Advances in regeneration and micropropagation

Anna Bach
Faculty of Horticulture
University of Agriculture
Kraków

In vitro cultures are increasingly important for the propagation of plants in modern agriculture, horticulture and forestry. For example, the application of genetic engineering for crop improvement cannot be successful without the routine, rapid and large-scale regeneration of "new plants".

Moreover, without these methods for rapid multiplication of vegetatively propagated crops (e.g. most fruit crops and ornamentals) and slowly growing plants (e.g. forest trees) breeding including genetic engineering would have an impact on the agricultural industry only after 20 to 30 years. Especially the new low-input agriculture — necessary for both environmental and economic reasons — requires the application of these biotechnological techniques (e.g. inbuilt pest resistance mechanism) in order to reduce fertilizer and pesticide use. Cost reduction is, however, essential to maintain the plant propagation industry in Europe. It seems that expensive laboratory facilities are no longer necessary (58). The objective of the present review is to summarize the recent advances in clonal propagation.

1. New methods for enhancement of regeneration

Plants can be regenerated *in vitro* from cultured meristems (micropropagation) or by the formation of somatic embryos or adventitious buds (52). The current methods of micropropagation are time consumig and depend heavily on manual labor.

When looking at factors such as increased costs of heating, restrictions regarding pesticides and fertilizers use or increasing labor costs it would seem that the odds are stacked against the *in vitro* industry as high costs and low profitability have restricted the market to the high value plant species only. They include medicinal, spice, forest and fruit species (29).

Each method of micropropagation which can provide substantial reduction in costs of production and help extend the technology, can be of particular use in biotechnology as well as in large scale propagation. There are a lot possible ways of stimulating propagation *in vitro* and reducing cost during growth stage, which may be divided into tissue factors and those connected with growing conditions.

1.1. Preparation of explants

1.1.1. Etiolation

In search for enhanced micropropagation methods a new technique has been worked out. According to this method, micropropagation of plants can be regarded as a two-step process with the multiplication of shoots followed by the etiolation treatment in darkness for 30-40 days. Chen Wei-Lun et. al., (14) have reported that *Populus tomentosa* produces in such conditions 3-5 times more shoots suitable for rooting than if "traditional methods" are used. Thanks to the etiolation technique, regeneration rates as high as fifteen plants per node of pineaple could be achieved (27). According to the authors, the etiolation technique has the advantage of avoiding callus production, thereby reduces the problem of genetic instability during micropropagation of trees.

1.1.2. Forced flushing

An original method of micropropagation of trees by means of forced flushing was described by Vieitez et al., from Spain (55). A 300 year-old *Quercus robur* tree was segmented into 25-30 cm fragments of branches, laid horizontally and forced to flush shoots in a growth chamber. Efficient multiplication of shoots was achieved by culturing decapitated shoot explants on medium supplemented with 0.89 μM of BAP. Similarly, Diner (15) reports that winter dormant buds of mature larch (*Larix europea*) can produce multiple shoots only when the branches are placed horizontally in water and buds are allowed to flush and grow for 4-5 days before explantation.

1.1.3. Laser irradiation

Among factors accelerating the regeneration process laser irradiation is indicated. At the 1991 Annual Meeting of the American Society for Horticultural Science, Al-Juboory (2) described reduced level of contaminations, increased growth, and regenerability of Pothos explants cultures treated with twenty five 30-second pulses of XCl eximer laser radiation. In addition, Portuguese scientists Rodrigues et al., (43) reported that the use of lasers to cut explants for the propagation of woody plants (*Persea indica*) resulted in

faster development of axillary buds as compared with explants cut with a blade. The authors suggest that the faster regeneration may have been caused by the stress temperature increase resulting from the application of laser.

1.1.4. Squashing and homogenization

In order to reduce the amount of manual labor required to divide and subculture tissue cultures, Molgard et al., (36) used homogenization of *in vitro* — cultured *Saintpaulia ionantha.*

The homogenization was done in sterile distilled water at 24,000 rpm then spread over agar medium with 0.5 mg BA/l and 0.1 NAA/l for five seconds. The method yielded an average of 64 shoots per 10 cm culture dish. This was the first report of adventitious shoots being formed directly from homogenized tissue of angiosperm. It is well known that ferns can also regenerate after homogenization of their tissues (11).

