population were grown in a yield trial in plots of 6 m^2, pop. 6454 and 76026 in three replications. 76023, 76024 and 76025 without replication. Yield components were analysed in the replicated trials. The stage of development of each plot was recorded four times with the Zadoks et al. decimal code, starting with stem elongation (±3.2) until ripening (±8.9). The rate of development and duration of a certain period can be expressed by a figure, obtained by substraction of the consecutive stages.

It was concluded that the rythm of development is heritable and selection for this trait is possible. It appeared that the optimal developmental rythm with respect to yield varied from one population to the other, depending on the parents of the cross.

In pop. 6454, time of booting (t_2) and rate of development at this growth stage, expressed as t_2–t_1, were significantly correlated with kernel yield (r=0.566 and r=–0.552). Lower, but significant coefficients of correlation were found for milk development (t_3) and duration of kernel filling (t_4–t_3) with r=0.415 and r=–0.445 respectively. Using these characteristics, the highest yielding lines could be selected. These lines were early booting, high tillering and relatively short strawed.

In 1982, the season was unfavorable and the differences between the parental varieties 'Effendi', 'Valeta' and 'Zephyr' were small and consequently, the variation within the pop. 76023 and 76024 was too small to be used for selection. The fourth parent 'Camila' had a quite different rythm of development and plant type (late, short straw) and thus, the variation within the populations 76025 and 76026 was large and correlation coefficients between the stages of development and kernel yield were significant with resp. r=–0.457 and r=–0.432 for stem elongation (t_1) and r=–0.435 and r=–0.472 for ripening (t_4). It appeared that most of the variation within these populations was due to the presence of 15, resp. 11 lines which strongly resembled 'Camila' and were the highest yielding. Apparently little recombination had taken place between 'Camila' and 'Effendi', resp. 'Zephyr': when these 'Camila-types' were removed from the populations, no significant coefficients of correlation between rythm of development and kernel yield were present.

Summarizing, in 1981, selection for yield through selection on rythm of development was possible and the highest yielding lines were early, particularly at the booting stage. In 1982 however, selection was only possible in two of the four populations and the selected high yielding lines were all of the – late – 'Camila-type'. These differences in optimal rythm of development in the two years give an indication of the limitations of the use of this type of selection criteria.

Descriptors: barley, *Hordeum vulgare,* rythm of development, yield, indirect selection

Effect of selection for harvest index on kernel yield in spring barley (Hordeum vulgare L.)

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The possibility to select for kernel yield through selection for harvest index at successive stages of selection has been investigated. During this investigation, the regular selection procedure from plant selection to yield trial was followed. Four F_5-populations (76023: Valeta × Effendi; 76024: Valeta × Zephyr; 76025: Camila × Effendi; 76026: Camila × Zephyr) were space-planted in 1979. In each population 400 plants were harvested at random and the harvest index of each plant was determined. In 1980 and 1981, progenies of about 50 high and 50 low harvest index plants of each population were grown in plots of 0.5 m² on the SVP trial field in Randwijk. In 1982, the same progenies were grown in plots of 6 m² (three populations without replication, one population with three replicates) on the SVP trial field in the Flevopolder.

Within the years, correlation coefficients between harvest index and kernel yield were significant and varied from $r=0.479$ to $r=0.533$ in 1981 and from $r=0.439$ to $r=0.616$ in 1982. Correlation coefficients between thousand kernel weights determined in 1981 and 1982 were significant in all populations ($r=\pm 0.480$).

In none of the populations, the harvest indices of the plant progenies determined in 1981 were significantly correlated with those of the single plants of 1979 and also the harvest indices determined in 1982 did not correlate with those of 1979 and 1981.

It thus appears that selection on the basis of harvest index in the early stages of the selection procedure does not lead to selection for kernel yield. Selection based on thousand kernel weight probably would have been more useful, although this trait is not the most important yield component.

Descriptors: barley, *Hordeum vulgare*, harvest index, yield

Luminescence method in screening for frosthardiness

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Frost resistance of cultivated plants and their hardening ability at low temperatures may be screened by postluminescence (PL) of the photosynthetic apparatus in the leaves. Experiments have been done on hardening of some winter wheat, winter rape and winter turnip cultivars.

Plants grew and were hardened under field and laboratory conditions. The pattern of postluminescence (PL) decay was a reliable indicator of plant resistance to low temperature.

As comparative method, electrical conductometry was used.

Descriptors: wheat, *Triticum aestivum,* rape, *Brassica napus,* turnip, *Brassica rapa,* postluminescence, frost resistance

Chilling effect on development of immature peach and sweet cherry embryos

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Embryo culture technique increases seed germination of early ripening peach and sweet cherry cultivars from which it is impossible to obtain seedlings by standard methods of stratification.

Our trials demonstrated that to achieve the best results it is essential:

- To peel off seed integuments in order to obtain a surface easy to sterilize and also to get germination.
- To apply a chilling period at + 5 °C (2 to 4 months) to the embryos already 'in vitro'. The length of this period positively affects germination.

Our researches further indicated that embryos of different cultivars, also if ripened under the same conditions, have different chilling requirements and that this requirement varies from year to year, since embryo development on the tree is affected by environmental conditions.

Descriptors: *Prunus,* embryo culture, chilling, germination

New planter for precision placement of small and large seeds

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A new planter and carrier developed recently by Walter and Wintersteiger KG, Austria, offers plant breeders, geneticists and others the facility consistently and precisely to place seeds at freely selectable distances apart in rows.

The mechanism responsible, a newly patented device using air suction, would also provide the means for automatic and complete self-cleaning between samples sown.

The new precision spaced planter is available in versions ranging in degree of automation, from a semi-automatic two-person operated design to a fully automatic electronically controlled one-operator system. The precision spaced planter is designed to use the 'Weihenstephan' Seedmatic system of seed magazines, or the Øyjord cone and spinner distributor. Two versions of the carrier provide the capability to match with either one or two sets of the six-unit single-head cup magazines of the Seedmatic system and one or two Øyjord cone and spinner units, as may be appropriate for individual research for crop improvement programs.

Descriptors: precision planter, field trials

Shoot tip culture in Vicia faba L.

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An important condition for physiological studies within breeding programs is homogeneous plants. By vegetative propagation, one can produce many identical copies of a mother plant. Shoot tip culture in vitro of the cultivar Herz Freya of *Vicia faba* showed problems in three areas, which we tried to overcome: disinfection, rooting and establishment of rooted shoots in soil.

It proved difficult to disinfect buds of seedlings emerging in soil. The following method of disinfection helped for introduction of shoot tips free from contamination for culture in vitro: disinfection of buds in $Ca(OCl)_2$ solution (5 g/l) at cool temperatures, and explanation of shoot tips from the buds in a film of this solution. Rinsing with sterile water was omitted. Disinfection damage was avoided by subsequent culture of the shoot tips at low temperature (4 °C) for 4 days.

After multiplication of shoots in vitro by enhanced formation of axillary shoots on a nutritional medium with benzyladenine, immediate rooting treatments failed. Subsequent culture for several months on hormone-free medium to allow rooting resulted in rooting, growth and even partially multiplication. Rooting was enhanced with increasing time from the benzyladenine-treated multiplication stage. Rooting rate reached about 20% in preliminary studies.

Direct transfer of rooted plants into unsterilized soil caused many losses. An intermediate period in non-sterile half-diluted mineral solution and subsequent culture in soil at low temperature (4 °C) enhanced the number of successfully established plants in soil.

The practical usefulness of *Vicia faba* clones is to be investigated.

Descriptors: field bean, *Vicia faba,* shoot tips, propagation in vitro

Early selection for yield and harvest index in bread wheat

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Feasibility of early selection for grain yield (Y) and harvest index (HI) was evaluated in a conventional pedigree breeding programme.

Biological yield, grain and straw weight were determined on the main culm of spaced F_2 plants and on F_3 head-rows. The F_2 plants were spaced 20 cm × 5 cm, the F_3 rows were 1.20 m long, at a seed density of about 300/m² and separated from each other by a row of a standard short-straw variety. Nine F_2 crosses for a total of 1 427 plants were evaluated. The 834 F_3 head-rows derived from plants which, within each cross, gave values for Y of HI greater than $\bar{x} \pm 1\sigma$.

The correlation coefficients between Y and HI were very high in F_2 generation and remarkably lower in F_3.

In addition a low heritability value for HI has been estimated by F_2/F_3 regression. As the present results disagree with the data already available in literature, a further study will be carried out on F_4 progenies grown in microplots at normal seed density.

Descriptors: wheat, *Triticum aestivum,* early generation selection, yield, harvest index

Significance of experimental heritability estimates in wheat breeding

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The heritability estimates of 19 traits, many of them important yield components, were extracted from studies in the wheat-breeding project at the Institute of Field and Vegetable Crops in Novi Sad in the period 1972-1982. Heritability was estimated by the methods of Mahmud & Kramer (1951), Mather & Jinks (1971), and from nested design I of Comstock & Robinson (1948) modified by Scossiroli & Pellegrini-Scossiroli (1962) and Borojević (1963).

Estimates showed that heritability of the majority of the traits such as plant height, spike length, number of grains per spike, weight of grains per spike, 1 000-grain weight, number of spikelets per spike, harvest index, content of chlorophyll and carotenoids sedimentation value, number of days to heading, flowering, milk stage, waxy stage, and full ripeness ranged from 50 to 60%. Heritability of total proteins in the grain was highest, 70%, and the lowest was leaf area, only 30%. The results obtained show that 50% of the breeding value may be predicted by heritability estimates of most of the studied traits.

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Descriptors: wheat, *Triticum aestivum*, heritability, breeding value

Some thoughts about the principles underlying honeycomb selection

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The principles of honeycomb selection are:
- selection is based on performance of plants in comparison with that of their neighbours;
- selection should occur in the absence of interplant competition.

As yet, the only experiences in favour of the method of honeycomb selection have been reported by its spiritual father Dr A. Fasoulas. Other studies yielded unequivocal results. This may have been due to inappropriate application of the principles, especially of the second, or it may have been that the quality of the principles was not as good as seemed at first sight. Thus it is conceivable that the average of the phenotypic values of the six neighbours of a central plant forms an unreliable measure for the growing conditions of the central plant. Further, it seems reasonable that the highest response to honeycomb selection will be obtained at some degree of interplant competition.

Descriptors: honeycomb selection, interplant competition

An efficient screening method for root knot nematode resistance in cucurbits and crucifers

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Meloidogyne species can cause high losses to important horticultural and agricultural crops. Resistance to *Meloidogyne* species of glasshouse cucumber is urgently needed because of legislation restricting the use of the soil disinfectant methylbromide and the high costs of other chemicals. Resistance is also searched for in rapid growing and deeprooting green manure crucifers, with the aim to employ resistance as a method of biological control by decreasing nematode populations.

A mass screening technique for resistance to sugar beet nematodes (*Heterodera schachtii*), see page 353, is therefore adapted for use in breeding for resistance to root knot nematodes (*Meloidogyne* spp.) in cucurbits and crucifers. The adapted method is also successfully applied to tomato and pepper. Seeds are sown in open-ended PVC tubes of 36 ml (one seed/tube) filled with moist silversand to which a nutrient solution is added (seeds of wild *Cucumis* species, tomato and pepper are pregerminated). 170 of these tubes are stood in an asbestos container. The asbestos containers are placed in a growth cabinet with 1-m^2 working surface accommodating 16 containers, thus a total of about 2700 plants. Seedlings are inoculated as soon as the cotyledons are expanded by adding about 50 prehatched larvae of *M. incognita* in 1 ml H_2O to each tube using a veterinary syringe. The larvae are hatched from galled cucumber or tomato roots with clearly visible eggmasses, by grinding the roots in a blender and depositing the pulp on a nematode filter with tap water. The temperature during the tests is 24 °C, the relative humidity 70%. Watering is hardly necessary. About four weeks after inoculation the roots are easily washed free of sand and number and size of galls recorded. The mean number of galls per plant is used for classifying accessions as to their resistance. The repeatability of the tests is good.

So far good levels of resistance have been found in accessions of the wild *Cucumis* species: *C. metuliferus, C. figarei, C. prophetarum* and *C. zeyheri* 2*x*. In cucumber (*C. sativus*) no resistant accessions have been found but in some accessions plants are found with very few galls. Progenies of these plants are being screened in order to ascertain their resistance. The heritability of the observed resistance levels will be studied. Preliminary correlation studies of this method with tests which resemble more the glasshouse situation are positive. Further studies are in progress. Resistant tomato cultivars can be distinguished very clearly from susceptible ones.

In crucifers resistant plants have been found in oilseed radish (*Raphanus sativus*) and in white mustard (*Sinapis alba*). Response to selection is promising.

The described method will be further refined in order to measure also eggmasses. Larval reproduction rate as the ultimate parameter of resistance in the field/glasshouse situation will also be studied.

