Chapter 4

Practical aspects of bioreactor application in mass propagation of plants

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Abstract: Bioreactors are an efficient tool for the production of plant propagules but, at present, their application is commercialized in only a few tissue culture companies. The present article reviews practical aspects of the use of bioreactors in the mass propagation of plants in relation to the responses of plant propagules in liquid medium, the characteristics of bioreactor culture techniques in plant propagation and discusses case studies of the use of bioreactors for several plant species including *Fragaria ananassa*, *Lilium* species, *Hippeastrum hybridum, Gladiolus grandiflorus, Spathiphyllum, Colocasia esculenta* and *Solanum tuberosum*. The establishment of plantlets from bioreactors and future prospects are also described.

Key words: bioreactor, liquid culture, mass propagation

Abbreviations: ABA - abscisic acid, MS - Murashige & Skoog medium (1962); FW - fresh weight; vvm - volume of gas per volume of liquid per minute

1. Introduction

The use of bioreactors in plant propagation is promising for the efficient production of propagules. As compared to conventional tissue culture techniques using solid or semi-solid medium, bioreactors require fewer culture vessels, less labor, utilities and space. At present, the use of bioreactor technology is starting to be commercialized in tissue culture nurseries, the results of laboratory-scale experiments suggest the practical applicability of the technique in plant propagation.

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The bioreactor is a vessel widely used in the cultivation of organisms such as microbes, animal or plant cells to produce metabolites or cells (Coombs, 1986; Takayama, 1997, 2000). Bioreactor technology is also applicable to the propagation of plants. The application for plant propagation was first reported in 1981 for *Begonia* (Takayama and Misawa, 1981). The techniques have been applied to many plant species since then (Styer, 1985; Takayama et al., 1986; Wheat et al., 1986; Akita and Takayama, 1988; Levin et al., 1988; Preil et al., 1988; Hale and Young, 1991; Preil, 1991, 1995; Preil and Beck, 1991; Sondahl and Noriega, 1991; Takayama, 1991; Ziv, M., 1991, 1992; Takahashi et al., 1992; Akita and Takayama, 1993a,b; Akita et al., 1994, Akita and Takayama, 1994 a,b; Harrel et al., 1994; Luttmann et al., 1994, Takayama and Akita, 1994; Tautorus et al., 1994; Ziv et al., 1994; Heyerdahl et al., 1995; Ziv, 1995; Ziv et al., 1995; Levinet et al., 1996; Moorhouse et al., 1996; Okamoto, 1996; Ziv and Shemesh, 1996; Chatterjee et al., 1997; Akita and Ohta, 1998; Hao et al., 1998; Takayama and Akita, 1998; Escalona et al., 1999; Abdullah et al., 2000; Akita, 2000; Correll et al., 2000; Gao et al., 2000; Ingram and Mavituna, 2000; Vinocur et al., 2000; Ziv, 2000; Honda et al., 2001; Lorenzo et al., 2001; Paek et al., 2001). In plant propagation using bioreactors, the process consists of an inoculation process using small propagules with multiple shoot buds that are aseptically inoculated into the bioreactor. These are cultured for one to two months submerged in liquid medium with forced aeration leading to the production of a large number of transplantable-size propagules such as plantlets, bulblets, microcorms or microtubers. In the case of *Spathiphyllum*, about 30,000 plantlets were obtained after two months of culture in a 10-litre glass bioreactor, among them about 3,000 plantlets were transplantable into soil. The advantages of the use of bioreactor in plant propagation are as follows (Takayama and Akita, 1994).

1. Large numbers of plantlets are easily produced and scaling up is easy.
2. Since handling of cultures, such as inoculation or harvesting is easy, labor cost is saved.
3. Cultures are always in contact with the medium, facilitating uptake of nutrients and growth rate.
4. Forced aeration (oxygen supply) is performed, which improves the growth rate and final biomass achieved.
5. Cultures are moving in the bioreactor, which results in the disappearance of apical dominance and in the development of numerous shoot buds into plantlets.

In spite of these advantages, there are some shortcomings such as hyperhydricity, plantlet size variation caused by different growth stages, and

microbial contamination (Takayama and Akita, 1998). In this report, we focus mainly on the fundamental and practical aspects of the use of bioreactors in plant propagation. *Spathiphyllum* plants have been used as a case study of propagation in a bioreactor and of the feasibility of reestablishment of the plants in soil.

