Modern Ways of In Vitro Propagation©

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INTRODUCTION

Ever since Murashige and Skoog (1962) published their famous nutrient medium based on the analysis of tobacco ashes in 1962, there has been a steady increase of in vitro propagated plants throughout the world. In the beginning only a modest number of plants could be propagated. Today, only a few species are still considered to be recalcitrant, and we believe that all plants can be propagated by in vitro culture — in principle. There are still many challenges to overcome, especially since productive in vitro propagation (hence plant transformation) in many species is genotype-dependant. There are still some recalcitrant species as well, e.g., *Cyclamen persicum*. This species can be propagated in vitro from leaf discs and through somatic embryogenesis from ovules. However, both these methods are still a challenge with obstacles to be overcome to be efficient. Our experience with somatic embryogenesis is that embryogenic callus is easily induced from immature embryos as described in Winkelmann et al. (1998). Still, it is difficult to synchronise somatic embryo development and give a predictable number of germinating embryos (Hvoslef-Eide, 2000). Nevertheless, the total number of plants produced in vitro had a steady increase through the seventies and eighties in Europe, like the rest of the world. In the nineties European laboratories experienced harder competition from countries with cheaper labour and European production started to decline, despite the fact that total world production was still on the rise. It was necessary to be innovative in Europe and look for production methods that reduced the labour input.

EXPLANATION OF TERMS IN USE

Propagation of plants is divided into generative (through seeds) and vegetative propagation. In this paper we will deal with vegetative propagation only. Traditional vegetative propagation includes cuttings, layering, or grafting. Cuttings can be without leaves (hardwood cuttings) or with leaves (softwood cuttings). A softwood cutting can be either just a leaf or a stem with leaves and with axillary buds in the leaf axils. Axillary buds are buds ready to burst given the right conditions, while adventitious buds break from a leaf with no preformed buds. This is an important distinction between adventitious buds and axillary buds. Only a few plants have the ability to form adventitious buds, e.g., *Begonia*, *Saintpaulia*, and *Streptocarpus*.

When propagating plants in vitro (= in glass), we use the same terms and call the shoots either adventitious if they break from leaf discs or axillary shoots if they originate from buds in the leaf axils or buds from a woody branch. By using in vitro culture, we can get adventitious shoots from many more species compared to traditional propagation, e.g., apples and other woody species. This is mainly due to the addition of plant growth regulators in vitro and the knowledge of how to manipulate cultures to form adventitious shoots. This ability to regenerate in vitro from leaf discs is an important prerequisite for most genetic transformation methods, especially using the soil bacterium *Agrobacterium*. Without a regeneration method

from leaf discs, where each shoot originates from one cell, it would be difficult to get solid transformants when using *Agrobacterium*. This method of transformation is the most common worldwide.

Chimeral plants have more than one genetic constitution in the same plant. Many plants are chimeric in nature. If the plant is chimeric with regards to flower colour, the chimeric nature is easy to spot by the different colours in the same plant or even same flower. An example could be the old cultivar of *B.* ×*hiemalis* 'Aida', which has a white rim around a salmon coloured flower. The colour of 'Aida' can be explained by the different cell layers in a plant. A plant has three to four (three is most common) cell layers (Preil and Engelhardt, 1982; Lineberger and Druckenbrod, 1985a; Preil, 1994b). The core of the plant is called LIII and can be compared to a hand, the next layer (LII) can be illustrated by a glove on the hand, while the last is the second glove over the first one. Hence a plant has two cell layers that cover the core (LIII) all around the plant. These two cell layers (LI and LII) have cell division periclinally, and this is why they cover the plant entirely in one cell layer each. In 'Aida' the outer cell layer (LI), and maybe even the next (LII), are without pigments and appear to be a white brim around each petal (Fig. 1). We can also often observe cultivars of *Saintpaulia* with dual-coloured flowers, so-called pinwheel flowering (Lineberger and Druckenbrod, 1985b). When propagating a plant, it is important to know if it is chimeral or solid. If it is solid all through the three (or four) cell layers, we can stimulate adventitious shoots and still get true-to-type plants back. Using adventitious shoots usually is more proliferative than stimulating only axillary buds to break into axillary shoots. If the plant is a chimera, we need to be very cautious; since adventitious shoots will arise from epidermis (LI) and the chimeral structure will be broken down. If this was a pinwheel-flowering *Saintpaulia*, almost all the flowers would be the colour of the epidermis and none of them would be pinwheel in the regenerated plants (Lineberger and Druckenbrod, 1985b). The only way to regenerate the pinwheel type is through axillary shoots from the buds in the axils in the flower peduncle, since axillary shoots keep the arrangement of the LI, LII, and LIII.