1.2. Media

Nutrient media, plant growth regulators and environmental factors are considered growing conditions for plants propagated *in vitro.*

1.2.1. New growth regulators

Plant growth regulators play the role of trigger compounds of media for increasing the potential number of regenerated organs.

At Hungarian University of Agricultural Sciences, Gyulai et al., (23) have developed a bioassay for auxins and cytokinins *in vitro* based on *in vitro* organogenesis from tobacco leaf disc. The new feature in the presented test, if compared to the pioneering methods of Skoog, is the individual use of auxins and cytokinins in the nutritive. This selectivity provides a new tool to determine whether a compound exerts auxin (IAA, ABA, NAA, 2,4-D, 2,4,5-T) or cytokinin (BA, ZEA, KiN, DPU, TDZ) activity.

1.2.2. Promotion of organogenesis and somatic embryogenesis

It seems that the mineral compounds of media, especially microelements such as copper ions, play an important role in the achievement of optimum regeneration.

Copper ions concentrations higher than normally used in plant tissue cultures ($CuSO_4$, 0.1 — 100 μM) enhance formation of shoots and roots in triticale and wheat calli as well as regeneration of shoots in cultured tobacco leaf discs (41).

It was found that root initiation, elongation and branching, as well as shoot initiation of wooddy plants were increased when cupric sulfate was replaced by moderate concentrations of cupric chloride (6).

Garcia-Sogo et al., (21) described the improvement of *Cucumis melo* plants regeneration from *in vitro*-cultured cotyledon-derived explants by increased levels of copper ions in the culture medium.

It was observed that some physical or chemical factors could improve mass production of somatic embryos (7). The increase in concentration of sucrose or glucose enhanced the number of ginseng and roses as well as asparagus somatic embryos. Morever, treating ginseng shoots with temperatures of 30-40°C could increase eightfold the number of somatic embryos. It was reported that somatic embryogenesis in *Citrus deliciosa* could be stimulated by substituting galactose for sucrose. The authors suggest that late-cycle utilization of phosphorus and somatic emryogenesis are related since utilization of phosphorus is a trigger element for the increased energy requirements of somatic embryos development (12). In contrast to cultures containing galactose, phosphorus appears to be rapidly depleted in sucrose-based medium and is thus unavailable for embryo development. Von Arnold, Egertsdotter and Mo (56) reported that extracellular proteins from *Picea abies* somatic embryos could regulate the development of other *P. abies* somatic embryos.

Recent reports have also described the ability of antibiotics (Cefotaxine, penicillin G or carbenicillin) to stimulate the process of somatic embryogenesis in *Dianthus cultivars* (37).

1.2.3. New media and containers

The use of media solidified with a starch-gelrite combination proved better to agar-solidified medium in enhancement of growth, differentation and secondary metabolite production by plant tissue cultures (10,24). The formulation of convenient liquid MS medium from the concentrate appears to be superior to powdered MS medium for both regeneration of nontransgenic and transgenic plant tissues (31). A novel method of micropropagation "in the bag" was described by Zacharias (58). Agristar, a USA company, manufactures a semipermeable membrane container marketed under the name CultuSAK. The recyclable plastic container is heat sealed to provide a totally enclosed, sterile environment that allows gas exchange while being impermeable to liquids. Plants in CultuSAK grew two to three times the amount they grew in conventional glass test tubes.

2. Micropropagation

2.1. Electrostimulation of *in vitro* cultures

Many researchers have reported that growth and regeneration can be stimulated by applying electric fields to plant tissue culture.

Dutta (17) from India has found that application of 1-2 microampers of

electric current to leaf-derived callus cultures of *Populus deltoides* results in an efficient increase of shoot proliferation.

