

HANDICAPS FOR THE LARGE SCALE COMMERCIAL APPLICATION OF MICROPROPAGATION

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Abstract

In the last 10 years micropropagation has shown a spectacular development. However, at present the widespread use of micropropagation is handicapped by the following facts:

1. Frequently mutations occur, particularly when applying the adventitious bud technique and callus systems.
2. Basic knowledge concerning factors affecting organ and somatic embryo formation in various types of cultures is often completely lacking.
3. Woody species (shrubs and trees) are extremely difficult to clone in vitro because rejuvenation can often not be induced; rooting of adult shoots is hardly possible.
4. Internal infections are still a serious handicap for commercial application.
5. Problems as vitrification (a physiological disease) and exudation of toxic compounds in the medium can hardly be solved.
6. In vitro (a relatively closed system) ethylene and carbon dioxide levels can increase to an unacceptable level, often resulting in plants of bad quality.
7. The role of the physical growth factors (light, temperature, humidity, the gas phase) is strongly neglected.
8. During the transfer from test tube or container to soil a high percentage of the plants can be lost, because the micropropagated plants are not well adapted to the in vivo climate.
9. Mass propagation in vitro is very labour intensive, resulting in too high a cost price. Real possibilities for mechanization are still missing.
10. The techniques developed are not always economically viable and therefore rejected.
11. During large scale micropropagation timing of production is often insufficiently controlled.

1. 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propagated in the years 1984-1987, and a list of plants micropropagated in numbers greater than 100 000 (Pierik, 1987, unpublished).

Number of commercial tissue culture laboratories in:

1984	1985	1986	1987
33	42	50	60

Number of plants propagated

	1984	1985	1986	1987
Pot plants	15,428,130	17,412,586	19,822,274	23,412,576
Cut flowers	10,036,990	11,420,824	12,639,758	16,202,707
Bulbs and corms	1,458,019	5,358,740	8,085,920	10,998,407
Orchids	1,534,500	1,116,740	1,449,190	1,659,530
Misc. ornamentals	242,383	301,865	375,805	564,906
Agricultural crops	280,000	300,000	311,825	309,200
Vegetables	60,040	71,205	68,825	73,233
Total	29,040,062	35,981,960	42,753,600	53,220,559

List of plants propagated in the Netherlands in numbers of more than 100 000

Bulbous and cormous crops	: L i l i u m , N a r c i s s u s
Orchids	: C y m b i d i u m , V u y l s t e k e a r a
Cut flowers	: G e r b e r a , S t a t i c e , R o s a , A n t h u r i u m a n d r e a n u m , A l s t r o e m e r i a
Pot plants	: N e p h r o l e p i s , H o r t e n s i a , S a i n t p a u l i a , P h i l o d e n - d r o n , A n t h u r i u m s c h e r z e - r i a n u m , C a l a d i u m , C o r d y l i n e , C a l a t h e a , D a v a l l i a , S y n c h o n i u m , B r o m e l i a c e a e , A l o c a s i a , F i c u s , S p a t h i p h y l l u m
Plants for aquaria	: C r y p t o c o r i n e , E c h i n o d o r u s
Agricultural crops	: S o l a n u m t u b e r o s u m

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 the success of micropropagation techniques is quite clear.
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. For many plant species, particularly vegetables, in vitro propagation is only used for a restricted purpose, such as breeding.
3. The most notable omission from the production figures is any significant number of hardy nursery stock plants and (forest) trees.

2. Basic problems when propagating plants in vitro

During in vitro propagation a number of problems arise which can be summarized as follows:

2.1. 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 (growth) 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 when aneuploid cells occur in the original explant.

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 somitic 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 induction of mutations by the application of in

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 (growth) 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 (growth) regulators can induce mutations.
3. Whether other factors (e.g. nutritional) are possibly responsible for mutations.

2.2. 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 from these families) have a relatively high capacity for regenerating organs in vitro: *Solanales*, *Cruciferales*, *Compositales*, *Liliaceales*. 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.

2.3. Physical factors

Due to the fact that the composition of the culture medium (in particular the growth regulators) strongly dominates in vitro culture, relatively little attention is being paid to the influence of the physical factors: day length, light intensity, spectral composition of the light, the gas phase (oxygen, carbon dioxide and ethylene), day and night temperature. Another argument for neglecting physical factors in growth and development in vitro is the relatively expensive growth rooms, measuring devices and other facilities that are required for experiments with physical factors.

Because test tubes, flasks, and plastic containers are more or less closed systems, it will be necessary to determine the carbon dioxide and oxygen concentration in the test tubes and to measure

photosynthesis and respiration. Oxygen is particularly important because it strongly determines organ regeneration. If the carbon dioxide concentration is too low for photosynthesis, or too high due to excessive respiration, gas exchange should be improved without endangering sterility. Nor should improved gas exchange promote water loss by evapotranspiration which can be a disadvantage.

2.4. Rooting problems with shrubs and trees

One of the most striking differences between herbacious and woody species, is that the latter are far more difficult to clone in vitro. 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 and 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 Citrus, Populus and Salix.

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.

2.5. Production of toxic compounds in vitro

A very common problem in plant tissue culture is the build-up of toxic brown and/or black substances on wounded surfaces which is generally due to oxydation of phenolic substances. The oxydation products 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.

2.6. 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. Hayashi and Kozai (1987) developed a facility for accelerating the acclimatization of micropropagated plants. 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. Rooting of micropropagated shoots in vivo is usually more economical than rooting 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.

2.7. 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 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).

2.8. Internal infections

Despite many precautions the starting material for in vitro culture can be infected internally. Endogenous 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 or 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 *Bacterium licheniformis* and/or *B. subtilis*); 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.

3. 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:

In vitro propagation systems described in 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.

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.

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.

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.

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 reduce 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.

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.

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.

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

There are a number of factors, which strongly influence the cost price of in vitro produced plants and the productiveness of a commercial tissue culture laboratory. These are summarized below:

4.1. The cost price of micropropagated plants is quite high because 70% consists of labour. More attention should be paid to automation in the tissue culture laboratory. Robotization and the use of fermentation culture and so-called synthetic seeds (encapsulated somatic embryos) can also save labour costs. To save labour in micropropagation liquid media can be added to exhausted cultures, instead of transplanting to a fresh medium (Maene and Debergh, 1985).

4.2. Timing of the production is 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.

4.3. 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. The most important factors in spreading of

bacterial contaminants are: (1) resistance of certain bacteria against (short) flaming; and (2) survival of bacteria in ethanol.

4.4. 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.

5. References

The reference indicated with * has mainly been used to prepare this manuscript and can be considered as a key reference.

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