Growing callus (undifferentiated cells) on a solid medium or plant cells in liquid suspension cultures, somatic embryos may be induced. Somatic means that the cells originate from somatic cells. These are in contrast to gametic cells that will go through meiosis and the egg cell will fuse with a pollen cell to become a seed with a recombination of characters. The somatic embryo is a clone of the parent plant — "clonal seeds." These originate from single cells or small cell aggregates and differentiate into bipolar structures that resemble zygotic embryos (without the endosperm), truly demonstrating the totipotency of plant cells. Totipotency was first described by Haberlandt in 1902 and means that each and every plant cell has the totipotency to dedifferentiate (lose their function) and develop (differentiate) into a new plant with root and shoot (Gautheret, 1983). All the cells have the genetic information necessary to form a new plant, and plants have the ability to switch genes on and off through redifferentiation. This separates plants from animals that do not have the ability to turn genes on again if they have been differentiated and turned off in the process. One could say that the plant cells have the ability to make stem cells from differentiated cells.

Cell cultures can be an effective way of propagating plants. Cell cultures need minimal manual handling. But they require oxygen, or else they would drown. Cell

Figure 1. *Begonia* × *hiemalis* 'Aida', showing one shoot with the original two-coloured flowers with the white brim and another shoot where the chimeral structure has been lost and the flower is white through all three cell layers. Photo: Erling Strømme.

cultures can be grown in Erlenmeyer flasks on a rotary shaker to shake oxygen into the suspension. This gives the opportunity to grow many cells in a small volume. Such cells would have their origin from all cell layers (LI, LII, and LIII) and would mean the breakdown of a potential chimera. Mutations are quite frequent in plants; on average there is a mutation for every 1 million cell divisions (Broetjes and van Harten, 1978). It is therefore highly possible that every plant consists of some mutation or other, which in principle means that very few plants are not chimeral in nature. In my opinion, this could be the reason that we observe a higher frequency of variation (somaclonal variation) after cell and callus cultures than when plants are derived from adventitious shoots. Axillary shoots have even less variation, which also makes perfect sense. One needs to be aware of such potential for somaclonal variation when propagating plants. It is necessary to test methods and know the plant material well. Some species and some selections are more unstable in cell and tissue culture than others. Such plants can only be propagated in suspension cultures if a certain degree of somaclonal variation is accepted or wanted (as in the case of plant breeding).

Adventitious and axillary shoots can also be propagated in liquid cultures, in advanced bioreactors, or in simple temporary immersion flasks. The important thing is to secure enough oxygen or reduce the time immersed in liquid if oxygen is limited. Too little oxygen could result in vitrified (glassy) shoots. These have a physiological damage that cannot be restored, and they will not grow from shoots. In short, they are useless. Ziv (1989) has described this phenomenon in detail and suggests using plant growth regulators in the medium if all else fails.

BIOREACTORS FOR LIQUID IN VITRO CULTURES

Bioreactors can be compared to the greenhouse compartments called phytotrons where the climate can be regulated in much more detail than a normal greenhouse. Bioreactors can control the environmental conditions for liquid suspension cultures to a very large extent, more so than Erlenmeyer flasks (Hvoslef-Eide et al., 2003). Depending on how advanced the bioreactor is, one can control temperature, oxygen, pH, light (quality, quantity, and day length), and the stirrer speed. The CO_2 content can also be controlled if one is willing to pay the costs of an electrode to monitor the concentration. There are many different bioreactors on the market, the simplest one hardly worth calling a bioreactor in my opinion. I would call them "bioreactors," since I think the term bioreactor should be reserved for the more advanced models where a true monitoring and control of parameters is possible. Since many include the simpler ones in the bioreactors category, they will also be included here. The simpler ones are useful for their purpose and deserve mentioning, even if the term bioreactor is somewhat misleading.

