* IN VITRO CULTURE OF HIGHER PLANTS AS A TOOL IN THE PROPAGATION OF HORTICULTURAL CROPS

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Abstract

In vitro culture of higher plants is the culture, under sterile conditions, of plants, seeds, embryos, organs, explants, tissues, cells and protoplasts on nutrient media. This type of culture has shown spectacular development since 1975, resulting in the production and regeneration of viable individuals of many plant species. In addition, since 1980 there has been an explosion in the development of genetic manipulation and biotechnology.

Various types of cultures and their applications are discussed: embryo culture, orchid seed culture, meristem culture, cloning of crops (single-node culture, meristem culture, axillary branching, adventitious shoot formation, callus culture, single cell and protoplast culture), anther and microspore culture, culture of ovules and excised flowers, and protoplast culture.

Vegetative propagation in vitro (also called micropropagation) will be dealt with in greater detail since it has yielded results which are of enormous importance in horticulture. A recent survey in the Netherlands has shown that the number of commercial tissue culture laboratories in this country has increased from 28 in 1983 to 50 in 1986; the total number of plants cloned in vitro increasing from 21 to 43 million over the same period.

The widespread use of in vitro cloning is an indication of its many advantages. However, there are also disadvantages. The criteria desired for the success of in vitro cloning can be itemized as follows: no induction of mutations; the starting material must be disease-free, or disease-free material must be produced by meristem culture; the transfer from test tube or container to soil should not be too difficult; the regeneration ability should not be lost; the technique should be economically viable and not too complicated (synthetic seeds!), otherwise it will be rejected. Woody species (trees and shrubs) are far more difficult to clone in vitro than herbaceous plants; the reasons for the differences are given.

In vegetative propagation and propagation by seeds, in vitro culture can also be a tool to: obtain disease-free plants; transport disease-free plant material; increase the genetic variation (by somaclonal variation and genetic manipulation); induce haploids; separate chimeras; obtain tetraploids; store plant material in gene banks where it can be kept disease-free under conditions which limit growth and development.

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1. Introduction

In vitro culture of higher plants is defined as the culture on nutrient media under sterile conditions, of plants, seeds, embryos, organs, explants, tissues, cells and protoplasts of higher plants. A list of different types of cultures and their possible applications is given below.

Type of culture:	Purpose:
Embryo culture	Shortening of the breeding cycle Prevention of embryo abortion Overcoming incompatibility Production of haploids As a source of callus formation
Orchid seed culture	Shortening of the breeding cycle Replacing symbiosis (mycorrhiza) Excluding competition with other micro-organisms
Meristem culture	Elimination of pathogens (viruses, fungi, bacteria) Vegetative propagation of orchids through protocorms Cloning of plants other than orchids Storage of disease-free plants Phytosanitary transport Germplasm collection
Micro-grafting	Grafting of meristems on virus-free (seedling) rootstocks to obtain virus-free propagation
Shoot tip and single-node culture	Orchid propagation Axillary branching as a tool to clone plants Cryo-preservation to create gene banks
Explant (without pre-existing buds) culture	Adventitious organ formation for cloning plants Obtaining disease-free plants Production of solid mutants (mutation breeding) Isolation of mutants Separation of chimeras Obtaining polyploids
Callus, suspension and single cell culture	Cloning of plants through organ and embryo formation Creation of genetic variants Obtaining virus-free plants As a source for protoplast production Starting material for cryo-preservation Production of secondary metabolites Biotransformation

Type of culture:	Purpose:
Anther and microspore culture	Production of haploids and to obtain homozygotes As a starting point for mutation induction Creation of all-male plants As a tool in genetic manipulation To breed at lower ploidy levels
Culture of ovules and excised flowers	Overcoming incompatibility To prevent precocious abscission of flowers Achievement of test-tube fertilization
Protoplast culture	Somatic hybridization Creation of cybrids Transplantation of nuclei, (fragments of) chromosomes and organelles Transformation studies Creation of genetic variants
Culture of protoplasts, cells, tissues and organs	As a tool in phytopathological research - Virus penetration and replication - Culture of obligate parasites - Host-parasite interactions - Culture of nematodes (excised root cultures) - Testing of phytotoxins - Nodulation studies As a tool in plant physiological research - Cell cycle studies - Metabolism - Nutritional studies - Morphogenetical and developmental studies