Populations of *Meloidogyne* species will be studied by the Research Institute for Plant Protection at Wageningen as to differences in their pathogenicity.

Descriptors: cucurbits, *Cucumis,* crucifers, root knot nematode, *Meloidogyne incognita,* resistance screening, biological control

Luminescence methods in investigation of physiological stress in plants

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In 1951, Strehler and Arnold found that photosynthesis is accompanied by a very weak red emission from chloroplasts. This emission, lasting several minutes after switching off the light, was named afterglow or postluminescence (PL). PL consists of several components with different decay times (from milliseconds to minutes). Afterglow intensity and decay depend on the mechanism of the photosynthetic processes and thus reflect plant response to environmental factors influencing photosynthesis (directly or indirectly). From PL data, one can obtain information about the mechanisms of physiological processes in plants – in particular about their resistance to stress factors of physical, chemical and phytopathological nature (low and high temperatures, drought, osmotic pressure, herbicides, fungi and microbes). For the detection of PL highly sensitive computer-programmed spectro-photometers equipped with special excitation devices are used. For studies on the influence of climatic factors on plants, special electronic thermostats were designed stabilizing temperature at −25 °C to +55 °C.

Descriptors: postluminescence, stress factors, resistance

Use of lethal and semilethal genes in hybridization to obtain short-stemmed competitive forms of wheat and triticale

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In nature, lethal genes are widespread. They usually are regarded as a disadvantage. However the presence of genes for hybrid dwarfness (D_1, D_2, D_3) in high-yielding varieties with wide ecological adaptability reflects their biological expedience. Such lethal and semilethal genes with low expressiveness may have great interest in breeding programmes for short-stemmed competitive forms with a high harvest index. In a suitable genetic background, genes of hybrid dwarfness can well be utilized and combined to new forms. The varieties of spring wheat Salute ($D_1d_2d_3$) and Druzhina-1 ($d_1D_2D_3$) with genes for hybrid dwarfness and triticale variety Inia Armadillo 'S' ($2n=42$) were used as parent materials in our present investigation. The cross between the two wheat varieties with the triticale variety resulted in 30-35% thin-leafed F_1 interspecific hybrids, which were not observed in crosses with wheat varieties, without genes for hybrid dwarfness (e.g. the variety Opal). Backcross of thin-leafed F_1 interspecific hybrids with Salute and Druzhina-1 gave 50-60% plants with extremely thin leaves and with a higher fertility than that of wheat and triticale. Moreover, the thin-leafed forms of dwarf and semidwarf wheats possessed some valuable characteristics, which originated from rye and triticale, namely pubescent upper internodes, hairy spikes and stem solidness. These characteristics were absent in the wheat varieties used and were not present in common breeding programmes of wheat. Thus it is possible to get short competitive forms of wheat and triticale suitable for higher seed rates and able to utilize the natural resources more efficiently. In the future, the use of lethal and semilethal genes allows the production of early-maturing varieties with thin leaves, which was not possible before.

Descriptors: wheat, *Triticum aestivum*, triticale, hybrid dwarfness, harvest index

Indirect selection for quality in cauliflower

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Cauliflower curds are often of poor cosmetic quality because of bracts interposed between the segments, and because of discolorations caused by anthocyanins. Selection against these defects is hampered by climatic effects on their expression. The defects can, however, be expressed in tissue culture irrespective of whether they have been induced under field conditions. In practice, plants are subjected to two tiers of selection – in the field, and then in culture.

Two autumn maturing cultivars, Pyramis and Hermia, which were bred using this technique were released in 1982.

Descriptors: cauliflower, *Brassica oleracea,* quality, tissue culture, indirect selection

Selection for micropropagation ability in Nerine and Gloriosa hybrids

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An interspecific hybridization programme was carried out with *Nerine* species. From the cross *N. angustifolia* × *N. sarniensis* about ten clones were obtained with promising ornamental characteristics. To test their production capacity under practical conditions it was necessary to have sufficient material. Therefore a procedure of micropropagation in vitro was established. Special attention was paid to genetic variation for micropropagation between the clones.

The aseptic culture was started with twin-scales from bulbs grown in a glasshouse. They were incubated on LS (Linsmaier & Skoog) medium with addition of casein hydrolysate 0.5 g/l, sucrose 30 g/l, and Difco Bacto agar 8 g/l.

The cultures were kept under 16 h of light (Philips TL 34, approx. 1500 lx) at a temperature of 21 ± 1.5 °C. The twin-scales developed bulblets from tissue just above the connecting basal plate. After subculture these bulblets produced daughter bulblets again and so on, but their growth vigour and vitality gradually decreased. Addition to the medium of an extra amount of sucrose of 10 g/l and indolebutyric acid (IBA) 0.3 mg/l solved these problems. On this medium young bulblets developed both leaves and roots and took about four months to attain subculturing size.

Using the tissue culture bulblets, the variation in multiplication rate between the clones was studied. The lowest average multiplication rate per original bulblet was found to be 0.3 every four months, whereas two clones reached an average rate of about 3 and one clone even a rate of 9. In following experiments addition of 6-benzylamino purine (BA) to the medium and destruction of the main growing point were studied to increase the multiplication rate. The high yielding clones responded much more positively to these means than the low yielding ones. Very high multiplication rates were obtained on BA 1-3 mg/l, but ultimately BA 0.1-0.3 mg/l was chosen as the best concentration for practical purposes, because this concentration still allows sufficient root formation from the daughter bulblets to enable their transplantation in the soil directly at the end of a multiplication cycle.

With *Gloriosa* a similar selection for genotypes with high ability for multiplication in vitro was performed, but it started earlier in the hybridization programme. Large differences were found between clones. This information may facilitate the choice of the parents for crosses.

The results indicate that it should be worthwhile in the breeding of vegetatively propagated ornamental crops to involve also micropropagation ability as a selection criterion.

Descriptors: Nerine, Gloriosa, interspecific hybrids, propagation in vitro, genetic variation

Evaluation of S_1 progenies for development of a synthetic population in winter barley (Hordeum vulgare L.)

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Development of a synthetic population of winter barley was initiated by intercrossing, in a half-diallele system, six two-way hybrids from seven six-row winter barley varieties. The first objective was to evaluate S_1 progenies from the initial cycle of recurrent selection for estimating the response to selection. The 329 selfed progenies from S_0 plants and the parents were planted in small plots in a randomized block design with three replicates.

The following traits were assessed: grain yield, 1000-grain weight, frost resistance, heading, plant height and lodging. The heritability estimates of the six traits were 0.34, 0.73, 0.21, 0.88, 0.89 and 0.62, respectively. The heritabilities could have been overestimated by the evaluation of the S_1 progenies in only one environment. The response to selection indicated a large genetic gain for all traits.

Because phenotypic correlations were not found between the five traits and yield, a combined index of selection was adopted. The index allowed selection of 16 of the 83 progenies previously selected for yield alone. These progenies will be used as parents for the next cycle and introduced into a pedigree system.

Descriptors: barley, *Hordeum vulgare*, synthetic population, S_1 selection

Testing perennial ryegrass (Lolium perenne L.) as spaced plants in swards

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In breeding perennial fodder grasses, the aim is selection of plants that are productive and are able to survive under the competitive conditions in the sward. A high disappearance of plants from grasslands results in open swards and is detrimental to production.

As a means of selecting plants that are effective in sward, testing of spaced plants in a sward of another grass is proposed, e.g. *Lolium perenne* plants in swards of *Phleum pratense* or *Dactylis glomerata.* Seedlings of *L. perenne* were raised in the greenhouse and planted out at wide spacing in a field sown with another grass at normal seed rate. After full establishment of planted seedlings and sown sward, individual plants can be assessed in sward conditions.

To test clones in this way rooted tillers were planted in a newly sown competitive grass.

The interspecific competition used in this procedure eliminated all plants that were weak in sward conditions.

Plants selected as productive survivors in swards gave progeny that produced swards with good regrowth after cutting and in which few plants disappeared, resulting in a high dry matter production.

Descriptors: perennial ryegrass, *Lolium perenne,* interspecific competition, testing in swards

Evaluation of physiological parameters for breeding for production under low temperature and light conditions

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Plant performance is determined by a large number of physiological processes each of which may be subject to genotypic variation. This variation has been inventorized for a number of physiological processes which are expected to be important for growth at low temperature and low light intensity.

Fifteen tomato genotypes originating from different climatic regions were grown under controlled conditions at three temperature regimes (19/14 °C, 19/10 °C and 19/6 °C, day/night) and low light intensity (24 W/m² visible radiation at the top of the plants during 8 h per day).

Growth of the plants was analysed. Net photosynthesis, dark respiration and stomatal resistance of the youngest fully developed leaf of each plant were measured. In addition to that, the contents of soluble sugars, starch, proline, nitrate and reduced nitrogen were determined in the dried plant material.

In separate experiments the sensitivity of the cell membranes of the fifteen genotypes to low temperature was investigated.

Significant genotypic variation was established with respect to the above characteristics.

In subsequent experiments the use of this variation for increasing the efficiency of breeding for low energy requirements, will be investigated.

Descriptors: tomato, *Lycopersicon esculentum*, low energy adaption, physiological traits

Shortening the selection cycle of reciprocal recurrent selection in maize (Zea mays L.)

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A serious disadvantage of reciprocal recurrent selection (RRS) in maize breeding is the long selection cycle. A method is proposed that can shorten the procedure of half-sib RRS from three to one generation. The first step is the production of two versions of the two basic populations of such a programme, i.e. a yellow-seeded version of each population (A_0 and B_0) and a purple-seeded version (A_0^p and B_0^p). The purple-seeded versions have to be homozygous for all seed-colour factors. The next step is to grow half-sib families of population A_0 in an isolation block with a tester composed of a mixture of A_0 and B_0^p and half-sib families of B_0 in a separate isolation block with the tester $B_0 + A_0^p$. All plants from the half-sib families have to be detasseled before silking. At harvest, ears are selected from the best families and subsequently the kernels of each ear have to be divided in a yellow-coloured and purple-coloured fraction. So each ear gives rise to two types of half-sib family.

The next year the half-sib families obtained from interpopulation crosses are tested in replicated field trials and simultaneously all half-sib families derived from intrapopulation crosses are grown in two isolation blocks. The first isolation block contains all half-sib families selected from population A_0 (= selection cycle A_1) with rows of a mixed tested ($A_1 + B_0^p$) in between and the second block all families selected from B_0 (= cycle B_1) with the other mixed tester ($B_1 + A_0^p$). Subsequently, ears have to be selected in the isolation blocks from the half-sib families selected on the base of their performance and the performance of the related testcross.

The next selection cycle consists of replicated field trials of $A_1 \times B_0^p$ resp. $B_1 \times A_0^p$, an isolation block of A_2 with the tester $A_2 + B_0^p$ and an isolation block of B_2 with the tester $B_2 + A_0^p$. Next cycles can be completed in a similar way in the subsequent years.

Descriptors: maize, *Zea mays*, reciprocal recurrent selection, seed colour

A biometrical approach to vegetable breeding

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In crosses within Brussels sprouts and bulb onions, information from the F_2 generations has been used to predict the likely agronomic performance of a random set of inbred lines which could be derived from these crosses. Large-scale inbreeding by single seed descent has begun to test the reliability of prediction and the best inbreds will be used to form new varieties, either as inbreds per se or as hybrids.

Descriptors: Brussels sprouts, *Brassica oleracea,* onions, *Allium cepa,* early generation selection, single seed descent, inbred lines

Portable device for measuring photosynthesis

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All crop production ultimately depends on photosynthesis (Evans, 1975; Good & Bell, 1980; Woolhouse, 1981).

Breeding for improvement of photosynthetic rate may increase biomass and thereby economical yield in grasses (Wilson, 1981), cereals (Planchon, 1976), sugar beet (Fick et al., 1975), soybean (Shibles et al., 1975), etc., although light penetration into the canopy, photorespiration, and translocation, cannot be disregarded.

So a portable device based on incorporation of $^{14}CO_2$ in leaves was constructed to measure the gross assimilation rate in situ.

In phytotron-grown plants, a good correlation was found between data obtained with this device and those with a gas-analyser exploring the complete process of net assimilation rate (wheat, $r = 0.800$; barley, $r = 0.497$). Thus, screening of genotypes for assimilation rate is now introduced in the barley and soybean breeding program in Toulouse.

A high level of photosynthetic activity led to the best compromise between yield and protein content, which were generally linked by a negative genetic correlation (barley, $r = -0.790$). In spring barley, for example, the total photosynthetic activity of the third leaf (gross assimilation rate $\times$ leaf area $\times$ life time) was strongly correlated with the protein yield ($r = 0.797$).

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Descriptors: cereals, photosynthesis, portable device, yield, protein production

Components for partial resistance of Lactuca to Bremia lactucae and Myzus persicae

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In *Lactuca* a large variation occurs for level of partial resistance to the downy mildew fungus *Bremia lactucae* and the leaf aphid *Myzus persicae*. Both for the host-pathogen and for the host-parasite relationship similar components are responsible for resistance.

