The production and evaluation of monohaploid potatoes
($2n = \times = 12$)
for breeding research on cell and plant level

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Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C. C. Oosterlee,
in het openbaar te verdedigen
op maandag 14 september 1987
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

STELLINGEN

1. De mening van Ross (1986) als zou zijn aangetoond, dat voor monohaploiden-inductie gynogenese veel minder effectief zou zijn dan androgenese, berust op onvolledige informatie.
   H. Ross, 1986. Potato Breeding; Problems and Perspectives. Verlag Paul Parey, p. 47

2. De voortschrijdende genetische-manipulatietechnieken vereisen weliswaar een uitbreiding van de reeds bestaande biologische terminologie, maar ook een vergrote waakzaamheid ten aanzien van het correcte gebruik van bestaande termen.

3. Zolang nog niet is bewezen dat somaclonale variatie fundamenteel verschilt van variatie, verkregen via traditionele methoden van mutatie-inductie, ligt het belang ervan voornamelijk in de gelegenheid die het biedt tot grootschalige selectie in vitro, in een volledig controleerbaar milieu.

4. Het waarnemen van callusontwikkeling vanuit protoplasten, rechtvaardigt niet het gebruik van de term regeneratie.

5. De reacties op de presentatie van de 'Banoni' getuigen zowel van de hoge verwachtingen die het publiek heeft van biotechnologie, alsook van de sterke invloed van de media en van de met academische titels beladen wetenschappers op deze verwachtingen.
   Televisiepresentatie 'Banoni', AVRO's Service Salon, maart 1987

6. De jarenlange discussies bij universiteiten en instituten over betere prestaties met minder middelen hebben de overheid al een groot deel van het krimpprobleem uit handen genomen door het vrijwillige vertrek van vele goede medewerkers naar het bedrijfsleven.

7. Het opnemen van een onderwijsverplichting in het takenpakket van een promotie-onderzoeker betekent een verkorting van de beschikbare onderzoekstijd en een extra aanslag op stressbestendigheid.

8. Voor het goed kunnen functioneren binnen een koor is het letten op de dirigent belangrijker dan het kunnen lezen van noten.

9. De aanleg van de nieuwe rijksweg door Amelisweerd is een voorbeeld van een eigentijdse methode om de mens dichter bij de natuur te krijgen.

Wageningen, 14 september 1987. Bert A. Uijtewaal

"False facts are highly injurious to the progress of science for they often endure long; but false views, if supported by some evidence do little harm, for everyone takes a salutary pleasure in proving their falseness, and when this is done, one path towards error is closed and the road to truth is often at the same time opened."

Charles Darwin (1871)
"The Descent of Man"
Ch. XXI, p. 852.

Aan: Liesbeth
Mijn ouders

Dit proefschrift is tot stand gekomen op de vakgroep Plantenveredeling (IvP) van de Landbouwuniversiteit Wageningen.
Het hierin beschreven onderzoek maakte deel uit van het onderzoeksprogramma van de Stichting voor Biologisch Onderzoek in Nederland (BION), als onderdeel van de Nederlandse Organisatie voor Zuiver wetenschappelijk Onderzoek (ZWO) en werd mogelijk gemaakt door financiële steun van de Stichting voor Technische Wetenschappen (STW).

De tekening op de omslag, gemaakt door Annet Posthumus, stelt een simplistische weergave van het onderzoek voor. De verschillende onderdelen hierin zijn gepresenteerd bij de aanhef van de desbetreffende hoofdstukken.

Dankwoord

Gaarne wil ik van de gelegenheid gebruik maken een dankwoord te richten tot een ieder die direkt of indirekt bijgedragen heeft aan de totstandkoming van dit proefschrift.
Alle medewerkers van de vakgroep Plantenveredeling, zowel wetenschappelijk, technisch als administratief, dank ik voor de prettige werksfeer. Een aantal mensen wil ik met name noemen.
Beginnende bij het opzetten van het onderzoekslaboratorium, denk ik met name aan Ton Florissen, de te vroeg overleden Ben Navest, Henk van de Scheur en Henk Sengers, die met enthousiasme veel praktische klussen hebben uitgevoerd.
Velen dank ik ook voor de hulp die zij geboden hebben in de periode dat het weefselkweeklaboratorium nog niet klaar was: het IVT, de vakgroep Tuinbouwplantenteelt en het Ital voor het tijdelijk onderkomen voor het weefselkweekmateriaal en Zachy Sawor voor zijn vrolijk optimisme in deze niet altijd makkelijke periode.
Tijdens het onderzoek heb ik voor het kaswerk steeds kunnen terugvallen op de praktische steun van Dirk Jan Huigen, Jan Rijksen en Joop Verdenius, waarbij de eerste zich steeds nauw bij het onderzoek betrokken toonde. Voor het laboratoriumwerk buiten de weefselkweek kon ik rekenen op zowel technische als praktische steun van met name Dr. Ramanna op cytologisch gebied en Jan Molenveld en Luuk Suurs op electroforetisch gebied. Voor het weefselkweekwerk heb ik de laatste twee jaren op vaste assistentie kunnen steunen. Jotien Falkena en Eelke Posthumus, beiden voor één jaar, hebben mij zeer kundig ontlast van vele praktische beslommeringen.
Gerrie van Eck en Annie Marchal wil ik danken voor het ter beschikking stellen van de onmisbaar gebleken tekstverwerker.
Mijn dank gaat ook uit naar de talrijke studenten die in meer of mindere mate tot de resultaten van dit onderzoek hebben bijgedragen: Theo Jacobs en Wiel Mattheij; beiden mede-pioniers in de rommelige beginperiode van het nieuwe lab., waren samen met Hedwig Boerrigter, Frans Tetteroo en Nico de Waard mede verantwoordelijk voor de reputatie die 'de Terp-clan' snel had opgebouwd betreffende de goede werksfeer, de sterke saamhorigheid en wellicht de opvallende luidruchtigheid. Ook daarna zorgden in chronologische volgorde Jan Joordens, Hans Dessens, Peter Goudappel, Piet van Gisbergen, Tonny van de Boom, Frans Bonnier, Herman van Eck en Peter Kolster voor een drukke maar altijd motiverende werkperiode.
Ik ben ook erg dankbaar voor de hulp die ik vanuit andere onderzoeksgroepen heb gekregen. Hierbij geldt het gehele Ital voor de prettige samenwerking, waarbij ik met name wil noemen Trudie Bokelmann voor haar praktische tips op protoplasten gebied en Wil ten Broeke voor zijn

onmisbare handigheid bij het gebruik van de doorstroomcytometer. Ook dank ik de vakgroep Cel- en Plantengenetica van de Rijksuniversiteit Groningen waarbij ik met name Laas Pijnacker en Margriet Ferwerda dank voor hun steun op cytologisch gebied.
Evert Jacobsen, mede werkzaam bij de vakgroep Cel- en Plantengenetica in Groningen, zal ik mij blijven herinneren door zijn onuitputbaar lijkende energie om, met zijn soms vernietigend lijkende, maar altijd opbouwend gebleken kritiek, steeds weer tegen mijn manuscripten 'aan te trappen' zoals hij het placht uit te drukken. Evert, voor je hulp in de vele vrije uren die jij voor mij beschikbaar stelde om op papier of via discussies problemen te bespreken ben ik je heel erg dankbaar.
Prof. Hermsen, onze verstandhouding tijdens het werk heb ik wel eens vergeleken met die zoals wij die op donderdagavond kenden als zanger en dirigent. Ik moest het zelf klaren en kreeg daarbij veel vrijheid zowel bij wat ik deed als hoe ik het deed, maar als een bepaalde stap in het onderzoek niet paste in het totale 'repertoire', als een stuk niet af of een bepaalde toon niet zuiver was greep u gebiedend doch persistent in, wat vaak resulteerde in een discussie. Ik heb uw rust en overzicht tijdens uw werkzaamheden zeer bewonderd en wil u bedanken dat u tijdens deze drukke werkzaamheden altijd tijd vond om mijn werk te dirigeren.
Bovenal wil ik echter mijn ouders bedanken voor de mogelijkheid die zij mij geboden hebben om te gaan studeren en voor de ondersteuning bij het werken aan dit proefschrift.

Contents

Abstract

The use of monohaploid potato clones in research in theory has many potential advantages in comparison with the use of heterozygous di- and tetraploids. Because in monohaploids each chromosome is single, recessive as well as dominant alleles are detectable and can be selected for. In addition, monohaploids offer a unique opportunity to produce homozygous clones. The aim of the study presented in this thesis was to determine which are the pros and cons of using monohaploids in practical breeding and fundamental research, and which are the consequences of monoploidy and homozygosity in a normally highly heterozygous crop such as potato.

Large-scale production of monohaploids appeared to be possible by prickle pollination, but turned out to be genotype-specific. It was shown that the ability for monohaploid production can be transferred to the progeny by crossing. During the investigation reported herein more than 500 monohaploids have been produced that could be used for further research.

In order to combine different selected monohaploid genotypes that will warrant high-level heterozygosity, methods for protoplast isolation, culture and fusion have been adjusted to the material used. Several monohaploid genotypes could be regenerated from protoplasts to plants but always the ploidy level raised to 2x or even 4x. After protoplast fusion, hybrid fusion products could readily be selected because of a differential regeneration capacity. By using isozyme markers for detection of hybridity the first regenerants after protoplast fusion were shown to be hybrids. A difference of at least four weeks in regeneration time was found between the first heterozygous (hybrids) and the first homozygous (parental) genotypes. Some regenerants were triploid. This indicates that at least one monohaploid protoplast was involved in fusion. The majority however was tetraploid and this was probably the result of somatically doubling x+x fusion products. Only in some genotype combinations also increased callus growth rates were found.

The plant vigour in homo- and heterozygotes suggests that dominance effects are stronger than additive gene effects. Owing to sterility problems and a relatively bad plant performance, homozygous and there-

fore also indirectly monohaploid potato clones are of little importance for practical breeding. However, for fundamental research, monohaploids are very useful: (i) they can be produced on a large scale from specific diploid lines, (ii) they can be maintained in vitro on the monohaploid level for at least 2-3 years via shoot tip propagation, and (iii) protoplast isolation and fusion, and plant regeneration from protoplast derived calli has proven to be possible.

Chapter 1.

General Introduction

Tetraploid level

Conventional potato breeding is a system of selecting in vegetative progenies of F_1 populations from crosses between tetraploid breeding clones (2n=4x=48; see framework 'Applied rules of nomenclature'). Five to 10 years of successive selection can result in new improved varieties. As soon as a clone has been identified as being superior to existing varieties in one or more characters, it may be registered as a new variety.

Applied rules of nomenclature

2n = chromosome number in diplophase (sporophyte, somatic cells)
n = " " in haplophase (gametophyte, gametal cells)
x = basic chromosome number

Tetraploid: 2n=4x=48 chromosomes
Diploid: 2n=2x=24 chromosomes
Monoploid: 2n= x=12 chromosomes
Dihaploid: haploid from a tetraploid, hence 2n=2x=24
Monohaploid: " " a diploid or a dihaploid, hence 2n=x=12

Intercrossing[1] di(ha)ploids gives rise to diploids (2x.2x=2x)
Fusion[2] of di(ha)ploids gives rise to tetraploids (2x+2x=4x)
Fusion[2] of mono(ha)ploids gives rise to diploids (x+x=2x)
1) Crossing = fusion of gametal cells, the natural fertilization process
2) Fusion = fusion of somatic cells, an *in vitro* process

Chromosome doubling through colchicine treatment or explant culture:
- di(ha)ploid → tetraploid
- mono(ha)ploid → homozygous diploid (DM=doubled monohaploid)
- homozygous diploid → homozygous tetraploid (DDM=doubly doubled monohaploid)

However, this way of potato breeding has many disadvantages.
The outcome of crosses between tetraploid parental lines is often unpredictable. Since the parents are highly heterozygous, the F_1 usually shows a large genetic variation for a lot of characteristics. Genotypes superior for a sufficient number of desired characters are extremely rare in such progenies; the more so, because the inheritance of most characters is unknown or unclear. Better knowledge of the genetics of the potato and of general and specific combining abilities of parental genotypes would be very helpful in making the selection process less laborious. In conventional potato breeding a lot of crosses between "promising" breeding lines are made to select and propagate many descendants per cross. The increasing number of characters to be taken into account diminishes the chance of finding a new variety which will improve the existing assortment. In the fifties the screening of 15,000 seedlings was sufficient to get a new variety introduced into the recommended list of varieties; at present this number is over 150,000 according to Pavek (1987). This may still increase in the years ahead, unless selection methods are improved, especially in the seedling and young clonal generations, in which under the present approach many valuable genotypes remain undetected and thus are eliminated by mistake.

Another alternative to present-day potato breeding is to switch partly from breeding at the tetraploid level to that at the diploid level, together with basic research in diploids.

Diploid level

Already in the thirties Ivanovskaja (1939) incidentally found dihaploids (2n=2x=24) among the progeny of a cross between tetraploid Solanum tuberosum L. and diploid S. phureja Juz. et Buk. However, not until 1958 systematic production of dihaploids was initiated (Hougas & Peloquin, 1958). In 1963 Chase proposed an "analytic breeding" scheme to breed potato at the diploid level. The scheme consisted of three stages: haploidization of tetraploids, breeding with diploids and finally restoring the tetraploid level. Breeding at the diploid level has potential advantages over that at the tetraploid level:

a) Improvement of breeding efficiency and shortening the time needed for breeding a new variety (von Wangenheim, 1962; Rowe, 1967; Haynes, 1972; Howard, 1978; Iwanaga, 1982).
b) Elimination of deleterious alleles.
c) Determination of the breeding value of tetraploids through analysis of their gametic samples (=dihaploids).
d) Facilitating genetic and basic breeding research.
e) More efficient introduction of desired characters from diploid wild and primitive species into diploid breeding programmes.
Mechanisms of 2n gamete formation are available for restoring the tetraploid level of selected superior diploids leaving their genotypes largely intact (Mendiburu et al., 1974; Hermsen et al., 1985).
Progress in the development of somatic hybridization techniques may result in the intact combination of two selected diploid genotypes.

An extensive review concerning the use of di(ha)ploids in an analytic breeding scheme is recently given by Ross (1986).

IN VITRO POTATO RESEARCH

In vitro propagation

In vitro techniques are being used in potato breeding for rapid propagation of advanced material and for healthy maintenance of germplasm. Many extensive reviews about in vitro potato culture have been published (Mellor & Stace-Smith, 1977; Miller & Lipschutz, 1983; Ross, 1986; Wang & Hu, 1986). In this introduction the developments in in vitro potato research, relevant to this thesis will be summarized.
Different techniques have been developed for the rapid propagation of potatoes as e.g. i) meristem, ii) shoot tip and nodal segment cultures and iii) the production of minitubers.
i) in vitro meristem culture, sometimes combined with temperature or antiviral chemical treatments, is the main method of eliminating viral infections from systemically infected potato cultivars without inducing undesired genetic changes (Wang & Hu, 1980). Cultured meristems are also the preferred material for cryopreservation of germplasm (Kartha 1981, 1982).

The explant used may be composed of the apical dome only, or of the apical meristem along with one to four subjacent young leaf primordia of the subapical meristematic section. In general, it is true that the larger the explant, the more likely it grows and produces whole plants. Yet, if viral elimination is the main concern, the size of the excised meristem should be as small as possible. An extensive potato meristem culture procedure has been published by Dodds and Roberts (1982).

ii) Shoot tip and nodal segment culture are mainly used for rapid in vitro shoot multiplication. Cultures can be obtained from in vitro derived shoots, greenhouse grown plants, or tuber sprouts. In vitro shoot tip and nodal segment cultures of disease tested potato material provide a rapid rate of clonal multiplication which is essentially shielded from reinfection. The multiplication rate generally varies among cultivars (Goodwin, 1980a,b).

iii) A method for mass in vitro tuberization for potato propagation was developed as an alternative to the laborious step of transplanting tender vegetative plantlets from in vitro conditions into the soil (Wang, 1978; Wang & Hu, 1982). This procedure has been adopted by the International Potato Center (CIP) for germplasm storage and international distribution for commercial field planting.

With proper induction, every axil of in vitro grown shoots can produce a microtuber. Except for their size (usually 1cm or less), these in vitro tubers are morphologically identical to in situ produced ones.

Explant, callus and cell suspension cultures

The first successful establishment of potato callus was reported by Steward and Caplin in 1951 and actively growing calli were produced. However, much later the regeneration of plants from callus of internodal segments (Wang & Huang, 1975), tuber discs (Lam, 1975) and rachises of compound leaves (Roest & Bokelmann, 1976) has been described.

Suspension cultures can be generated readily from friable callus cultures (Bajaj & Dionne, 1967; Austin & Northcote, 1973; Lam, 1977). Plantlets may be regenerated from single, isolated suspension cells (Lam, 1977) and from cell suspension derived protoplasts (Wenzel et al., 1979), but the regeneration frequency and ploidy of the regene-

rated plants appeared to be dependent, at least in part, on the age of the cell culture (De Vries & Bokelmann, 1985). Plants regenerated from cultured leaf explants or cell suspensions may display a tremendous variation (van Harten *et al.*, 1981; Austin & Cassels, 1983; Cassels *et al.*, 1983; Sree Ramulu *et al.*, 1984; Wheeler *et al.*, 1985). Somaclonal variation of regenerants is strongly dependent on the genotype and the length of time in callus stage (Sree Ramulu, 1986).

Ploidy instability of explant cultures may also be an advantage: *in vitro* mitotic chromosome doubling may provide an alternative to *in vivo* colchicine doubling (Jacobsen, 1981; Wenzel *et al.*, 1979, 1982).

Regeneration of virus-free plantlets from explant cultures (Wang & Huang, 1975; Wang, 1977) may also serve as an adjunct to the more traditional meristem tip culture methods used for virus elimination from potato (see reviews Mellor & Stace-Smith, 1977; Wang & Hu, 1980).

The main potential of the use of explants, calli and cell suspension cultures is the *in vitro* selection for mutant characters. In this way Behnke (1980a,b) succeeded in isolating plants with greater quantitative resistance to fungal pathogens. Van Swaay *et al.* (1985) reported the isolation of plants with an increased frost tolerance from cell suspensions. The induction and selection, at the University of Groningen, of a waxy potato mutant with starch consisting of amylopectine only , has opened new perspectives for the starch industry (Hovenkamp *et al.*, Subm.).

The value of somaclonal variation for breeding research will not be discussed here extensively. Up to now no striking results have been obtained indicating that somaclonal variation is fundamentally different from variation that occurs *in vivo*. Sanford *et al.* (1984) mentioned for Russet Burbank that at least part of the somaclonal variation obtained also occurs *in vivo*. The opportunity of selection for all kinds of variation *in vitro* is, more than somaclonal variation itself, one of the promising aspects in potato research.

In potato, in contrast to *Nicotiana tabacum* and *N. plumbaginifolia*, so far little progress has been made with the isolation of marker lines necessary for *in vitro* genetic complementation studies and large scale selection of hybrid calli after protoplast fusion. Only recently Jacobsen (1986) reported the regeneration of plants resistant to high con-

centrations of S-(2-aminoethyl) cysteine (AEC), an analogue of lysine, from protoplasts isolated from AEC-1-resistant calli.