2. In vitro cloning in the Netherlands

It is especially notable in horticulture that people have quickly responded to the results obtained from research on micropropagation. Since there are no accurate statistics of the extent to which cloning in vitro is being used commercially, first of all an analysis will be given of the Dutch horticultural industry. The tables below show the number of companies producing micropropagated plants in Holland, the number of plants propagated in the years 1983-1986, and a list of plants micropropagated in numbers greater than 100 000 (Pierik, 1987).

Numbers of plants produced:	Number of commercial tissue culture laboratories:			
	1983	1984	1985	1986
Less than 10 000	6	3	12	14
10 000-100 000	9	15	14	18
100 000-500 000	4	4	6	6
500 000-1 000 000	1	2	1	2
1 000 000-5 000 000	8	9	7	7
More than 5 000 000	0	0	2	3
Total	28	33	42	50

Number of plants propagated	1983	1984	1985	1986
Pot plants	15 243 327	15 428 130	17 412 586	19 822 274
Cut flowers	3 634 497	10 036 990	11 420 824	12 639 758
Bulbs and corms	590 392	1 458 019	5 358 740	8 085 920
Orchids	1 347 350	1 534 500	1 116 740	1 449 190
Miscel. ornamentals	8 952	242 383	301 865	375 805
Agricultural crops	279 400	280 000	300 000	311 825
Vegetables	27 053	60 040	71 205	68 825
Total	21 130 771	29 040 062	35 981 960	42 753 600

List of plants propagated in the Netherlands in numbers greater than 100 000 in 1986:

Bulbous and cormous crops	: Lilium
Orchids	: Cymbidium
	Vuylstekeara
Cut flowers	: Gerbera
	Anthurium andreanum
Pot plants	: Nephroplepis
	Saintpaulia
	Anthurium scherz.
	Cordyline
	Davallia
	Synchonium
	Bromeliaceae
	Alocasia
	Ficus
	Spathiphyllum
Plants for aquaria	: Cryptocorine
	Echinodorus
Agricultural crops	: Solanum tuberosum

What conclusions can be drawn from these tables?

1. The number of plants propagated in numbers greater than 100 000 is relatively low, indicating that there are still problems in micropropagation even in an important horticultural country such as Holland. Undoubtedly for a number of crops such as G e r b e r a, lily, N e p h r o l e p i s, potato, etc. the success of micropropagation techniques is quite clear and has been accepted commercially.

2. Many plants reported (Pierik, 1987) in a Dutch survey are produced in very low numbers (under 3 000 per year), indicating either that the techniques have not yet been perfected, or that the cost price of these plants is too high. It is true for many plant species, particularly vegetables, propagation is only used for a restricted specialized purpose, such as breeding.

3. The most notable ommission from the production figures is any significant number of hardy nursery stock plants and (forest) trees.

3. Analysis of micropropagation

In connection with the figures presented in the previous paragraph, an analysis will be made of micropropagation; this analysis will be divided into:

1. Requirements of vegetative propagation in vitro.
2. Advantages of cloning in vitro.
3. Cloning systems in vitro.
4. Basic problems when plants are propagated in vitro.
5. Handicaps for commercial application of in vitro cloning.
6. Factors affecting cost price of in vitro produced plants.

4. Requirements of vegetative propagation in vitro

When plants are cloned in vitro, the following requirements should be met:

- Genetic stability (no mutations).
- Thorough selection of plant material, in particular freedom from diseases.
- Reversion from the adult to the juvenile phase should be possible, particularly when propagating shrubs and trees.
- Retention of the regenerative ability.
- Economical justification in comparison to the production of the same plant in vivo.
- The transfer from test tube to soil should not be too complicated and must be carried out without too many losses.
- In vitro propagation should not be too complicated, otherwise it will be rejected in practice.