In the *Lactuca-Bremia lactucae* relationship resistance level increased if the latent period was longer, the number of primary infections was smaller, the size of the lesions was smaller and the number of infected leaves was smaller just like the spore production.

In various lettuce genotypes these components are associated. However, it is still uncertain whether they are governed by the same genes with pleiotropic effects, by more or less closely linked genes or by associated genes. Results of analyses of F_2 populations suggest that at least in part of the partially resistant genotypes, various components are governed by different, non-linked genes.

Through genetic recombination after intercrossing partially resistant genotypes the resistance level could be enhanced.

In the *Lactuca-Myzus persicae* relationship the ultimate resistance level is influenced by acceptance, larval mortality, larval period, adult mortality, the frequency of winged aphids and the larvae production. Here too, there was a correlation between the various components.

Application of a *Myzus* population simulation model and sensitivity analyses revealed that for instance a reduction of the larval period and an increase of larval mortality contributed significantly to enhance the resistance level of lettuce genotypes.

Tests for investigation of differential interactions between lettuce genotypes and aphid biotypes did not indicate such interactions for resistance components. Significant main effects were found. For instance on the partially resistant PIVT 47 the predominant biotype wM_2 had a larval period of 12.0 days while this biotype had a larval period of only 8.7 days on the susceptible PIVT 313. If these lettuce genotypes were tested with the more virulent biotype wM_1, the differences in larval period were smaller.

Through genetic recombination and accumulation of resistance genes for various components it is tried to enhance the resistance level.

Descriptors: lettuce, *Lactuca sativa*, partial resistance, *Bremia lactucae*, *Myzus persicae*

Gametic competition in Cichorium intybus and the induction of self seed formation on self-incompatible genotypes by double or triple pollinations

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In *Cichorium intybus* research is done on the occurrence of gametic competition after mixed or double pollinations with self and foreign pollen. This phenomenon is exploited for the production of F_1 hybrids by intercrossing self-compatible inbred lines (Eenink, 1982).

In part of the experiments flower heads were emasculated before pollination. Gametic competition was deduced from the percentages of hybrid seeds in the various experiments with flower color functioning as a marker.

Among self pollen parent (SPP) clones large differences occurred for frequency of germinated pollen grains after self pollination. These differences were positively correlated with the degree of self-compatibility (SC) of the clones. No significant differences for pollen germination rate were found. Among SPP clones large differences occurred for general hybrid producing ability (GHPA). There was a significant negative correlation between GHPA and SC of the clones. Among foreign pollen parent (FPP) clones, used for pollination of SPP clones, large differences occurred for general hybrid inducing ability (GHIA). Results from removing the stigma and a part of the style at varying intervals after mixed pollination and results from double pollination with a delayed second, FPP pollination suggest differences in pollen tube growth rate in the apical part of the style and in the basal part and/or in the ovary. Besides general HPA and general HIA also specific HPA (SHPA) and specific HIA (SHIA) occur.

F_1 hybrids may also be produced by intercrossing self-incompatible (SI) lines. However as yet SI genotypes could not be stimulated to form reasonable numbers of self seeds.

In experiments with double or triple pollinations, including the use of incompatible self pollen and compatible one-day-old foreign pollen, the frequency of self seeds per flower head increased significantly. For instance if an SI genotype was pollinated with old pollen grains of a compatible foreign genotype, at about 240 min after selfing at anthesis, self seed production increased from 0.08 seeds per flower head to 2 seeds per flower head. After a mixed pollination with self and foreign pollen at anthesis followed by a second, delayed selfing, the number of self seeds increased from 0.08 seeds to 4 seeds per flower head. So these pollination methods might offer possibilities to produce reasonable numbers of self seeds on SI genotypes.

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Descriptors: witloof-chicory, *Cichorium intybus*, gametic competition, hybrid seed production, self-incompatibility, multiple pollination

Relationships between stomatal frequency, stomatal resistance and growth rate in lettuce

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Among lettuce cultivars and wild species significant and consistent differences were found for stomatal frequency.

In different environments similar ranking orders of lettuce genotypes for this frequency were observed, which means that no significant genotype × environment interactions occurred. However, at low temperatures stomatal frequency was lower than at high temperatures.

A positive correlation occurred between stomatal frequency and growth rate of lettuce genotypes cultivated at favourable growing conditions. Under temperature stress, however, no correlation existed.

Stomatal resistance was measured by using a leaf porometer. This resistance appeared to be influenced by various disturbing factors like leaf age, place of measurement on the leaf, time of the day and light intensity. At a temperature of e.g. 17 °C there was a negative correlation between stomatal resistance and stomatal frequency. This implies that leaves with a high stomatal frequency had a low stomatal resistance and vice versa. At 17 °C stomatal resistance was negatively correlated with growth rate.

It is now investigated whether the parameters stomatal frequency and stomatal resistance can be exploited as criteria of selection in a breeding programme for adaptation of lettuce to poor energy conditions.

Descriptors: lettuce, *Lactuca sativa*, stomatal frequency, stomatal resistance, low energy adaptation

Agronomic performance of androgenetic doubled haploid spring barley (Hordeum vulgare L.)

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Anther-derived doubled haploid lines from spring barley (*Hordeum vulgare* L.) F_1 hybrids (Foroughi-Wehr et al., 1982; Friedt & Foroughi-Wehr, 1983) were tested in replicated field trials at two places in two successive years. Variation and mean performance in agronomic characters such as grain yield, grain weight, plant height, lodging resistance, earliness and mildew (*Erysiphe graminis* f.sp. *hordei*) resistance were determined. As expected, large differences between doubled haploid cross progenies (DH families) were observed for all characters studied. Within DH families, the variation between lines was similar to that usually found within conventionally bred cross progenies. Beyond that, there was no variation for morphological characters detected within the vast majority of DH lines (95%). These are therefore considered to be completely homozygous diploid lines, and the frequency of residual or additional variation (somaclonal variation) derived by tissue culture is definitely not more than 5%.

The overall results demonstrated that highly productive true-breeding genotypes could be produced from each kind of barley hybrid in very early generations (F_1, F_2) of a breeding programme compared with conventional procedures such as pedigree-selection or bulk-breeding methods. A comparative field trial of androgenetic and conventionally selected cross progenies demonstrated that highly competitive lines could be established by either method. However they were obtained 2-4 years earlier by haploid methods with much smaller populations than usually needed in conventional breeding.

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Descriptors: barley, *Hordeum vulgare*, androgenesis in vitro, doubled haploids, performance, somaclonal variation

Improvement of harvest yield in maize

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Harvest yield can be defined as the yield of a given variety picked up by a given harvest machine during the harvest period. It represents the biological yield decreased by the grain losses occurring during the period from maturity to harvest as well as in harvest process.

Harvest yield in maize depends on several characteristics of the plant: (a) ability of the stalk to stay vertical until harvest; (b) position of the ear at the time of harvesting; (c) resistance of the ear to destruction during shelling; (d) resistance of the grain to mechanical damage.

Plant characteristics that contribute to a higher harvest yield and that could be improved by breeding are: (a) rind thickness – to be increased from 0.9-1.1 mm to 1.2-1.5 mm for higher stalk-crushing strength and lodging resistance; (b) ear height – more than 30 cm from the ground during late harvest, to avoid ear losses and shelled grain in harvesting; (c) ear attachment to the stalk – to be strong enough to avoid dropped ears before and during picking; (d) ear-crushing strength – enough to prevent ear destruction and unshelled grains; (e) husk number – to be 6-9 instead of 12-16, open after maturity for rapid grains drying; (f) grain-top texture – semiflint or flint, instead of soft dent; (g) grain form – elongate deep; (h) pericarp thickness – 50-70 instead of 100-150 μm, for rapid drying, easy shelling, and low mechanical damage.

Descriptors: maize, *Zea mays*, harvest yield

Culture of sugar beet (Beta vulgaris L.) in vitro

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The aim of our study was to establish methods for culture in vitro for: induction of haploids, conservation and multiplication of desirable genotypes, plant regeneration from callus and suspension cultures, culture of protoplasts, and characterization of isozyme systems.

- Induction of haploids. We tried to induce haploids by androgenesis and gynogenesis. Nuclear divisions were induced in cultures of isolated microspores. Microspores cultured in anthers could develop into microcalluses, which sometimes differentiated into heart-shaped structures (probably proembryos). Some plants were obtained by culture of excised ovaries.
- Vegetative multiplication. The most suitable explants were shoot meristems from young plantlets and flower buds.
- Plant regeneration from callus and suspension cultures. As explant sources, petiole segments of clones multiplied in vitro proved to be most appropriate. Media for efficient maintenance of callus and cell-suspension cultures were developed. Established cell lines with high regeneration capacities were isolated from primary and secondary calluses. For a certain strain, plants were regenerated from cell-suspension cultures. The initial concentration of cells was critical for control of growth and morphogenesis. If it fell below a critical concentration, the lag phase sometimes became unlimited and cells failed to grow and finally died. In suspension cultures inoculated with high concentrations of cells, we obtained large undifferentiated cell aggregates. We obtained good plating by mixing a suspension (of single cells and small cell aggregates) with melted agar medium.
- Protoplasts. Viable protoplasts were isolated from established callus, cells of leaf mesophyll and suspension cultures. The protoplasts regenerated a cell-wall and developed to oval-shaped cells with clear cytoplasmic strands and streaming, and a large nucleus.
- It is planned to develop isozymes as genetic markers for plants in vitro and in vivo.

Descriptors: sugar beet, *Beta vulgaris*, culture in vitro, haploids, propagation in vitro, callus culture, suspension culture, protoplast, isozymes

Chlorophyll fluorescence, a useful selection method for plants with better growth abilities at suboptimal temperature?

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Hardening of plants against low temperature conditions causes changes in membrane lipid composition. These changes result in a shift of the phase separation temperature of the membrane to a lower value which enables plants to function at lower temperatures. Changes in lipid composition and phase separation temperature were also observed during hardening of cucumber lines to suboptimal temperature conditions.

It is well-established that chlorophyll-a fluorescence of intact leaves shows temperature-dependent changes which may depend on the lipid composition of the chloroplast membrane. We compared temperature-dependent variable chlorophyll-a fluorescence transients of cucumber plants grown at normal (25 °C day, 20 °C night) or at suboptimal (20 °C day, 15 °C night) conditions in order to investigate the possibility of using temperature-dependent chlorophyll fluorescence as a selection criterion for adaptation to suboptimal conditions.

Preliminary experiments showed that curves of the fluorescence intensity of the peak (P) as well as (P-S) against temperature showed two breaks between 27 °C and 0 °C. When plants were grown at normal conditions, breaks were observed at 6 °C and around 20 °C. Plants grown at suboptimal conditions also showed two breaks but the high temperature break was shifted to a lower value (from 20 °C to 16 °C). The temperature shift was most pronounced with leaf discs from line 45 plants selected for better growth at suboptimal conditions, indicating that phase transitions in the chloroplast membrane occurred at a lower temperature in these plants. The low temperature break around 6 °C was hardly affected by growth at suboptimal conditions. Further experiments are underway to investigate the correlation between chlorophyll fluorescence transients and adaptation to suboptimal growth conditions.

Descriptors: cucumber, *Cucumis sativus*, cold resistance, chlorophyll fluorescence

Adaptation to competition and breeding of forage crops: grass-legume mixtures

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Breeding of forage crops adjusted to growth in associations is concerned with interspecific competition.

The perplexity of agronomists confronted with variation in such associations is well evidenced by considering some aspects of crop biomasses. This is mainly due to interaction effects in grass-legume mixtures (i.e. in comparison with the pure stand of the grasses, with reference to nitrogen level). In fact, knowledge of the comparative functioning of pure stands or associations, in terms of dynamics of production, with contrasting levels of resource, is very poor. We need to know more about physiology of components (e.g. nitrogen fixation of legume) (Chalamét et al., 1983).

Another approach to the function, that considers individuals as a 'meta-population' of organs is promising. Environmental and genetic effects on growth (in terms of morphogenesis) of red clover are presented (Maitre, 1981).

Interferences have interest only if they result in a better performance, so we need to screen the response to association; examples are given of variation: between families (Maitre, 1977), between genotypes within a natural population (Wacquant et al., 1979; El Chahatah, 1983).

Definition of ideotypes and search for co-adaptation must be balanced during selection. This needs more research on morphogenesis and functioning of individuals in a canopy. The use of natural populations (for grasses, white and red clover) will result in a more systematic collection on a small scale at the margin of contrasted sites.

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Descriptors: grasses, legumes, forage crops, interspecific competition

The effect of selection for low temperature flowering ability in Chrysanthemum morifolium

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The chrysanthemum is a hexaploid, vegetatively propagated crop with an optimum temperature for rapid flowering of 17-18 °C. This temperature is maintained in commercial production of cut flower chrysanthemums and the breeders have always selected at this temperature.