The pulsed microcurrent intensities in the range of 0.6-2.6 μA promote shoot differentation in cabbage (*Brassica oleracea var. capitata*). The presence of BA is essential for the promotive effect (51).

Another example of electrostimulation of regeneration in tobacco leaf-derived callus, especially development of roots, was observed (4). It has recently been reported that pulsed electromagnetic field can stimulate growth of *in vitro*-cultured *Castanea sativa* embryos (44,40). It is suggested that electrostimulation may facilitate the uptake of ions and nucleic acids from the surrounding culture medium by inducing pore-like openings in the plasma membrane. It enhances cell wall formation and cell division (40).

2.2. The effect of mycorrhized fungi on cultures *in vitro*

A number of recent studies have been conducted on the effects of mycorrhizal fungi on growth and development of various micropropagated plants. Improved strains of vesicular arbuscular (VA) mycorrhizal fungi have been used to improve plant performance on soils of low biological activity. These symbiotic fungi are associated with the roots of 90% of all plants. They magnify the effective surface area of plant roots up to tenfold and can produce a 20-200% increase in plant growth. A close look at the publication discussing the effect of mycorrhizal fungi on *in vitro* cultures shows the discrepancy in results obtained.

Wang (50) reported that inoculation with VAM fungi resulted in reduced mortality and increased leaf and root dry weight of gerbera and *Nephrolepis*, as compared with uninoculated controls. The mycorrhizal gerbera plants flowered faster than control plants.

Quince (*Cydonia oblonga*) rootstock, inoculated with mycorrhizal fungus *Glomus intraradices* showed increased nematode tolerance in low-phosphorus soil (13). It was found that the effect of the mycorrhizal fungus *Glomus intraradices* which inoculated root system of several micropropagated fruit shrubs depends on the species and variety. The effects ranged from beneficial (hortensia) through neutral (early maturing strawberry and raspberry) to negative (late-maturing strawberry and raspberry). It seems that specific effects of each species of the arbuscular mycorrhizal fungus on individual prospective host plants must be determined (53). At the University of Maine — Canada the effect of inoculating *in vitro* of regenerated microcutings of *Vaccinium angustifolium* (lowbush blueberry) with mycorrhizal fungi was tested. It has been found out that one of the fungi, namely *Hymenoscyrphus ericacae* significiantly increases growth of seedlings (32).

2.3. Improvement of rooting *in vitro*

Rooting *in vitro* can be promoted by *Agrobacterium rhizogenes*. The stimulation of root formation in apple shoots and eucalyptus shoot explants was observed (39). In the case of eucalyptus clones, difficulties in killing the bacteria after transformation of the explants were noted. To solve the problem, South African scientists Mac Rea and nan Staden used the inverted explants technique.

On the other hand, McAffee et al., (34) described the improvement in induction of roots on micropropagated forest trees shoots (*Pinus monticola*, *P. banksiana*, *Larix laricina*) after coculture with *Agrobacterium* strains, as compared with untreated controls and with NAA- or IAA treated controls. Exogenous polyamines were used for improveming rooting of hasel shoots (42).

2.4. Improved acclimatization

The procedure that allows to shorten the micropropagation period of blueberry from two years to six months has been developed by Cornels University scientists, Isutsa, Prits and Mudge (25). In this method, shoots obtained from *in vitro* culture were rooted in a fog chamber at relative humidity of 94-100% under a photon flux density of 100 $\mu mol/m^2/s$ for seven weeks. Then, the plantlets were passed through a fog tunnel containing a gradient of light and humidity (two weeks), and finally grown in a greenhouse for seven more weeks. The blueberry plants were 30-60 cm tall and suitable for planting in the field.

2.5. Hypoxic long-term storage of micropropagated plantlets

An original method for long-term storage of micropropagated plantlets was described by Dorion et al., (16). The exposure to low oxygen concentration can substitute for low temperature storage of plantlets of peach and a peach x almond hybrid. An interesting interaction between oxygen concentration and storage temperature was observed. At 25°C storage under 0.25-0.5% oxygen was most effective whereas at 12°C 1-2% oxygen was best. Micropropagated shoots can survive for 8-10 months at 0°C either in the air or under hypoxic conditions, whereas at 25°C for 2-4 months and at 12°C for at least 4-6 months.