5. Advantages of cloning in vitro

- When classical methods of in vivo vegetative propagation fall short of that required, in vitro cloning is an important tool in speeding up propagation.

- Adult plant material, which often cannot be cloned in vivo, can sometimes be rejuvenated in vitro and then subsequently cloned.

- The growth of in vitro propagated plants is often stronger than those cloned in vivo; this is mainly due to rejuvenation and/or the fact that they are disease-free.

- When the existing methods of cloning are too slow or too complicated to be profitable, in vitro cloning can be applied to produce large numbers of plants more quickly and at a competitive price.

- By in vitro cloning expensive methods such as grafting or budding on a root stock can be rendered obsolete.

- In vitro cloning enables the uncovering of chimaeras and the isolation and cloning of spontaneous or induced mutants. Mutation induction and regeneration of adventitious buds in vitro makes it possible to obtain solid mutants. The reason for this is that adventitious plants often find their origin in one cell.

- In contrast to in vivo propagation, in vitro cloning of herbaceous plants can be continued all the year around and so become independent of the seasons.

- In vitro storage and cloning facilitates the creation of gene banks (preservation of valuable plant material) and the storage of plants under pathogen-free conditions on a relatively small surface. By use of low temperature storage and freezing, time spent on cloning as well as the space required can be drastically decreased. Low temperature storage also makes it possible to stagger production more effectively than under in vivo conditions.

- In vitro cloning enables the production of disease-free plants and thereby the phytosanitary transport from country to country.

- Greenhouses are expensive and energy prices high. These costs can be reduced by in vitro cloning as very few stock plants are required for starting material and much less greenhouse space is required for making cuttings.

- For plant breeders, propagation in vitro facilitates cloning of parent plants as starting material for hybrid seed production. Another argument for plant breeders to apply in vitro cloning is that a new cultivar can be cloned much faster and consequently sold earlier than when only applying classical propagation techniques.

- In vitro cloning enables genetic manipulation (engineering), which would be impossible when there are no methods available for

regenerating protoplasts, cells and tissues.

6. Cloning systems in vitro

Several methods have been developed to propagate plants in vitro. Since the reliability of these methods is very different, as is their convenience of application, it is best to summarize them as follows.

6.1. Single-node culture and rooting of ensuing shoots

The most obvious, simple and secure method of cloning plants in vitro is the single-node culture method, with the production of shoots and the regeneration of roots on these shoots.

With typical rosette plants, this method is difficult to realize due to problems of infection when isolating in vitro. However, those plants which elongate, forming a stem with leaves and buds in the axils of these leaves, can be very easily propagated using this method. As soon as a bud or a shoot tip grows out into an elongated stem, the single nodes with dormant buds are subcultured to form an elongated stem, and the apical shoot tips can be rooted directly. Quite recently this system was successfully applied (Pierik, 1987 , Pierik and Steegmans, 1985; Pierik et al., 1986) to lilac (cultivars and root stocks) and various vegetables (tomato, cucumber and sweet pepper). The rate of propagation with this system is strongly dependent on the number of nodes (leaves) formed in vitro within a certain time period.

This method is the most natural in vitro propagation method that exists. The method is also very safe, because the integrity of the plant (in particular meristems and buds) is not disturbed. It should be noted that in most cases the method can be successfully applied to herbaceous plants. However, when cloning of shrubs and trees is attempted with this method, serious problems with dormancy of buds and elongation of shoots can arise.

6.2. Axillary branching

The second, slightly more artificial, method is the induction of axillary branching in isolated shoot tips.

In nature, apical dominance can be broken by removing the apical bud, resulting in a release of dormancy of the axillary buds; as soon as the apical bud is removed, basipetal flow of auxin which normally keeps the axillary buds dormant, stops.