2. Responses of plant propagules in liquid medium

In order to propagate plants in bioreactors, plantlets should be firstly cultured in liquid medium. For most terrestrial plants, growth is limited in water. In spite of this fact, growth of *in vitro*-cultured plantlets in liquid medium is stimulated in many plant species when aerated. The reasons are based on the physiological characteristics of cultures as described above (items 3, 4 and 5 above). From our experiences, many species of ferns and herbaceous angiosperms grow well, but the propagation of tree species, especially gymnosperms, is difficult in liquid medium. Many monocotyledonous plant species are suitable for propagation in liquid medium. Plantlets grown in liquid medium tend to have a hyperhydric nature (vitrification) of leaves and stems. This hyperhydric nature can be partly overcome with the use of a modified medium with lowered concentrations of salts and sugar and also by increasing the light. The morphology of propagules in the liquid medium is influenced by culture conditions such as medium components, irradiation, temperature, aeration, and differences between genera or species. Regulation of morphology and physiological status of cultures in liquid medium is important for the successful production of propagules in the bioreactor.

3. Solid culture, shake culture and bioreactor culture

In order to establish protocols for propagation of plants in the bioreactor, culture conditions should be examined precisely using solid medium and then using liquid medium in shake culture. Shake culture can be used to evaluate the suitability of a plant species for cultivation in a bioreactor. The shake culture method itself is rather easy and requires less labor than solid culture and is efficient for the production of a large number of plant propagules. However, the operation cost is still high, and a large number of culture vessels are required. Shake culture is the transition stage from solid culture to bioreactor culture**.** The efficiency of propagation of large number of plantlets is quite high in the bioreactor compared to solid culture or shake culture and results in a savings of labor cost (1/12.5). The number of plants

produced and the manual labor required for plantlet dividing and transplant were almost the same between bioreactor and culture bottle. The efficiency of reestablishment of plants in soil is almost the same for the bioreactor and culture bottle.

Table 1: Comparison of the specifications of *Spathiphyllum* propagation in bioreactor with liquid medium and in culture bottle with solid medium

Items	Bioreactor	Culture bottle
Equipment:		
Vessel volume	20 l	500 ml
Medium volume (litre per vessel)	16.6 l (liquid)	100 ml (solid)
Number of vessels	6	1000
Inocula (number of test tubes)	96	150

4. The characteristics of bioreactor culture techniques in plant propagation

Many plant genera and species have been investigated in shake and bioreactor cultures. Cultures that grow well in liquid medium can be scaled up for bioreactor culture. This chapter will describe a case study of mass plant propagation focused mainly on the preparation of inocula, types of propagules, methods of bioreactor culture and cultivation in soil.

4.1 Preparation of 'seed cultures' (inocula)

In order to produce a large number of plantlets in a bioreactor, large numbers of propagules developing new shoots are prepared as inocula. The appropriate propagules include a) multiple shoot buds, b) regenerative tissues such as protocorm-like bodies, embryogenic or meristematic tissues, c) somatic embryos, or d) stems or shoots with a number of axillary buds. Here multiple shoot buds and stems or shoots were used as inocula because of their genetic stability**.** Small pieces of tissue with multiple shoot buds that have been propagated in the test tubes were the most preferable inoculum for the bioreactor. For example, in the case of *Spathiphyllum*, a piece of tissue cultured on 10 ml of agar medium was used to inoculate 1 to 2 litres of liquid medium in the bioreactor. Optimum inoculum size differs between genera or species, but usually a small inoculum size is sufficient. Multiple shoot bud cultures serially subcultured in shake flasks were used as inocula,

but sometimes they only remained as multiple shoot buds without growing into plantlets even in the bioreactor. Cases like these should be considered when establishing propagation schemes.