To cope with rising costs of energy one approach was to lower the temperature in the greenhouse. Existing cultivars did not perform well at lower temperatures and breeding programmes have sprung up to develop cultivars adapted to low temperature.

Existing cultivars were screened for ability to flower at temperatures below 18 °C. Crosses were made between those that would flower at 14 °C though unvariably late and with few flowers. The F_1 plants grown to flowering at 12 °C to 15 °C showed large genetic differences in ability to flower. The majority of the seedlings did not flower at all, a considerable number flowered later than the mid-parent and only incidentally were plants recovered that flowered earlier than either parent. The number of flowers on the F_1 plants generally exceeded that on the mid-parent.

Selected F_1 plants were clonally propagated and tested for flowering ability at a range of temperatures. The optimum temperature was still 18 °C but the selections, although delayed, could flower at a wider range of low temperatures and produced more flowers per stem.

Descriptors: chrysanthemums, *Chrysanthemum morifolium,* low temperature, adaptation, flowering, selection

A method for the selection of first division restitution gametes during megasporogenesis in diploid potato

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For polyploid crops, the promising potentials of 'Analytic Breeding' systems have been emphasized by many plant scientists. In the autotetraploid potato, 'Analytic Breeding' involves induction of dihaploids, breeding at the diploid level and return to the tetraploid level by Sexual Polyploidization (SP). Sexual polyploidization can be achieved by means of $4x \times 2x$ (Unilateral SP) and $2x \times 2x$ (Bilateral SP) crosses, making use of preferably First Division Restitution (FDR) gametes formed by the selected diploid parental clones.

The potential value of Sexual Polyploidization through $4x \times 2x$ crosses has already been demonstrated unambiguously by American and Canadian researchers. The potentials of $2x^{FDR} \times 4x$ crosses are basically equal to those of $4x \times 2x^{FDR}$ crosses, whereas the expectations of $2x^{FDR} \times 2x^{FDR}$ crosses are even better. However, in both cases diploid clones producing a high and stable frequency of FDR $2n$-eggs are needed, but not available at present, mainly because of the difficulties involved in cytogenetic analysis of female meiosis.

In the Department of Plant Breeding (Agric. Univ.) at Wageningen a correlation between the occurrence of desynapsis during micro- and megasporogenesis in diploid potato clones was recently established. Both normal meiosis and Second Division Restitution (SDR) in association with desynapsis lead to aneuploid sterile spores. Only FDR in association with desynapsis will lead to euploid fertile $2n$-spores! Therefore the most important potential application of the aforementioned correlation is the possibility to select diploid potato clones with, theoretically, sole FDR $2n$-egg formation, simply by screening for stable desynapsis during microsporogenesis and testing female fertility of thus selected clones. Female fertility can be tested by pollination with either male fertile, desynaptic diploid plants or tetraploid varieties, both contributing $2n$-pollen to the progeny. As the testcross progeny may be expected to consist of tetraploids only, the mean number of seeds/pollination or seeds/berry obtained may serve as a measure for the level of FDR $2n$-egg formation.

The female fertility of desynaptic clones is usually extremely low. However, the moderate female fertility of some of such clones and the production of tetraploid progeny through testcrossing illustrates the practical value of the above described concept. Still to be selected, highly fertile desynaptic diploid clones with good agronomic characters may be used both to apply 'Analytic Breeding' in autotetraploid potato and to produce vigorous and uniform tetraploid progeny from $2x^{FDR} \times 2x^{FDR}$ crosses for growing potatoes from true seed.

Descriptors: potato, *Solanum tuberosum*, first division restitution, dihaploids, desynaptic mutants

Characterization of self-incompatible genotypes in cabbage and kohlrabi by isoelectric focusing of stigma extracts

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Extracts from stigmas of *Brassica oleracea* L. were analysed by isoelectric focusing (IEF) on polyacrylamide gels, 0.3 mm thick. We examined the relationship of IEF patterns, ranging from pH 3.5 to 9.5, to self-incompatibility (S) of genotypes. After electrophoretic separation, Coomassie staining of stigma protein or visualization of glycoproteins by various methods both elicited poor information. Restricted patterns or even no bands appeared in some plant material. Improved silver staining, however, led to typical protein profiles. The positions of the bands remained stable regardless of the physiological state of the flowering plants.

Stigma analysis for S characterization were used in cabbage and kohlrabi families, resulting in protein bands specific to breeding stocks and lines. In segregating populations, individuals could be distinguished by their distinct patterns. Studies of inbred lines and their corresponding hybrids showed a codominant inheritance of the marker profiles.

In some cases, S alleles were directly identified without crosses by comparing their IEF patterns with those of known S homozygotes.

Descriptors: cabbage, kohlrabi, *Brassica oleracea,* self-incompatibility, isoelectric focusing

Research plot harvesting and data acquisition by one person

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The Walter & Wintersteiger Nurserymaster plot combine was adapted by mounting a large cyclone grain collector within easy reach to the right side of the operator, and a sample carrier bin was attached to the combine to the left side of the operator.

An electronic balance placed beneath the cyclone collector was attached to a 1-cm metal base above shock mounts fixed to the battery box and the balance connected by its RS 232 interface to a portable electronic data terminal. Accessories were constructed for providing easy and rapid access to sample bags of polypropylene mesh grouped ahead of time in sets by harvest sequence. The waiting time for combine clean-out between plots is used for acquiring data, and also, in sequence, for tying the sample bag and transferring it to the carrier bin. Then the next bag is attached to the cyclone collector, and the grain emptied into it before the next plot is harvested. Even without the electronic devices the system saves labor and allows greater control over interplot contamination, but with these devices greater efficiencies and quicker access to yield data can be achieved.

Descriptors: plot combine, accessories, data acquisition, efficiency, field trials

Cytoplasmic variations associated with cytoplasmic male-sterility in Petunia hybrida plants

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Cytoplasmic male-sterility (CMS) in plants is of importance for commercial production of hybrid seed because it eliminates self-fertilization of the seed parent plant (Van Marrewijk, 1979). Our research is aimed at increasing the applicability of this trait, which is as yet limited because this trait is only found in few plant species. We are studying the molecular basis of CMS in *Petunia hybrida* and investigating whether CMS can be transferred by somatic cell hybridization and cybridization from petunia to other, economically important species of the family Solanaceae.

To elucidate the molecular basis of CMS, we have compared chloroplast (cp) and mitochondrial (mt) DNA from CMS and fertile plants. Restriction endonuclease patterns, obtained after digestion of cpDNA (Bovenberg et al., 1981) from normal and CMS *Petunia hybrida* with the enzymes Bam HI, Bgl I or Sal I did not indicate any variation among these cpDNAs, making a possible role of chloroplasts in CMS less likely. We therefore focused our attention on mitochondria, and developed a procedure for isolation of mitochondrial DNA from *P. hybrida* cell suspension cultures (De Haas & Kool, 1982).

Comparison of DNA fragment patterns obtained upon restriction endonuclease digestion of mtDNA from fertile and cytoplasmic male-sterile *Petunia hybrida* cv. Rosy Morn, indicated distinct variation between the mtDNA from fertile and CMS plants.

Differences between the mitochondria of the two cytoplasmic types were also observed at the level of protein synthesis in isolated mitochondria. The most prominent difference was that mitochondria from the CMS plants synthesize an additional polypeptide of molecular weight 34000 dalton that was not observed in mitochondria from fertile plants. The occurrence of this polypeptide was accompanied by the disappearance of a polypeptide of molecular weight 38000 dalton that is synthesized in fertile mitochondria. Differences in the translation products of chloroplasts from fertile and CMS plants were not observed.

The results obtained so far support the hypothesis that the mitochondria rather than the chloroplasts are the coding site of factors that condition cytoplasmic male-sterility in *Petunia hybrida*.

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Descriptors: petunia, *Petunia hybrida,* cytoplasmic male-sterility, cytoplasmic variation, mitochondrial DNA, somatic hybridization

Maintenance and expression of mutant properties (resistance to 6-fluorotryptophan) in plants regenerated from mutant cells of Petunia hybrida

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Mutant cells resistant to 6-fluorotryptophan (6-FT) were isolated from suspension cultures of *Petunia hybrida* cells after treatment with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (final concentration 40 mg/l, incubation for 4 h). A number of these cell lines resistant to 6-FT grew on auxin-free medium. This suggests that, in analogy to the carrot cell lines resistant to 5-methyltryptophan (Widholm, 1977), the mutation responsible for the resistance to 6-FT causes an increase in the concentration of free tryptophan, which in turn may lead to increased auxin synthesis.

Plants were regenerated from one of the resistant and auxin autotrophic cell lines (AK 5069). Calluses obtained from these regenerated plants were resistant to 6-FT, indicating that they still expressed the mutant phenotype. The mutant plant AK 5069 was crossed with one of the sensitive parental plants. Explants of the progeny were placed on callus-inducing medium. The calluses obtained from about half the progeny were resistant to 6-FT. Most of them were also auxin-autotrophic. These results indicate that mutations leading to resistance to 6-FT can be induced and selected for in cell cultures of *Petunia hybrida*. The mutant property is maintained after plant regeneration and is inherited by the M_2 progeny as a single, dominant nuclear trait.

The resistant plants looked abnormal. Tumor-like structures formed on stems and leaves, perhaps by increased auxin synthesis as a result of a higher concentration of free tryptophan. We are measuring concentrations of free tryptophan in wild-type and resistant mutant plants, and in calluses to determine the mechanism of the resistance and also to verify the expression of the mutation at the plant level.

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Descriptors: petunia, *Petunia hybrida*, selection in vitro, 6-fluorotryptophan, plant regeneration

Effect of seed size on performance of cereal varieties in small plots

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Spring wheat, barley and oat varieties were selected on the basis of differences in seed size. Of some varieties, the seeds were graded into two size fractions. This facilitated the evaluation of the effect of seed size on performance in the absence of genetic differences.

All entries were planted in small plots, where intergenotypic competition could exert its influence, as well as in yield trials. The purpose of this approach was to study the bias in grain yield as a result of seed size in intergenotypic interference.

Improvement in field testing procedures is suggested on the basis of the results.

Descriptors: cereals, seed size, intergenotypic competition, field trials

Competition and progeny selection in chrysanthemums

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An investigation was carried out to determine whether chrysanthemum cultivars differ in competitive ability and, if so, the likely effects of this on progeny selection when each plant is of a different genotype.

Individual 'target' plants of four contrasting cultivars were interplanted within pure stands of each of the four cultivars to give 16 replicated combinations of four 'target' genotypes with four competitive backgrounds. Using criteria such as flowering date, flower number and plant dry weight, the cultivars were found to differ significantly in their competitive ability.

In a subsequent experiment, the 'strongest' and 'weakest' competitors, 'Pollyanne' and 'Hurricane' respectively, were grown together on a square lattice in such a way that the competitive effects of differing numbers (0-4) of nearest neighbours of unlike genotype could be determined. Significant linear trends confirmed the relative competitive abilities of these two cultivars.

To quantify the effects of competition during progeny selection, 'target' plants of identical genotype were grown in pre-determined positions within clonally replicated plots of segregating full-sib families and their variance due to position (distinguished by different sets of segregating neighbours) for each of several characters was compared with that of other 'target' plants growing in corresponding positions within monoculture plots (with similar sets of uniform neighbours).

In only one of four such trials did the effect of segregating neighbours contribute more to total variance than the effect of uniform neighbours. Averaging over all four trials and all characters, position in segregating plots contributed 17% to total 'target' plant variance compared with 21% for position in monoculture plots. This lack of effect was probably due to full-sibs having a restricted range of competitive ability and the tendency for the joint competitive effect of a set of segregating neighbours to approach an overall average value for the family as a whole. In practice, therefore, competition is likely to have little deleterious effect on selection efficiency. Wider plant spacing to reduce the effect of competition in chrysanthemums would greatly increase growing costs (or reduce progeny numbers) and cannot be justified.