Removing or killing the apical bud also breaks apical dominance in vitro, resulting in axillary branching, but in most cases this is realized by application of cytokinins, which are capable of antagonizing the auxin produced by the apical meristem.

As soon as axillary buds or sprouts are formed, they can be separated individually from the original shoot tip; they are placed on a fresh medium again with cytokinin to repeat axillary branching, etc. When enough shoots have been obtained, the process of axillary branching is stopped by ommitting cytokinin; subsequently auxin is applied to promote rooting of the individual shoots in vitro or

sometimes in vivo.

The axillary branching method is frequently applied, being relatively simple and quite safe. If no adventitious buds are formed, no mutations generally occur. Regeneration of adventitious buds is in most cases an unwanted side-effect of too high cytokinin levels in the medium during axillary branching.

It is thought that shoot tips from plants in the adult phase gradually rejuvenate during repeated axillary branching in vitro; after a number of transfers shoots appear to be able to regenerate roots, which is an indication (indirect proof) of rejuvenation. As soon as rejuvenation has taken place, rooted shoots can be produced. However, in many woody plants, the reversion from adult to juvenile cannot be obtained for reasons as yet unknown.

In practice the axillary shoot method has become easily the most important propagation method in vitro because:
- The method is generally simpler than the other methods of propagation.
- The rate of propagation is relatively fast.
- The genetic stability is usually preserved.
- The growth of the resulting plants is very good, due to rejuvenation and/or the lack of micro-organisms in the plants.

6.3. Regeneration of adventitious buds or shoots

The third important method that is used to clone plants in vitro is called the adventitious bud/shoot technique. An explant (from leaf, petiole, stem, stalk, corm, etc.) without pre-existing buds or shoots is isolated in vitro. In most cases cells in the explant dedifferentiate and subsequently start to divide; under appropriate conditions, cell division can be directed in a very special way so that adventitious buds arise, which can develop into shoots. These shoots can be rooted or used to continue shoot formation according to methods 3.1, 3.2, or 3.3.

When adventitious bud formation can easily be induced in vitro, the method has many advantages. The number of plant species that can regenerate adventitious buds on excised explants is relatively small and often restricted to herbaceous plants; woody plants, particularly in the adult phase, are almost incapable of regenerating buds.

The greatest difficulty with the adventitious bud technique is that the chances of obtaining mutated plants is much higher than when method 3.1 or 3.2 is applied. This holds particularly true for (crypto)chimaeric plants and also for plants from single cell origin.

The adventitious bud technique is quite successfully applied in the following plant species, which are relatively easy to regenerate: lily, S a i n t p a u l i a i o n a n t h a, hyacinth, B e g o n i a, A c h i m e n e s, S t r e p t o c a r p u s, C i c h o r i u m i n t y b u s, chrysanthemum.

The auxin and cytokinin requirement for adventitious budding can be very different for various plant species and even cultivars. A small number of plants such as lily and hyacinth react positively to auxin application, whereas many species require cytokinin to form adventitious buds. Others require both cytokinin and auxin. In many species a relatively high cytokinin level and a low auxin level promote adventitious budding.

6.4. Regeneration of plants from callus, single cells and protoplasts

Callus is a strongly dividing and more or less undifferentiated tissue. It can be obtained by isolating tissues, organs or embryos in vitro; differentiated tissues generally first undergo dedifferentiation before cell division starts. Under appropriate conditions, callus can be subcultured in vitro without organ or embryo formation. However, it is also capable of regenerating somatic embryos or adventitious buds.

Although many research workers claim that cloning of higher plants through callus is a very attractive method, the reverse has been found to be true. The greatest difficulty with callus, and also with single cell and protoplast cultures, is that the genetic stability is extremely low. Plants originating from protoplasts, single cells and callus are very often mutated. Another handicap of callus culture is that during prolonged subculture the organ(embryo)-forming capacity is lost. Only in a few plant species, such as *Anthurium andreanum*, and coffee, is callus culture a satisfactory and rather safe method, applicable by commercial tissue culture companies.