The different types of growth chambers for liquid cultures are:

- Temporary immersion
  - No stirring
- Simple "bioreactors"
  - Stirred by air through an inlet from the bottom (airlift)
- Advanced computer controlled bioreactors
  - Stirred by propellers
  - Stirred by Vibromix

Through European network cooperation, COST (COST is a non-commercial cooperation in the field of scientific and technical research, funded by the European Commission), a lot of knowledge on mass propagation through shoot and cell cultures have been acquired, e.g., propagation of poinsettia (*Euphorbia pulcherrima*) through somatic embryos (Preil and Engelhardt, 1982; Brandau et al., 1997; Osternack et al., 1999; Saare-Surminski et al., 2000), cyclamen (Hohe et al., 1999a; 1999b; Hvoslef-Eide and Munster, 1998), *Eustoma grandiflorum* (syn. *Lisianthus*), carnation (*Dianthus caryophyllus*), *Clematis*, *Anthurium*, *Phalaenopsis*, and *Gentiana* (Hvoslef-Eide et al., 2004), and shoot cultures of *B.* ×*cheimantha* (Christmas begonia) (Hvoslef-Eide, 2000). Geier et al. (1992) demonstrated that there is less variation after a suspension culture of *Euphorbia pulcherrima* (poinsettia), than in the callus culture of the same genotype that was the start culture of the suspension. This may possibly be explained by a selection towards embryos with all their genetic information intact; that cells without genetic changes have greater chance of producing a normal embryo.

Temporary Immersion ('Ebb and Flo' System). This system takes into consideration that most plants are not designed to be submerged in liquid, like aquatic plants are. The name temporary immersion speaks for itself; the plant tissue or plant cells are in one container and the medium in another container. With regular intervals, liquid nutrient medium is pumped into the vessel with the plants and the plants are submerged a very short while, for minutes only. Thereafter, the medium is pumped back into the reservoir vessel for the medium. This procedure is repeated a certain number of times through the 24-h cycle (Berthouly and Etienne, 2005). If this is repeated too often, the risk increases of obtaining vitrified shoots (shoots

damaged by hyperhydricity). At the same time, it is important to secure the plant's demands for enough moisture and nutrients. There is usually a fine balance, which is optimal. Saare-Surminski and colleagues (2000) have used temporary immersion for mass propagation of *Gentiana* over a propagation period of 12 weeks. By submerging the plants for 1 min 16 times per 24-h cycle, more shoots were damaged through hyperhydricity than when reducing the number of 1-min immersions to 8 (Hvoslef-Eide et al., 2004). Preil and his coworkers in Ahrensburg have designed homemade "ebb and flow" systems in 5-L glass containers. Using this system for as little as 1 min every 24 h gave excellent propagation of *Phalaenopsis* shoots (Hempling and Preil, 2005). This system secured good quality plants and a high proliferation rate at the same time as it reduced the input of manual labour substantially. In Sweden the Swedish University of Life Sciences has done experiments in similar containers using Preil's recommendations. They have very good results with only 1-min immersion per 24 h for raspberries, strawberries, rhubarb, and bilberries (Welander and Zhu, 2004). There are several commercial systems for temporary immersion on the market. They all have in common that the volumes of the containers are rather small, and hence the time saved for manual labour is less than when using Preil's large 5-L containers. Some of these commercial systems are also very expensive.

Simple "Bioreactors." The simplest bioreactors are just plastic bags with sterile filters and a bubble aeration from the bottom. The air serves two functions in such a system: one is to supply oxygen to the plants, the other to provide mixing of the cultures. Surplus air is let out through another sterile filter through the top. These are the cheapest types of bioreactors and are much used in Israel for a number of shoot cultures (Ziv et al., 1998; Ziv, 2005). Shoots cultures can withstand such conditions better than more shear-sensitive suspension cultures. The inconvenience with such a system is the burst of the air bubbles when they reach the surface, where the burst causes cell death and foam on the cell suspensions. Problems with hyperhydricity are often overcome by using different types of growth retardants in the medium (Ziv, 2005).