Descriptors: chrysanthemums, competition, selection

Combination of results from several data sets: some possibilities for a better understanding of genotype-environment interaction

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Genotype sensitivity to environment is important for characterizing parents in yield breeding and for defining environments advantageous to select lines stable or specifically adapted. A series of performance trials on 25 durum wheat varieties, spread over several sites in central and southern Italy from 1975 to 1981, was used to assess genotype-environment (GE) interaction. Varieties and sites were not the same in all years; a site-year combination constituted an environment. From these trials, two data sets were obtained: the first with 13 varieties in 85 environments from 1975 to 1979, the second with 17 varieties in 60 environments from 1979 to 1981. A third derived set consisted of 8 varieties, common to both sets, grown in 128 environments from 1975 to 1981. Linear and two multi-phase regression models were used in studying the relationship between GE interaction and environmental indexes. When a single linear regression fails, the multi-phase regression models can resolve the non-linear relation by two or three straight lines. The multi-phase regression models applied were: two intersecting straight lines (Jinks & Pooni, 1979) and three straight lines (Mariani et al., 1983). The goodness of fit of these models was tested. The same environmental indexes, as means of 25 varieties in each environment, were used for regression analysis of all sets. Joint regression analysis (Perkins & Jinks, 1968; Eberhart & Russell, 1966) for linear and multi-phase regression models was applied to each set; the third set was analysed in three conditions: 128, 85, 60 environments, respectively. For the 8 varieties in the three conditions, all the regression models applied showed similar efficiency values and analogous stability parameters for each variety. Consequently, the response to environmental improvement of the other varieties (not common) of the two sets could be assumed valid also if obtained from data of 85 and 60 environments only. The non-orthogonality of data was so overcome and it was possible to generalize the results on varietal response as if all the varieties had been grown in all environments. As relative efficiency values revealed that multi-phase regression models applied to the three sets were not significantly different from linear regression, this was used to measure the average sensitivity over all environments for each variety. In addition we verified by multi-phase regression models whether a specific response to poor, average or rich environments arose for a variety.

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Descriptors: genotype-environment interaction, wheat, *Triticum durum*, environmental index, stability parameters, multi-phase analysis.

Long-term cold storage of Prunus in vitro

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Trials on long-term cold storage of different clones of *Prunus* in vitro (proliferation phase) were performed.

Different temperatures were tested, +8 °C, +4 °C and -2 °C, with or without low-intensity light (0.5 klux) at a photoperiod of 16 h. Best results (up to 12 months storage) were achieved with the lowest temperature in darkness.

The time elapsed from the date of subculture to the beginning of the storage plays also an important role. Survival in cold storage of established cultures (1 or 2 weeks old) was much higher than for cultures cold-stored just after transplanting.

Descriptors: *Prunus*, storage in vitro, temperature, light

Is early generation selection necessary?

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Single seed descent (SSD) is being compared with the pedigree breeding method (PED) for the production of glasshouse lettuce varieties at the G.C.R.I. Direct comparison of the two methods permits an assessment of the effects of delaying selection to near-homozygosity, whilst initiating SSD after one or two generations of PED allows the effects of early generation selection to be studied.

297 SSD progenies were generated from two crosses which had already been extensively tested using PED. Cross I had led to the release of four new varieties. No varieties had resulted from Cross II although it had appeared equally promising in the early PED generations.

The SSD lines, their parents, and the four PED varieties were evaluated in late spring trials in polythene structures using an agronomic index. This index was based on relative rankings for ground cover, hearting, shape, size and general performance and was weighted in favour of general performance.

Index values for SSD lines transgressed those of the parents for both crosses. In Cross I, lines equal to the four varieties resulting from PED were obtained. The SSD lines with the best agronomic index from Cross I were superior to those from Cross II, confirming the results found with PED.

Delaying the onset of SSD to allow selection in F_2 (both crosses) and F_3 (Cross I) resulted in higher mean indices indicating that early generation selection was effective. Examination of the distributions showed that selection was largely successful in removing inferior genotypes.

Replicated yield trials of SSD lines selected on the basis of agronomic index are described and discussed.

Descriptors: lettuce, *Lactuca sativa,* early generation selection, single seed descent, pedigree breeding, selection methods

Squish hybridization for the detection of viral infection of plant leaves

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In order to breed plants for resistance to viral infections, a reliable, sensitive assay for virus (such as ELISA) that is at the same time facile and relatively rapid is required. However, for a variety of reasons, some viruses can not be reliably assayed by ELISA or similar methods. We report on an alternate simple assay procedure which is based on nucleic acid hybridization. Small circles of leaf tissue of turnips (*Brassica rapa* L.) either healthy or infected with cauliflower mosaic virus (CaMV) were squished with a wooden dowel onto filter paper. Standard amounts of CaMV and viral DNA were spotted onto the same paper. After successive 15-min treatments in 1.5 mol/l NaCl, 0.5 mol/l NaOH and in 3.0 mol/l NaCl, 0.5 mol/l tris HCl, pH 7.0, the papers were incubated for 18 to 24 h at 65 °C with ^{32}P-CaMV DNA (obtained by nick translation) in Denhardt's solution for nucleic acid hybridization. After four washes of the filter paper at 65 °C with 2×SSC, an autoradiograph of the paper was made. Increasing standard amounts of DNA or virus spotted resulted in an increased autoradiographic spot density. All squishes from healthy turnip leaves so far tested failed to bind radioactivity while all squishes from diseased plant leaves gave dark spots on the film. The technique is suitable, after some modifications, for facile screening of large numbers of plants for resistance to viral diseases. Several other applications of squish hybridization will also be demonstrated.

Descriptors: turnip, *Brassica rapa*, cauliflower mosaic virus, assay for virus, squish hybridization, resistance, breeding

Effects of mass selection for crown height in sugar beet, Beta vulgaris L.

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In sugar beet crops heterogeneity for crown height, which is the length of the above soil surface part of the beet, causes losses by overtopping and toptare by undertopping during mechanical harvesting. Although the variation is partly due to cultural practice, genotypic variation within varieties and populations plays a considerable role. As selection pressure on crown height has been low, mainly mass selection is applied. It is carried out in a kind of stratified selection per replication, on a uniform soil without wheelings, and in a very regular stand.

Five generations of divergent selection in diploid populations have resulted in mean differences in crown height between the high and low selections of about 11 cm. Although selection differentials are of the same magnitude, the responses in the low direction are smaller than in the high direction. This asymmetry arises from reaching the limit of zero crown height in the low direction. The results obtained in five different years, using the unselected original population as a reference, are in very good agreement with those from the five generations compared in one experiment. The responses in two tetraploid populations are similar.

In our selection experiments a higher crown is found to be positively correlated with a longer epicotyl and a longer hypocotyl (its component parts), a greater total length of the beet, a shorter root length, a higher root yield, a higher sugar yield, higher contents of K, Na and α-amino-N, somewhat more losses by overtopping, less toptare by undertopping and considerable less dirt tare.

It appears that mass selection, carried out with great accuracy, can still be a powerful method of intrapopulation improvement.

Descriptors: sugar beet, *Beta vulgaris*, crown height, mass selection

Selection within progenies of interspecific Brassica hybrids

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The aim of the work was to obtain genetically differentiated initial material for breeding of new seed and leafy fodder forms of *B. napus* suitable for the Polish climate and soils.

Material for this work was derived from our own collection of winter and summer forms of *Brassica* species. The method was hybridization between *B. oleracea* ($2n=18$), *B. campestris* ($2n=20$) and *B. napus* ($2n=38$). Crosses were made by conventional methods, or by ovary excision and embryo culture.

Hybrids were identified by cytological analysis. If genomes were similar (equal number of chromosomes in parental forms), immunochemical analysis was also applied. Differences in relief of seed coat were also used as an additional criterion of hybridity.

In the evaluation of hybrids, such agricultural features as rate and vigour of vegetative grówth in autumn, winterhardiness, rate and vigour of vegetative growth in spring, earliness of generative development and seed production were taken into account, as well as qualitative traits, fatty acid composition of seeds, and glucosinolates, protein and fibre contents of seeds and green matter.

Hybrids were compared with forms in the collection. Some had no analogues. The most valuable forms for desired features were selected.

Descriptors: Brassica, interspecific hybrids, embryo culture

Use of planting dates to select stress tolerant and yield stable triticale genotypes for the rain-fed Mediterranean environment

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Mediterranean cereal agriculture suffers from several agro-climatic constraints. Early planting exposes the crop to moisture stress during the seedling stage and to frost during tillering and flowering, while late planting negatively affects the tillering capacity and consequently reduces the yield. However, it was possible in two seasons (1980/81 and 1981/82) to select triticale genotypes that tolerate the effects of early and late plantings.

Descriptors: triticale, stress tolerance, Mediterranean, planting date, rain-fed environment

Pollen competition in the cucumber, Cucumis sativus L.

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Yields of hybrids from interspecific crosses in the genus *Cucumis* aided by γ-irradiated mentor pollen (IMP) have been disappointingly low. IMP greatly stimulates fruit development, and it may aid pollen tube growth of the pollen of the male parent through the style, but it also competes with the latter for the available ovules. In cucumber, IMP induces development of ovules to full-sized, but empty 'seeds'. Some of these 'pseudoseeds' contain endosperm but never an embryo. A higher irradiation dose lowers the ability of the irradiated pollen to compete with the fresh pollen in fertilization.

The pollen germination percentages in vitro of several marker lines were similar, but average pollen tube lengths differed by a factor two. Germination was hardly affected by 1 kGy irradiation, but pollen tube lengths were strongly reduced. Higher irradiation doses caused a decline in germination and pollen tube growth.

Mature cucumber fruits may be cut transversely as well as longitudinally. Segregations of markers or of more complicated characteristics can thus be studied per segment. It is assumed that ovules closest to the style are the first to be fertilized, and some microscopic evidence for this is presented. Therefore deviating segregation ratios indicate differences in fertilizing ability. Results of testcrosses with several marker lines show that significant deviations from the expected rations may occur depending on the segment. Observation of deviating segregation ration per fruit segment also facilitates distinction between somatic and gametophytic causes for an observed deficit of recessive offspring in testcrosses.

With simultaneous double pollinations, the order in which two pollen types are applied has a large effect on the relative fertilizing success of the pollen types. The pollen which is applied first occupies most ovules, possibly because it makes the most intimate contact with the stigmatic surface. Alternatively, two pollen types may be separately applied to different stigmatic lobes, whereafter the two bundles of pollen tubes fuse in the style and compete on equal footing for the ovules. The pollen appears to fertilize ovules in either locule of the ovary, irrespective of the stigmatic lobe on which it was applied.

The techniques developed with the marker genes are used in breeding for better growth at low temperature via selection of pollen in response to temperature stress.

Descriptors: cucumber, *Cucumis,* interspecific hybridization, pollen competition, marker genes, fruit segments

Water transport in cucumber plants measured by nuclear magnetic response

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The root system is a largely neglected part of the plant in most breeding programs of non-tuberizing crops. Also our breeding program at the IVT to improve growth and production of the glasshouse cucumber at lower temperatures in winter has so far concentrated on shoot growth and fruit production. The importance of the root system for the growth of the cucumber is reflected by the strong stimulation of growth at higher soil temperatures. The decrease in growth at lower air temperatures may possibly be compensated by increased soil temperature, but more insight in shoot/root interactions at different temperatures is needed. The water transport through the plant is the result of interactions between shoot and root, each in their environment. Water transport in growing cucumber plants and in plants grafted onto rootstock *Cucurbita ficifolia* was measured with a pulsed nuclear magnetic resonance technique (NMR). The method uses the weak magnetic moment of the protons of the water molecules themselves. It is therefore a direct, non-destructive measurement of flow, as opposed to determinations based on tracers, heat or weight reduction. The linear and volumetric water flow as well as the water content can be determined simultaneously, with a time resolution of a few seconds.

Measurements were carried out in the laboratory. Plants were grown in a temperature-controlled aerated solution culture during the measurements. Ambient temperature, light intensity and relative humidity were kept constant, and the root temperature was gradually lowered from 20 °C to 4 °C at a rate of 1-1.5 °C/10 min. Thereafter the plants were kept for 2-5 h at this low root temperature. Shoot temperature was 25 °C and relative humidity about 50%.

The results so far show that there was no immediate reaction of the water transport velocity on the lowering of the root temperature even as low as 4 °C. The linear flow velocity per leaf area of grafted plants was twice as high as that of non-grafted plants raised at the same temperature. The volumetric flow of grafted plants was also higher, indicating a higher activity of the root system of the rootstock *Cucurbita ficifolia*. Prolonged exposure to low root temperature depressed water transport in all plants. The rate of flow decrease was lower for plants raised at 16 °C than for those raised at 20 °C. The relative rate of flow decrease of grafted plants was lower than that of non-grafted plants at the same temperature.

The relative flow decrease may serve as an indication of low temperature damage. A high value of this coefficient results from poor adaptation of the plant at low temperature. The heavy equipment (magnet system) thus far precluded measurements in the glasshouse or field. Miniaturization of the apparatus is in progress to enable large scale measurements of individual plants in situ. The water transport of individual plants of segregating breeding populations grown under defined root and shoot environments may indicate the efficacy of their root system. The contribution of the roots to growth and production can thus be analyzed.

Descriptors: cucumber, *Cucumis sativus*, root system, water transport, nuclear magnetic resonance, low energy adaptation

Fertilization studies on winter rape with low glucosinolate content

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Fertilization and seed set were studied in three 00 winter rape varieties, Librador, NPZ St. 1657/79 and DSV St. 154/81, with Jet Neuf and Garant as standard varieties. In 1982 the material was grown in the field at two plant densities (40/m²; 20/m²); in a third treatment, pollination by bees was excluded by planting the material in a cage.