7. Basic problems when propagating plants in vitro

During in vitro propagation a number of problems can arise which can only be solved by fundamental research. These basic problems can be summarized as follows:

7.1. Mutations

One of the most important barriers to the propagation of plants through cell and tissue culture, is the chromosomal instability of cells. We are largely ignorant of the causes of the frequent occurrence of these mutations. Experience, in research as well as in commercial tissue laboratories, has shown that the occurrence of mutations is strongly dependent on the following factors:

- The propagation system chosen.
- The type of regulator used.
- The type of tissue used; differentiated or undifferentiated.
- The genotype.
- The number of subcultures.
- Whether a plant arises from one or more cells.
- If the plant used is a chimaera or not.
- The ploidy level of the plant.

When the organizational structure of a plant, an organ or a tissue is broken down, quite often cell lines, tissues and plants are obtained which are genetically not identical to the original genotype; when the organization pattern is maintained and a shoot tip with apical and lateral meristem remains functioning, usually no mutations occur. In principle the genetic integrity and functioning of an organ should be maintained in vitro in the same way as in natural methods of

cloning (in vivo), e.g. by runners, division, etc. It is generally thought that dedifferentiation of differentiated cells in vitro, followed by intensive cell division is primarily responsible for the mutations.

There are, in principle, two explanations for the occurrence of mutations:

1. In the original explant, mutated cells (especially polyploid cells as a result of endomitosis or nuclear fusion) already occur and are expressed as a result of in vitro culture.
2. Mutations are directly induced by in vitro culture; this holds true particularly for the occurrence of aneuploid cells.

In both cases the practical question arises how mutated cells and later mutated plants can be avoided?

When the original explants contain deviating cells, this is in most cases the result of genetic instability during somatic cell division. The only possibility, in this case is to choose starting material in which there are hardly any or no mutated cells present. Since it has been shown that the frequency, particularly of endopolyploid cells, is much greater in differentiated tissues, it is advisable to always start with undifferentiated tissues such as meristems. The question of how to prevent cell divisions in pre-existing mutated cells cannot yet be answered. There are rather vague indications that certain synthetic regulators are responsible for selective cell divisions in already mutated cells.

The question of how mutation induction by the application of in vitro culture can be avoided, is very difficult to answer. There are again indications that synthetic regulators such as BA, NAA, and 2,4-D are responsible for mutation induction in vitro.

Since we are largely ignorant of the fundamental causes of mutations in vitro, basic research will be necessary to demonstrate:

1. Whether synthetic regulators really can selectively induce cell divisions in cells which are already in the mother (ex)plant genetically different from the genotype as a whole.
2. Whether synthetic regulators can induce mutations.
3. Whether other factors (e.g. nutritional) are possibly responsible for mutations.

7.2. Organ and somatic embryo formation

Basic knowledge concerning factors affecting organ and somatic embryo formation in various types of cultures is often completely lacking. In most cases we only know how to balance the many essential factors to obtain organ or embryo formation, but the fundamental cause of regeneration is unknown.

It is well known that certain families and genera have a relatively high capacity for regenerating organs in vitro: Solanaceae (Solanum, Nicotiana, Petunia, Datura, Lycopersicum), Cruciferae (Lunaria, Brassica, Arabidopsis), Compositae (Cichorium, Lactuca, Chrysanthemum), Liliaceae (Lilium, Haworthia, Ornithogalum). However, it

is completely unknown why such families and genera and not others possess such an ability to regenerate organs and/or somatic embryos in vitro.

Since we know that in a number, although certainly not in all plants, auxins can induce root formation and cytokinins shoot formation, the mechanism of action of these two regulators at the molecular level is completely unknown. What is the reason that in so many plants, and particularly in tissues of adult trees and shrubs, adventitious organ formation cannot be induced?

The decrease of organ formation with increasing age is also a difficult and unexplained phenomenon. In practical terms it means that adventitious organ and embryo formation in explants of adult shrubs and trees, which is essential to realize rejuvenation, can in most cases not be obtained. A better understanding of the difference between the juvenile and adult condition in physiological, biochemical and molecular terms is required to solve the regeneration problems in shrubs and trees.