4.2 Types of propagules produced in the bioreactor

Various types of plant propagules such as shoots, bulbs, microtubers, corms and embryos have been successfully propagated in bioreactors. Several examples are as follows:

a) Shoots: *Atropa belladonna, Begonia×hiemalis, Chrysanthemum morifolium, Dianthus caryophyllus, Fragaria ananassa, Nicotiana tabacum, Scopolia japonica, Spathiphyllum, Stevia rebaudiana, Zoysia japonica, Primula obconica*.
b) Bulbs: *Lilium* species*, Fritillaria thunbergii, Hippeastrum hybridum, Gladiolus, Hyacinthus orientalis*.
c) Corms: *Colocasia esculenta, Pinellia ternata, Caladium* sp.
d) Tubers: *Solanum tuberosum*
e) Embryos or adventitious buds: *Atropa belladonna*

The propagules produced in the bioreactor should be easily to reestablished in soil, with as little adaptation to *ex vitro* conditions as possible. Storage organs such as bulbs, corms or tubers seem to be the best choice for proliferation in bioreactors.

4.3 Configuration and operation of bioreactors: practical aspects

The bioreactors used for research are usually quite expensive, and are not practical for commercial plant propagation. In order to reduce the costs, simplicity of structure and handling, long-term maintenance of aseptic conditions, and efficient growth of propagules must be considered in designing the bioreactor. Practical bioreactors for plant propagation will provide batch cultures without sensors or controlling devices (Figure 1). This type of bioreactor is easily applicable to many plant genera and species including *Spathiphyllum* propagation as indicated in figure 2. At the end of the culture period, propagules should be of sufficient development and easily harvested, transplanted and reestablished in the soil. At present, plant genera or species with these characteristics are limited, but in the future when the regulation of the growth and physiological characteristics become highly developed, bioreactors may be more widely used in the mass propagation of plants.

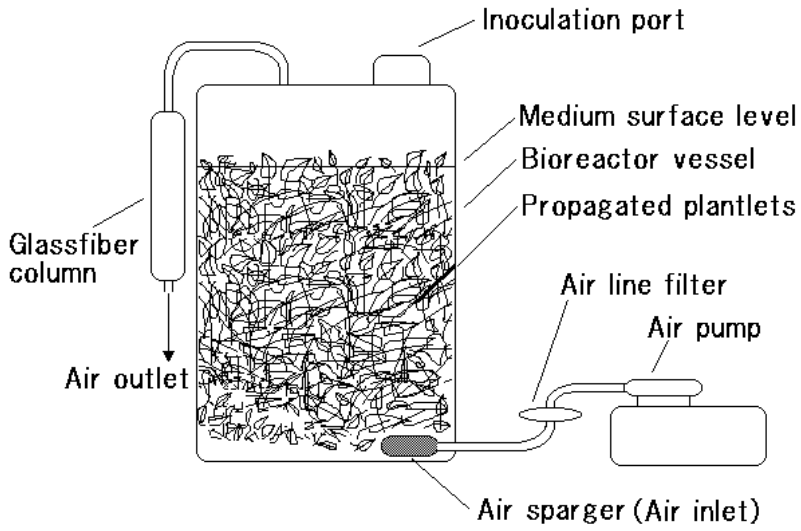

Figure 1: Simple aeration bioreactor for various plant propagation using liquid medium.

5. Case studies of the use of bioreactors in plant propagation

5.1 Strawberry (Fragaria ananassa)

Strawberry plants propagated in the field are easily infected by viruses, and so virus-free plants propagated by tissue culture are widely utilized by the growers. The propagation of strawberry is rather easy in conventional tissue culture (Boxus, 1974, 1976; Boxus et al., 1977; Damiano, 1980). The method is primarily performed to produce virus-free stock plants, but the production of large numbers of plants required for commercial cultivation in glasshouse or in the field can not be supplied trough tissue culture, so virus-free plants propagated by tissue culture are further propagated by *ex vitro* cultivation. The ultimately aim of mass propagation using bioreactors is to produce large numbers of virus-free plants to distribute directly to farmers. We reported the mass propagation of strawberry plantlets using bioreactors (Takayama et al., 1985a).

Tissue segments of multiple shoot buds induced on 10 ml of agar medium supplemented with 1 mg l^{-1} 4PU (N-(2-chloro-4-pyridyl)-N'-phenylurea) in 25 mm (diameter)×125 mm (length) test tubes were

inoculated into liquid medium in a bioreactor operated at an aeration rate of 0.25 vvm under illumination of fluorescent light at 12.5 μmol $m^{-2}s^{-1}$.