Protoplast culture

Potato protoplasts were first isolated in the early 70's from S. tuberosum tubers (Lorenzini, 1973; Tomiyama et al., 1974 cit. Miller & Lipschutz, 1983) but no cell divisions were observed. Dunwell and Sunderland (1973) succeeded in regenerating plants from potato protoplasts. Since then protoplast isolation, culture and regeneration have been achieved for numerous tetraploid cultivars (Shepard & Totten, 1977; Butenko & Kuchko, 1978; Shepard, 1980; Gunn & Shepard, 1981; Thomas, 1981; Shepard, 1982; Karp et al., 1982; Bokelmann & Roest, 1983) and di(ha)ploid clones (Binding et al., 1978; Wenzel et al., 1979). Shoot formation on callus obtained from protoplasts from a monohaploid potato clone has been reported only once (Roest & Bokelmann, 1983) as far as the author is aware.

Most groups of research workers published their own protocol for protoplast isolation, culture and plant regeneration. To be successful, attention has to be paid to numerous factors, particularly (a) the source plant culture conditions (b) composition of the protoplast isolation and culture media and (c) protoplast culture conditions, i.e. temperature and illumination.

Somatic hybridization

Progress in the area of somatic hybridization within S. tuberosum L. has been hampered by lack of suitable selection markers. Wenzel et al. (1982) did not find one single somatic hybrid among 2000 plants regenerated after protoplast fusion between two dihaploid S. tuberosum clones. Regenerated calli were preselected on the basis of increased vigour, a characteristic of hybrid fusion products between different Datura species (Schieder & Krumbiegel, 1980). However, somatic hybrid cells of heterozygous diploid potato clones were apparently not more vigorous than cells of the parental clones (Wenzel et al., 1982).

The use of dominant mutations (De Vries et al., 1987), or the mechanical isolation of fusion products (Puite et al., 1986) may overcome these selection difficulties. The identification of individual chromo-

somes (Puite et al., 1986) and the use of phenotypically distinguishable fusion partners (morphologically or by isozyme banding patterns) may be a good help for the detection of hybrids after plant regeneration.

S. tuberosum L. has proven to be amenable to somatic hybridization with other Solanum species such as S. chacoense Bitt. (Butenko & Kuchko, 1980), S. nigrum L. (Binding et al., 1982) and S. brevidens Phil. (Austin et al., 1985) or other solanaceous genera such as Lycopersicon esculentum Mill. (Melchers, 1978, 1980) or Nicotiana tabacum L. (Skarzhynskaya et al., 1982, cit. Gleba & Evans, 1983).

In contrast to research with tetraploid and diploid potatoes no report has been made concerning fusion between monohaploid potato protoplasts.

Anther culture

Unlike many solanaceous species, the cultivated potato has not responded well to anther culture. Dihaploid plants have been extracted from tetraploid cultivars (Dunwell & Sunderland, 1973; Johansson, 1986) and monohaploids from di(ha)ploid clones (Foroughi-Wehr et al., 1977; Sopory et al., 1978; Wenzel et al., 1979), but the frequency is generally low.

Several factors have shown to be of critical importance: (a) the composition of the culture medium (Sopory, 1979; Wenzel & Uhrig, 1981; Johansson, 1986), (b) the stage of development of the cultured microspores (Sopory et al., 1978; Dunwell & Sunderland, 1973; Weatherhead & Henshaw, 1979) and especially (c) the genotype of the donor plant (Dunwell & Sunderland, 1973; Foroughi-Wehr et al., 1977; Jacobsen & Sopory, 1978; Wenzel et al., 1979; Wenzel & Uhrig, 1981; rev. Maheshwari et al., 1982).

The failure of some genotypes to produce viable embryos in anther culture may be caused by recessive lethal genes (Wenzel, 1980). Wenzel and Uhrig (1981) suggested that the differences between genotypes may also result from differences in sensitivity to components of the culture medium, especially the growth regulators. They made several crosses between genotypes that responded well in anther culture and genotypes that responded poorly. The percentage of good response in the F_1's differed for each cross. In two F_1's the authors could isolate

several clones that produced many plants in anther culture. Yet, in later experiments more than 99% of the regenerated plants appeared to be di- or tetraploids (Uhrig, 1985) originating from unreduced gametes, sporofytic tissue or mitotically doubled monohaploid cells. This implies a lot of screening work, which again is hampered by the lack of useful parental markers.

POTENTIALS OF MONOHAPLOIDS

Baerecke and Frandsen (cit. Frandsen, 1968) came across the first monohaploid potato clone after pollinating a dihaploid with S. phureja Juz. et Buk. Later on, monohaploids were induced systematically both via gynogenesis (van Breukelen et al., 1975, 1977) and androgenesis (Sopory, et al., 1978; Jacobsen, 1978; Binding et al., 1978; Sopory & Tan, 1979). This created new potentials for fundamental potato breeding research since monohaploids possess a number of advantages over heterozygous di- and tetraploids:

a) Since each chromosome is single, all genetic alterations, both recessive and dominant, will become visible immediately. Therefore monohaploids are of great potential value for the induction and isolation of both recessive and dominant mutations.

b) Since recessive alleles can not remain hidden behind dominant ones anymore, deleterious recessives will automatically be eliminated in monohaploids.

c) Through mitotic doubling of monohaploids, homozygous di- and tetraploids can be obtained.

d) Crossing homozygous di- and tetraploids, obtained from the same monohaploid, can be the first step in the production of a homozygous trisomic series.

e) Homozygous parental clones may produce uniform hybrid offspring.

f) Series of monohaploids, together with the diploid source material, are ideal tester material for genetic analysis, e.g. of isozyme markers.

The potentials of monohaploids, together with the improved monohaploid induction technique and the opportunities offered by somatic cell

genetics, enabled the development of an analytic synthetic breeding scheme as presented in Fig. 1 (after Wenzel et al., 1979; slightly modified).

This hypothetic breeding scheme shows that with routine induction of monohaploids, a tetraploid clone may be produced containing all four desired characters that were scattered in the source material.

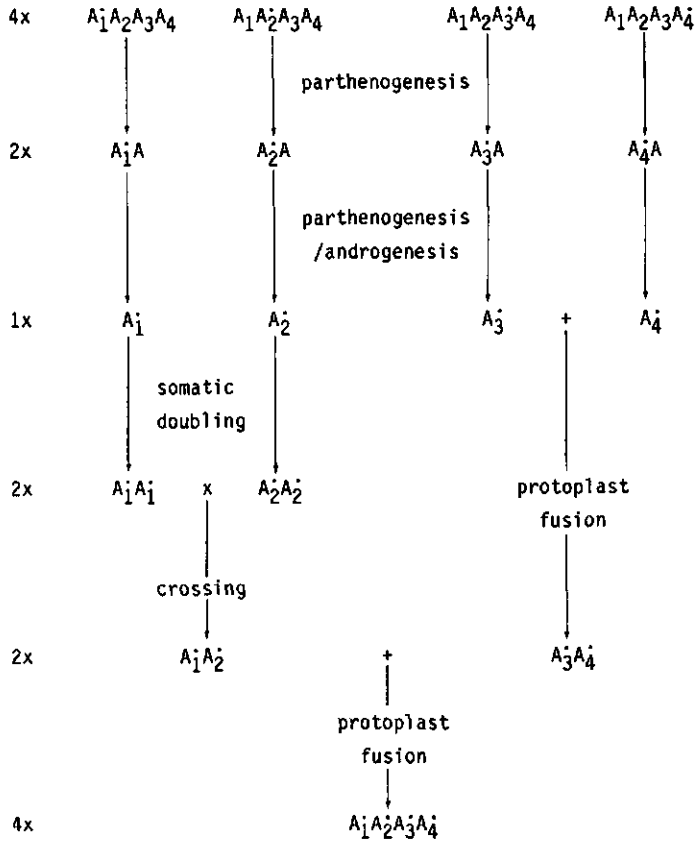

Fig.1. Analytic synthetic breeding scheme. In this scheme selection for monohaploids carrying major genes for resistance '•' to four different pathogens is combined with polyploidization via somatic doubling and subsequent crossing, and via protoplast fusion (Wenzel et al., 1979, slightly modified).

The investigations on monohaploid potato described here were started i) to test the feasibility of such a scheme and ii) to study the effects of gene dosage and heterozygosity on the performance of a potato clone. Research was focussed at:

1) Large-scale gynogenetic production of monohaploids from many different diploids (chapter 2).

2) Fusion of protoplasts from monohaploid clones and the subsequent detection of hybrid regenerants (chapter 3).

3) Stability in ploidy level of the monohaploids during axillary bud multiplication (chapter 4).

4) Screening of monohaploid clones for *in vitro* growth and the ability of protoplast isolation, culture and plant regeneration (chapter 5).

5) Comparison of growth characteristics of monohaploids with the diploid parent clone and with the mitotically doubled homozygous diploids and tetraploids (chapter 6).

Chapter 2.

PRODUCTION OF POTATO MONOHAPLOIDS (2n=x=12) THROUGH PRICKLE POLLINATION

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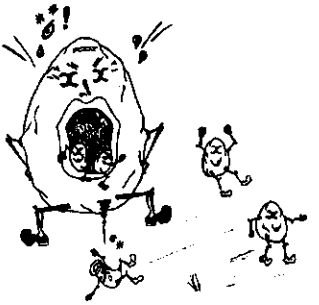

Summary

Data are presented on the potential of gynogenesis for the production of monohaploids and on factors affecting their frequency and relative vigour. Diploid Solanum tuberosum L. and S. tuberosum x S. phureja Juz et Buk hybrids were used as maternal parents and selected S. phureja clones as prickle pollinators with embryo-spot and nodal band as dominant seed and plant marker.

About 2 million seeds were screened for absence of embryo-spot. After raising plants from phenotypically spotless seeds further screening for absence of nodal bands and for ploidy level was carried out. Finally more than 500 monohaploid plants from three genetically different groups of maternal parents were obtained.

Frequency and vigour of the monohaploids were clearly dependent on their maternal genotypes. The data also indicated an effect of the pollinator genotype, the physiological stage of the maternal plant and the environment on monohaploid frequency.

On the basis of these results the possibility of breeding for a higher monohaploid production rate and for more stable and vigorous monohaploids is discussed. Furthermore, gynogenesis and androgenesis are compared. It is suggested that both should be used in order to obtain monohaploids from sufficiently various diploid breeding material.

Introduction

The first successful experiments for the specific purpose of producing monohaploid potato plants (2n=x=12) took place in the early seventies. Van Breukelen et al. (1975, 1977) used prickle pollination (gynogenesis) and Irikura (1975) and Foroughi-Wehr et al. (1977) successfully tried anther culture (androgenesis).

Owing to their high level of heterozygosity, autotetraploid potato cultivars may comprise deleterious alleles at many loci without any harmful effects on growth and performance. On the other hand, such alleles are fully expressed in monohaploid plants. The same holds true for mutant alleles, whether dominant or recessive. If monohaploids survive, they not only are free from deleterious genes, but also have

a harmonious combination of genes. Hence selection for viable and relatively vigorous monohaploids implies selection for exceptional, excellent gene combinations.

It is only through identical doubling of monohaploid genomes that homozygous diploid or polyploid potatoes can be produced. Such homozygotes have special potentialities for potato breeding and research as pointed out by Van Breukelen et al. (1975).

After the pioneering work in the early seventies, anther culture has been studied most extensively as to its potential for the production of monohaploids, mainly at the Max Planck Institute for Breeding Research at Cologne, W. Germany (Sopory, 1977; Jacobsen, 1978; Binding et al., 1978; Sopory & Tan, 1979; Uhrig, 1983, 1985; Wenzel & Uhrig, 1981; Wenzel et al., 1979, 1981, 1982). This led Ross (1986) to the premature conclusion that "gynogenesis, as compared with androgenesis, is much less effective". In the present paper it will be attempted to restore the balance by presenting data on the potential of gynogenesis for monohaploid production and to discuss some factors affecting the frequency of gynogenetic monohaploids and to examine the merits and limitations of both gynogenesis and androgenesis.

Materials and Methods

The diploid plant material taken for the production of monohaploids derives from the species Solanum tuberosum L. and S. phureja Juz. et Buk. Three independent (groups of) diploid clones are included in this study:

(i) M9.

M9 is a diploid hybrid. Its ancestry is presented in Fig 1.

The US-W clones (5293-3 and 7589-2) were produced and selected by Dr. S.J. Peloquin and his coworkers in Wisconsin, USA and kindly provided via Dr. H. de Jong, Canada. These clones were intercrossed and M9 selected by the junior author in 1981.

(ii) PS10.

PS10 is an F_1-hybrid from the cross S. phureja x diploid S. tuberosum. It was selected by the senior author for its monohaploid producing ability via gynogenesis. The S. phureja parent was bred by Dr. B. Maris for adaptation to long day conditions; the diploid S. tuberosum parent

was bred by Ir. N. van Suchtelen from pure S. tuberosum dihaploids. Both parental clones were kindly provided by the Foundation for Agricultural Plant Breeding, Wageningen.

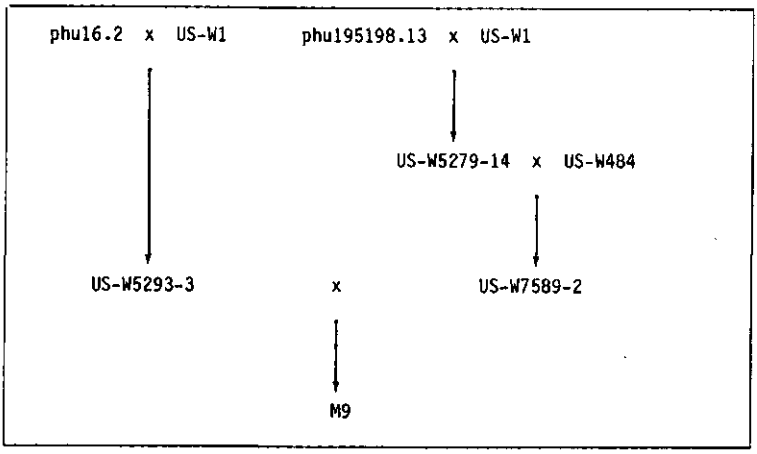

Fig 1. Ancestry of the diploid hybrid clone M9. phu = S. phureja; US-W1 and 484 are dihaploids from cv. Katahdin and Merrimack respectively.

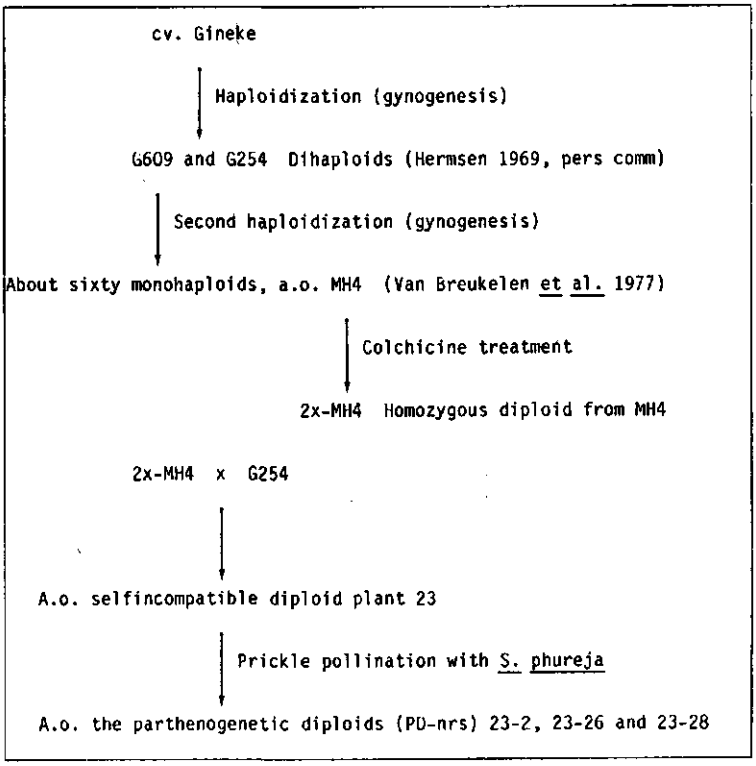

Fig 2. Origin of three parthenogenetic diploid clones (PD-nrs).

(iii) PD23-2, PD23-26, PD23-28.
These parthenogenetic diploid (PD) clones derive from cv. Gineke according to the scheme in Fig 2.

In addition to the three independent (groups of) clones, the next F_1 hybrid populations were included:
PS10xM9 (9 genotypes) and M9xGPG (11 genotypes).
GPG is the code for F_1-plants from spotless seeds of the backcross (G609xIvP48)xG609. G609 is a highly fertile dihaploid from cv. Gineke with a high monohaploid producing ability (Van Breukelen et al., 1975). IvP48 is a S. phureja clone selected by Hermsen & Verdenius (1973) for its superior dihaploid inducing ability and its homozygosity for embryo-spot.

The experiments were carried out during the period 1982-1985. All plants were grafted onto tomato rootstocks and grown in an air-conditioned greenhouse at high relative humidity.
The pollinators for monohaploid induction via gynogenesis were the S. phureja selections IvP35 and IvP48 (Hermsen & Verdenius, 1973) and a recently selected improved pollinator IvP101 (Hermsen, in prep.). All three pollinators are homozygous for the dominant seed marker embryo-spot, which is indispensable for selecting monohaploids via gynogenesis.
The selection procedure of monohaploids was as follows. From the bulk of seeds obtained from crosses between diploids and pollinators, spotless seeds were selected and sown in flats. After emergence the plants with "nodal bands" (controlled by the same genes as embryo-spot) were removed. In non-hybrid seedlings the ploidy level was first estimated by counting the number of chloroplasts in the guard cells of the stomata according to Frandsen (1968); later the number of chromosomes was counted in root tip cells according to Henderson & Lu (1968).
All monohaploid lines were maintained in vitro in shoot tip cultures on MS-medium (Murashige & Skoog 1962) with 20g sucrose/l and 20g mannitol/l at 10°C and low light intensity. In this way monohaploid lines could be maintained from 1983 till now without the occurrence of spontaneous doubling. Maintenance via tubers was often not possible since

most lines failed to produce them.
Monohaploid plants were screened for vigour in the greenhouse and under in vitro conditions on MS-medium with 20g sucrose/l. As vigour strongly depends on environmental conditions, the data for vigour can only serve as a measure for the relative vigour of the monohaploids in a given environment.

Results

About 2 million seeds have been screened for absence of embryo-spot. Seeds with no clear embryo-spot were also classified as "spotless". About 33,000 spotless seeds were selected. Of these only 75% germinated, probably because the seeds containing a partly developed embryo or no embryo at all were unavoidably classified as spotless. About 19,000 seedlings could easily be recognized as hybrids owing to the presence of nodal bands. In addition to 1,500 lethals, 4,000 diploids and a few tetraploids , more than 500 monohaploid plants were obtained from the 6,000 parthenogenetically developed seedlings. From the five parental lines that have been pollinated in more than two years the results are summarized in Table 1. In 1982-1984 a mixture of pollen from both IvP35 and IvP48 was used and in 1985 also IvP101 was included as a pollinator.

It is apparent that the parental genotypes differ in their ability of producing viable monohaploids. One can also note that both the frequency of monohaploids per 100 berries and monohaploids per 1000 seeds differ in the successive years. This may be due to (i) the physiological stage of the parental plants, (ii) the environmental conditions and/or (iii) the types of pollinator used. Table 2 and 3 demonstrate that these possibilities are relevant. In Table 2 large differences in monohaploid frequencies can be observed when pollinations are carried out during different stages of plant development or at different times during the season.

Table 1. Numbers and frequencies of monohaploids from five different diploid clones obtained over the years 1982 through 1985.