7.3. Loss of the ability to regenerate organs and somatic embryos

It is well known that frequently-subcultured cells and tissues loose the ability to regenerate organs and embryos. At present there is no good explanation for this phenomenon. The fundamental question can be raised whether the loss of organ formation is caused by mutation or by selective cell division in those cells which probably do not possess the ability to regenerate. Are polyploid cells in a normal diploid plant the reason for the lack of totipotency or are we completely ignorant of the causes of organ regeneration in certain 'non-totipotent' cell types?

7.4. Rooting problems with shrubs and trees

It is frequently impossible to regenerate roots on excised or subcultured shoots from adult shrubs and trees. Root initiation in vitro appears confined to those species and cultivars whose cuttings can be successfully rooted in horticulture or forestry practice; even if rooting is possible in vivo, problems can arise with rooting in vitro. The adult status of the plant material is the largest handicap. At present the rooting problems in vitro with shrubs and trees can only be solved by inducing rejuvenation so restoring the rooting ability. The most efficient method of inducing rejuvenation is adventitious bud or shoot regeneration. However, since adult plant material of shrubs and trees is practically incapable of forming adventitious shoots in vivo or in vitro, this is not a very realistic possibility, except e.g. in C i t r u s, P o p u l u s and S a l i x.

In a number of plant species such as apple, lilac, etc. it is possible to induce rejuvenation in vitro by repeated axillary shoot formation. During a number of transfers shoots go back step-by-step from the adult to the juvenile state by which the rooting ability is restored. This system of continued subculturing of shoots is comparable to the in vivo applied system of repeatedly making cuttings from cuttings.

7.5. Production of toxic compounds in vitro

A very common problem in plant tissue culture is the build-up of toxic brown and/or black compounds on wounded surfaces which is generally due to phenolic oxydation. The quinones (the oxydation producs of phenols) are very toxic for higher plants, and may severely limit growth and differentiation.

In practice preventing the production of these toxic compounds by the plant is extremely difficult. Use of anti-oxidants such as ascorbic acid and citric acid to stop phenolic oxydation are generally unsuccessful. Various other procedures to inhibit phenolic oxydation (culture in darkness, liquid media, etc.) have also been unsuccessful. The most efficient way of eliminating toxic compounds in the media is the use of activated charcoal (in most cases charcoal from Merck, nr.2186). However, a big disadvantage of activated charcoal is that it adsorbs not only toxic compounds, but also very essential organic substances such as auxins, cytokinins and many other compounds in the media (Misson et al.,1983). In a number of cases PVP (polyvinylpyrrolidone) has been successfully used as an adsorbant for toxic substances.

7.6. The transition from test tube to soil

The transfer from test tube to soil is often very difficult because the in vitro produced plants are not well adapted to the in vivo climate. The adaptation problems can be summarized as follows:

1. Root regeneration in vitro appears to be vulnerable; in vitro formed roots do not function properly (fewer root hairs) in vivo and are rather weak and often die off; in soil in vitro formed roots often have to be replaced by newly formed subterranean roots. As a consequence of the non-functional roots, transpiration outside the test tube is too high and can result in the loss of many plants. To promote functional root formation, it is necessary to regenerate roots in vivo instead of in vitro.
2. Leaves formed in vitro are not well adapted; they are thin, soft and photosynthetically not very active; the cuticular wax layer is poorly developed, resulting in excessive cuticular transpiration in vivo; stomata do not operate properly; there are poor vascular connections between the shoots and the roots.

Due to the very special environment in vitro, it is extremely difficult to produce plants, which are well adapted to the life outside the test tube. A decrease in the relative humidity in vitro can result in a better cuticular wax formation and hence less cuticular transpiration.

3. In vitro grown plants are completely sterile; inoculation with micro-organisms (bacteria, fungi) may be subsequently necessary in vivo for plants normally living symbiotically.