The growth of plantlets in the bioreactor was stimulated by the addition of cytokinin, but they were hyperhydrated (vitrified) when harvested from the bioreactor. Therefore, the medium used for bioreactor culture contained no cytokinin or was supplemented with only a low concentration of cyotokinin.

After about one month, cultures were of transplantable size. The plantlets were taken out of the bioreactor and directly transplanted in vermiculite, covered with transparent film and cultivated in a glasshouse in shady conditions (acclimatization). The plantlets just after harvest from the bioreactor were slightly etiolated comparing to intact plants growing in the field (total content of chlorophyll was about 1/3). However, during acclimatization, chlorophyll content increased and after 2 to 4 weeks, new leaves emerged and plants were successfully acclimatized. More than 90% were successfully established in vermiculite.

5.2 Lilium species

Lilium species are important flowering plants and consist of 80 species (Willis, 1978) including over 10,000 varieties or strains. Most of strains are clonally propagated, reflecting a practical need for mass clonal propagation by tissue culture.

The process for clonal mass propagation of *Lilium* was patented and applied to bioreactor culture (Takayama and Misawa, 1982, 1983; Misawa and Takayama, 1985; Takayama et al., 1991**;** Takayama et al., 1996). The process consists of a) induction of multiple bulbscales by cytokinin, b) stimulation of the growth of each bulbscale in aggregated form in liquid medium, c) dissection of each bulbscale, and d) regeneration of newly formed bulblets in liquid medium. The bulblets thus obtained were easily transplanted in soil without acclimatization, and were grown to the flowering stage (Takayama et al., 1982, Takayama and Ohkawa, 1990).

The time to flowering was dependent on the species or cultivars, e.g. one year in *L. longiflorum* and *L.* × *formolongi*, or several years in *L. auratum* and *L. speciosum.* The duration also depended on bulb size. In order to shorten the time to flowering, production of larger bulblets is required. An efficient method for production of larger bulblets utilized higher medium concentrations (Takayama and Misawa, 1979), longer incubation time, and larger volume of culture medium per bulblet (Takayama et al*.,* 1982; Takayama and Ohkawa, 1990). Culture methods satisfying these conditions were used in the bioreactor. In order to produce large numbers of bulblets in the bioreactor, large numbers of regenerative tissue segments must be

inoculated. Regeneration ability of bulbscales revealed traverse gradient in one bulbscale. The most regenerative segment dissected from one bulbscale was the proximal portion while low regeneration ability was seen on the distal portion (Takayama and Misawa, 1983). In spite of these observations, cutting the bulblets randomly into pieces simplifies the process and is satisfactory. Use of such bulbscale segments as inocula resulted in the production of a large number of newly formed bulblets, and is the preferred inocula for bioreactor culture (Tsumaki and Takayama, 1992).

Although the efficiency of the production of bulblets in liquid medium (shake or bioreactor) was different between species, almost the same culture conditions were optimal within the same species or genera. However, sometimes quite different culture conditions were optimal in different strains within same species of *L. auratum* (Takayama and Okuyama, 1996).

5.3 Amaryllis (Hippeastrum hybridum)

Hippeastrum hybridum is a well known ornamental bulbous plant and is usually clonal propagated by twin scaling (Hanks, 1986; Okubo et al. 1990, Huang et al., 1990a, Stancato et al., 1995), but because of virus disorders and a low propagation rate, alternative tissue culture methods have been developed by several authors (Mii et al., 1974; Hussey et al., 1975a,b; Yanagawa et al., 1977, 1980; Fountain and Rourke, 1980; Huang et al., 1990b), but the propagation efficiency is still not adequate for production of large numbers of plants. Tissue culture of *Hippeastrum* is not difficult, but the multiplication rate is usually not high enough to produce a sufficient number of propagules for bioreactor culture. The use of cytokinin was not effective in the stimulation of the regeneration of newly formed bulblets, in fact cytokinin inhibited the growth of bulblet. In order to stimulate the regeneration and growth of *Hippeastrum hybridum in vitro*, the authors employed the noching method on the basal end of the small bulblet propagated *in vitro*. The bulblets were vertically sectioned into 4 to 8 divisions, which was easily performed and stimulated the formation of bulblets. The use of MS liquid medium containing 30 g l^{-1} sucrose in light was required. Based on this result, sectioned bulblets were cultured in the light in a 10-litre glass jar fermentor containing 5-litre of MS liquid medium. This resulted in the efficient propagation of bulblets in the bioreactor (Takayama and Yokokawa, 1996). After 4 months of cultivation in a bioreactor, 167 bulblets of 637 g FW were produced on average. When the culture period was extended to more than one year, the size of the bulblet became larger. After about one year of cultivation, bulb diameter increased to over 2 or 3 cm (Takayama and Yuasa, unpublished result).