For calculation of fertility, the number of seeds per siliqua was counted and related to the number of ovules, which is assumed to be a measure of potential seed set.

Siliquae of the 5th side-shoot showed the same number of ovules as those from the main shoot. There were practically no differences in number of ovules in relation to plant density or growing in the cage.

Marked reduction in fertility was found in the lower part of the crop measured as fertility of the 5th side-shoot. This cannot be explained by reduced pollination by bees. The same reduction in fertility was observed on plants grown in the cage free of bees.

Up till now U.V. microscopic analysis gave no evidence for significant differences in pollen germination, pollen-tube growth and fertilization.

These results suggest that reduction in fertility could occur after fertilization by ovule abortion or seed degeneration, which should be analysed in the 1983 trial.

Descriptors: rape, *Brassica napus,* glucosinolate content, seed set

Hybrids, hybridization and taxonomy of cultivated plants

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In order to obtain new cultivars, plant breeders create countless numbers of hybrids. The nature of these hybrids depends on:
- relationship between parental plants;
- mode of origin of the hybrid (e.g. single cross, backcross, somatic hybridization, wide hybridization).

With regard to intergeneric and interspecific hybridization, only a few first generation hybrids will be 'ready made' cultivars. Most hybrids will need further breeding to develop cultivars from them.

Before a newly developed cultivar can be put on the market, it has to be named and it must be classified within a species or genus. This is necessary, since both botanical and cultivar names are needed in communication for science, trade and legislation. It is therefore essential to have a clear set of internationally accepted nomenclature rules and to avoid unnecessary changes of names.

Formation of cultivar names is regulated by the International Code of Nomenclature for Cultivated Plants (ICNCP, 1980). Several countries have additional restricting rules, set by law in relation to Plant Breeders' Rights.

A cultivar resulting from intergeneric or interspecific hybridization that resembles one of the parental species should be assigned to the corresponding parental species. However, if such a cultivar is quite different from either parental species, it should be classified under a new taxon to be named.

Formation and publication of botanical names is regulated by the International Code of Botanical Nomenclature (ICBN, 1978). In the naming of new cultivated intergeneric and interspecific hybrids, this has following drawbacks:
- the ICBN has no legal force;
- description (Latin) and typification, as required to validate a new name, are often impossible.

References

ICBN (International Code of Botanical Nomenclature), 1978. F.A. Stafleu et al. (Eds.), Regnum Vegetabile 97, pp. 457.

ICNCP (International Code of Nomenclature for Cultivated Plants), 1980. C.D. Brickell et al. (Eds.), Regnum Vegetabile 104, pp. 32.

Descriptors: taxonomy, cultivated plants, interspecific hybrids

Genetic variation among plants of birdsfoot trefoil regenerated from callus cultures: preliminary results

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Leaflets of *Lotus corniculatus* L. were induced to form callus on UM (Uchimiya & Murashige) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) 2 mg/l and kinetin 0.1 mg/l. Since birdsfoot trefoil is an outcrossing species, a large variation was observed in callus formation and suitable plant genotypes were selected. Plant regeneration through somatic embryogenesis was obtained from callus cultured on MS (Murashige & Skoog) medium with 2-isopentenyl adenine (2-iP) 1 mg/l and indoleacetic acid (IAA) 0.1 mg/l.

About 200 plants, regenerated from callus which derived from a single plant, were checked for chromosome number and some morphological traits.

Preliminary results indicate constancy of chromosome number and a noteworthy variation for morphological traits. In order to measure and compare genetic variation induced by culture in vitro with that in natural populations, 100 regenerated plants and as many from seed were cloned, grown in greenhouse, moved to the field and evaluated for some morphological and agronomic traits.

Descriptors: birdsfoot trefoil, *Lotus corniculatus*, callus culture, plant regeneration, genetic variation

Modifying plant architecture of maize (Zea mays L.)

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Modifying the maize plant in accordance with parameters of the ideotype for grain and biomass production is a progressive system of maize breeding. Our ideotype scheme demonstrates that the maize plant modified for grain production should be about 150-160 cm tall with strong stalk and small tassel. Leaves should be shorter, narrower and upright, mainly at upper insertions, and 1-2 ears should be inserted higher than on plants of the present hybrid maize type.

The use of mutant dwarfing genes is one way of modifying plant height. The possibilities of employing the brachytic-2 and brevis-2 mutants were demonstrated with a diallele cross of five brachytic-2 and five brevis-2 inbred lines. They show that both mutants might be successful genetic sources to modify plant height. The effect of erect leaf position on grain yield was investigated by comparison of isogenic single crosses of liguleless-1, liguleless-2 and liguleless-3 at three plant densities. The results showed that upright leaves (liguleless) might positively influence grain and dry matter yields, but soil moisture or precipitation was a limiting factor. So breeding for drought resistance should be combined with breeding for erect leaves.

Descriptors: maize, *Zea mays*, ideotype, plant height, erect leaves

Irradiated pollen in barley breeding

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Studies with irradiated pollen in *Nicotiana* (e.g. Pandey, 1975, Nature 256; Caligari, Ingram & Jinks, 1981, Heredity 47) have shown that variation for a single character can effectively be transferred from one genotype to another using this technique. An investigation was therefore started to see whether the same would hold true in barley (*Hordeum vulgare*), some results of which are presented here.

An obvious application of such a technique would be to improve specific weaknesses in a genotype. The cultivar Golden Promise, which commands 60% of the Scottish acreage, was chosen since it is susceptible to several diseases, in particular mildew. This cultivar was thus used as the female parent and 'Magnum', which carries mildew resistance, was the pollen donor. Pollen was subject to 4 different doses of gamma (γ) rays namely: 5, 10, 15 and 20 Gy to produce an M_1 generation, while at the same time unirradiated pollen was used to produce the F_1 for comparison.

A sample of the parents, M_1 and F_1 were scored in the glasshouse for mildew resistance, juvenile growth habit and height. Variation in all these characters was observed in the M_1, mostly in the form of plants expressing certain of the recessive maternal characters, while the F_1 was uniform.

The M_2 generation was produced by selfing the M_1 and the F_1 selfed gave the F_2. These were grown in the field in 1982. In general the M_2 showed a greater resemblance to the maternal cultivar than the F_2 did. Some of the M_2 plants which appeared identical to 'Golden Promise' were tested electrophoretically for their hordein patterns and of those some showed the paternal characteristics.

Thus the technique would appear to give results similar to those reported for *Nicotiana* and additionally showed the effect with electrophoretic variation. Also, among the M_2 plants it was possible to achieve the objective of selecting plants which were apparently identical to 'Golden Promise' but were mildew resistant. These will, obviously, be examined further in the next generation.

Descriptors: barley, *Hordeum vulgare*, pollen irradiation, transformation in vivo

Manipulation of the first division restitution gamete formation in potato through the use of meiotic mutants

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Homogeneous populations of first division restitution (FDR) gametes are expected to be of great value for the production of uniform and highly vigorous progeny in polyploid crops.

Because the cultivated potato is an autotetraploid, there is scope for FDR gametes in potato breeding, and their use is attractive. In view of this an attempt was made to select diploid clones of potato that can produce 2*n*-pollen in high frequencies, and exclusively through FDR. To achieve this, diploid desynaptic mutants of potato with a tendency for a high degree of meiotic nuclear restitution were selected. The attractiveness of using a desynaptic mutant is that, because of the failure of chromosome pairing at metaphase I, only those restitution nuclei that result from FDR are functional.

The SDR gametes in a desynaptic mutant, if they arise, are expected to be non-functional because of chromosome imbalance. This means the fertile 2*n*-pollen from a desynaptic mutant constitute relatively homogeneous population of FDR gametes.

The high level of fertility of mutants that is observed in this study, as well as their crossability as male parents indicate that fertile desynaptic mutants may be a good source of FDR gametes for practical breeding.

Descriptors: potato, *Solanum tuberosum,* first division restitution, desynaptic mutants

Stability of periclinal chimera in loganberry plants obtained by micropropagation

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Thornless loganberry is a periclinal chimera; 3644 plants of this clone, obtained by micropropagation, were observed in the field. After one season of growth, none of them was thorny in the principal shoot, though 1.3% of the plants showed some degree of thorniness in one or two lateral shoots, ranging from totally thorny to a mutated sector of varying length and width. Observations were also conducted on thorniness of S_1 seedlings originated from 724 plants, to see if layer substitution could be detected. Similar observations were also conducted on plants originated from roots on two-years-old plants in the field.

Descriptors: loganberry, periclinal chimera, propagation in vitro, stability

Stability and performance of two micropropagated peach rootstocks

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Plants of two micropropagated peach rootstocks, GF 43 and GF 677, were tested in the nursery for stability and also compared with plants obtained by standard propagation. In the second year, scions budded on these rootstocks were also observed.

Plant growth, shoot length and leaf area were recorded. Micropropagated plants of GF 43 had less vigour; leaf area, shoot length and final growth of the scions in micropropagated material were inferior to normally propagated GF 677.

Descriptors: peach, rootstocks, propagation in vitro, performance

Effect of indoleacetic acid (IAA) on the meiotic process in anthers of Secale cereale L. cultured in vitro

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Anthers of rye (*Secale cereale* L.) of the diploid cultivar Elbon were cultured in vitro, in T (Vázquez & Rueda, 1982) medium and in a medium supplemented with indoleacetic acid (IAA) 4 mg/l (T1). As the three anthers in each flower of rye are almost synchronous in development, one was used to establish the meiotic stage and the other two were cultured. The cultures were initiated in different leptotene or zygotene periods and were kept for 6, 18, 24, 30, 38 and 58 h in the medium. The effect of the hormone was deduced by comparing the behaviour of the pollen mother cells (PMCs) cultured in the two media, T medium being used as control medium.

Two meiotic stages, the early and mid-leptotene were affected by the culture and the early zygotene was affected by the hormone, the effect being a noticeable decrease in the number of chiasmata per cell in metaphase I. However very little effect was observed upon anaphase or telophase, abnormalities being infrequent in these stages, though the number of anthers with some affected PMCs increased in the presence of the hormone.

Descriptors: rye, *Secale cereale,* anther culture, indoleacetic acid (IAA), meiosis

Cell selection in vitro in cultures with different ploidy levels

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When cultured in vitro, plant tissues and cells could modify their original chromosome numbers. The different cell populations originating in these cases change their frequency by competition during culture between the cells with different chromosome numbers, the cells with the normal chromosome number of the species on some occasions being eliminated.

To study the evolution of the chromosome number during the time in cultures with different ploidy levels, two experiments were carried out:

- Diploid ($2n=14$) and tetraploid ($2n=28$) embryos of rye (*Secale cereale* L.) were cultured in a solid medium, the predominance of the initial chromosome number being observed during the time. An aneuploid cellular line was detected.
- Embryos of diploid barley (*Hordeum vulgare* L.), $2n=14$, were cultured in colchicine-containing media with the object of inducing non-diploid cells. In this case, it was observed that high concentration of colchicine had a deleterious effect upon the cultured explants, aneuploid cells being induced with lower concentrations. These cells were eliminated in the cultures, the diploid chromosome number being re-established in the time.

Descriptors: rye, *Secale cereale*, barley, *Hordeum vulgare*, cell culture, chromosome number, colchicine

Production of homozygous lines of triticale by anther culture

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Hexaploid (6*x*) spring types and octoploid (8*x*) winter types of triticale were grown as bulk populations in Finland through 8-10 generations. Populations consisted of early and hardy European and Canadian materials that had been grown in bulk. These very heterogeneous populations underwent general climatic adaptation by selection for early maturity and overwintering. In addition, mild selection for seed characteristics was applied.

The populations exhibited wide variation in morphological traits and had become well adapted to climatic conditions. Thus efforts were being made to select lines for agronomic characters.

To study variation between lines, anther culture techniques were applied to selected individuals or specially made paired crosses. Methods were mainly as in Chinese investigations on triticale with N_6 medium.

Results were promising with 6*x* spring types, which revealed high levels of variation between lines in the F_2 generation after chromosome doubling. Results were even more promising on 8*x* winter types of triticale, however the F_2 generation after chromosome doubling has not yet been produced.

The anther culture haploid programme was promising for effective selection of pure lines in the F_2 and later generations after chromosome doubling. The breeding programme could thus be accelerated and particularly in triticale selection could be done on pure lines. Limitations are mainly caused by large individual differences in the initiation of embryoids and by the appearance of many albino plants, lacking chlorophyll.

Descriptors: triticale, anther culture, doubled haploids, selection efficiency

Plants resistant to Phoma lingam regenerated from cell cultures of haploid rape, Brassica napus L.

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Cell suspension cultures of haploid rape (spring line H1) were treated with a mutagen and then plated on medium containing the toxin produced by the pathogen *Phoma lingam*. This toxin has a drastic inhibitory effect both on seedlings and on cell cultures of rape. Cell colonies which survived and grew further after more than two passages through medium containing the toxin were selected as resistant.