Year	Source	Number of berries	Average number of seeds per berry	Number of monohaploids		
				total	per 100 berries	per 1000 seeds
1982	M9	415	277	76	18.3	0.66
1983	M9	483	260	84	17.4	0.67
	23-2	8	268	1	12.5	0.47
	23-26	159	187	12	7.5	0.40
	23-28	218	115	7	3.2	0.28
	PS10	104	263	3	2.9	0.11
Total and means		972	216	107	11.0	0.51
1984	23-2	781	214	65	8.3	0.39
	23-26	130	218	11	8.5	0.39
	23-28	214	198	11	5.1	0.26
	PS10	411	252	27	6.6	0.26
Total and means		1536	223	114	7.4	0.33
1985	M9	361	314	42	11.6	0.37
	23-2	97	278	14	14.4	0.52
	23-26	10	237	1	10.0	0.42
	PS10	150	384	22	14.7	0.38
Total and means		618	324	79	12.8	0.39
'82-'85	M9	1283	227	202	15.7	0.57
	23-2	886	222	80	9.0	0.41
	23-26	299	202	24	8.0	0.40
	23-28	432	156	18	4.2	0.27
	PS10	665	284	52	7.8	0.27
Total and means		3565	244	376	10.5	0.43

Table 2. Numbers and frequencies of monohaploids obtained from clone M9 during 1983. *: during the day of pollination the maximum temperature in the greenhouse was 43°C.

Date	Number of berries	Average number of seeds per berry	Number of monohaploids		
			total	per 100 berries	per 1000 seeds
3/6	73	247	29	39.7	1.61
29/6	57	254	1	1.7	0.07
5/7	43	128	3	7.0	0.55
12/7*	24	56	0	-	-
19/7	156	247	25	16.0	0.65
2/8	128	322	21	16.4	0.51
29/8	26	307	5	19.2	0.63

Table 3. Numbers and frequencies of monohaploids obtained from six different lines using different pollinators.

Source	Polli-nator	Number of berries	Number of berries per 100 pollinated flowers	Average number of seeds per berry	Number of monohaploids		
					total	per 100 berries	per 1000 seeds
M9	IvP35/48	230	28	304	27	11.8	0.38
	IvP101	131	33	331	15	11.4	0.35
PD23-2	IvP35/48	61	68	242	2	3.3	0.13
	IvP101	36	77	340	12	33.3	0.98
PD23-26	IvP35/48	6	75	297	0	-	-
	IvP101	4	80	147	1	25.0	1.70
PS10	IvP35/48	98	84	388	9	9.2	0.24
	IvP101	52	76	377	13	25.0	0.66
PS10xM9-1	IvP35/48	20	59	83	1	5.0	0.60
	IvP101	21	68	68	4	19.0	2.80
PS10xM9-2	IvP35/48	12	32	382	3	25.0	0.65
	IvP101	13	41	294	2	15.4	0.52
Total	IvP35/48	427	38	306	42	9.8	0.32
	IvP101	257	43	315	47	18.3	0.58

In Table 3 a comparison is made between the monohaploid inducing ability of IvP(35+48) and IvP101 in the year 1985. From these data it may be concluded that for the parthenogenetic induction of monohaploids in diploid S. tuberosum and S. tuberosum x S. phureja hybrids, IvP101 is the better pollinator, i.e. has a higher monohaploid induction rate (MIR) than the combination of IvP35 and IvP48. IvP101 exceeds the IvP(35+48) mixture in (i) number of berries per 100 pollinated flowers, (ii) number of monohaploids per 100 berries and (iii) number of monohaploids per 1000 seeds. These results correspond with those obtained from the induction of dihaploids in tetraploid potato cultivars using the same pollinators (Hermsen, in prep.).

A large variation was found among the monohaploid plants obtained. The most vigorous plants reached a length of 170 cm and branched profusely, whereas the least vigorous stopped growing already at a height of 10 cm. Between these extremes there was a large number of plants that either grew well but did not branch, or branched excessively but did not grow taller than about 40 cm. Most of the morphological characters were variable: length of the fifth leaf varied from 1 to 18 cm; the length/width ratio of the top leaflet of the fifth leaf ranged from 1.4 to 2.8 and the length of the internodes varied between 1 and 4.5 cm.
Tuberization was also variable. Some plants did not tuberize at all, whereas others did, even under long day conditions. Whenever tubers were formed, they generally varied in size from 3 to 13 mm but tubers of 25 mm in diameter were also observed.
About 60% of the plants produced flower buds, which generally dropped before anthesis. Three monohaploids flowered. They did not produce viable pollen and did not form berries after pollination of eight flowers with diploid pollinator lines.

The parental lines differed greatly in monohaploid production rate (MPR) as well as in quality of the monohaploids they produced (MQL = monohaploid quality level) (Table 4).

Table 4. Data showing the quality of monohaploids from five diploid lines (pooled data of the years 1982-1985). n = number of viable plants after three months; (sub)lethal = dying within three months. For calculation of vigour see text.

Source	n	Vigour			Percentage
		max.	min.	mean	(sub)lethal
M9	168	9	1	4.3	17
PD23-2	70	9	1	4.4	12
PD23-26	23	9	1	4.4	18
PD23-28	17	8	1	4.1	6
PS10	34	4	1	1.4	35

To qualify the MQL of the diploid lines, estimates of maximum as well as minimum relative vigour are given for the monohaploids from each parental line. The relative vigour is expressed in a scale from one to ten. It has been calculated as the height of the plants in cm after 3-4 months in relation to their age in days ([heigth/age]x10). A correction of at most two scale units was carried out depending on their degree of branching. A non-destructive way of estimating vigour was thus obtained.

Since a large part of the planned research on the obtained monohaploids had to be carried out *in vitro*, all lines were screened for vigour in shoot tip cultures and for the potential of protoplast isolation and fusion. Material in both the greenhouse and *in vitro* was screened for stability of ploidy level. Stem and leaf pieces were incubated *in vitro* for callus induction and chromosome doubling to obtain, from each monohaploid, a homozygous series of ploidy levels (x,2x,4x). The results of both the *in vitro* work and the production and selection of homozygous lines will be published in future papers.

Conclusions and Discussion

Only one diploid clone (M9) was studied for monohaploid production rate (MPR) during one entire season (1983). The results suggest a clear intra-seasonal variation of this character. An adequate explanation for this phenomenon cannot be given at this stage of research. No correlations could be found between MPR and environmental conditions, like greenhouse temperature and light intensity in the period from five days before to two days after pollination. The physiological condition of the plants is likely to be important. In the 1983 season, with a relatively high average outdoor temperature (17.8°C) in June, July and August, the very early pollinations seemed to be most successful in inducing parthenogenetic development of embryos. Besides intra-seasonal also inter-seasonal variation could be detected.

Little is known about the mode or modes of origin of monohaploids in potato. In theory two processes are possible: (i) one sperm nucleus fertilizes the secondary embryo-sac nucleus, whereas the other one, which normally fertilizes the egg cel, degenerates leaving an unfertilized egg cell in a vital triploid endosperm. This is the process known for maize (Chase 1969); (ii) both sperm nuclei fertilize the secondary embryo-sac nucleus leading to tetraploid endosperm or, when dealing with 2n pollen, to hexaploid endosperm. This is the process suggested for the induction of dihaploid potato lines (Von Wangenheim et al., 1960). The involvement of both processes may be most likely.

Since monohaploids obtained from one parental line are a gametic sample of that line, the quality of monohaploids may provide an estimate of its breeding value. The high frequency of vigorous monohaploids from the clones M9, PD23-2, PD23-26 and PD23-28 may suggest a large number of genes for vigorous growth in these clones. On the other hand, PS10, which itself is highly vigorous, did hardly produce any well-growing monohaploids. This line probably owes its large vigour to its highly heterozygous condition (intra-locus interaction). In monohaploids there is no heterozygosity and in the PS10 monohaploids this lack is not compensated for by positive gene action nor by inter-locus interaction. On the contrary, recessive genes for (sub)lethality reveal themselves in these monohaploids (Fig. 3).

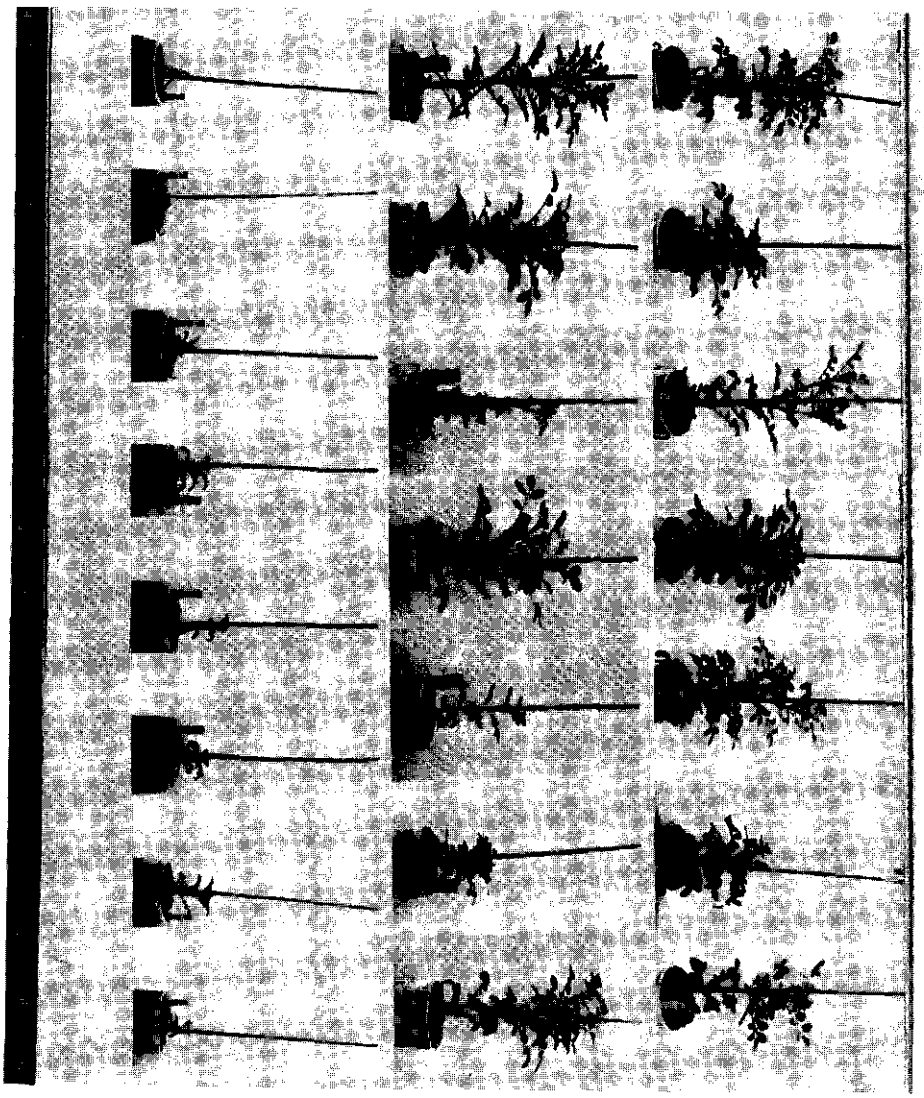

Fig. 3. Representative monohaploid seedlings from two diploid potato genotypes photographed 4 months after sowing (bar = 10cm). Upper and middle row: two random samples each of 7 different monohaploids from diploid M9, each sample photographed from a different angle; lower row: random sample of 8 different monohaploids from diploid PS10. For M9 and PS10 see text.

To investigate which will be the result of crossing a line with average MPR and low MQL with a line of high MPR and high MQL, we crossed PS10 with M9. Twelve random plants from the F_1 were taken for testing MPR. Since three plants died, only nine could be investigated. The results are shown in Table 5.

Table 5. Frequencies and quality of monohaploids of nine F_1-plants from the cross PS10xM9 in the year 1985. n = number of viable plants after three months; (sub)lethal = dying within three months. For calculation of vigour see text.

Source	n	Number of monohaploids		Vigour			Percentage
		per 100 berries	per 1000 seeds	max.	min.	mean	(sub)lethal
PS10xM9-1	2	6.9	0.41	1	1	1.0	50
-2	3	6.0	0.16	2	1	1.3	25
-3	4	5.6	0.53	5	2	2.5	33
-4	14	11.9	0.74	4	1	1.4	26
-6	1	1.3	0.08	1	-	1	50
-8	1	11.1	1.05	5	-	5	0
-9	0	-	-	-	-	-	-
-10	16	18.6	1.98	9	1	2.5	47
-11	1	1.8	0.10	2	-	2	0
Total	42	7.7	0.43			2.0	37

Whereas over the years the monohaploids from PS10 and M9 showed an average vigour of 1.4 and 4.3 respectively, the mean MQL of the F_1 PS10xM9 (MQL=2.0) is lower than the midparent vigour (MQL=2.9). In fact only one monohaploid was as vigorous as the better monohaploids of M9. So the diploid F_1 plants, although vigorous, were hardly able to pro-

duce well growing monohaploids. Apparently the positive genes from M9 could not compensate for the negative genes from PS10. Some F_1-plants were able to produce monohaploids in high frequencies, but this was associated with low average vigour and high mortality in the first three months.

The results of a cross between a line with high MPR and high MQL (M9) and a line with low MPR and high MQL (line GPG) are shown in Table 6.

Table 6. Frequencies and quality of monohaploids of 11 F_1-plants from the cross M9xGPG in the year 1985. n = number of viable plants after three months; (sub)lethal = dying within three months. For calculation of vigour see text.

Source	n	Number of monohaploids		Vigour			Percentage
		per 100 berries	per 1000 seeds	max.	min.	mean	(sub)lethal
M9xGPG-1	2	3.3	0.15	6	4	5.0	0
-2	4	7.4	0.24	8	1	4.0	0
-3	4	20.0	0.45	5	3	3.8	0
-4	5	11.6	0.59	7	2	5.0	0
-5	0	-	-	-	-	-	-
-6	1	16.7	0.40	2	-	2	0
-7	0	-	-	-	-	-	-
-9	1	1.1	0.42	9	-	9	0
-10	1	3.8	0.07	4	-	4	0
-11	1	2.0	0.05	1	-	1	0
-12	6	4.5	0.24	4	1	2.7	33.3
Total	25	4.2	0.15			4.6	10.7

Although in this experiment the total number of monohaploids obtained is low, it may be concluded that the quality of the monohaploids is rather good, while the production rate is lower than the mean rate of both parents.

In summary, the tendency is that

(i) high MPR x low MPR gives low MPR;

(ii) high MQL x high MQL gives high MQL;

(iii) low MQL x high MQL gives low MQL.

When potato monohaploids have to be produced on a large scale, whether through anther culture or prickle pollination, these conclusions are very important. Even when the induction mechanisms for androgenesis and gynogenesis are totally different and coded by different genes (resulting in different MPR-values), the MQL of the parental line is a critical trait for the frequency and potential use of monohaploids. This means for the scheme of Wenzel et al., (1979) that, in trying to obtain monohaploids or homozygous diploids from lines that have some desired characters (e.g. genes for resistance) but low MQL, a lot of problems have to be dealt with. When crossing such lines with high MQL lines, only slow progress will be made, if conclusion (iii) would turn out to be generally valid.

Using the anther culture technique, Uhrig (1985) got 2 monohaploids out of 313 regenerants that were obtained from about 700 anthers. This means 6.4 monohaploids per 1000 regenerants and 0.29 monohaploids per 100 anthers. All plants had to be regenerated and screened for ploidy level as there was no marker available for the selection of monohaploids. The supposed advantage of producing homozygous doubled lines directly via anther culture (Wenzel et al. 1979) ceases to exist if one takes into account that a series of markers is necessary to detect completely homozygous diploids. Besides homozygous diploids, also heterozygous diploids (originating from 2n gametes) and parent-like diploids (originating from somatic anther tissue) may be obtained. It is obviously more efficient to insert one marker in a pollinator, than to insert several in the parental material, because one pollinator probably is sufficient to obtain monohaploids from a number of parental lines. Thanks to the available marker embryo-spot in the pollinator lines used, it was relatively easy to obtain 0.6 monohaploids per 1000 seeds and 18 monohaploids per 100 berries (Table 3) from several diploid lines using the prickle pollination technique. The statement that androgenesis in itself has more potentials for monohaploid production than gynogenesis because an anther contains more gametes than an ovary is depreciated by these results.
Furthermore, using the anther culture technique, a lot of valuable genotypes may be lost because they lack the in vitro regeneration

capacity, which as such is a useless selection criterion in a breeding scheme starting with homozygous lines.

The genetic aspects of monohaploid production deserve careful consideration. Both gynogenetic and androgenetic monohaploids can be obtained from a limited number of specific genotypes. The genetic basis of gynogenesis and androgenesis may be different. If so, both prickle pollination and anther culture should be applied in order to obtain monohaploids from sufficiently various diploid breeding material.

Chapter 3.

Protoplast fusion of monohaploid (2n=x=12) potato clones; identification of somatic hybrids using malate dehydrogenase as a biochemical marker

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Summary

The isolation, fusion and successful culture of protoplasts from monohaploid potato clones is described. These monohaploids were obtained from two diploid lines coded M9 and PD23-2. M9 was homozygous for malate dehydrogenase(MDH) (one slowly moving band; banding pattern A) and PD23-2 was heterozygous (three bands; banding pattern AB). The nine monohaploids X1,1 - X1,9 obtained from M9 all showed banding pattern A and the 49 monohaploids X2,1 - X2,49 from PD23-2 showed banding pattern A or B (the latter showed one fast moving band). Protoplasts of X1,1 with banding pattern A and of X2,1 with banding pattern B were fused. Some calli of the heterofusion experiment showed plant regeneration. Based on MDH tests these regenerants could be characterized as somatic hybrids. From the results presented it can be concluded that MDH can be used as a biochemical marker in fusion experiments with monohaploids and that plant regeneration ability can be increased by fusion of different plant clones.

Introduction

The potential use of monohaploid potato clones for breeding and fundamental research in somatic cell genetics has already been mentioned in literature (Wenzel et al., 1979; Uijtewaal & Hermsen, 1986). However, difficulties with instability in ploidy level during tissue culture may cause too many problems for practical use (Jacobsen et al., 1983; Karp et al., 1984; Tempelaar et al., 1985; Pijnacker et al., 1986; Uijtewaal, 1987). It has been shown that protoplast isolation from monohaploid potato is possible and that the protoplasts occasionally can be grown to callus and regenerated to shoots (Bokelmann & Roest, 1983). Successful protoplast fusions between different monohaploids and successive regeneration have not been described till now. The most simple way to select for such fusion products is using selection markers such as recessively inherited nitrate reductase deficiency or dominantly inherited amino acid analogue resistance (De Vries et al., 1987b). Another way to detect fusion products in potato is selection of fusion partners with one or more mitotically visable distinct chromosomes (Puite et al., 1986). In this paper detection of fusion products at the plant stage is described by using malate dehydrogenase as an

intrinsic biochemical marker.

Materials and Methods

Plant material

For MDH-analysis the diploid clones M9 and PD23-2, F_1-plants from M9xPD23-2 and parthenogenetically obtained monohaploids from M9 (X1,1 up to X1,9) and from PD23-2 (X2,1 up to X2,49) were used. The diploid clones are genetically unrelated; M9 originates from a cross between Solanum tuberosum and S. phureja, whereas PD23-2 is a parthenogenetic diploid of pure S. tuberosum origin. More details about the genetic background have been given by Uijtewaal et al. (1987a). For isozyme analysis as well as protoplast isolation, plant material was cultured in vitro in 9cm high glass containers on solidified MS-medium (Murashige & Skoog, 1962) with 2% sucrose (25°C, 16h daylight, 7000 LUX), or in the greenhouse.