3. New systems

3.1. Advances in synthetic seed technology

The idea of producing synthetic (artificial or somatic) seeds by embedding somatic embryos or other meristematic structures in a synthetic coating has

stimulated a great deal of research in recent years (22). Most attempts to use plant somatic embryos as synthetic seeds have concentrated on encapsulation of hydrated embryos. Such coated embryos do not remain viable for very long, which limits their usefulness. Immature somatic embryos can be induced to mature by culturing in the presence of abscisic acid or a high molecular weight osmoticum such as polyetylene glicol (54). This treatment produces high quality mature conifer embryos with increased storage of lipids and proteins (8,35).

Takahata et al., (46) have found that microspore-derived embryos of broccoli (*Brassica oleracea var. italica*) and Chinese cabbage (*Brassica campestris*) can be made tolerant to dessication to about 10% water content by exogenous application of abscisic acid. Survival of embryos depends on the abscisic acid concentration used and on the development stage of the embryo. Optimal desiccation tolerance was obtained with cotyledonary-stage embryos exposed to 0.1 μM abscisic acid. Such conditions allowed 27-48% of the embryos to imbibe and germinate to form plantlets and maintain that ability for as long as six months when stored at room temperature and humidity. Desiccation tolerance can also be induced by osmotic stress from high concentration of sorbitol but with a lesser effect than that of ABA (59).

Production of synthetic (artificial) seeds containing somatic embryos usually requires a callus culture, resulting in varying degrees of somaclonal variation. Methods have recently been developed for the elimination of unwanted variation using plant organs that have been directly induced from explants. Yao Yuguang et al., (57) have induced bud formation (97%) from leaf explants of two eucaliptus species and encapsulated in alginate beads. After storage at 4°C for 45 days germination rates up to 90% were achieved. Similarly, Ganeshan et al., (20) reported that axillary buds and shoot tips isolated from micropropagated grapevine plants appeard to be a proper material for encapsulating as synthetic seeds.

An original method for the production of artificial seeds using fragments of *Agrobacterium rhizogenes* transformed horseradish (*Armoracia rusticana*) hairy-roots in alginate beads was described by researchers of Nagoya University (49). As regards synthetic seed production of rare orchids, Tandon et al., (1994) have recorded a 100% conversion frequency under *in vitro* conditions and 88% conversion frequency when the encapsulated protocorm-like bodies of *Cymbidium giganteum* and *Dendrobium wardianum* were sown directly in sand.

An artificial endosperm has recently been developed in carrot synthetic seed industry (38). The endosperm consisted of sustained-release microcapsules contained in self-breaking gel beads. The microcapsules gradually release sugar, swell and split after they are sown under humid conditions. The beads contain 0.1% Topsin M as a fungicide. Gel beads are sorted automatically; those containing low-quality embryos are rejected. The percentage of germinated carrot embryos in a greenhouse is 52%. The successful results, i.e. 90% of the somatic embryos of sweet potato forming roots and up to

50% regenerating plantlets were obtained after being suspended them in a viscous gel (fluid drilling) (45). Automated production of somatic embryos or artificial seeds requires that morphologically well-developed embryos be separated from malformed or immature ones. At the University of Tokyo, Kurata (28) has developed a computer vision algorithm that can evaluate the shapes of somatic embryos in an automated production system. Ling et al., (30) of Rutgers University — U.S.A. described the use of computer vision sensing techniques to evaluate the quality of somatic coffee embryos. The application of computer vision for predicting somatic embryo quality suggests a great potential for using such a system in the development of synthetic seed technology (30).