Plants regenerated from selected and from control cultures were tested for resistance by inoculating them in the greenhouse with a defined number of pathogen spores. As comparison, test plants of the susceptible variety Lesira and of the tolerant variety Jet Neuf were simultaneously infected with the same spore inoculum. Those plants that at maturity (3-4 months after infection) did not show visible symptoms or only light and strictly local lesions (similar to 'Jet Neuf') were considered resistant or tolerant.

Among the regenerants from selected cultures, 22% gave a tolerance response to infection with the pathogen. Few plants (4%) regenerated from control cultures, showed an increased tolerance to the fungus too.

Although derived from originally haploid cells, no regenerant was haploid, the majority being diploid, with frequent hypodiploid chromosome numbers between 34 and 37. There were marked morphological differences between these plants.

The progenies of some susceptible and tolerant regenerants were tested for resistance to *P. lingam*. Among the progeny of tolerant regenerants (450 plants tested), 30% proved tolerant like the parent plant, whereas the proportion of tolerant plants among the progeny of susceptible regenerants (400 plants tested) was 4%.

Segregation in the progenies, usually also recognizable by morphological and physiological features, can only be explained on the basis of heterozygosity or chromosomal instability of the regenerated plants.

Descriptors: rape, *Brassica napus*, haploids, selection in vitro, *Phoma lingam*, plant regeneration

Gynogenesis in vitro and biometric studies of doubled haploids obtained by three techniques in Hordeum vulgare L.

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Generally the mature or nearly mature embryo sac gave a positive response to gynogenesis in vitro, as shown for barley, wheat, maize, sugar beet and lettuce. All nuclei of the embryo sac (egg, synergids, polar nuclei, antipodal cells) could divide and give embryos or callus. Mitosis of these nuclei started on the second to the fifth day of culture. Fairly often, it was the egg or the egg and the antipodal cells that were induced to divide (wheat, barley, lettuce). The embryogenic ovaries gave rise to one or several green plantlets. In barley, all gynogenetic plants were haploid; in other species, they were haploid, mixoploid or diploid (lettuce, wheat).

Some haploid plants of *Hordeum vulgare* L., originating from androgenesis in vitro, gynogenesis in vitro and interspecific crosses with *Hordeum bulbosum* L. were obtained from the fixed line of spring barley 'Bérénice'. These haploid plants were doubled with colchicine and then self-pollinated. The control line was 'Bérénice'. The doubled haploid plants and the control line differed significantly.

Discriminant multivariate analysis shows a difference between groups of doubled androgenetic haploid plants, doubled gynogenetic haploid plants and the control line. This difference persisted in offspring of all these doubled haploid plants (2nd, 3rd, 4th generation of selfing).

Diploid plants from interspecific crosses and gynogenetic plants were found to be closer to the control line than androgenetic plants. Androgenesis produced a greater variation than gynogenesis or interspecific crosses. The origin of the haploids (microspores or haploid embryo sac cells) and an effect of culture in vitro may cause the differences observed.

Descriptors: barley, *Hordeum vulgare*, gynogenesis in vitro, androgenesis in vitro, interspecific hybridization, *Hordeum bulbosum*, doubled haploids

Flower and pollen biology studies at the University of Bologna

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The Institute of Fruit Science and Tree Growing and the C.N.R. Research Center for Fruit Technique, University of Bologna, developed a program of research on fruit-tree fertility related to the fruit-breeding projects in Italy.

- Research on sterility. In nectarine peach, male sterility has been discovered on cvs Ruby Gold and Rhone Gold, which have atrophic anthers and poor pollen. In apple, cv. Starkrimson shows gametophytic sterility; despite the high germination capacity of the pollen in vitro, its tube growth is normally arrested at the upper third of the style tissue, which is characterized by very rich cytoplasm, large rough endoplasmic reticulum (RER) and secretion of proteins and polysaccharides.
- Overcoming self-incompatibility in apple. Using mentor pollen it was possible to overcome the self-incompatibility in 'Starkrimson'. The compatible pollen was devitalized with ethanol, gamma and laser rays so that it maintained its germination capacity, but was not able to fertilize. A few incompatible pollen tubes mixed with the devitalized compatible ones reached the end of the style and could fertilize the ovules, as demonstrated by seeded fruits. The pioneer pollen technique (pollination with viable incompatible pollen preceded by pollination with viable compatible pollen) was successful and several self-pollinated leaf-marked seedlings were obtained.
- Research on pollinators. For peach, nectarine, cherry, plum, apple and pear a list of suitable pollinators for the most important varieties was prepared, as well as of incompatible groups.
- Ecological aspects of pollination. Some fungicides can affect the germination of apple and pear pollen in vitro and in vivo, and can harm fruit set, but not necessarily yield.
- Taxonomic means for genetic research. By scanning electron microscope, it was possible to distinguish pollen of different apple varieties morphologically and the standard clone from the spur clones. Identification of different genera seems possible by electrofocusing regarding total protein and the enzyme esterase.

Descriptors: fruit crops, flower biology, pollination, taxonomy, male sterility, self-incompatibility

Towards selection in vitro in Lilium

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Callus cultures of *Lilium* can be grown in liquid media for several years while retaining good regenerating ability. In principle, such stability in vitro would make *Lilium* a good candidate for model studies on the feasibility of selection in vitro, e.g. for horticulturally important plant traits. However the type of callus that has been used by other workers, usually obtained from bulb scales for rapid propagation, is not suitable for selection purposes because of its compact and slow growth. So far, we have not been able to improve this by varying the growth medium. However callus formed unintentionally from certain embryos in an embryo-rescue program did grow much faster and had a more friable appearance. We intend to investigate the stability and regenerating ability of this type of callus, and the feasibility of initiating a protoplast culture from it.

Elimination of lily latent virus, another useful aspect of culture in vitro, could be done quite effective by inducing buds on small bulb scale explants and transplanting these as soon as possible. This would seem much more economic than transplanting apices from whole bulbs.

Descriptors: lilies, *Lilium*, selection in vitro, virus elimination

Induced quantitative variation and selection effects in barley

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No abstract available

Anther culture and haploid regeneration in various lines, crosses and varieties of Brassica campestris L.

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Between genotypes of *Brassica campestris* L., a very large variation occurs in haploid production by anther culture. For practical breeding, it is, however, important that, in anther culture, haploids can be recovered in high frequency from every donor plant.

Altogether 120 crosses, lines and varieties were used for haploid production. The number of anthers excised was about 61 700. In the following examples, the numbers of plantlets were calculated per 1 000 cultured anthers.

Haploid production in commercially available varieties is so far interesting that their agronomic properties are well known. Another point is that plantlets produced by anther culture show extremely large genetic variation even in the most homogenous varieties. The double-zero-varieties DF-15, Candle and Ante produced 0, 2 and 21 plantlets, respectively, and our own lines Jo 3 100, Jo 3 101 and Jo 3089 gave 40, 54 and 13 plantlets. High-glucosinolate varieties Torch (38 plantlets) and Span (31 plantlets) were slightly better than commercial 00 varieties.

It has been postulated that the auxin-like character of glucosinolates could play some role in androgenesis. When donor plants with a high content of glucosinolate in fat-free meal (>30 μmol/g) were compared with plants with a low content (<30 μmol/g), the production of plantlets in anther culture was 66 and 30, respectively. The vitality of donor plants was, however, an important factor and donor plants lacking glucosinolate often suffer selection pressure. Media containing extract from high-glucosinolate seeds did not increase haploid production in donor plants with either high or low glucosinolate content.

When plants derived from anther culture were crossed with each other or with commercial varieties, a very strong change in plantlet production was recorded in F_1. Double-zero variety Candle produced about ten times as many (22) plantlets when crossed with line H1. The cross H11/407 × H5/283A gave an average value of 222 plantlets per donor plant. Six donor plants gave plantlets and ten procuced from 28 plantlets up to 1 176 plantlets per donor plant.

The F_1 hybrid between Torch and Sarson (Torch × Yellow Sarson PJ 173849) produced 1 318 plantlets per 1 000 anthers, being the highest rate. Both are high-glucosinolate types but haploid production in parent lines was not much higher than in other varieties. The hybrid vigour of donor plants will be further studied.

Descriptors: Brassica campestris, anther culture, haploids, glucosinolate content

Methods of cereal breeding used in East Siberia

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The agricultural part of East Siberia has a very extreme climate. Short growing season, early drought, excessive moisture at the end of summer, lack of heat, strongly pronounced zonality and rapid spread of diseases of roots and spikes are factors influencing methods of cereal breeding.

Breeding of spring wheat, barley and oats is carried out in the Agricultural Research Institutes of Krasnojarsk and Burjatsk, in Experiment Stations at Tulun and Khakass. The main method of breeding is intraspecific crossing. Selection in progeny from crosses between local flexible old and modern varieties, and specimens from Povolzhe, Canada, the United States and northern Europe is most effective. Interspecific and intergeneric hybridization is also carried out. Besides simple-pair crosses, three and four-way crosses and backcrosses are used in the breeding programmes. During the last ten years these methods gave rise to highly productive varieties, which are now grown in agricultural practice. These varieties are very early, have a yield potential of 5 000–7 000 kg/ha, good quality and other valuable characteristics.

Mutagenesis has not yet given stable success, but in breeding nurseries there are promising mutant lines, which are being used in crosses. Attempts to grow hybrid embryos between wheat and barley, and barley and rye on artificial medium have failed. More promising results have been obtained with breeding spring triticale. Selection of wheat and barley varieties resistant to loose smut was also successful. Investigations on haploid selection, cell and tissue culture, heterosis breeding and genetic engineering need to be developed further.

Descriptors: cereals, breeding method, interspecific hybridization, East Siberia, triticale

Breeding varieties of oilseedradish and white mustard resistant to sugar-beet nematodes

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Both oilseedradish, a non-bulbing form of *Raphanus sativus,* and white mustard (*Sinapis alba*) are rapid growing and deep rooting green manure crops which are highly appreciated by sugar-beet producers in Germany and the Netherlands. Unfortunately, until recently, these crops could not be used in sugar-beet rotations because they are host of the beet cyst nematode (*Heterodera schachtii*) and a biotype of *Heterodera trifolii.* Apart from its effect on legumes, the latter pathogen also causes damage to sugar beets and related plants and to cruciferous crops. So far this pathogen was only found in the Netherlands. Breeding research to identify resistance and to increase resistance levels against the beet cyst nematodes in oilseedradish, white mustard and *Brassica* crops was initiated at the SVP in 1973 with the development of a mass-screening method. Two methods are presently in use:

- The SVP sand culture method employs a suspension of larvae, prehatched in a $ZnCl_2$ solution, for inoculum. Inoculation is done with a veterinary inoculation gun. The plants are grown in 36 ml pots with moist silversand.
- The Van der Have method employs the cavities in pieces of double wall acrylate panel as pots which are filled with diseased soil.

Both tests are carried out in a growth cabinet at a density of 2500 plants per m^2 at a temperature of 22 °C. By the end of 1977 the methods became operative. Response to selection of oilseedradish and white mustard was good, and high levels of resistance were achieved after several generations of selection.

Field testing was started in 1980 and the resistant plant material did decrease nematode populations (*Heterodera schachtii*) as much as a fallow treatment. The resistant variety Sereno (oilseedradish) of Van der Have was included in the Dutch descriptive list of varieties of field crops in 1982. Other, improved, varieties of oilseedradish have been submitted for inclusion. Submissions for testing and registration of resistant varieties of white mustard have also been made by the firms of Zwaan en de Wiljes and Van der Have.

Descriptors: oilseedradish, *Raphanus sativus,* white mustard, *Sinapis alba,* resistance, *Heterodera,* screening technique

Breeding Asiatic lilies for low light requirement

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For the forcing of Asiatic lilies during the winter period, additional lighting is necessary to prevent flowerbud abscission and flowerbud blasting. Experiments were carried out under controlled climate conditions to determine the minimal amount of light needed for winter flowering of a number of parental lily varieties. The variation between the varieties for this character was considerable. Of the eight varieties which were tested during three successive years under controlled climate conditions and under natural winter light conditions, 'Connecticut King' and 'Enchantment' appeared to be the most light sensitive while 'Uncle Sam', 'Scout', 'Pirate' and 'Tabasco' were the least sensitive varieties.

For the selection of lilies with a low light requirement both induced mutation and seedling populations were tested under winter light conditions in the greenhouse. Out of M_1-mutation populations of 'Enchantment' and 'Connecticut King' 726 plants were grown under winter light conditions; 16 plants which produced more than three flowers per stem were selected.

A diallel analysis of intervarietal crosses with nine varieties resulted in significant general combining abilities (GCA) for the observed characters: bud abortion (bud abscission + bud blasting), forcing time, leaf scorch, number of buds and stem length. 'Enchantment' gave the highest positive GCA for bud abortion indicating the high light sensitivity of the hybrid progenies from this variety, while progenies from 'Uncle Sam' were the least sensitive. Out of the 1874 seedlings which were used for the progenies from diallel analysis, 21 plants with no bud abortion were selected.