Isolation of protoplasts

Protoplasts were isolated from 4-weeks old axenic shoots according to the procedure originally described by Binding et al. (1978) and Bokelmann and Roest (1983) but modified for monohaploid potato material: 1.5g of plant material was cut into fragments and left for plasmolysis in 15ml of a solution of 0.53M mannitol (ca 550mOsm) for 30 minutes. After sucking off the mannitol and so removing most of the cell debris, 15ml enzyme medium (EM, Table 1) was added. After the enzyme treatment, which was carried out overnight at 27°C in the dark, the digested plant material, gently stirred with a pipette for protoplast release, was filtered through a double filter with pore sizes of 297um and 88um respectively. The filtrate was diluted with an equal volume of washing medium (WM, Table 1) and centrifuged for 3 minutes at 65g. The pellet was resuspended in washing medium and centrifuged again. For the purification of the protoplast solution the pellet was resuspended in 10ml of a 0.45M sucrose solution (ca 550mOsm) and a small layer of about 0.5ml of washing medium was put on top. After centrifugation at 30g for 15 minutes the viable protoplasts were concentrated in a band between the sucrose and the washing medium (Fig. 1). Because of the presence of the washing medium even small amounts of protoplasts could easily be

collected with a pasteur pipette without sucking up sucrose. After a last washing step the protoplasts were resuspended in 2 to 3ml of culture medium (CM, Table 1) and counted in a haemocytometer.

Table 1. Media used for the isolation, fusion and culture of protoplasts; 1) half strength V-KM medium as composed by Binding & Nehls (1977) and fully described by Bokelmann and Roest (1983); 2) both macerozyme and cellulase were of the type Onozuka R10 (Yakult Honsha Ltd, Japan); 3) components of the V-solution were dissolved in a 0.2M glycine/NaOH buffer pH 10.4.

	Media					
Components	CM	WM	EM	W-sol	PEG-sol	V-sol
V-KM[1)]	x	x	x			
coconut milk (ml)	20		20			
glucose (g/l)	35		35	0.901		
mannitol (g/l)	45		35			
2.4D (mg/l)	0.2		0.2			
NAA (mg/l)	1.0		1.0			
zeatin (mg/l)	0.5		0.5			
NaCl (g/l)		17,53		4.91		
macerozyme[2)] (g/l)	2					
cellulase[2)] (g/l)	10					
$CaCL_2.2H_2O$ (g/l)				18.38	1.76	7.35
KCl (g/l)				0.373		
PEG 4000 (g/l)					450	
glycine/NaOH buffer[3)]						x
osmolarity (mOsm)	500	500	550	525	800	
pH	5.7	5.7	5.7	5.7	6.0	10.4

Protoplast fusion

Protoplast fusion was carried out using the phase-boundary technique of Binding and Nehls (1978) modified for the fragile monohaploid protoplasts. For fusion, protoplasts of both lines in a density of 5.10^5 protoplasts/ml (0.5 ml of each) were mixed in a coated plastic culture tube (Greiner Ltd.) till a final amount of protoplasts of 10^6 was obtained. By addition of 5ml W-solution (Table 1) and pelleting the

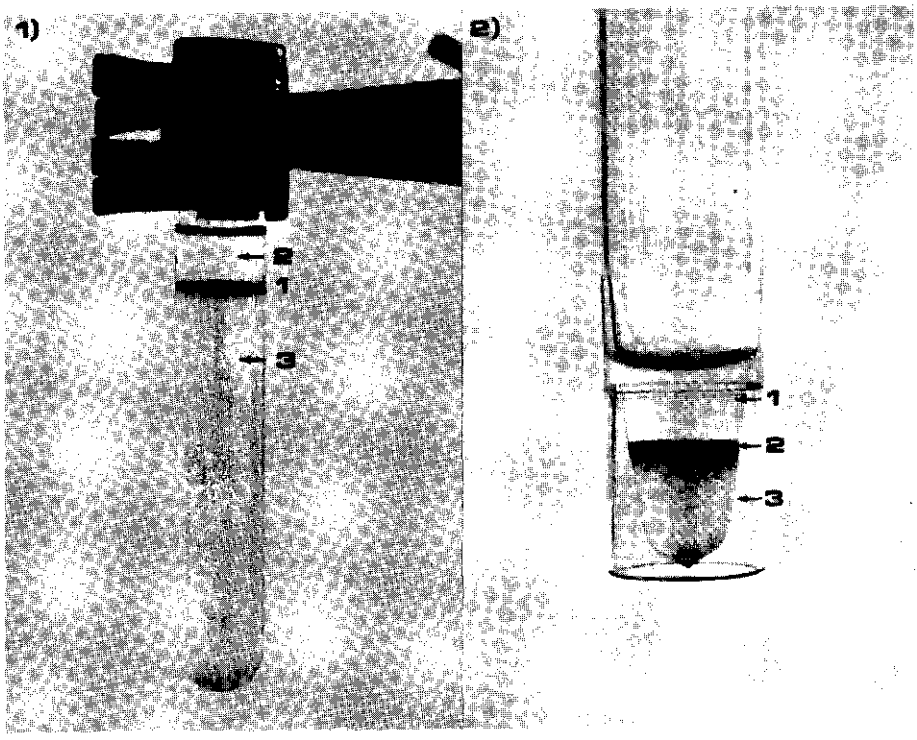

Fig. 1. Purification of the protoplast solution using sucrose. Concentration of the protoplast fraction in a sharp band (1) in between sucrose (3) and a layer of washing medium (2) by centrifugation, thus separating the floating protoplasts from the heavier cell debris.

Fig. 2. Protoplast fusion using a phase-boundary technique. By addition of a V-solution (1) the PEG could gently be washed out from the protoplasts (2) floating on top of the superfluous PEG (3).

protoplasts by centrifugation for 3 minutes at 30g the culture medium was washed out. The supernatant was removed till about 0.2ml with a pasteur pipette. The protoplasts were resuspended in the remaining solution and 0.4ml PEG-solution (Table 1) was added from a height of ca 10 cm in small drops on top of the protoplast suspension, waiting 10 seconds for the solution to stabilize after each drop. In this way the protoplasts were coated with PEG while the redundant PEG-solution sank to the bottom of the culture tube. Incubation with PEG solution during

20 minutes allowed the protoplasts to make close contact with each other. Then 0.5ml V-solution (Table 1) was gently added twice, with an interval of about 15 minutes, on top of the protoplasts. In this way the protoplasts assembled in the contact zone of both solutions (Fig. 2) allowing a gentle dilution of the PEG. Immediately after addition of the second 0.5ml V-solution the PEG solution under the protoplast layer was removed with a pasteur pipette. After another 15 minutes 5ml W-solution (Table 1) was added and all material was homogenized by gently turning over the culture tube. The protoplasts were pelleted by centrifugation for 2 minutes at 17g. The remaining protoplasts were washed three times with W-solution to remove the PEG, and after that they were transferred to 1.6ml culture medium and cultured in a Petri dish (0.6cm).

Protoplast culture and plant regeneration

Protoplast culture was carried out according to Roest and Bokelmann (1983) with minor modifications, at 25°C under continuous light (1800 LUX). The protoplast suspension was diluted on the third and the sixth day with 0.8ml auxin free culture medium. After two weeks the cultures in which the microcalli had developed were 'softed' by dilution with agarose dissolved in auxin free culture medium up to a final agarose concentration of 0.2% and the density of the growing microcalli was reduced to a concentration of about 400 calli per 9cm Petri dish. After a month the obtained calli were transferred to solid callus growth medium (CG, Table 2) and cultured at 25°C and 16 hours daylight (3500 LUX). After another three weeks these calli, depending on their size, were transferred again to callus growth medium (<2mm) or to shoot initiation medium (≥2mm)(SI, Table 2). For shoot initiation the light intensity was increased to 9000 LUX. Regenerating calli were transferred onto shoot elongation medium (SE, Table 2).

Ploidy level determination of regenerants

Regenerants were checked for ploidy level by counting the number of chloroplasts in the guard cells according to Frandsen (1968) and the number of chromosomes in the root tip cells according to Henderson and Lu (1968).

Table 2. Media used for the growth of protoplast derived calli (CG) and for shoot initiation (SI) and elongation (SE) from these calli. All components, except for the agar, were filter sterilized; 1) MS plant salts (Murashige & Skoog, 1962) without NH_4NO_3; 2) N vitamins (Nitsch & Nitsch, 1969); 3) agar type: K 267 (Flow Company).

Components	Media		
	CG	SI	SE
MS plant salts - NH_4NO_3[1]	x	x	x
N vitamins[2]	x	x	x
NH_4NO_3 (g/l)			0.82
sucrose (g/l)	5	5	10
mannitol (g/l)	35	35	
agar[3] (g/l)	8	8	8
zeatin (mg/l)	1	1	
IAA (mg/l)	0.2	0.01	

Electrophoresis

Sample preparations and polyacrylamide (PAA) gel electrophoresis were carried out according to Suurs and Jongedijk (1987). However, a different pore size gradient was used. The gel was prepared with a gradient controller as described by Altland and Altland (1984). The gradient was composed as follows: gel length 0-2cm, 5-10% PAA; 2-12cm, 10-14% PAA; 12-13cm, 14-20% PAA.

The gel was stained for malate dehydrogenase at 30°C in the dark in a mixture of 30mg NAD, 20mg MTT(3-(4,5-dimethyl-2-thiazyl)2,5-diphenyl-2,4-tetrazoliumbromide) and 4mg PMS(phenozine methosulfate) dissolved in 100ml 0.1M tris-HCl buffer pH=7.5 containing 0.4g DL malic acid until bands appeared (± 1 hour).

The gels were photographed with a polaroid MP-4 camera (Pac-film type 665) and the negatives were used for scanning with an ultrascan LKB laser densitometer (scan speed 10mm/min; paper chart 1mm/sec).

Results

MDH-analysis of source material and selection of fusion partners

Since shoot tip propagated plants showed sharper bands after MDH analyses than greenhouse grown plants, the former were used for MDH analyses. With callus, the banding patterns obtained were too vague for MDH analyses.

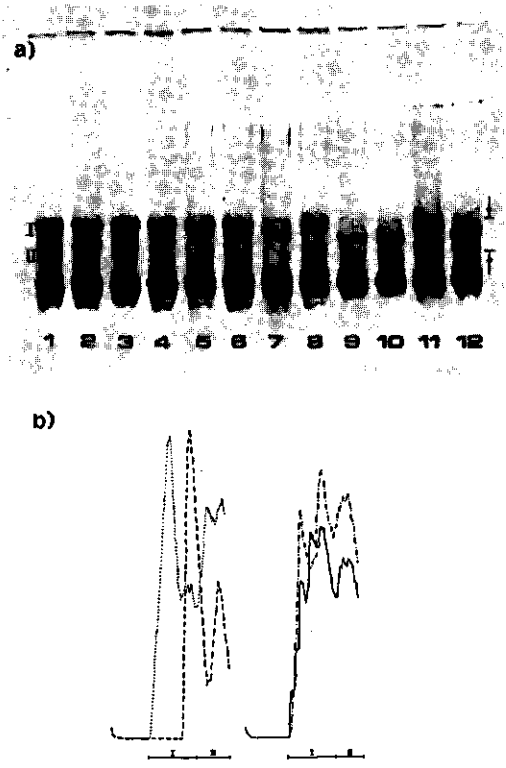

Fig. 3a. MDH-allozyme banding patterns of two diploid lines and their monohaploid derivatives. D1=M9; D2=PD23-2; X1,n=monohaploids from M9 and X2,n=monohaploids from PD23-2. The composition from left to right is: 1=X1,1; 2=X1,1; 3=X1,3; 4=X1,4; 5=X1,5; 6=D1; 7=D2; 8=X2,5; 9=X2,4; 10=X2,3; 11=X2,2; 12=X2,1. The space between the arrows indicates the part of the gel that was taken for scanning (b). I=sharp staining bands; II=diffuse staining bands.

Fig. 3b. Densitograms of MDH-banding patterns of the three banding types A (....), B (----) and AB (——) and the mixture of A and B (.-.-). In fig. 3a and fig. 5 is indicated (space between arrows) which part of the gel is taken for scanning. Scan speed = 10mm/min; Paper chart = 1mm/sec. I= sharp staining bands; II=diffuse staining bands.

The diploid clones M9 and PD23-2 showed the banding types A (one slow band) and AB (three bands) respectively as illustrated in Fig. 3a. Attention was paid only to the three sharply staining slower bands (I) and not to the more diffuse staining faster bands (II). The monohaploids obtained from M9 all showed the A-banding pattern, whereas the monohaploids from PD23-2 displayed a 1:1 segregation in banding pattern types A and B (one fast band)(Table 3, Fig. 3a). The scannings of the three banding types A, B, AB and the mixture of A and B are shown in Fig. 3b.

Table 3. Results of MDH-analysis using diploid and monohaploid potato plant material.

Clone	Banding pattern A	B	AB	
M9	1			
PD23-2			1	
F1(M9xPD23-2)	26		22	($X^2$1:1=0.33; p>0.50)
M9 monohaploids	9			
PD23-2 monohaploids	31	18		($X^2$1:1=3.52; p>0.05)

The 48 F1-plants of the cross M9xPD23-2 (AxAB) segregated 1:1 for A:AB MDH-banding patterns suggesting a dimeric structure of the MDH-allozyme. It must be concluded that the A and B banding types represent plants which are phenotypically homozygous for one allele and that the AB banding type represents a plant containing two different alleles. Based on these MDH-analyses, the vigorously growing monohaploids X1,1 and X2,1 with banding patterns A and B respectively were selected for protoplast fusion experiments. It is expected from the results shown in Table 3 and Fig. 3a that the hybrid fusion products will show banding pattern AB.

Analysis of plants obtained from fusion experiments

Monocultures of protoplasts from X1,1 and X2,1 showed cell divisions and produced calli. After more than five months of culturing, regene-

rants could be obtained from X1,1, whereas after even one year no shoots were regenerated from X2,1. After fusion of protoplasts from X1,1 and X2,1 no clear differences could be detected between the growth rate of the calli originating from homo- and heterofusions. Eleven weeks after the fusion treatment however, only the potential hetero-calli showed shoot initiation and plant regeneration. From three out of 50 different well growing calli shoots have been regenerated. All these regenerants appeared to be tetraploid after chromosome counting, and were tested for hybrid nature using MDH as a biochemical marker. Besides the monohaploid starting material, also in vitro doubled homozygous 2x and 4x plants of X1,1 and homozygous 2x plants of X2,1 were used as controls. The results of these MDH-tests are shown in Fig.4. It is apparent that the ploidy level did not influence the MDH banding pattern and that the mixture of both X1,1 and X2,1 plant material showed a summation of the homozygous banding types A and B respectively. All tetraploid regenerants from the three shoot producing calli showed banding pattern AB. Therefore it must be concluded that all these regenerants are true hybrids.

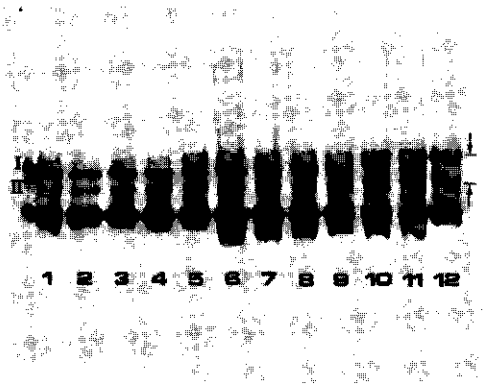

Fig. 4. MDH-allozyme banding patterns of three protoplast fusion regenerants (R1,R2,R3), both monohaploid parental clones (X1,1 and X2,1), mixtures of plant material of both parental monohaploid clones (M) and the homozygous di- and tetraploid derivatives of X1,1 (DX1,1 and TX1,1) and the homozygous doubled X2,1 (DX2,1). The composition from left to right is: 1=X2,1; 2=TX2,1; 3=DX2,1; 4=X2,1; 5=M; 6=R1; 7=R2; 8=R3; 9=M; 10=X1,1; 11=DX1,1; 12=TX1,1. The space between the arrows indicates the part of the gel that was taken for scanning (fig. 3b). I=sharp staining bands; II=diffuse staining bands.

Discussion

In the plant material used, MDH appeared to be a suitable isozyme marker for the pre-selection of monohaploid potato clones to be included in fusion experiments in order to allow an identification of the hybrid fusion products at the plant level. The hybrids could be recognized by the presence of an additional dimeric band that could not be obtained by in vitro doubling of the ploidy level nor by mixing plant material of both monohaploid clones before electrophoresis. The MDH allozyme used appeared to be inheritable in a Mendelian way. Callus appeared to be unsuitable for MDH analyses since only results too vague for classification could be obtained.

Since it has been shown that in Datura species hybrid fusion products can be selected for by hybrid vigour at the callus level (Schieder, 1980), attention was paid to the growth rate of the calli obtained after protoplast fusion. Although the appearance of heterozygous hybrid calli on the one hand and monohaploid and/or fully homozygous calli on the other hand might have justified the expectation of hybrid vigour, no differences in growth rate at the callus level could be detected. However, the hybrid calli apparently have a better regeneration capacity than the parental ones since all regenerants obtained showed the AB banding pattern and thus were hybrids. The parents could not, or only after a long period of time, be regenerated to shoots.

Although variation in plant growth conditions does not change the MDH-banding pattern, it is advisable to use in vitro plant material under standard conditions. The reason is, that, contrary to minor changes of laboratory techniques and chemical lots (Goodman & Stuber, 1983), plant condition may have great influence on the purity of the electrophoresis samples (Suurs, unpublished).

The phenomenon of weaker staining 'traiding' bands, as found for the allozyme studied (Fig. 3, 4), was also mentioned by Newton (see review Goodman & Stuber, 1983). She noticed that these traiding bands, classified as modified forms of one MDH allozyme, are eliminated after purification of the soluble MDH's. Since they were not interfering with our characterization of hybrid plants, no attention was paid to them.

Using MDH-allozyme as an example, this paper shows that by selecting the fusion parents for naturally available variation at isozyme level

it is possible to identify the hybrid regenerants after protoplast fusion. This is an addition to the existing methods using mutants (De Vries et al., 1987b) or cytotypes (Puite et al., 1986) for the characterization of potato protoplast fusion products.

Chapter 4.

Ploidy variability in greenhouse cultured and *in vitro* propagated potato monohaploids (2n=x=12) as determined by flow cytometry.

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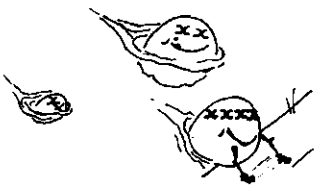

Abstract

The DNA distributions of 23 different monohaploid potato clones were investigated by flow-cytometric measurements. All monohaploid clones differed in DNA distribution but none of them contained only monoploid cells in the leaves. All were highly stable on the monohaploid level for 2-3 years. Investigation of the influence of different factors on the DNA distribution in leaf cells showed that the material derived from *in vitro* shoot tip propagation contained a lower proportion of polyploidized cells than greenhouse grown plants. With protoplast isolation the enzyme treatment of *in vitro* cultured plant material induced a striking shift of DNA distribution towards the lower C-value whereas the mechanical purification steps caused a selective loss of monoploid nuclei. Seasonal influence on the DNA patterns could be detected.