3.2. Bioreactors — micropropagation via Temporary Immersion System (SIT)

With the goal of finding a simple, rapid and effective method which could be used in a genetic transformation program as well as for mass propagation, a temporary immersion system was introduced. This new culture system was described for the first time by Alvard et al. (5), as a very efficient propagation technique. Specially modified autoclavable filter units are used as culture vessels. At the 8th International Congress of Plant Tissue and Cell Culture, held in Florence, the same simple, inexpensive, easy-to-operate temporary immersion system was described. It uses a commercial filtration unit in which explants are cultured and a laboratory air pump which flushes the liquid medium from the filtration unit. The system has been successfully used with various types of explants derived from a number of different plant species (*Citrus*, *Coffea*, *Eleis*, *Hevea*, *Musa*). By varying the frequency and length of immersion, embryo development as well as hyperhydricity can be controlled (48). Embryogenic cultures are placed in the upper compartment and immersed only when the liquid medium from the lower compartment is pushed up by the air pressure. When the pressure is released, the liquid medium is flowed back by gravity, keeping the explant in a condition of high relative humidity. The medium is refreshed each month. Beneficial effects of the treatment on the rate of proliferation and fresh matter weight are unquestionable. The SIT system could be used in propagation of different species (9). According to Teisson and Alvard, "the temporary immersion technique can profitably replace costly biofermentors for both research and mass production"(48).

The SIT showed its superiority over the classic liquid or the semisolid medium in multiplication of triploid banana and plantain cultivars (18). Somatic embryos derived from male *Musa* flowers were proliferated on semisolid medium or immersed in liquid medium for one minute every six hours. The coefficient of multiplication was nine in the temporary-immersion system and three on semisolid medium.

At the 1995 Congress on *In Vitro* Biology, the use of the SIT system for

micropropagation of pineapple was described (19). The pineapple shoot axillary meristem explants inside a 10-liter Nalgane culture vessel were multiplied. The shoots were immersed in a liquid culture medium for six minutes every hour. The SIT allowed 6.000 — 8.000 plants to be produced from three initial pineapple shoots within 4-5 months.

Recently a temporary-immersion system for improved bioreactor production of potato tubers has been described (1). At present, more than 200 units of SIT are used in different laboratories.

4. Novel application of plant tissue

4.1. Detection of water pollutants

Recent studies have suggested that plant tissue cultures may be useful for bioremediation of metal pollutants. It was observed that plant cell suspension cultures could remove environmental contaminants from aqueous solution. The suspension-cultured *Datura innoxia*, *Citrus citrus* and sweet corn show significant ability to reduce the level of barum, iron and plutonium in media (26). At the Czech Institute of Organic Chemistry & Biochemistry in Prague it was found that *in vitro*-cultured, *Agrobacterium rhizogenes* — transformed hairy-roots of *Solanum nigrum* were very efficient in removing cadmium (33). According to the authors, plant hairy-root cultures may be useful for removing heavy metals from wastewater or as a model for evaluating the availability of heavy metals from sewage sludge.

4.2. *In vitro* induced sex-shifted plants

Al-Khayri et al. (3) have found that under certain *in vitro* conditions male spinach plants could shift-sex. In the experiment normally dioecious male plants become monoecious and produce seeds. The benefit of this phenomenon is great: it would achieve selfing within dioecious species.

The recent advances in micropropagation and regeneration of plants showed the wide range of the tissue culture applications into commercial propagation. Therefore, the constant development of these methods seems necessary.

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Summary

The ability to regenerate plants from cultured cells, tissues or organs provides a tool for mass propagation or plant transformation of crop species.

The review discusses the recent advances in regeneration and micropropagation. The examples include:

— new methods for enhancement of regeneration (etiolation of shoots, forced flushing, laser cutting of tissus, squashing and homogenization),

— progress in preparing of media,

— effect of electrostimulation or mycorrhizal fungi on plant micropropagation,

— new system of mass propagation (advances in synthetic seed technology, micropropagation via SIT — temporary immersion system),

— novel application of plant tissue culture.

Key words:
micropropagation, regeneration *in vitro*, explants, bioreactors, synthetic seed.

Address for correspondence:
Anna Bach, Faculty of Horticulture, University of Agriculture, Al. 29 Listopada 54, 31-425 Kraków, Poland, fax: (48) 12-11-13-22.