Advanced Computer-Controlled Bioreactors. The COST group has experimented with two different commercial bioreactors, both controlled by computers, of the same size (2 L): Braun Biostat and Aplikon. In both these commercial bioreactors, the COST members have most often modified the oxygen supply according to the recommendations by Walter Preil and coworkers (Luttman et al., 1994). The supply of oxygen is secured by diffusion through very thin silicone tubing (0.2 mm) in a 2-m long tube by various systems. The common feature of all these modified oxygen systems is that they were all fixed installations in the bioreactors. The thin walls of the silicone tubes allow diffusion of gasses (oxygen, CO_2, and ethylene) to enter and escape according to the difference in concentrations of the medium and the tubes. This allows non-sterile air enriched with oxygen to be used, since the contaminants are too large to pass through the pores of the tubes. This is an efficient way of supplying oxygen and removing exhaust gasses as long as the tubes are not too old. We have reported earlier of problems with clogged up tubes (Hvoslef-Eide and Munster, 1998). The recommendation is thus to replace all the tubes at regular intervals to ensure good diffusion of gasses.

Several types of stirring mechanisms have been used by the COST members, both propellers and a type of mixer used for mixing paint called Vibromix. This a metal plate with conical perforations that vibrates at high speed. Although they were designed to mix paint, they are quite effective also for cell cultures. Some cultures that are especially sensitive to shear forces may respond negatively to the Vibromix, but these mixers are better than the normal stirrer provided in commercial bioreactors (Preil, 1988; 1991).

Using computers to control the temperature, oxygen level, stirrer speed, etc. is very accurate. The temperature is controlled through water baths with a water flow in a double jacket around the bioreactor. The oxygen level is controlled by an electrode measuring the oxygen saturation in the medium and a valve enriching the air of the silicone tubes with pure oxygen. If required, the pH can also be controlled through adding acid or base through sterile filters in the lids of the reactors (Hvoslef-Eide et al., 2005).

Development of Novel Bioreactors for Mass Propagation of Plants. At the Norwegian University of Life Sciences, UMB (formerly Norwegian University of Agriculture, NLH) we had a strong motive to establish methods of mass propagation, which required less manual input and less work in the flow hood and are more effective than the tissue culture methods on solid medium. Preil and his coworkers in Ahrenburg in Germany had already introduced the idea of using suspensions to produce somatic embryos in liquid cultures (Preil and Engelhardt, 1982; Preil et al., 1982). He also introduced the use of commercial, computer-controlled bioreactors for mass propagation in the COST cooperation (Preil, 1988; 1991). At first, poinsettia was introduced as a model plant but plants from somatic embryos of poinsettia turned out to be without the important branching factor. Today we know that this branching factor is a plant pathogen (a phytoplasm), which was removed through cell cultures. Without the branching factor, the plants were almost 2 meters tall with a single star at the top (Preil 1994a). At the Norwegian University of Life Sciences (UMB) we made use of Preil's expertise and came to visit him in his laboratory to study bioreactor design and the pros and cons of the commercial types he had there. Then we designed our own bioreactors. The disadvantages of the commercial types were many, since they have been designed to grow bacteria and not plants:

- Stirring by propellers gave high shear forces, causing stress and cell death
- Supply of oxygen yielded bubbles that burst on the surface, causing cell death and foam
- Growth of cells on stationary oxygen suppliers inside
- Lastly, but not least, they were very expensive to purchase

The workshop at UMB used these experiences to construct and build a series of six identical bioreactors, taking into account the special requirements of successful growth of plants in liquid cultures. Comparable, computer-controlled bioreactors were available commercially at the time (1992) for approx $30,000, while ours cost approximately $10,000 each. Detailed descriptions of how our UMB bioreactors are constructed can be found in Hvoslef-Eide et al. (2005). In short; our bioreactors have been constructed so that there are no quiet zones were cells can settle and grow on the instruments and still not be subjected to high shear forces. This is obtained through installing the thin silicone tubing (2 m long) in loosely hanging