Introduction

It is known that differentiated plant tissues *in vivo* may contain a mixture of cells with nuclei containing normal DNA-levels and cells with duplicated levels of DNA (Langlet, 1927; D'Amato, 1952). Recent research in potato has made clear that in monohaploid potato clones a relatively high proportion of leaf cells with elevated C-values can occur as a consequence of differential triggering of additional chromosome reduplication (Jacobsen *et al.*, 1983; Karp *et al.*, 1984; Tempelaar *et al.*, 1985; Sree Ramulu & Dijkhuis, 1986; Pijnacker *et al.*, 1986). Since genotypic differences in cytogenetic stability of monohaploids have been detected (Jacobsen *et al.*, 1983; Karp *et al.*, 1984; Tempelaar *et al.*, 1985), ploidy studies were carried out on a collection of monohaploid potato clones from different sources.

The influence of culture conditions on the DNA-distribution of the nuclei was studied by screening material of plants grown in the greenhouse or *in vitro*.

Since genetic manipulation work is often carried out on protoplasts, attention is paid to the DNA-distribution among nuclei of isolated protoplasts and to the influence of different steps of the protoplast isolation procedure on the DNA-distribution among the nuclei of such protoplasts.

To avoid misjudgement of phenotypic differences caused by plant age or

seasonal influence, ploidy determinations were repeated during a period of more than one year. To cope with the large amount of work involved, the rapid method of nuclear DNA-content determination by flow cytometer was chosen (Galbraith et al., 1983), along with some cytological examinations.

Materials and Methods

Plant material

Twenty-three monohaploid (2n=x=12) potato clones selected from a population of about 200 parthenogenetic monohaploids, were propagated both in vivo and in vitro and screened for DNA distribution pattern. The selected clones, which originated from three unrelated diploid genotypes, showed striking differences in vigour. The monohaploids coded as 839-.. and 849-.. were derived from the diploid hybrid M9. The monohaploid 13-3-2 and those designated as 851-.. are monohaploids from the diploid clones coded PD13-3 and PD23-26 respectively. The monohaploids 855-1 and 855-2 were obtained from a diploid hybrid (L9xG609)xM9, with L9 and G609 being dihaploids of the cultivars Libertas and Gineke respectively. For further information about genotypic background see Uijtewaal et al. (1987). To enable a comparison with the results obtained by other research groups (Jacobsen et al., 1983; Karp et al., 1984; Tempelaar et al., 1985; Pijnacker et al., 1986), the androgenetic monohaploid clone MN977322 (abbreviated to 7322) was also included. This clone has been obtained at the Max Planck Institute, Cologne, West Germany.

All plant material was maintained in plastic containers via shoot cultures on MS-medium (Murashige & Skoog, 1969) with 20g/l sucrose and 8g/l agar, at 25°C and 16h light (4000 LUX) and 8h darkness. In vivo plant material was grown under standard greenhouse conditions (20-25°C; 24cm pots) during the period of March - July.

Material for determination of DNA-content

The upper 3-4cm (till about the sixth leaf) of four-week-old in vitro grown shoots and young mature leaves of four-months-old greenhouse plants were used for determinations of DNA-content. In contrast to in vitro grown plants the leaves of greenhouse grown plants were more

composed, and thus as a rule more differentiated.

Protoplast isolation

The upper 3-4cm of four-week-old *in vitro* grown shoots were used for protoplast isolation. Protoplasts were isolated according to the procedure of Bokelmann and Roest (1983), modified with respect to enzyme incubation time (overnight, without rotation) and osmolarity of the media (550mOsm).

Flow cytometry

For flow cytometric analysis nuclei were isolated from non-fixed plant material according to Galbraith *et al.* (1983) in combination with the buffer used by Blumenthal *et al.* (1979), with some minor modifications according to Sree Ramulu and Dijkhuis (1986). In contrast to the procedure of Ramulu and Dijkhuis (1986) samples from plant material were prepared with 0.1% triton and stained with ethidium bromide up to a final concentration of 10mg/l.

Protoplast samples were normally taken after a single filtration through a 87um nylon filter, directly after the enzyme incubation (unpurified). Exceptionally, nuclei of saturated protoplasts (i.e. after several centrifugation steps for purification; purified) were measured in order to check the possible influence of the saturation procedure on the DNA-distribution pattern. Both purified and unpurified protoplasts were pelleted for 1 min at 1000rpm (180g) and resuspended in 500ul chopping buffer (Sree Ramulu & Dijkhuis, 1986) and stained with ethidium bromide.

DNA-Measurements and chromosome counts

The DNA-measurements were carried out at the Foundation Ital, Wageningen with a Fluorescence Activated Cell Sorter (FACS)-IV (Becton Dickinson, Sunnyvale, U.S.A.) equipped with a spectra Physics argon ion laser, model 164-05 operated at 0.3W/488nm with a LP 620 filter in the emission beam.

To calculate the fraction of nuclei per fluorescence peak the number of nuclei per fluorescence peak, as measured by the Cell Sorter, was divided by the total number of nuclei measured. All measurements were

carried out within three hours after sample preparation. All histograms of DNA-content have been obtained without applying a correction method for background debris.
Chromosomes were counted in Feulgen squashed cells.

Results

1. Ploidy level and DNA-distribution patterns of in vitro grown shoots

In all 23 monohaploids the root tips showed only monoploid dividing cells. Genotypic differences in DNA-distribution pattern were apparent as clearly illustrated in Fig. 1 for leaf material from two in vitro grown monohaploid clones.

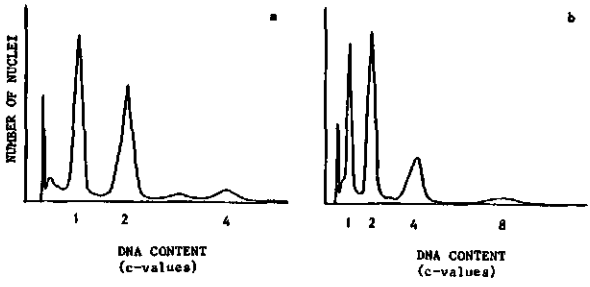

Fig. 1. DNA distribution of nuclei from in vitro grown leaf material of the monohaploid clones 13-3-2 (a) and 851-23 (b)

In 851-23 the frequency of 2C and 4C nuclei is much higher than in 13-3-2 and even 8C nuclei were found. The data in Table 1 show the DNA-distribution of all 23 in vitro propagated monohaploid clones. They were screened in the period from 3-12-1985 to 25-2-1986.
It is apparent that genotypic differences in DNA-distribution patterns can be detected between the monohaploid clones screened, especially between those from PD23-26. However, all showed a distribution pattern in which 1C, 2C, 4C and sometimes even 8C peaks were present. No clear difference in the mean C-value was found between the groups of monohaploids originating from M9 and PD23-26 respectively. DNA-Distribution pattern and plant vigour were compared but no relation could be detected.
In none of the monohaploids any alteration in DNA-distribution pattern was found after two to three years of shoot tip propagation.

Table 1. DNA-determinations of *in vitro* grown shoots from 23 different monohaploid potato clones during the period of 3-12-'85 to 25-2-'86. All figures are the means of two measurements.

Diploid parent	Monohaploid clone	Fraction of nuclei per C-value			
		1C	2C	4C	8C
M9	839-16	.42	.42	.16	-
	839-19	.40	.46	.14	-
	839-61	.47	.40	.13	-
	839-79	.37	.46	.17	-
	849- 7	.42	.45	.13	-
	849-30	.42	.45	.13	-
	means	.42	.44	.14	-
PD13-3	13-3-2	.40	.48	.12	-
PD23-26	851- 7	.31	.46	.19	.04
	851-12	.26	.45	.23	.06
	851-20	.34	.47	.19	-
	851-23	.30	.45	.20	.05
	851-31	.40	.49	.11	-
	851-32	.36	.46	.18	-
	851-34	.46	.45	.09	-
	851-49	.43	.47	.10	-
	851-50	.38	.49	.13	-
	851-54	.45	.45	.10	-
	851-56	.38	.49	.13	-
	851-59	.46	.46	.08	-
	851-63	.43	.48	.09	-
	means	.38	.47	.14	.01
	7322	.40	.48	.12	-
(L9xG609)M9	855-1	.39	.51	.10	-
	855-2	.54	.38	.08	-

2. The influence of different factors on ploidy level and DNA-distribution

-Seasonal influence on DNA-distribution

All *in vitro* material was cultured under controlled conditions. Yet slight differences in DNA-distribution were detected within genotypes between the samples examined throughout the year as shown for the clone 7322 in Table 2a. Although not all clones varied in the same way, there

was a tendency of higher numbers of 2C and 4C nuclei during November, December and January. Hardly any variation was found for DNA-distribution in replicates sampled on the same day (Table 2b).

Table 2. Comparison of DNA-distribution in shoot tip cells of *in vitro* grown shoots of clone 7322 at different periods of the year a) and of different plant samples on one day b).

a) Date	C-value			b) Date	C-value		
	1C	2C	4C		1C	2C	4C
7/4	.49	.42	.09	7/4	.48	.43	.09
9/7	.52	.40	.08		.52	.40	.08
18/11	.49	.43	.08		.49	.42	.09
9/1	.40	.48	.12				

-DNA-distributions of greenhouse grown and in vitro cultured plants

Table 3 shows the DNA-distribution of seven monohaploid clones that were cultured both in the greenhouse and *in vitro*. From the Figures it appears that three genotypes show hardly any difference in DNA-distribution, whereas four display higher 1C-peaks with *in vitro* material. The mean values indicate that in shoots of *in vitro* grown material the 1C peak is higher than in just fully developed leaves of greenhouse grown plants.

Table 3. Comparison of DNA distribution in the upper part of *in vitro* grown shoots and in fully developed young leaves of greenhouse grown plants.

Clone	Fraction of nuclei per C-value					
	in vitro grown plants			greenhouse grown plants		
	1C	2C	4C	1C	2C	4C
849- 7	.42	.43	.15	.35	.47	.18
843- 6	.47	.39	.13	.35	.53	.12
13-3-2	.55	.38	.07	.40	.49	.11
839-16	.45	.43	.12	.48	.38	.14
839-19	.40	.46	.14	.42	.44	.14
839-61	.50	.38	.12	.51	.40	.09
839-79	.57	.33	.10	.44	.43	.13
Means	.48	.40	.12	.42	.45	.13

-DNA-Distribution in cells versus isolated protoplasts from in vitro grown plants

Table 4 shows the DNA-distributions of nuclei from in vitro plant material before protoplast isolation and from isolated protoplasts.

Table 4. Comparison of DNA distributions of nuclei from in vitro plant material and unpurified protoplasts isolated from the same plants.

Clone	Fraction of nuclei per C-value							
	plant material				protoplasts			
	1C	2C	4C	8C	1C	2C	4C	8C
839-16	.44	.45	.11	-	.61	.39	-	-
839-79	.37	.46	.17	-	.51	.43	.05	-
849-30	.50	.42	.08	-	.58	.42	-	-
7322	.53	.39	.08	-	.66	.34	-	-
13-3-2	.38	.52	.10	-	.61	.39	-	-
851- 7	.31	.46	.19	.04	.36	.53	.11	-
851-12	.26	.45	.23	.06	.30	.55	.15	-
851-18	.29	.52	.19	-	.37	.51	.12	-
851-23	.30	.45	.20	.05	.21	.52	.23	.04
Means	.38	.46	.14	.02	.47	.45	.07	.01

These protoplasts were sampled and screened for DNA-distribution directly after the enzyme treatment without any purification. From these data it is apparent that in almost all cases the frequency of the DNA-values of the nuclei of the cells after the enzyme treatment have shifted from the higher (4C and 8C) to the lower (mainly 1C) DNA-levels. For the clones 13-3-2 and 839-79 this shift is illustrated in Fig. 2 showing the DNA-measurements from plant material (Fig. 2a and 2c) and from unpurified protoplasts (Fig. 2b and 2d). It is apparent that an almost complete disappearance of the 4C-peak may occur after the enzyme treatment.

Variation in enzyme incubation conditions like omission of hormones or osmotic level variations showed no alterations in the resulting DNA-histograms. The protoplast purification procedure itself however can cause considerable changes in DNA-distribution pattern. For genotype

13-3-2 Table 5 shows a clear shift in DNA-distribution of a protoplast suspension before (B) and after purification (C) from 1C to 2C. Apparently the mechanical steps (before C) cause a differential loss of the, perhaps more fragile, monohaploid protoplasts.

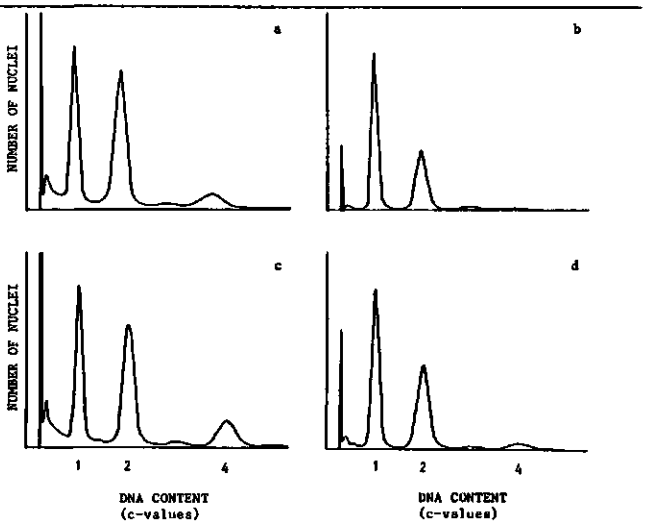

Fig. 2. DNA distribution of nuclei from in vitro plant material (a and c) and purified protoplasts isolated from it (b and d) of the monohaploid clones 13-3-2 (a and b) and 839-79 (c and d).

Table 5. Comparison of DNA distributions of nuclei directly isolated from the in vitro grown leaves (A), after enzyme treatment (unpurified protoplasts,B) and after the entire protoplasts isolation procedure (purified protoplasts,C).

Clone	Treatment	Fraction of nuclei per C-value		
		1C	2C	4C
13-3-2	A	.55	.38	.07
	B	.74	.26	-
	C	.60	.40	-

3. Chromosome counts in shoot tips and young leaves of in vitro grown shoots

The data of chromosome counts in shoot material of *in vitro* grown plants of the monohaploid clones 13-3-2 and 839-79 are shown in Table 6. It is apparent that metaphases were found most frequently in the shoot tips and that, starting from the youngest leaf, the number of metaphases decreased to zero in leaf 4. Neither in the shoot tips nor in the leaves of 13-3-2 diploid dividing cells could be detected. In clone 839-79 diploid dividing cells were found in the second and third leaf but not in the shoot tips. Apparently, in these monohaploids, chromosome doubling does not occur in the meristematic tissue of the shoot tip and the youngest leaves, but may occur in the more differentiated older leaves.

Table 6. Ploidy level of metaphase cells in different parts of *in vitro* grown shoots.

Clone	Plant part	Number of cells in metaphase	Percentage 1x	Percentage 2x
13-3-2	top	40	100	0
	leaf 1	40	100	0
	leaf 2	30	100	0
	leaf 3	5	100	0
	leaf 4	0	-	-
Total and means		115	100	0
839-79	top	40	100	0
	leaf 1	40	100	0
	leaf 2	30	90	10
	leaf 3	5	60	40
	leaf 4	0	-	-
Total and means		115	96	4

Discussion

Since cells can be in different stages of the cell cycle, interpretation of the DNA-histograms is rather complex. The 2C-peak may consist of mitotic monohaploid cells in G2, mitotically active reduplicated cells in G1 or non-mitotically active reduplicated cells (apparently differentiated G0-cells)(van't Hof & Kovacs, 1972; Evans & van't Hof, 1974; Galbraith et al., 1983). The 4C-peak may contain mitotic reduplicated cells in G2, mitotic double-reduplicated cells in G1 or non-mitotic double-reduplicated cells. Despite the lack of knowledge about the mitotic state of the 2C and 4C nuclei, one should be aware of the presence of a considerable number of 2x nuclei when working on protoplasts of monohaploid material. The differentiated doubled nuclei that are present in the starting material may have a selective advantage in being triggered to mitosis, as was demonstrated in leaf explants by Pijnacker et al. (1986). When the lead of mitotic activity of doubled nuclei is enlarged at the moment of shoot regeneration, the first regenerated plants will be diploid or tetraploid. If this is the case indeed, special attention should be paid to the slower developing and later regenerating calli.

By measuring the DNA-distribution at different dates during the year, it became clear that some genotypes may show a seasonal variation in DNA-distribution. In order to obtain unambiguous results, tests in several successive years are necessary. Nevertheless the results obtained during one year showed higher C-values during the winter season. One has to be aware of this when comparing DNA-distribution patterns obtained in different periods of the year.

Comparing plant material grown in the greenhouse with that cultured in vitro only minor differences can be seen. Yet, some conclusions can be drawn. For the in vitro plants, the tops of which are used for DNA-measurements, a considerable part of the 2C-peak can be ascribed to mitotically active monohaploid cells in G2. For the plants grown in the greenhouse of which some young expanded leaves were taken for DNA-measurements, the 2C-peak will mainly consist of reduplicated nuclei since this material contains hardly any dividing cells. The presence of

doubled nuclei may be due to the fact that differentiation often is involved with polyploidization (Nagl, 1978). So, working on monohaploid potato, it is better for protoplast isolation to use *in vitro* grown plants which are less differentiated.

The difference in variation in DNA-distribution before and after protoplast isolation may be explained, by assuming that cells with nuclei in G2-phase (2C or 4C) and/or endoreduplicated nuclei (2C or 4C) are more difficult to isolate or are selectively broken down by technical damage. The latter is in line with the observation of Meadows (1982) that protoplasts in G2 are selectively lost during the first three days of culture.
The shift may also be explained by assuming inhibition of DNA-synthesis during enzyme incubation. In this case 1x and eventually 2x cells in late G2/M (2C and 4C resp.) pass through mitosis to G1 (1C and 2C resp.). This hypothesis may be supported by the data presented in Fig. 2. Clone 13-3-2 containing no diploid dividing cells (Table 4) shows a large 2C-peak probably existing of 1x nuclei in G2/M and 2x nuclei in G1 and a small 4C-peak of 2x nuclei in G2 (Fig. 2a). After enzyme incubation the nuclei in G2/M may have passed through mitosis to G1 resulting in a disappearance of the 4C-peak and a large reduction of the 2C-peak despite the fact that the 2C-peak was also 'filled' with 2x nuclei passing through mitosis (Fig. 2b). Leaf material of clone 839-79 containing metaphases with 24 chromosomes (Table 4) also showed a larger 4C-peak (Fig. 2c) than 13-3-2 resulting, after enzyme incubation (Fig. 2d), in a smaller decrease of the 2C-peak. The 2C-peak is 'filled' with relatively more 2x nuclei passing through mitosis.

It has been shown that enzyme incubation and protoplast purification may both affect the distribution of DNA. This hampers the interpretation of the DNA-histograms. Profound cytological research is necessary to obtain more detailed information about the constitution of the nuclei in the leaves.

Karp *et al.* (1984) and Tempelaar *et al.* (1985) found that their monohaploid material was subject to instability of ploidy level. However, we

investigated a large collection of monohaploid clones and found that all clones could be stably propagated and maintained both *in vitro* and *in vivo*, on the monohaploid level for at least two to three years. The only example of polyploidization of dividing cells in this study was the occurrence of diploid cells in young leaves of 839-79.

Chapter 5.

Relative performance of monohaploid potato clones and their diploid parents at plant level and after protoplast isolation and subsequent fusion.