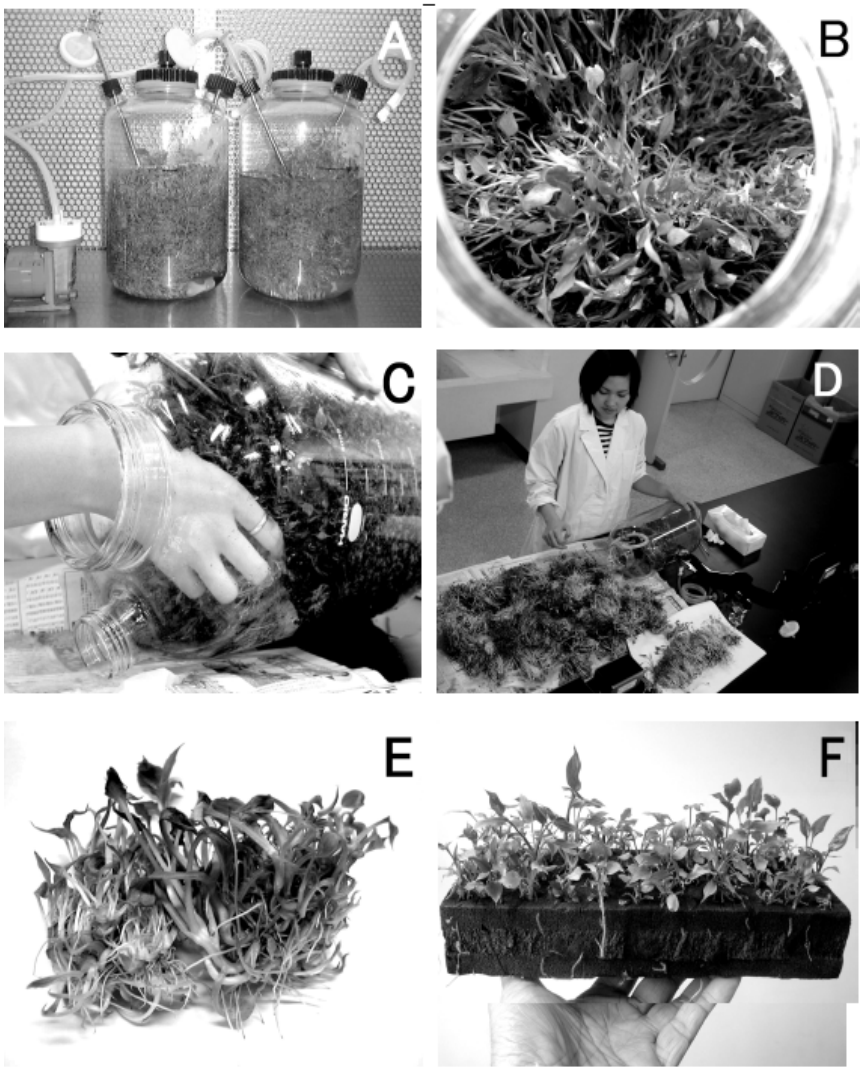

Figure 2: Propagation of *Spathiphyllum* plants using bioreactors: (A) *Spathiphyllum* plantlets in bioreactors. (B) Close-up of plantlets growing in bioreactor. (C. and D) Harvesting the plantlets. (E) Plantlets from bioreactor. (F) Plants reestablished in Oasis growing medium after cultivated with plastic cover under irradiation.