Descriptors: lilies, *Lilium*, flowering, low light requirement, mutation breeding

Early generation selection for yield in cereals

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By virtue of lower environmental variability and minimum effects of intergenotypic competition, selection for grain yield may begin as early as the F_2 generation via single plant selection for grains/tiller and 1000-grain weight. In contrast selection for tillers/plant, yield/plant, harvest index or total biomass is likely to be successful only in rows as in the F_3 generation and later (Valentine, 1979; 1981; 1982).

Visual assessment of yield has often been overlooked or misrepresented in discussions of breeding strategies. The efficiency of visual assessment of yield and yield components in spring barley and winter oat rows has been found to be moderately high. Assessments of tillers/row and to a lesser extent 1000-grain weight were most effective in barley, and yield/row and tillers/row most effective in oats. In both cases, assessments of yield were biased towards the most easily assessed characters: tillers/row in barley and grains/row in oats. The value of breeding experience in each crop was demonstrated (Ismail & Valentine, 1983; Valentine & Ismail, 1983).

Yield can also be estimated from unthreshed ear weight, since the proportion of grain to total weight of the ear (in a given environment) is extremely consistent. By counting the grains in single ears, it is also possible to derive an accurate estimate of 1000-grain weight.

An alternative breeding method, referred to as accelerated pedigree selection (APS), bases initial selection on the assessment of families. These are derived from unselected individual plants by accelerated generation procedures. There is less risk of genetic shift than in single seed descent. APS should result in better varieties in less time.

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Descriptors: yield, early generation selection, cereals, visual assessment, accelerated pedigree selection

Seed and forage productivity of lucerne hybrid and inbred plants under competitive conditions

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A study was conducted at Perugia (Italy) in 1981/1982 to evaluate the importance of intraspecific competition in lucerne (*Medicago sativa* L.) and to investigate the consequence of different percentages of inbred seeds on seed and forage yield. Three mixtures with ratios of inbred seed to hybrid seed (S_1/F_1) of 1/9, 1/2.33, 1/1 were sown, seed by seed, in micro-plots (50 cm×30 cm) at a seed rate of 1600/m².

In 1981, data were collected on establishment; green matter yield per plant and dry matter yield per plot at 1st, 2nd and 3rd cut; height and number of stems per plant at the 3rd cut. In 1982, data were collected on green matter yield per plant and dry matter yield per plot at 1st and 3rd cut; seed yield per plant at 2nd cut; height and number of stems per plant at 1st and 3rd cut. Survival (number of plants alive) was recorded in 1981 and 1982 in autumn.

The results can be summarized as follows. (1) Forage yield of S_1 was much lower than that of F_1 plants in each of the three mixtures; the inbreeding effect was higher than that usually shown in space plant trials. (2) Since there were no differences in dry matter yield per plot between mixtures, differences in S_1 seed percentage would not seem important for productivity of lucerne. (3) S_1 plants were selectively eliminated, particularly during the seeding year. One year after sowing, only a few S_1 plants survived. (4) Only 3% of the seed produced in the 2nd year came from S_1 plants. (5) The correlation coefficient between forage yield and seed yield per plant ($r=0.79^{**}$) confirms that selection for forage yield in lucerne seems not incompatible with seed yield selection and can be done under competitive conditions.

Descriptors: lucerne, *Medicago sativa*, competition, inbreeding, seed production, forage production

Interaction between compatible and incompatible pollen in apple and pear

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Using untreated – instead of dead or irradiated – mentor or pioneer pollen to overcome self-incompatibility has the advantage of a much higher fruit set and so may increase the chances to obtain self seeds. To distinguish these seeds, the mentor/pioneer pollen has to be equipped with a marker gene, in our case a gene for red leaf colour. This principle was used to study single and double (24-h interval) pollinations with compatible 'marker' pollen (C) and self-incompatible (S) pollen and the mixtures 1:1 (1C+1S) and 1:9 (1C+9S).

For three apple trials it appeared that the mean PI (= pollination index, expressing seed set per pollinated flower) decreased with increasing amounts of self pollen: from 1.09 for the control C to 0.52 for 1C+1S and 0.08 for 1C+9S (S only: PI = 0). Selfing done 24 h after applying the mixtures increased the PI to 0.66 for (1C+1S)/S and to 0.29 for (1C+9S)/S (PI = 0.89 for C/S). Pollinating twice with these mixtures increased the PIs still further to 1.26 for (1C+1S)/(1C+1S) and 0.49 for (1C+9S)/(1C+9S) (PI = 1.19 for S/C).

Accordingly, an abundance of self pollen usually depressed the outcome, the extent of which depended on the combination in which it arrived on the stigma. For instance, when used as 'pioneer pollen' – as in S/C – the self pollen has even a positive effect. On the whole, it is clear that the compatible and incompatible pollen interact, though the contribution of the latter to seed set was not so large (in most cases less than 10% selfed seeds).

With the aid of 'marker pollen', the contribution of the 1st and 2nd pollen in a double pollination was investigated in relation to the interval between pollinations. At a not too long interval – in relation to temperature – the 2nd pollen was found to produce 2-3 times as many seeds as the 1st pollen. However, the contribution of the 1st and 2nd pollination to seed set was about equal at an interval of 24 h between them when the weather during pollination was warm (>20 °C), at an interval of 48 h when it was moderately warm (16-18 °C) and at an interval of 70 h when it was fairly cold (11-15 °C). This shows again the promoting influence of the first – so called – pioneer pollen, while the advantage to the second obviously decreases when the interval becomes larger and the weather warmer.

The fact that a relatively long interval between pollinations still works, suggests that the first pollen provides a signal, which is related to the growth of the pollen tube.

Descriptors: apple, pear, mentor pollen, self-incompatibility, double pollination

Genetic adaptation of cutroses to low light intensity

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In the year-round rose culture, growers suffer heavy financial losses owing to blind shoots during winter. Blindness in roses is the delayed effect of flower bud abortion in an early stage of shoot development, as a result of low light intensity. Large varietal differences as to blindness occur.

As a basis for a breeding programme, growth and development of hybrid Tea-rose seedlings under controlled light conditions were studied; irradiance varied from 4-24 W/m^2, day length was 8 h, temperature 20 °C. Like in varieties, in seedlings the flower bud could abort. With decreasing irradiance, mortality increased, percentage of flowering seedlings decreased, time to flower increased, plant length at flower decreased, the leaf area decreased. In flowering seedlings leaf number was constant (6); aborting seedlings had fewer leaves with a smaller area.

For the populations studied, genotype-light interactions did not affect the percentage of flowering seedlings. Inheritance of flowering ability under low irradiance was mainly controlled by additive gene action. 'Prominent' and 'Zorina' had a positive, 'Baccara', 'Ilona' and 'Sonia', a negative GCA for flowering under low light intensity (8 W/m^2).

Comparing the flower production in the greenhouse of seedlings flowering or aborting under controlled low irradiance, it was shown that previously flowering seedlings produced more flowers than previously aborting ones. This was due to a lower percentage of blind shoots in winter, in previously flowering plants.

Descriptors: cutroses, flower production, flower-bud abortion, low light intensity, selection

Merits of S_1-family relative to combined S_1-family and test-cross selection in improvement of rye populations

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In hybrid rye, recurrent selection aims to enhance general combining ability to an opposite population. Usually efficiency of selection is judged by rate of gain for equivalent amounts of effort. Effort depends on the reproductive properties of the crop and testing intensity. In self-fertile rye populations, S_0 candidates can be either selfed or selfed and outcrossed to a male-sterile tester to produce seed for the evaluation of S_1 family and combined S_1 and test-cross family, respectively.

In a simulation study, several modifications of the two selection methods were compared. Comparisons were based on equivalent effort and the same inbreeding rate per year. Procedures differed for plot size (observation plots, micro plots and regular yield plots) and cycle length (two and three years). To allow for diverse operational and genetic situations, we considered various levels of effort and characters differing in heritability. The parameters such as genetic variances, genotype-environment interaction variances, correlation between target and selection criterion were varied within a range based on estimates from several trials. By determining the optimum allocation of resources for number of candidates, sites and replications, the maximal genetic gain for each combination of parameters and selection procedure was calculated. These gains served as a criterion for the relative merits of the selection methods and their modifications.

Descriptors: rye, *Secale cereale,* S_1-family selection, test-cross selection, combining ability, simulation

Sperm cells in pollen and pollen tubes of spinach

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The sperm cytology and its structural relationship with the vegetative nucleus in the mature pollen grain and in the growing of pollen tubes in vivo was investigated in *Spinacia oleracea*.

In mature pollen, the sperm cells were elongate long-tailed cells with a nucleus, mitochondria, endoplasmic reticulum, ribosomes and some small vesicles. Plastids and dictyosomes were not observed. The two sperm cells in a pollen grain were directly linked by a short transverse cell wall with plasmodesmata. They were together enclosed by the plasma membrane of the vegetative cell. Reconstruction of many serial sections by electron microscopy revealed consistent occurrence of the sperm cell's vegetative nucleus association in the pollen grains. These associations suggest that these structures were structurally connected at that time.

In the growing pollen tube this association persisted during the first part of the penetration of the female tissues.

What happens to this association and the connected sperm cells when the pollen tubes penetrate the nucellus and when the tube contents are discharged in the degenerated synergid is the following topic of study.

Descriptors: spinach, *Spinacia oleracea*, pollen grain, pollen tube, sperm cell, (ultra)structure

Genotypic differences in relocation of temporarily stored carbon-14 from the stem to the grain in spring wheat

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The object of this study was to determine genotypic differences in the use of soluble carbohydrate reserves in the stem for grain filling.

In a field trial with four spring wheat genotypes, the assimilates of the flag leaf-blade were labelled with carbon-14 at anthesis. Plants were harvested 3, 22 and 55 days (maturity) after labelling. Dry weight was determined for the different plant parts which were then analysed for total non-structural carbohydrates (TNC) and carbon-14 in TNC.

The genotypes showed significant differences in accumulation and depletion of TNC in the stem. At 22 days after anthesis, TNC content in the peduncle averaged 13.6% (range 9.5-16.8) and in the first internode 26.8% (23.0-30.6). At maturity, the peduncle contained 2.0% TNC (1.3-3.5) and the first internode 1.5% (0.9-2.6).

Total carbon-14 in TNC was determined 3 days after labelling. At that time, 78% of it (range 76.6-81.5) was found in the stem. At maturity, 33% was found in grains. This proportion varied from 27 to 44%, indicating genotypic differences in relocation of temporarily stored carbohydrates from the stem to grains.

Descriptors: wheat, *Triticum aestivum*, relocation of carbohydrates, stem, grain

Simulation studies on factors in pedigree breeding

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Pedigree breeding was simulated for five sizes of F_2 population (5000, 1000, 500, 200 and 100), four levels of environmental variance resulting in heritability (h^2) for the F_2 of 0.05, 0.2, 0.5 and 0.9 in six dominance types (complete and partial recessive, no dominance, partial and complete dominance and overdominance) and three recombination values between adjacent loci (1/2, 1/8 and 1/32). The aim was to study the influence of these factors on response to selection. Twenty loci of equal effect were assumed to control the character.

From the results of 10 runs, we found a steady increase of genotypic means in response to selection for completely recessive to partially dominant types. The resulting genotypes were homozygous for all loci. For complete dominance and overdominance, the response to selection was erratic and genotypes were still heterozygous after 10 generations of selfing. This was more evident for high heritability or tight linkage. A population size of 100 to 200 F_2 plants may be large enough, if the aim is to look for recombinants that retain 80-85 % of the desirable genes from the F_1 generation.

Descriptors: pedigree breeding, simulation, population size, genetic gain, type of dominance, linkage

Plant regeneration from protoplast-derived callus of winged bean

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Protoplasts were isolated from one-week-old suspension cultures of winged bean (*Psophocarpus tetragonolobus*) with an enzyme mixture of cellulysin 50 g/l, macerase 10 g/l, sorbitol 0.5 mol/l and $CaCl_2$ 0.1 mol/l at pH 5.5. Protoplasts were then cultured in modified B5 (Gamborg B5) medium.

Cell division occurred within 3-4 days in protoplast populations of $150\text{-}300 \times 10^6$/l. Cell colonies formed callus when transferred to MS (Murashige & Skoog) medium containing sucrose 30 g/l and the growth regulators naphthaleneacetic acid (NAA) and 6-benzylamino purine (BA). Regeneration of plantlets was achieved by manipulating the various combinations of the growth regulators. The present suggest that winged bean can be a model system for investigation genetic modification in legumes.

Descriptors: winged bean, *Psophocarpus tetragonolobus*, protoplast culture, plant regeneration

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Index of descriptors