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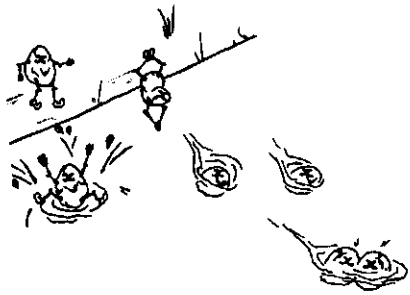

Summary

Plant growth performance was studied in 118 potato monohaploids and in their diploid parents. Of these monohaploids 76 were also investigated at the protoplast level and eight of these were used in protoplast fusion experiments as well.

No correlation was found between relative performance of greenhouse grown and *in vitro* grown plants. No or only weak correlations were found between different *in vitro* characteristics such as plant growth, protoplast yield per gram plant material, plating efficiency and callus growth. This indicates the unpredictability of these characters.

The protoplast fusion experiments indicated that only in some genotype combinations increased callus growth rates may be found. However, it is not clear whether such calli were hybrids or not. In protoplast monocultures only diploid and tetraploid regenerants were obtained. After fusion, tetraploids but also some triploids could be regenerated. The finding of triploids indicates that monoploid protoplasts were involved in fusion. Isozyme analysis and morphological assessment of the plants pointed out that the majority of the fusion regenerants was hybrid. The implications of these results are discussed.

Introduction.

The performance of plants is genotype dependent. Research in potato has made clear that also tissue culture ability is genetically determined and can be transferred after crossing to progeny plants (Wenzel *et al.*, 1979; Uhrig, 1985). Knowledge about the relation between relative performance of genotypes under greenhouse and under *in vitro* conditions is poor. Such knowledge is of great importance since , in general, plant material is tested under greenhouse conditions first, before it is used for *in vitro* experiments. In this study five diploid clones have been screened for their relative performance at the plant level in the greenhouse and at different levels of *in vitro* culture. In addition the relative performance of these diploids was compared with that of their gynogenetic monohaploid descendants (=gametic sample) in order to test the quality of their gametes.

The relative performance of monohaploids in the greenhouse and at different levels of *in vitro* culture has been investigated.

So far, only little report has been made about protoplast culture experiments in monohaploid potato. Regenerants obtained from monohaploid potato derived protoplasts, all appeared to be tetraploids (Sree Ramulu et al., 1986; Uijtewaal et al., 1987b) and the only fusion experiment described resulted in tetraploid hybrids. In this study protoplast isolation and culture have been investigated of a large number of monohaploids and from some of them plants could be regenerated. In addition fusion experiments between monohaploids will be described. Since a better regeneration capacity of hybrid fusion products was observed in such combinations (Uijtewaal et al., 1987b) diploid, triploid and tetraploid hybrid regenerants could be expected. If this better regeneration capacity after fusion of monohaploids would be generally valid, then a useful selection criterion for hybrids would be available. In this study regenerants obtained after fusion of monohaploids were screened for hybridity using isozymes as genetic markers.

Materials & Methods.

Plant material

118 monohaploids were investigated at plant level and 76 of them also at protoplast level (Table 1). They originated from five different (groups of) diploid Solanum tuberosum L. or S. tuberosum x S. phureja Juz. et Buk. hybrids:

i) M9 is a diploid hybrid, partly derived from S. phureja.

ii) The parthenogenetic diploid (PD) clones are derived from cv. Gineke

iii) Clone LGM-1 is a cross product of dihaploids of cv. Libertas (L) and cv. Gineke (G), and the diploid hybrid M9.

iv) The clones MGPG-2; -3 and -12 are cross products of M9 and clone GPG. The latter is derived from (G609xS. phureja)xG609, where G609 is a dihaploid from cv. Gineke.

v) 7322 is an androgenetic monohaploid derived from the diploid clone H78.01. It has been obtained at the Max Planck Institute, Cologne, FRG.

The detailed ancestry of the first four (groups of) genotypes has been presented in Uijtewaal et al. (1987a); that of 7322 in De Vries et al. (1987). The origin and number of monohaploid genotypes studied on plant level and in protoplast experiments have been included in Table 1.

Table 1. Source and number of gynogenetic g) and androgenetic a) monohaploids investigated on plant level and in protoplast experiments.

Diploid source	Monohaploids	Investigated number on plant level	Investigated number in protoplast experiments
M9	839-..;849-..[g]	36	9
PD23-2	851-..[g]	52	52
PD23-26	854-..[g]	16	6
PD23-28	852-..[g]	7	2
LGM-1	855-..[g]	4	4
MGPG-12	8522-..[g]	2	2
H78.01	7322[a]	1	1
Total		118	76

The plant material was cultured *in vivo* in 24cm pots in the greenhouse under normal daylight conditions and temperatures varying from 20-30°C and *in vitro* in 15cm high glass tubes or in 9cm high glass containers *in vitro*, on solidified MS-medium with 2% sucrose (25°C, 16h daylight, 7000 Lux). Plants grown in glass tubes were used for determination of relative vigour; those in glass containers were used for phenotypic classification and protoplast isolation.

The relative vigour is expressed in a scale from one to ten. For the greenhouse grown plants it was calculated from the height in cm (h) after 3-4 months of growth and the plant age in days (a), according to the formula (10xh/a). Dependent on the degree of branching a value of at most two scale units was added to the calculated vigour. For the *in vitro* grown plants, relative vigour was calculated from the parameters h = height in cm after about one month of growth, la = leaf area as measured with a photo-electric area meter, both parameters being related to a = the age in days of the plants when the assessments were made. The calculation was according to the formula (H+LA)x0.5, where H = 2xh/a and LA = 0.4xla/a.
The phenotypic classification is shown in Fig. 1.

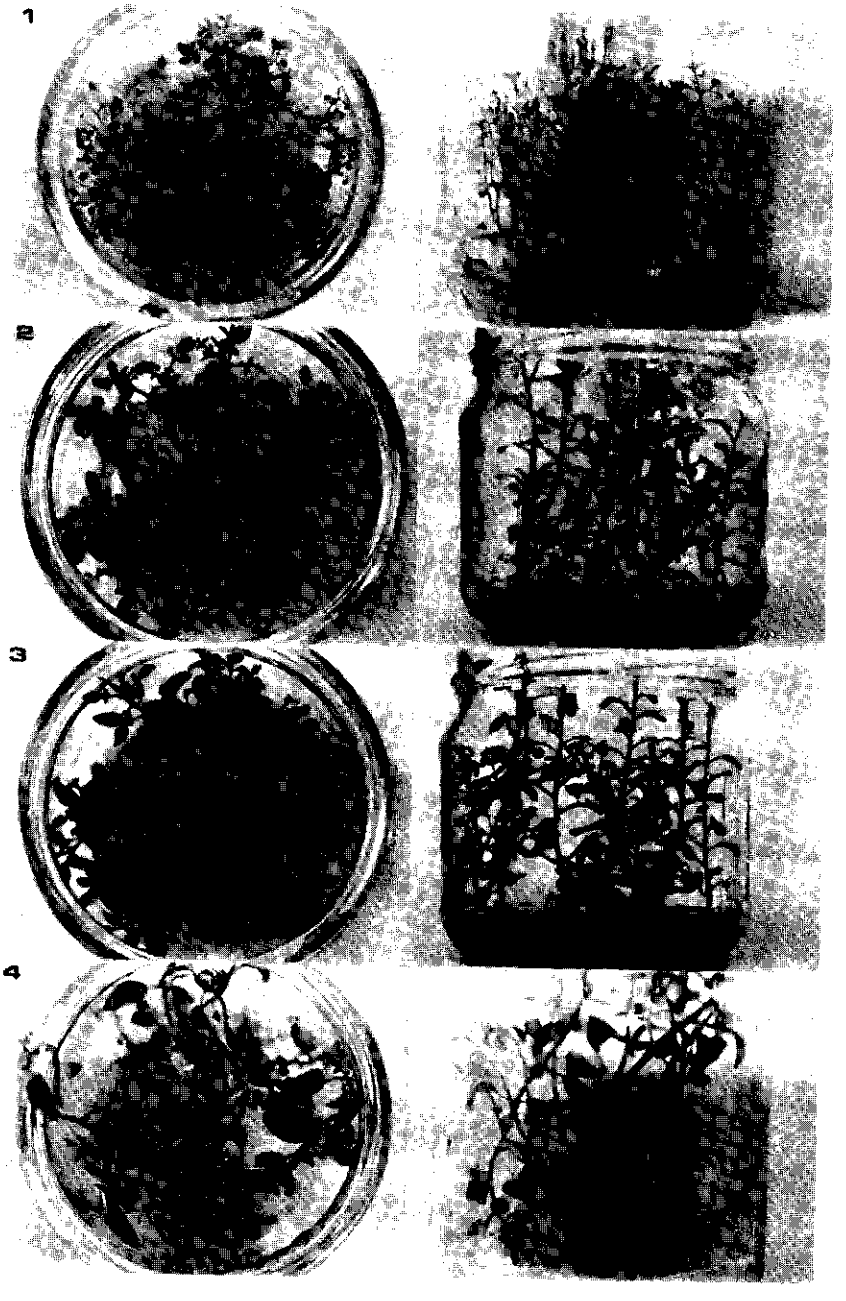

Fig. 1. Phenotypic classification (1=small leaves, thin stems; 4=big leaves thick stems) of *monohaploids* grown in shoot cultures in glass containers.

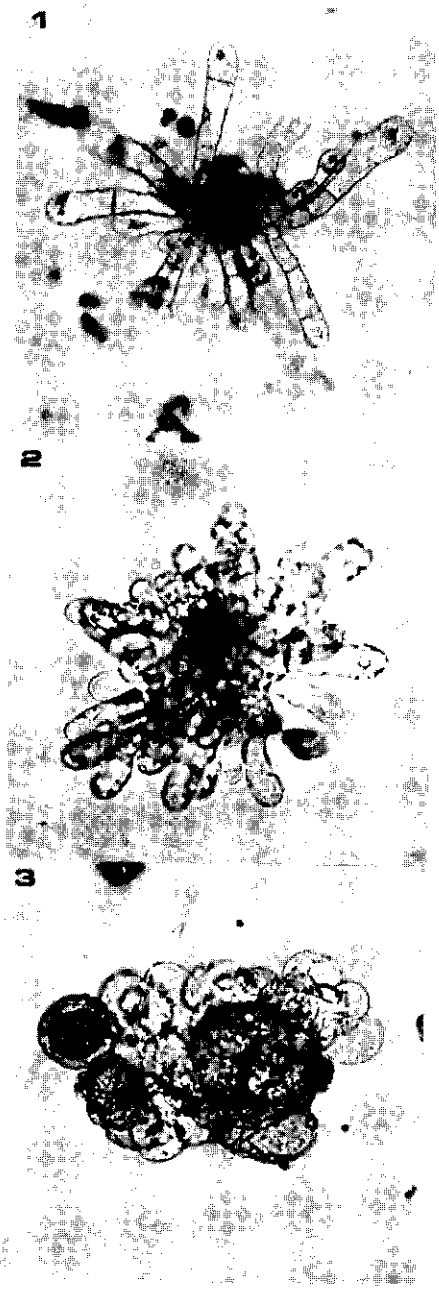

Fig. 2. Growth type classification of calli (1=loose; 3=compact) derived from protoplasts of monohaploid clones.

Protoplast isolation and culture

Protoplasts were isolated from four weeks old axenic shoots. The procedures for protoplast isolation, culture and plant regeneration were according to Uijtewaal et al. (1987b).

The yield of protoplasts was determined per gram plant material. The plating efficiency was calculated as the percentage of calli formed per number of cultured protoplasts. The callus growth was determined visually two months after protoplast isolation by estimation of the callus size, and classified from 0 to 4. All observations were carried out twice. The callus growth type classification is shown in Fig. 2 (loose, loose/compact and compact).

Protoplast fusion

Protoplast fusion was carried out according to Uijtewaal et al. (1987b). All vital protoplasts still present after the fusion procedure (starting with $5x10^5$ protoplasts of each clone) were cultured in 6cm Petri dishes. Depending on the number of calli formed, the cultures were diluted in 9cm Petri dishes to final colony concentrations of ca. 400 per Petri dish, i.e. ca. 30 colonies per ml.

Ploidy level determination in regenerants

Regenerants were checked for their ploidy level by counting the number of chloroplasts in the guard cells according to Frandsen (1968), and the number of chromosomes in the root tip cells according to Henderson and Lu (1968).

Isozyme analysis

For control of hybridity of the fusion products the isozymes 6PGDH and MDH were used. Sample preparation, polyacrylamide gel electrophoresis and staining procedure were according to Suurs and Jongedijk (1987).

Results

Genotype comparison for relative performance in the greenhouse and in shoot culture.

Large differences in performance, both in the greenhouse and in vitro, were detected among monohaploids originating from one diploid and

between groups of monohaploids originating from different diploids. In Table 2 the data are given for the diploid parental clones themselves as well as for their monohaploid derivatives.

Table 2. Data showing the greenhouse and in vitro performance of five different diploid clones and their monohaploid derivatives. n=Number of monohaploid genotypes. The greenhouse data are the means of two measurements carried out during one season; the in vitro data are the means of three measurements carried out during two years of shoot tip propagation; standard deviations in parentheses. r=Coefficient of correlation for greenhouse and in vitro performance of individual monohaploids; p=one-sided critical level.

Diploid source	n	Greenhouse			In vitro			Coeff. of correlation
		Max.	Min.	Mean	Max.	Min.	Mean	
M9	-			14			8	
M9	36	9	1	4.9(2.4)	6	2	3.6(1.5)	r= .11;p=.39
PD23-2	-			10			2	
PD23-2	52	9	1	5.2(1.9)	7	2	3.7(1.3)	r= .03;p=.42
PD23-26	-			10			3	
PD23-26	16	9	1	5.0(2.7)	9	2	4.2(1.6)	r= .31;p=.13
PD23-28	-			10			3	
PD23-28	7	8	1	4.1(3.2)	4	2	3.0(0.8)	r=-.26;p=.21
LGM-1	-			14			no data	
LGM-1	4	3	1	2.0(0.8)	9	3	6.3(2.5)	r=-.16;p=.42
	115			4.9(2.2)			3.8(1.4)	r= .00;p=.48

It is apparent that the diploids M9 and LGM-1 have a better greenhouse performance than PD23-2, PD23-26 and PD23-28. M9 exceeded the PD-clones also under in vitro conditions. Unfortunately LGM-1 got virus infected and had to be removed before the in vitro performance could be determined. The largest variation (1-9) in relative greenhouse performance between monohaploids originating from one diploid was found for the monohaploids originating from M9, PD23-2 and PD23-26, and the smallest (1-3) for the monohaploids from LGM-1. The mean relative greenhouse

performance was similar for the monohaploids from M9 and PD23-2, somewhat lower for the monohaploids from PD23-28 and much lower for those from LGM-1. The latter is in contrast with the results from the *in vitro* grown plants, where the mean performance of the monohaploids of LGM-1 exceeded that of the other diploids. No correlation was found between the growth performance of individual monohaploids in the greenhouse and *in vitro* ($r=0.00$; $p=0.48$; $n=115$).

Genotype comparison for protoplast culture ability.

All genotypes tested yielded protoplasts that started to divide within six days of culture. However, the number of protoplasts obtained per gram plant material varied greatly (Table 3). Large genotypic variation could be detected for the plating efficiency (PE: $0\text{-}3.10^{-3}$) and the callus growth rate (CGR).

Table 3. Genotype comparison for protoplast culture ability of five diploid clones and their monohaploid derivatives. P=mean relative in vitro performance of the monohaploids. n=Number of monohaploids; Callus growth was classified from 0 to 4. Standard deviations in parentheses. n.d.=Not determined.

Diploid source	P	n	Protoplast yield ($x10^5$)	Plating efficiency ($x10^{-3}$)	Callus growth	No. of regenerants
M9	-	-	5	3.0	3	1
M9	4.3	7	7.7(3.1)	1.5(1.5)	2.9(1.1)	3
PD23-2		-	15	1.0	3	-
PD23-2	3.7	52	7.7(4.2)	1.5(1.5)	2.8(1.0)	6
PD23-26		-	11	1.0	3	-
PD23-26	5.0	6	9.2(3.8)	0.5(0.5)	2.4(0.9)	-
LGM-1		-	n.d.	n.d.	n.d.	n.d.
LGM-1	6.3	4	7.5(5.3)	0.5(0.5)	2.3(1.5)	-

It is apparent that the relatively vigorous diploid M9 had a much lower protoplast yield per gram plant material than PD23-2 and PD23-26. This can be due to the thicker stems of M9 as compared with the other diploids. The monohaploids from PD23-2 had a better general performance for PE than their diploid parent. Monohaploids from M9 and PD23-26 showed an equal or lower performance than their diploid parents. The callus growth rates of monohaploids and their respective diploid parents were comparable. Within each group of monohaploids individual genotypes were detected with an equal or better plating efficiency and callus growth than their diploid parent. Plants could be regenerated out of protoplast derived calli, of three monohaploids from M9 (43%) and of six monohaploids from PD23-2 (12%). This difference in regeneration percentage suggests a genotypic influence, but careful consideration of these results is required since the number of regenerants is low.

Within the group of monohaploids from PD23-2 coefficients of correlation have been calculated between the different plant and cell growth characteristics (Table 4). It is apparent that no correlation was found between plant growth in the greenhouse and different characteristics of in vitro growth ($p>0.25$). Also, no correlation was found between shoot culture growth (including leaf area) and the yield of protoplasts per gram plant material ($p>0.25$). The relatively high correlation between plant vigour in vitro and growth type ($r=0.74$; $p=0.00$) was expected, since the latter was more or less dependent on the personal interpretation of the former. Small but significant correlations were found between the protoplast yield and plating efficiency ($r=0.29$; $p=0.02$), between plating efficiency and callus type ($r=-0.39$;$p=0.01$) and between callus type and callus growth ($r=0.34$; $p=0.02$). Also significant correlations were found between the callus growth and the in vitro plant vigour ($r=0.31$; $p=0.02$) and plant growth type ($r=0.42$; $p=0.00$).

Table 4. Coefficients of correlation (r) between different levels of plant and cell growth calculated for 52 monohaploids originating from the diploid clone PD23-2. n=Number of plants; p=one-sided critical level. Growth type and callus type classification as shown in Fig. 1 and 2 respectively.

	1)Growth in vivo	2)Growth in vitro	3)Growth type	4)Yield protopl.	5)Plat. eff.	6)Callus type	7)Callus growth
2)	r=.03 (n=52) p=.42						
3)	r=0.01 (n=52) p=.48	r=.74 (n=52) p=.00					
4)	r=.07 (n=52) p=.32	r=-.09 (n=52) p=.25	r=-.02 (n=52) p=.43				
5)	r=-.05 (n=49) p=.37	r=.66 (n=49) p=.33	r=-.09 (n=49) p=.27	r=.29 (n=49) p=.02			
6)	r=-.06 (n=44) p=.35	r=.19 (n=44) p=.11	r=.22 (n=44) p=.08	r=-.08 (n=44) p=.31	r=-.39 (n=44) p=.01		
7)	r=.11 (n=42) p=.25	r=.31 (n=42) p=.02	r=.42 (n=42) p=.03	r=-.08 (n=42) p=.31	r=-.12 (n=42) p=.23	r=.34 (n=42) p=.02	

Protoplast fusion

Fusion experiments have been carried out with 8 relatively vigorous monohaploid clones. In all fusion combinations the monohaploid 7322 was one of the fusion partners, because (i) protoplasts of monohaploid 7322 could not divide under the standard culture conditions used, and

(ii) 7322 could be distinguished from the other monohaploids by its banding pattern for at least one of the isozymes used. In Table 5, the protoplast culture results of the monohaploids in monoculture (controls) and after fusion with 7322 are shown. From six out of the seven combinations plants were obtained; in three out of these six, plant regeneration did not occur in monoculture. It is apparent that in the combinations 7322 with 849-30 and 8522-9 plant regeneration occurred much sooner than in the monocultures.