5.4 *Gladiolus grandiflorus*

The conventional propagation method of *Gladiolus* is to use microcorms regenerated around the basal disc of the cormels. Stocks should be chosen carefully to prevent spread of diseases (Wilfret, 1980). Tissue culture techniques have enhanced the propagation efficiency (Bajaj et al., 1982; Dantu and Bhojwani, 1995; Sen and Sen, 1995; Jager et al., 1998; Park et al., 2001,). The use of bioreactors overcomes the problems encountered in conventional propagation or in tissue culture propagation on agar medium. Ziv (1990, 1991, 1992) has investigated bioreactor culture of *Gladiolus* and reported that *Gladiolus* buds proliferated profusely to clusters in bioreactor cultures supplemented with either ancymidol, paclobutrazol or uniconazol. Mechanically separated meristemoid clusters were induced to form cormlets which could either be stored or transplanted *ex vitro* without acclimatization (Ziv 1992). We also established a propagation protocol for *Gladiolus* using bioreactors in a two stage culture (Takayama et al., 1987). Shoot cultures were established in the bioreactor using MS medium with 10 g l^{-1} sucrose, and after shoots were fully grown in the bioreactor, concentrated sucrose solution was added to a final concentration of 90 g l^{-1}. By this method, microcorms from the basal position of the shoot were produced in the bioreactor. Using two stage culture in 1-litre bioreactor, the number of corms produced was about 300.

5.5 *Spathiphyllum*

Spathiphyllum is a foliage *Araceae* plant native to tropical regions and accepted as an ornamental plant because of its sweet aroma and flower shape. The propagation of *Spathiphyllum* is generally carried out by dividing, but the efficiency is not high. Therefore, tissue culture was performed (Watad et al., 1997; Ramirez-Malagon et al., 2001). Acclimatization is required for establishment of plants in soil.

We are propagating *Spathiphyllum* using bioreactors as a model experiment for direct transplant of bioreactor-cultured plants into soil under open conditions (without film cover). In our study, shoots were taken out of the bioreactor, transferred aseptically to glass bottles, and dried for one week in a glass bottle covered with paraffin-coated paper or dried gradually for several weeks using glass bottles covered with aluminum foil and paraffin-coated paper. The plants were acclimatized during desiccation. After an ambient desiccation period, plants were transferred to Oasis growing medium (Smithers-Oasis Co.), watered, with or without plastic cover, and cultivated under fluorescent light in the culture room. Almost 100% of the plants survived. Even when the plants were cultivated without plastic cover,

plants could also be reestablished in Oasis growing medium, but the survival rate was lower. The maximum survival rate in open cultivation was 70%, and among them over 80% were rooted on Oasis growing medium. Treatment with ABA or mannitol (osmotic agent) also stimulated the reestablishment of the plant in Oasis growing medium in open cultivation (Takayama and Miura, Takayama and Inoue, unpublished results).

Culture conditions in the bioreactor and the subsequent treatment enhancing the acclimatization and reestablishment of tissue-cultured plantlets without protected cultivation will be important for commercial use of bioreactors.

5.6 Colocasia esculenta (Taro)

Colocasia esculenta is an important food crop, especially in tropical regions. The propagation of this plant is easily performed by division**,** but the commercial production of corms of this plant and transmission of virus disorders is the main problem. Tissue culture is an alternative way to propagate virus-free microcorms (Chand and Pearson, 1998; Akita and Ohta, 1996).

We have established an efficient method for propagation of microcorms of *Colocasia* using bioreactor (Takayama et al., 1989 a, b). The process of *Colocasia* propagation in a bioreactor consists of two steps.

-Step 1: Propagation of shoots in a 10 to 20-litre glass bioreactor using half-strength MS medium supplemented with 30 g l^{-1} sucrose (Takayama et al., 1989a). *Colocasia* shoots produced in this step were transplanted in soil and easily reestablished, but the manual transplantation of the shoots is laborious. To solve this problem, conditions for the production of microcorms in the bioreactor were examined. Microcorm production was quite difficult, but after serial experiments, specific conditions for the induction of microcorms were established, which were satisfactorily applied to bioreactor cultures (Takayama et al*.,* 1989b).