7.7. Vitrification

In certain plants in vitro propagation is severely handicapped by a morphological and physiological disease, called vitrification. In the literature, names such as glassiness, translucency and vitrescence are also used instead of vitrification. The disease is often (Debergh, 1983) caused by a high relative humidity in the gas phase of the test tube and an excessive uptake of water from the agar. In general there is no satisfactory way of avoiding vitrification, although in a number of cases it can be prevented or decreased by increasing the agar concentration in the medium (Debergh, 1983).

7.8. Internal infections

Despite many precautions the starting material for in vitro culture can be infected internally. Endogenous, internal, or systemic bacterial or fungal contamination forms a serious handicap for in vitro culture. These internal infections cannot be eliminated by external sterilization. In principle there are two ways of combating this problem: addition of antibiotics to the medium and application of meristem culture.

Application of antibiotics is usually unsuccessful and even dangerous. High concentrations of antibiotics often have phytotoxic effects on (higher) plants. Antibiotics also select the most resistant strains of a particular micro-organism. The only efficient but very complicated and laborious method to solve the problem of internal infection is the application of meristem culture.

It is often thought that plant material in vitro is free from bacterial or fungal diseases when no contaminations can be detected by the naked eye. This is a dangerous assumption; only an appropriate microbiological screening can show if it is justified. Often media enriched with pepton ot trypton are used to check whether plant material is free from fungi and/or bacteria.

Special attention has to be paid to a bacterium which, in the USA is called the white ghost (most probably B a c t e r i u m l i c h e n i f o r m i s and/or B. s u b t i l i s); this is a frequently occurring pathogen both in and on the plant. The white ghost is very difficult to attack and is often the principal cause of infection explosions in tissue culture laboratories.

8. Handicaps for the commercial application of in vitro cloning

There are a number of restrictions on the replacement of current methods of in vivo propagation by in vitro methods. These restrictions which can in principle be eliminated, are summarized below:

8.1. One of the most striking differences between herbacious and woody species, is that the latter are far more difficult to clone in vitro. The main reason is that woody species have a relatively weak regenerative capacity when compared to herbaceous species. Induction of rejuvenation by adventitious bud formation is extremely difficult to obtain in trees and shrubs.

8.2. In vitro propagation systems described in so-called scientific papers have often not been fully optimized, resulting in a too slow propagation rate. Therefore these systems are rejected by horticultural practice, where there is often no time and money for a systematic optimization of cloning in vitro.

We have demonstrated in several crops that by systematic experimentation and step-by-step optimization of various factors an increase of the propagation rate of 100% can often be achieved. Slow propagation rates result in a cost price per plantlet which is too high in comparison to in vivo methods, so that the in vitro system is economically not feasible.

8.3. Since only a very restricted number of commercial tissue culture laboratories have a good insight into the cost price of each individual species or cultivar, cost price calculation of in vitro produced plants remains urgently needed.

8.4. In scientific papers a model study on in vitro propagation is often described for only one genotype (cultivar) of a particular species. When commercial tissue culture laboratories try to propagate other genotypes according to the described system, the results are often disappointing. In a number of cases this is due to the fact that research workers have forgotten that hormonal requirements may be very different for various genotypes (cultivars) of one species.

8.5. In vitro propagation systems are sometimes rejected because the plants have too strong a branching habit when transferred to soil. The main reason for this problem is, usually an excessively high cytokinin level in the culture medium during axillary branching or adventitious bud formation. To prevent excessive branching, the cytokinin level should be lowered during shoot production, or a transfer (just before the rooting in vitro) to a medium without cytokinin may be necessary.

8.6. Since the cost price of a test tube plant is not only determined by the price to produce shoots in vitro, but also by the price to root these shoots, more attention should be paid to reduction of the price of rooting. For this reason more attention should be paid to optimizing rooting in vitro e.g. by replacing agar by liquid media (better oxygen supply). Another possibility is to transfer minishoots formed in vitro directly onto artificial substrates such as rockwool in plug plates and to root in vitro produced shoots outside the test tube. Conventional rooting procedures of micropropagated shoots in vivo not only increase the number of roots that are functional in soil, but can also contribute to a lowering of the total cost price.