Table 5. Data of 8 monohaploid clones for callus growth (scale 1-9), shoot regeneration time in weeks (regen.) and ploidy level of the regenerants (ploidy), in protoplast monocultures and after protoplast fusion. n=Number of regenerants.

Clone	Monoculture				After fusion with 7322			
	callus	regen.	ploidy	n	callus	regen.	ploidy	n
7322*	0	24	4x	10	-	-	-	-
839-79	4	30	4x	10	3	0	-	-
849-30	3	25	2x/4x	10	3	12	4x	20
855-1	1	0	-	-	4	19	4x	2
855-2	3	0	-	-	3	16	3x/4x	4
855-3	1	0	-	-	3	16	4x	8
8522-9	3	14	2x/4x	10	4	7	4x	20
851-22	4	24	2x/4x	10	3	20	4x	4

* Protoplasts of 7322 did not start first cell divisions under normal auxin concentrations (0.2mg/l 2,4D and 1mg/l NAA) but did so in the presence of high auxin concentrations (0.2mg/l 2,4D and 6mg/l NAA) during the first 12 days of culture until a multicellular stage was obtained. After that, the high auxin concentration started to become toxic and had to be lowered to a concentration as used for the other genotypes after 12 days of culture (0.1mg/l 2,4D and 0.5mg/l NAA). Callus culture and plant regeneration were carried out under the same conditions as used for the other genotypes.

The ploidy level of the plants regenerated from monocultures of different monohaploids was 4x or 4x and 2x. Also with 7322, cultured under other conditions (see foot-note Table 5), only tetraploid regenerants were obtained. Some of the regenerants were screened for isozyme banding pattern but did not show any differences with the original monohaploid parent.

The ploidy level of the regenerants of five different fusion combinations was 4x, but from the combination 855-2 with 7322 also 3x regenerants were found (Table 5). The latter must have originated from either homo- or heterofusion.

Isozyme analyses of all first regenerants from the five combinations mentioned showed the hybrid banding pattern. For the combinations 7322 with 8522-9 and 855-2 also homozygous regenerants were found, after 17 and 19 weeks respectively. Calli tested for banding pattern for 6 PGDH also showed the homozygous banding type. In no fusion experiment homozygous 7322 calli or regenerants were obtained. In Fig. 3 the determination of the hybrid character of a regenerated plant in two particular combinations compared with that of the parents is shown.

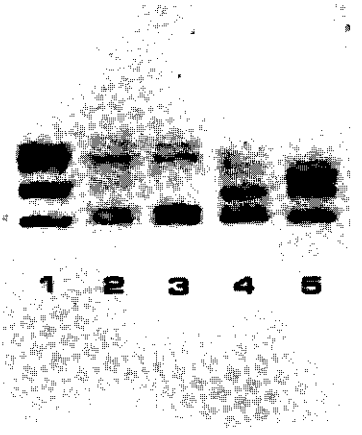

Fig. 3. 6PGDH banding patterns of parental clones and regenerants after protoplast fusion. 1=849-30; 2=7322+849-30; 3=7322; 4=7322+8522-9; 5=8522-9.

Morphological determination of a sample of the regenerants of the fusion 7322 with 849-30 and 8522-9 showed that all regenerants had intermediate leaf hairiness and flower colour; all showed a better greenhouse growth performance and male fertility than the homozygous tetraploid counterparts.

Discussion

Uijtewaal *et al.* (1987a) already pointed out for greenhouse plant vigour that for obtaining vigorous monohaploids not only the monohaploid production ability of a diploid, but also the genetic basis of its vigour is of importance. In other words, a vigorous diploid is not always able to produce relatively vigorous monohaploids when its vigour is mainly based on heterozygosity. In this paper it is shown that it also holds true for *in vitro* plant growth (Table 2). Furthermore no correlation was found between relative performance of greenhouse grown and *in vitro* grown plants. This has implications for the technique to be used for monohaploid production. Such a consideration should be based, not only on practical considerations, but also on the research objectives with the monohaploids obtained. So, it may be better to use the prickle pollination technique (*in vivo*) when practical breeding or gene dosage research with homozygous plants is the main objective. On the other hand, it may be recommendable to use the anther culture technique to produce monohaploids for *in vitro* research. In both cases it will be better to use both techniques in order to obtain monohaploids from sufficiently various diploid breeding material.

The fact that no correlation could be detected between *in vivo* plant growth, *in vitro* plant growth and the yield of protoplasts per gram plant material, and only weak correlations were found between the other *in vitro* growth characteristics underlines the unpredictability of these characters. The weak correlation found between *in vitro* plant growth and callus growth ($r=0.31$; $p=0.02$) and between plant growth type and callus growth ($r=0.42$; $p=0.03$) suggests that anther culture, which includes a callus phase, may imply a positive selection for *in vitro* performance. The weak positive correlation between the yield of protoplasts per gram plant material and the plating efficiency ($r=0.29$;

p=0.02) may be genotypically determined but may also be a result of some co-protection or mutual stimulation during the protoplast isolation procedure. The weak negative correlation between plating efficiency and callus growth type (=compactness) suggests that the latter is a purely environmental effect based on mutual competition in the first weeks of culture. However, there appeared to be a weak positive correlation between the compactness of the calli and the callus growth rate. The lack of a negative correlation between plating efficiency and callus growth rate excludes the possibility that the callus growth rate is purely an effect of the lower number of calli developing in the first weeks of culture.

The protoplast fusion experiments indicated that only in some genotype combinations increased callus growth rates were found. However, it is not clear whether such calli were hybrids or not.
It has already been mentioned in literature that starting with monohaploid material the majority of explant or protoplast derived shoots are diploid or tetraploid and only rarely octoploid (Karp et al., 1984; Tempelaar et al., 1985; Sree Ramulu et al., 1986). This suggests that the diploid and the tetraploid level are near to optimal for plant regeneration under the culture and regeneration conditions applied. Probably this is also the case for the material and culture conditions discussed here. The in vitro culture period was probably too long to enable monohaploid regeneration.
This may also explain the regeneration of mostly tetraploid plants after protoplast fusion. Presuming, in the population of freshly isolated protoplasts, a ratio 1C:2C of 2:1 with a neglectable percentage of 4C (Uijtewaal, 1987) and an equal chance to take part in fusion for both, the number of C+C fusions will be four times higher than the number of 2C+2C fusions. This suggests that at least part of the tetraploid hybrids are the result of somatically doubled C+C fusion products. The appearence of some triploid regenerants, probably originating from 2C+C fusion products, suggests that the triploid level is stable enough to pass the callus phase without doubling. The only possibility to check for the occurrence of multiple fusions (C+C+C or C+C+C+C) to result in triploids or tetraploids is to use three or more

fusion partners, all with a different dominant marker, presuming the regeneration percentage is high enough to detect the multiple hybrids. In these experiments the majority of the fusion products appeared to be hybrids. This is remarkable since no such reports are known from literature. The fact that homozygous regenerants occurred later than the heterozygous hybrids and that a lot of calli tested showed the homozygous 6PGDH banding pattern suggests that after fusion the regeneration capacity of homozygous clones is influenced more negatively than that of heterozygous hybrids. This seems likely since homofusions carried out in other experiments showed a microcallus development comparable to that of unfused controls, but showed less and delayed plant regeneration.

Chapter 6

Morphology and vigour of monohaploid potato clones, their corresponding homozygous diploids and tetraploids and their heterozygous diploid parent.

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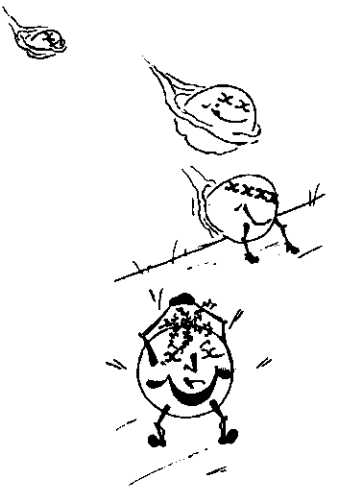

Summary

To study the joint effects of homozygotization and polyploidization in potato, the performance has been examined of five potato genotypes at three (x,2x,4x) and two genotypes at two (x,2x) ploidy levels. Six out of the seven genotypes studied were compared with their heterozygous diploid parental clone. In this way comparisons could be made between i) the heterozygous diploid and its monohaploid derivatives, ii) three or two ploidy levels per genotype and iii) homozygous di- and tetraploids and their heterozygous diploid source.

Large variation could be detected between monohaploids obtained from one diploid source. A striking increase in vigour was observed with somatic chromosome doubling from x to 2x, but less clearly from 2x to 4x. The relatively vigorous diploids showed a weaker response to tetraploidization than the less vigorous ones. The heterozygous diploid exceeded all homozygous di- and tetraploid derivatives in performance.

The results of this study suggest positive gene dosage effects for tuber production more than for leaf area and plant height. The observations on plant vigour in homo- and heterozygotes suggest that dominance effects are stronger than additive gene effects. Owing to sterility problems, homozygous potato clones will presumably be of little importance for practical breeding.

Introduction

In the autotetraploid potato homozygotization by repeated selfing is an extremely slow process: 27 generations of selfing are needed to reach 99% homozygosity. Furthermore severe inbreeding depression and decrease of fertility are common barriers to selfing for more than 3-5 generations. Therefore the pure effect of homozygotization can be studied to a limited degree only. Haploidization of a tetraploid has the same effect on homozygotization as three times selfing of that tetraploid and hence is accompanied by a considerable decrease in vigour and changes in performance regarding leaf shape, tuber production and yield (Peloquin & Hougas, 1958; v. Suchtelen, 1966; Gorea, 1970; Caroll & Low, 1975, 1976). Besides the effect of 4x to 2x haploidization, that of mitotic 2x to 4x doubling on relative performance and tuber yield has been studied by Frandsen (1967), Ross et al. (1967), Rowe (1967)

and De Maine (1985). In both directions the change in ploidy level is associated with a reduction in the degree of heterozygosity. This makes it hard to establish which proportion of the haploidization effects is due to the reduced ploidy level and which to the reduced level of heterozygosity.

The possibility of obtaining monohaploids from di(ha)ploids via gynogenesis or androgenesis opens up new perspectives. On the one hand this offers the opportunity to study the effect of haploidization of heterozygous diploids to monohaploids. On the other hand, pure polyploidization or gene dosage effects can be studied by comparing monohaploids with their corresponding homozygous diploids and tetraploids. In this paper attention is paid to both aspects by studying the relative performance of several monohaploids from one diploid clone, and by comparing the relative performance of the monohaploids, their heterozygous diploid parent and homozygous diploid and tetraploid derivatives of the monohaploids.

Materials and Methods

Homozygous di- and tetraploid potato clones have been obtained via adventitious shoot regeneration on stem explant cultures (Roest & Bokelmann, 1980) of one androgenetic and six gynogenetic monohaploids (Uijtewaal et al., 1987a).

Table 1. The diploid source parents M9 (Uijtewaal et al., 1987a) and H78.01 (De Vries et al., in prep), seven monohaploids and their corresponding ploidy series.

Source	Monohaploids	Ploidy series
M9	839-19	(x,2x,4x)
	839-45	(x,2x,4x)
	839-61	(x,2x)
	839-79	(x,2x)
	849- 7	(x,2x,4x)
	849-30	(x,2x,4x)
H78.01	7322	(x,2x,4x)

Table 1 summarizes the material used in this study. The clone 7322, of which the origin is fully described by De Vries et al. (1987a) was obtained at the Max Planck Institute, Cologne, West Germany.
All clones were propagated in vitro via shoot tip culture and after rooting transferred to the greenhouse at the end of January. The experiment includes 12 plants per genotype at each ploidy level. The plants were cultured in 24cm pots in the greenhouse under normal daylight conditions and temperatures varying from 20-30°C.
All observations, except tuber production, were carried out early July. For comparison of plant vigour, the parameters plant height, leaf area per leaf, leaf area per plant and tuber production were examined. Flower bud development and male and female fertility were examined to study the potential use of homozygous clones in crosses. For effects on plant morphology, length/width-ratio of the terminal and lateral leaflet and internode length were examined. For the determination of leaf area per leaf the mean value of the fifth and the sixth fully-grown leaves as measured with a photo-electric leaf area meter were taken. Pollen fertility was estimated by lactophenol acid fuchsine staining according to Sass (1964). Female fertility was tested by pollination of open flowers with mixtures of pollen from the S. phureja pollinator lines IvP48 and IvP101 (Hermsen & Verdenius, 1973; Hermsen, in prep.).

Results

The performance of six monohaploid clones relative to their common diploid parental clone M9 has been determined (Table 2). For comparison the absolute values of M9 were used. It is apparent that the ploidy change from diploid to monohaploid is accompanied by a striking loss of vigour. As expected, the monohaploids showed a significantly lower relative value for plant height (0.54), internode length (0.39), leaf area per leaf (0.10) and leaf area per plant (0.12) than the heterozygous diploid (1.00). Tuber production in terms of tuber weight per plant was almost completely lost. A small difference was found between M9 and the monohaploids for the number of leaves per plant. Within the group of monohaploids considerable variation could be detected for the characters investigated. It is apparent that for plant height the clones 849-7 and 839-61 were superior, a phenomenon which was only

Table 2. Performance of the heterozygous diploid M9 (part A) and relative performance (in relation to M9) of six monohaploid derivatives (part B) for nine quantitative characters. The standard deviation is given in parentheses. L/W=length:width.

Genotype	Plant height	Leaf area per leaf	Number of leaves	Leaf area per plant	L/W-ratio of the terminal leaflet	L/W-ratio of the lateral leaflet	Internode length	Frequency of tuber producing plants	Tuber weight per plant
A) M9	92.6(cm)	7963(mm^2)	65	5241(mm^2)	1.80	1.90	3.8(cm)	1.00	33.5(g)
B) 839-19 (x)	0.48	0.09	1.00	0.09	0.91	1.05	0.39	0.27	0.01
839-45 (x)	0.50	0.07	1.02	0.07	1.02	1.13	0.29	0.29	0.01
839-61 (x)	0.67	0.20	1.35	0.27	1.26	1.14	0.47	0.08	0.02
839-79 (x)	0.51	0.08	1.17	0.09	0.95	1.07	0.42	0.18	0.01
849- 7 (x)	0.65	0.07	1.05	0.08	0.87	0.98	0.42	1.00	0.10
849-30 (x)	0.44	0.07	1.40	0.10	1.04	1.07	0.32	0.00	0.00
Mean (x)	0.54(0.09)	0.10(0.05)	1.16(0.18)	0.12(0.08)	1.01(0.14)	1.07(0.06)	0.39(0.07)	0.30(0.36)	0.02(0.04)

partly due to a larger internode length. For leaf area per leaf as well as total leaf area per plant, 839-61 was superior. This was mainly due to a relatively large number of relatively big leaves. In general the length/width (L/W) ratio of the terminal leaflets was somewhat smaller than that of the lateral leaflet, again with the exception of clone 839-61.

The relative values for nine characters of mitotically doubled homozygous di- and tetraploids in relation to the original monohaploid (Table 3A) and the performance of these homozygous di- and tetraploids in relation to the heterozygous parental clone M9 (Table 3B) have been determined. It is apparent that the mean values of the homozygous clones showed hardly any increase by raising the ploidy level from 2x to 4x. In fact the homozygous 4x appeared, only in leaf area per leaf and tuber weight per plant, to be superior over the homozygous 2x plants. In general it can be said that, except for tuber weight, the increase in vigour from x to homozygous 2x is much larger than the increase from 2x to 4x. The relative increase in tuber weight per plant with raising ploidy levels from 2x to 4x was larger than the increase from x to 2x (Table 3 and Fig. 1). Except for the number of leaves per plant the heterozygous diploid parental clone had a better overall performance than the homozygous diploids and tetraploids. The most important effect of homozygotization was detected for leaf area per leaf and per plant and for tuber production (Fig. 1).

The terminal leaflet showed hardly any differences in L/W-ratio at different ploidy levels. However, for the lateral leaflet a negative relation between L/W-ratio and ploidy level was observed.

Observations on plants of the ploidy series of genotype 7322 showed the same tendencies (Table 4) as found for the polyploidized monohaploids of M9 (Table 3). Because of the absence of the diploid parental clone of 7322, its homozygous di- and tetraploids could not be compared with their own heterozygous source.

Table 3. Performance of homozygous di- and tetraploids relative to their original monohaploid (partA) and to their heterozygous diploid source M9 (partB) for nine quantitative characters. The standard deviation is given in parentheses. L/W=length/width.

Genotype		Plant height	Leaf area per leaf	Number of leaves	Leaf area per plant	L/W-ratio of the terminal leaflet	L/W-ratio of the lateral leaflet	Internode length	Frequency of tuber producing plants	Tuber weight per plant
A) 839-19	(2x)	0.90	1.24	1.18	1.45	1.02	0.81	1.27	0.74	1.00
	(4x)	1.11	2.30	0.94	2.13	0.89	0.68	1.87	1.85	12.67
839-45	(2x)	1.40	4.67	1.14	5.29	0.99	0.83	1.64	3.45	3.67
	(4x)	1.81	4.79	0.68	3.27	0.96	0.67	1.91	1.00	17.33
839-61	(2x)	1.46	1.38	1.05	1.44	0.91	0.78	1.11	1.14	0.33
839-79	(2x)	2.06	2.46	1.13	2.80	1.02	0.79	1.56	0.55	5.33
849- 7	(2x)	1.67	2.46	1.43	3.49	1.01	0.91	1.25	0.45	2.33
	(4x)	1.06	4.79	0.99	4.69	0.98	0.72	1.13	0.82	4.79
849-30	(2x)	1.53	2.43	0.70	1.71	0.91	0.90	1.25	0	0
	(4x)	1.72	1.99	0.89	1.79	1.06	0.87	1.92	0	0
Means	(2x)	1.51(0.38)	2.44(1.23)	1.11(0.24)	2.70(1.51)	0.98(0.05)	0.84(0.06)	1.35(0.21)	1.22(1.22)	2.11(2.08)
	(4x)	1.43(0.40)	3.47(1.53)	0.88(0.14)	2.97(1.31)	0.97(0.07)	0.72(0.11)	1.71(0.39)	0.92(0.76)	6.80(7.77)
B) Means	(2x)	0.82(0.26)	0.21(0.08)	1.26(0.18)	0.26(0.12)	0.98(0.09)	0.89(0.05)	0.55(0.13)	0.31(0.37)	0.05(0.09)
	(4x)	0.73(0.15)	0.26(0.10)	0.98(0.23)	0.24(0.08)	0.93(0.14)	0.76(0.13)	0.61(0.11)	0.39(0.35)	0.19(0.20)

Table 4. Relative performance of the genotype 7322 on the homozygous diploid and tetraploid level in relation to its monohaploid origin for nine quantitative characters. L/W=length/width.