-Step 2: After *Colocasia* shoots were fully grown in the bioreactor, according to step 1, the aeration rate was elevated two to five times**.** At the same time, the concentration of sucrose was elevated to 90 g l^{-1}. During the drying process by aeration, the cultures exhibit morphological changes to form microcorms, which developed at the base of the shoots.

Using an 8-litre bioreactor containing 6 litres of medium, 2977 microcorms were harvested after one month for step 1 and another one month for step 2. The microcorms produced were easily transplanted to soil and cultivated. After one season, a large number of well-grown corms were harvested. When storage of microcorms was required, microcorms were preserved for more than 6 months in the refrigerator.

Several *Araceae* species related to *Colocasia* such as *Anthurium andreanum, Caladium bicolor, Pinellia ternata,* and *Amorphophallus konjac* have similar *in vitro* culture characteristics.

5.7 Potato (Solanum tuberosum)

Virus-free potato microtubers are important for potato farmers because they are necessary for production of high quality potato tubers, and microtubers are easily stored, and distributed. Factors, which affect the tuberization of potatoes have been reported by many researchers (for example, Hussey and Stacey, 1981; Estrada et al., 1986) and to date, several important results have been reported (for example, Hulscher et al., 1996; Jimenez et al., 1999). Among them, we first reported the use of bioreactors for efficient production of potato microtubers (Akita and Takayama 1988, 1993a, b, 1994a, b).

We developed an efficient process that consists of two phases, including semicontinuous liquid medium surface-level control (Akita and Takayama 1994b). Virus-free shoots subcultured in test tubes were used as inocula. The process of tuber propagation in bioreactors consists of two phases: Shoot multiplication (Phase 1) and microtuber formation (Phase 2).

In Phase 1, shoots were multiplicated in MS medium containing 30 g l^{-1} sucrose under continuous irradiation. In this process, shoot cultures were grown favorably in the air space with the use of a small volume of the liquid medium. In Phase 2, liquid medium was fed to completely submerge the shoots, and at the same time, the concentration of sucrose raised to 90 g l^{-1}. After one month of cultivation in Phase 2, microtubers were produced around the medium surface. The process was further revised and was established as the semicontinuous medium surface level control method (Akita and Takayama, 1994b). Using this revised method, the microtubers were induced and developed in the entire space of the bioreactor. The transplant and cultivation conditions of microtubers produced in the bioreactor were examined and are applicable to commercial propagation (Akita and Takayama, 1993a).

Recently, a more simple system was reported by Akita and Ohta (1998). The system used a plastic bottle placed on an air-permeable sheet and potato tubers were successfully formed by using a rotary culture system without forced aeration. This type of system was scaled-up to a 10-litre bioreactor and tubers were efficiently produced (Akita, unpublished data). Moreover, it could be important for practical application of culture techniques to choose inexpensive vessels and systems for cost reduction.

6. Cultivation of bioreactor cultured plantlets *ex vitro*

The ultimate aim of the use of bioreactors for plant propagation is to produce plants for cultivating in outdoor conditions. Various types of propagules from bioreactors have been subjected to soil cultivation. The authors' experiences of the cultivation of plants in soil provided the background for the efficient establishment of plants in soil, especially in the case of *Lilium* microbulbs, *Hippeastrum* bulbs, *Solanum tuberosum* microtubers, and *Spathiphyllum* plants. The morphology of the propagules from the bioreactor, handling of the propagules in transplantation, dormancy of microtubers, requirement of the acclimatization process, growth of the shoots and roots *ex vitro*, uniformity of plant size and genetic characteristics, in relation to practical cultivation of the propagules will be published in the future (Takayama, in preparation).

7. Prospects of bioreactor technology in mass propagation

The high efficiency of plant propagation using bioreactors has been revealed, and bioreactor technology seems to be applicable to commercial propagation in the several plant species discussed herein. However, many problems still exist in scale-up and in application to other plant species. The main cause for the problems comes from the difficulty in the preparation and handling of the bioreactors, in preparation of 'seed cultures' and in finding the optimum culture conditions, which depends mainly on the types of culture, different genera or species of plants, etc. The advantage of bioreactor technology exists in the high efficiency and ease of operation . The problems have to be overcome before the general use of the technique for commercial propagation . It is important to note that the bioreactors will be a most promising system for industrial plant propagation, including process automation and robotics.

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