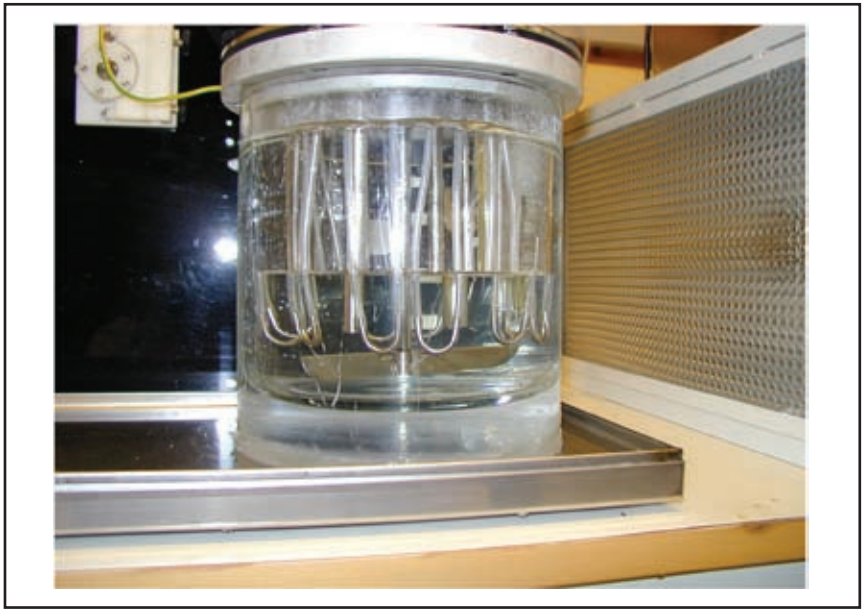

Figure 2. One of the bioreactors constructed and built at the Norwegian University of Life Sciences showing the loosely hanging silicone tubing and the pitch-blade propeller inside the bioreactor. The double jacket container to provide temperature control is also visible. The reactor has just come out of the autoclave where it has been sterilised together with one litre liquid medium. It has been calibrated and is now ready for inoculation of cells.

loops under the lid in a way to provide an inlet and an outlet after the whole length has been dipped into the medium, and able to move with the movement inside (Fig. 2). The other special invention is the pitch-blade propeller that changes direction at regular intervals (10 sec has been a good timing). By changing direction, the stirring can be so much gentler than if the stirring is in one direction only. Also the change of direction secures movement and no quiet zones where cells can settle. Otherwise, our bioreactors have exactly the same functions that the commercial bioreactors have for control of temperature, oxygen, and pH. They are different also in the mixing of the gasses and how the temperature is kept at set point (see Hvoslef-Eide et al., 2005 for details). We now have six identical bioreactors that can be used in factorial experiments, which gives much more information regarding effects and interactions than just comparing a bioreactor with the growth in Erlenmeyer flasks. We have grown suspension cultures of *Daucus carota* (carrot), *Betula* (birch), *Cyclamen*, and *Picea abies* (Norway spruce), obtaining somatic embryos (in Norway spruce only after plating the cultures on solid medium for embryo formation) in these bioreactors. We have also grown shoot cultures of Christmas begonia (*B.* ×*cheimantha*) with success.

CONCLUSIONS

It is extremely important to know the plant material to choose the right method for vegetative propagation to get true-to-type plants in the regenerated plants. If

the plants are chimeras, the only method that can be used is regeneration from axillary buds, a method that ensures all three cell layers to be intact in the regenerated plants (e.g., in pinwheel-flowering *Saintpaulia*). Regeneration in liquid cultures gives a high proliferation rate and can be used when propagating genetically stable plants. Simple and advanced bioreactors have been successfully used for mass propagation of plants for both adventitious shoots and somatic embryogenesis. Often a combination of proliferation in bioreactors and temporary immersion is the most effective and gives good quality plants and a sound economy in commercial production.

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