Genotype	Plant height	Leaf area per leaf	Number of leaves	Leaf area per plant	L/W-ratio of the terminal leaflet	L/W-ratio of the lateral leaflet	Internode length	Frequency of tuber producing plants	Tuber weight per plant
7322 (2x)	1.43	2.12	1.42	3.04	1.08	0.91	1.11	0.91	0.06
(4x)	1.43	2.73	0.98	2.69	1.12	0.75	1.22	1.81	0.41

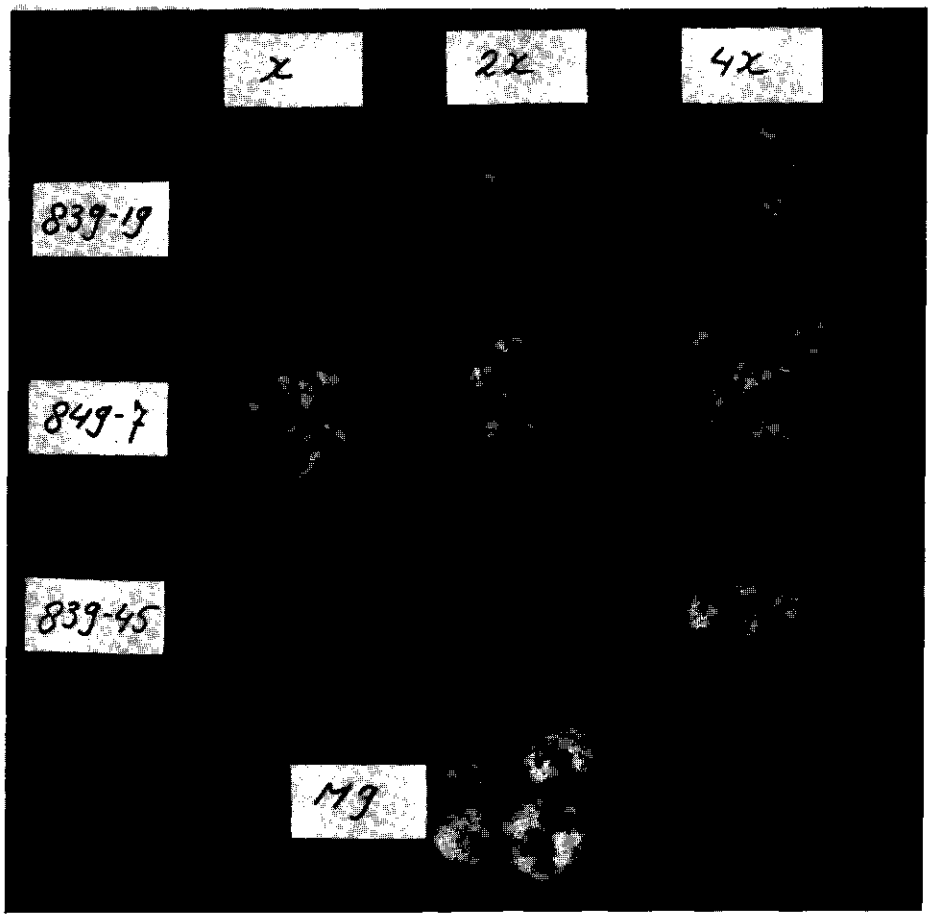

Fig. 1. Mean tuber production per plant of three different genotypes at three ploidy levels and of their heterozygous diploid parent M9.

All clones produced flower buds, and three of them produced open flowers. Pollen stainability of the homozygous clones was very low: 25% for 849-30(2x), 10% for 7322(2x) and 15% for 7322(4x), whereas the heterozygous diploid M9, cultured under the same conditions as the homozygous clones showed 85% stainability. Female fertility of these open flowers was tested by pollination. Pollination of about 100 flowers of 7322(2x) gave two berries containing 15 seeds in total. These seeds gave rise to diploid hybrid plants. Pollination of tetraploid 7322 (50 flowers), and diploid 849-30 (ten flowers) did not give any berry set.

Discussion

Although this study has been carried out in only one season, tendencies in the relative performance of potato plants can be indicated.

It appeared that reducing the ploidy level from diploid to monohaploid has a dramatic effect on plant growth performance and tuber formation. Similar effects were found for the change from the tetraploid to the diploid level by Peloquin and Hougas (1960). In the present experiment the extent of this effect was clearly dependent on the parameter studied. Compared to the diploid parental clone, plant height and internode length decreased with 46 and 61% respectively. The decrease was much stronger for leaf area per leaf and per plant and for tuber production, 90%, 88% and 98% respectively, whereas the number of leaves per plant and their L/W-ratio remained more or less the same.

The six monohaploids derived from M9 represented a gametic sample visualized on the plant level. The variation observed within the group of monohaploids is a direct consequence of the heterozygosity of M9 and hence of the variation of its gametal genotypes. The monohaploid clone 839-61 showed the smallest decline in plant growth performance, yet tuber production was low.

Comparison of the monohaploids with their mitotically doubled and twice doubled homozygous counterparts showed that this pure polyploidization effect strongly varies per genotype. For some genotypes the optimal ploidy level, in terms of plant height or leaf area, seemed to be the diploid, and for others the tetraploid level. No or only minor differences in growth performance, including tuber production, were found by Rowe (1967) and De Maine (1985) when comparing heterozygous diploids with their mitotically doubled counterparts.

There is an overall increase in mean performance with raising the ploidy level from x to 2x. Yet, hardly any increase and sometimes even a decrease in performance was detected for the polyploidization step from 2x to 4x, except for tuber production. This suggests positive gene dosage effects for tuber production. All genotypes showed a reduction of L/W-ratio of the lateral leaflet with raising ploidy level. This is in agreement with the data presented by Van Breukelen *et al.* (1975). However, large variation could be detected for L/W-ratio of the lateral leaflets of the monohaploid genotypes, i.e. 2.17 to 1.86. L/W-Ratio of

the terminal leaflet did not show any relation with ploidy level. The fact that the heterozygous diploid M9, except for the number of leaves per plant, exceeds all homozygous di- and tetraploids in performance, suggests that for these combinations of alleles heterozygotization is more important than polyploidization. In other words dominance effects may have a larger influence on performance than additive or gene dosage effects.

Fertility was very low in the doubled and twice doubled homozygous clones. Only in the diploid 7322 some female fertility was detected. This strongly limits the use of homozygous clones in crossing schemes. It may also imply that protoplast fusion will be necessary to obtain homozygous triploids and the production of a homozygous trisomic series may not be possible by crossing a homozygous triploid with a corresponding homozygous diploid.

In further analytic breeding research, apart from the polyploidization effect as described in this paper, numerous heterozygotization effects can be studied by the combinations of genomes of 2, 3 or 4 different monohaploids. If a sufficient level of fertility is available, a number of these genome combinations may be obtained by crossing. For other combinations protoplast fusion will be necessary. This means that for this material an efficient protoplast fusion system and a suitable detection method for hybrid fusion products are needed. Isozymic markers and preferential plant regeneration of hybrid fusion products may offer some opportunities (Uijtewaal et al., 1987b). However, ploidy instability as determined in leaf material of protoplast source plants (Uijtewaal, 1987) and after plant regeneration from protoplast derived calli (Uijtewaal et al., 1987c) may still be a critical factor.

Chapter 7

Algemene samenvatting en discussie

Aan het werken met monohaploide aardappellijnen zijn een aantal potentiële voordelen verbonden boven het werken met heterozygote di- en tetraploiden. De basis van deze voordelen is het in enkelvoud voorkomen van alle chromosomen. Dit heeft enerzijds tot gevolg dat zowel recessieve als dominante allelen worden blootgelegd en eventueel kunnen worden uitgeselecteerd. Anderzijds vormen monohaploiden de enige mogelijkheid tot het verkrijgen van homozygote lijnen. Het belang hiervan voor de aardappelveredeling wordt belicht in hoofdstuk 1. De succesvolle productie van monohaploiden en de inherente nieuwe mogelijkheden van monohaploidie brachten aardappelonderzoekers tot het opstellen van het theoretische schema, dat is weergegeven in Fig. 1 op blz 14. Aan de hand van dit schema zullen alle knelpunten met betrekking tot het gebruik van monohaploiden worden aangegeven. Het hier gepubliceerde onderzoek diende o.a. om te toetsen of dit schema voor de aardappel een reële optie is voor een nieuwe veredelingsmethode alsook om te onderzoeken wat de directe gevolgen zijn van monoploidie en homozygotie bij een normaliter zeer heterozygoot gewas als de aardappel.

De eerste hindernis die genomen moest worden was de grootschalige productie van monohaploiden. Het onderzoek was hierbij gericht op gynogenese, enerzijds omdat aan androgenese (antherencultuur) reeds door verschillende groepen werd gewerkt en anderzijds omdat op het IvP ruime ervaring betreffende gynogenese (prikkelbestuiving) aanwezig was. Er is bij dit onderzoek (hoofdstuk 2) uitgegaan van diploide Solanum tuberosum L. en S. tuberosum x S. phureja Juz. et Buk. hybriden als maternale ouders en geselecteerde S. phureja klonen als prikkelbestuivers met kiemstip (en 'nodal band') als homozygoot-dominante embryo (en plant) merker. Uit meer dan 2 miljoen zaden werden de kiemstiploze zaden geselecteerd, waaruit uiteindelijk meer dan 500 monohaploide planten werden verkregen en wel uit vijf genetisch verschillende groepen van maternale ouders. Het is hierbij gebleken dat frequentie en groeikracht van de monohaploiden duidelijk afhangen van het genotype van de ouder. Ook bleek de monohaploidenfrequentie te worden beinvloed door het genotype van de bestuiverlijn, de fysiologische conditie van de moederplant en het milieu. Hoewel in principe monohaploiden op grote schaal geproduceerd kunnen worden, is de invloed van het moederlijke

genotype op de productiefrequentie waarschijnlijk de beperkende factor voor de praktische toepassingsmogelijkheden van monohaploiden. Indien zou blijken dat androgenese en gynogenese ook genetisch gezien onafhankelijke processen zijn, -hetgeen waarschijnlijk is-, biedt een gecombineerde toepassing van beide technieken wellicht perspectieven voor verbreding van de genetische basis van monohaploiden.

Een volgende vraag was: kunnen uit monohaploiden protoplasten worden geisoleerd en gefuseerd en kunnen eventuele somatische hybriden worden geselecteerd en gedetecteerd? Aangezien er geen selectieve genetische merkers in het materiaal voorhanden waren, is onderzocht of het mogelijk is de heterozygote hybride fusieproducten te scheiden van de homozygote fusieproducten en van de ongefuseerde ouderprotoplasten op grond van een verschil in groeikracht op callusniveau. Het gebruik van isozym-merkers is onderzocht als mogelijkheid voor de detectie van hybride regeneranten. Hierbij is in een eerste experiment (hoofdstuk 3) uitgegaan van twee diploiden: M9 en PD23-2, de F1 hiervan en de populaties van monohaploiden van beide diploiden. De beide ouders en de F1 zijn onderzocht op hun banderingspatroon voor malaatdehydrogenase (MDH). Hierbij bleek dat M9 homozygoot was voor een MDH allozyme (A: één band), PD23-2 heterozygoot (AB: drie banden) en de F1 een uitsplitsing gaf in homozygote en heterozygote nakomelingen duidend op een monogeen systeem met een dimeer karakter. De monohaploiden van M9 vertoonden alle dezelfde band (A), terwijl de monohaploiden van PD23-2 uitsplitsten in twee banden (A en B). Twee relatief groeikrachtige monohaploiden, een van elke ouder, die te onderscheiden waren door hun MDH-banderingspatroon zijn gekozen voor protoplastenfusie. Hierbij is gebruik gemaakt van een protoplastenisolatie- en fusietechniek (zgn. fase-grens techniek) als beschreven in hoofdstuk 3. Er zijn in dit experiment geen aanwijzingen gevonden, dat er sprake is van hybride groeikracht op callus niveau, maar de regeneratie van de fusieproducten (na 11 weken) verliep sneller dan die van de ongefuseerde ouders (na 21 weken). Alle regeneranten, afkomstig van drie calli, vertoonden het hybride (AB) banderingspatroon. Uit controle-analyses van homozygote somatisch verdubbelde 2x en 4x planten bleek, dat het hybride banderingspatroon geen gevolg was van een veranderd ploidieniveau. Hiermee

was aangetoond dat, uitgaande van monohaploiden, hybride fusieproducten kunnen worden verkregen. Alle regeneranten, zowel de hybriden als de ongefuseerde ouders, bleken echter tetraploid. Vraag was dus nu: kan selectie op stabiele en groeikrachtige monohaploide klonen een oplossing bieden voor dit (in)stabiliteitsprobleem?

Om dit te onderzoeken zijn van 23 verschillende monohaploide lijnen DNA-hoeveelheid analyses uitgevoerd met behulp van een doorstroomcytometer (hoofdstuk 4). Hierdoor kan indirect een aanwijzing worden verkregen voor het aantal chromosomen in de cel. Hoewel tussen de getoetste klonen grote verschillen in het DNA-distributiepatroon werden gevonden, bleek geen enkele kloon alleen monoploide cellen in de bladeren te bevatten. Onderzoek naar de invloed van verschillende opkweekomstandigheden zoals kas- of in vitro-condities toonde aan, dat in vitro-materiaal de minste verdubbeling vertoonde. Hoewel in de winterperiode een verlaagd percentage monoploide kernen kon worden geconstateerd, bleek gedurende het hele jaar een zeker percentage di- en tetraploide cellen aanwezig in het blad. De groeitopjes bleken echter wel monoploid, waardoor de planten via scheuttop-cultures minimaal 2-3 jaar stabiel in stand konden worden gehouden. In het algemeen wordt echter het relatief instabiele bladweefsel gebruikt als bron voor protoplasten. DNA-analyse van bladprotoplasten direkt na de enzymbehandeling toonde een relatieve verhoging van het percentage monoploide cellen, mogelijk als gevolg van een blokkering van de celdelingscyclus in de G_1 gedurende de enzymbehandeling. De mechanische zuiveringsstappen, die na de enzymbehandeling volgen, veroorzaakten daarentegen een verlaging van het percentage monoploide cellen, mogelijk als gevolg van een selectief verlies van de zwakkere monoploide protoplasten.
Door het gebruik van in vitro opgekweekt plantmateriaal en een voorzichtige behandeling tijdens de isolatie kan het percentage verkregen monoploide protoplasten zo hoog mogelijk worden gehouden.

Voor groeikrachtanalyses zijn meer dan 100 monohaploiden samen met hun diploide ouder onderzocht en beschreven met betrekking tot groeikracht op plantniveau in de kas en in vitro en een groot deel van de genotypen tevens met betrekking tot de isolatie en fusie van protoplasten (hoofd-

stuk 5). Doel hierbij was te onderzoeken of er relaties bestaan tussen de verschillende niveau's van plant- en celgroei bij monohaploiden onderling en met hun diploide ouders, en of het mogelijk is een diploide hybride te regenereren na protoplastenfusie van relatief groeikrachtige monohaploide planten.
Noch op monoploid, noch op diploid niveau is er een correlatie gevonden tussen de relatieve groeikracht van in de kas en in vitro opgekweekte planten. Geen of slechts zwakke correlaties zijn gevonden voor de verschillende in vitro groeieigenschappen als plantontwikkeling, protoplastenopbrengst per gram plantmateriaal, 'plating efficiency' en callusontwikkeling. Dit onderstreept de onvoorspelbaarheid van en de problemen bij de selectie op dergelijke eigenschappen.
Protoplastenfusie-experimenten hebben aangetoond, dat bij sommige genotypecombinaties een verhoogde callusgroeikracht kan optreden, waarbij echter het hybride karakter van de afzonderlijke calli niet is vastgesteld. In monocultures van protoplasten bleken alle geregenereerde planten di- of tetraploid te zijn. Dit is wellicht een aanwijzing, dat de protoplasten- en callusfase te lang is om regeneratie zonder verdubbeling mogelijk te maken. Na fusie werden voor het merendeel tetraploiden alsook enkele triploiden verkregen. Deze laatste zijn een aanwijzing, dat homologe of heterologe fusie van monoploide protoplasten is opgetreden. De ontstane triploiden bleken wel groeikrachtig genoeg voor een stabiele regeneratie. Dit toont aan, dat de fusie op zich geen probleem hoeft te zijn voor monoploide protoplasten, maar dat wellicht de mediumsamenstelling en de cultuuromstandigheden daaropvolgend nog verder geoptimaliseerd moeten worden om een stabiele regeneratie mogelijk te maken. Wellicht biedt een alternatief voor het nu nog noodzakelijke groeihormoon 2,4D hierbij perspectieven.

Teneinde de gezamenlijke effecten van homozygotie en haploidie/polyploidie in de aardappel te onderzoeken is de habitus bestudeerd van vijf aardappelgenotypen op drie (x,2x,4x) en twee op twee (x,2x) ploidieniveau's (hoofdstuk 6). Zes van deze zeven genotypen zijn vergeleken met hun heterozygote ouderkloon. Op deze manier kon een vergelijking worden gemaakt tussen i) de heterozygote diploid en monohaploiden van deze diploid, ii) twee of drie ploidieniveau's per genotype en iii)

homozygote di- en tetraploiden en hun heterozygote diploide ouder. Grote variatie werd gevonden tussen monohaploiden verkregen van één diploide bron. Een opvallende toename in groeikracht werd waargenomen bij somatische chromosoomverdubbeling van x naar 2x, maar minder duidelijk van 2x naar 4x. De relatief groeikrachtige diploiden vertoonden een zwakkere respons op tetraploidisatie dan de minder groeikrachtige. De heterozygote diploide ouder overtrof al zijn homozygote di- en tetraploide afgeleiden in habitus. De resultaten van deze studie vertonen de tendens van een positief gendosiseffect vooral voor knolproductie maar ook, zij het in wat mindere mate, voor bladoppervlak en planthoogte. De waargenomen verschillen in groeikracht tussen homo- en heterozygote klonen tonen aan, dat dominantie-effecten sterker zijn dan additieve effecten. Als de steriliteitsproblemen van het onderzochte materiaal inherent zouden zijn aan homozygotie bij de aardappel in het algemeen zullen homozygote aardappellijnen waarschijnlijk van weinig betekenis zijn voor de praktische aardappelveredeling.

Kort samengevat kan gesteld worden dat monohaploide aardappels voor de praktische veredeling voorlopig van weinig belang zullen zijn door de genetische beperkingen in de productie ervan en de instabiliteit van het chromosoomaantal op celniveau. De hieruit voortkomende homozygote lijnen vertonen veelal beperkingen in de fertiliteit en de knolproductie. Een schema als in Fig. 1 kan dus vooralsnog als een utopie worden beschouwd. Voor fundamenteel wetenschappelijk onderzoek echter zijn monohaploiden van grote waarde temeer daar monohaploide planten gedurende tenminste 2-3 jaar stabiel in stand kunnen worden gehouden en voor dit materiaal veel van de technische problemen ten aanzien van protoplasten-cultuur en -fusie nu zijn opgelost.

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Curriculum vitae

Bernardus Antonie Uijtewaal werd geboren op 12 oktober 1959 te Utrecht. Van 1971 tot 1977 doorliep hij de VWO-opleiding aan de Scholengemeenschap "de Klop" te Utrecht, waarna hij de studie Plantenveredeling begon aan de toenmalige Landbouwhogeschool te Wageningen. In 1983 behaalde hij het ingenieursdiploma cum laude met de doctoraalvakken: plantenveredeling, taxonomie van cultuurgewassen en erfelijkheidsleer. Van juni 1983 tot juni 1987 was hij als promotie-assistent werkzaam op de vakgroep plantenveredeling aan de tegenwoordige Landbouwuniversiteit Wageningen, wat ondermeer resulteerde in het hier gepresenteerde proefschrift. Sinds juli 1987 is hij in dienst van de firma Nunhems Zaden te Haelen en tijdelijk gedetacheerd bij de firma Hoechst in Frankfurt (Dld.).