8.7. When chimaeric plants are propagated in vitro, the chance of losing the original genotype is much greater than in non-chimaeric plants. In most cases so-called uncovering of chimaeras occurs in vitro when adventitious buds (although not wanted) are formed. Careful axillary branching is the only way of safely propagating chimaeric plants in vitro.

8.8. Another factor, which is often neglected, is the enormous variability of isolated explants in vitro. Often this variability is

caused by the original position of the explant on the plant. It is necessary to gain a better insight into the so-called physiological gradients, which reflect themselves, particularly in hormonal gradients. The auxin and cytokinin level in our culture media should therefore be adjusted in relation to the endogenous hormone level at a particular location.

8.9. The necessary equipment required for setting up a professional tissue culture laboratory is quite expensive, particularly for developing countries.

8.10. In vitro produced plants may display troublesome effects in vivo: bushiness (the repeated production of side shoots) and complete reversal to the juvenile phase.

9. Factors affecting the cost price of in vitro produced plants

There are a number of factors, not yet mentioned in previous sections, which strongly influence the cost price of in vitro produced plants and the productiveness of a commercial tissue culture laboratory. These are summarized below:

9.1. The cost price of micropropagated plants is quite high because the technique is very labour intensive; 70% of the cost of in vitro plants is labour. More attention should be paid to automation in the tissue culture laboratory. The first intelligent robotic system for in vitro plantlet production was recently described by Deleplanque et al. (1985). An automated plant culture system controlled with a microcomputer was recently described by Tisserat and Vandercook (1985,1986); this system is relatively inexpensive to construct, and provides a labour-saving method of culturing plants in vitro. The use of fermentation culture and so-called synthetic seeds (encapsulated somatic embryos) can also save labour costs.

9.2. Staggering and timing of the production are sometimes not correct so that in certain periods labour, space (laboratory and culture rooms) and further equipment are not optimally used. In most cases production peaks and periods of little work can be avoided by broadening the range of plant species. Another way of staggering production and so ensuring optimal use of labour and space, is the application of low temperature storage.

9.3. Infections during isolation and production can greatly increase the cost price of in vitro produced plants. Infection at the moment of the isolation in vitro can be decreased by raising the starting material in clean greenhouses, where pests (bacteria and moulds) and pest vectors (aphids, white flies and mites) are thoroughly eliminated. Another essential prerequisite for sterile isolation is that plants in the greenhouse should only be watered on the pot or soil and not on the plants themselves. A further possibility is to decrease the relative humidity in the greenhouse.

To reduce the number of infections during the production it is essential to work under strictly sterile conditions: regular cleaning

of the rooms and especially floors, use of laminar air-flow cabinets, etc. It is also essential not to bring infected plant material into a tissue culture laboratory.

9.4. The loss of a relatively high percentage of plants during the transfer from test tube to soil is another factor which strongly influences the cost price. The high relative humidity in the test tube is primarily responsible for big losses of plants when they are transferred to soil. It is regrettable that in most scientific papers, no hardening-off procedures are described. In principle, although rarely achieved in practice, in vitro plants should be produced that are well adapted to in vivo conditions. Good facilities should be available to protect in vitro plants in soil against too much transpiration; this can be achieved by increasing the relative humidity in the atmosphere, by reducing the light level and also by lowering the air temperature.

Another important factor accounting for losses of in vitro produced plants in soil is the fact that test tube plants have been growing under sterile conditions and once in soil are not protected against attacks of micro-organisms. This problem can be solved by using sterilized soil (gamma irradiation, steaming) and applying fungicides and bactericides during the first weeks.

10. Conclusions

It is clear that more basic and applied research will be necessary to solve the problems mentioned. The gap between basic research and application should be bridged, otherwise the number of plants species cloned in vitro in large quantities will remain low.

11. Bibliography

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