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Advanced Technology in Micropropagation of Some Important Plants

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ABSTRACT

Novel methods and advanced culture systems concretely discussed in this chapter range from mutual relationship between microorganisms and plant root system, *in vitro* flowering, micropropagation for recalcitrant plants, synseed production, direct somatic embryogenesis and protocorm-like body formation, and several applied culture systems for plant regeneration, micropropagation, and materials for genetic transformation. All of the investigations have shown to be applicable for other cultivars/varieties and been considered to undergo further researches and to be up-scaled for commercial goals. Each investigation/study is presented with its materials and methods, the obtained results and suggestion for further research. Progress of plant biotechnology involved in these new methodologies has been reviewed, showing their advantages in modification of plant culture media, optimization of physical, chemical and physiological conditions for *in vitro* flowering, advanced micropropagation of recalcitrant plants, standardization of synseed production and preservation, applications and modification of advanced culture systems, and studies on shoot morphogenesis and regeneration, somatic embryogenesis and protocorm-like body formation. In addition to potential application and further research, a few problems that have arisen and disadvantages are also recorded with some solutions provided by presenting methodologies or other research ideas to be executed *a posteri* for improvement of these micropropagation methods and culture systems.

1. INTRODUCTION

The progression of plant biotechnology has been increasingly involved in finding novel methods and culture systems for quality and quantity improvement of plant production. These findings should be applicable for either basic researches or commercial production. They mainly focus on how to optimise the regeneration, organogenesis, survival rate, growth and development fashion of valuable medicinal, ornamental and food crops. In this chapter, we have some discussion on several innovative methods in plant regeneration, morphogenesis and propagation. These themes involve (i) positive effect of contaminating microorganisms in plant culture media; (ii) determination of physical, chemical and physiological conditions for *in vitro* flowering of different floral plants; (iii) novel methods for micropropagation of recalcitrant plants, with the case study of *Paphiopedilum delenatii*, an endemic and endangered slipper orchid in Vietnam; (iv) investigation and standardization of synseed production in propagation and preservation of some valuable ornamental plants; (v) applications and modifications of some advance culture systems, namely aeration, temporary immersion and shake culture systems; (vi) control of direct somatic embryogenesis and protocorm-like body formation in *Lilium* spp.; (vii) microponic culture system for rooting improvement in micropropagation; and (viii) *in vitro* hydroponics towards a simple and effective culture system for hygrophilous plant material production.

2. POSITIVE EFFECT OF CONTAMINATING *BACILLUS* SPP. IN *CHRYSANTHEMUM* SPP. CULTURE MEDIA

Positive effects of *Bacillus* spp. on the *in vitro* and *ex vitro* growth of *Chrysanthemum* spp. Inside the tissue of nearly all healthy plants, many microorganisms are present, the endophytes. Endophytes are mutualistic to their host; at least some of them are thought to produce specialized substances such as secondary metabolites to protect the host from attack by fungi, pests and mammals and in return, obtain nutrients from the plant. In fact, endophytic metabolites were reported to inhibit a number of microorganisms. A positive effect of *Bacillus* spp. on

Abbreviations: AC, activated charcoal; **BA**, 6-benzyladenine; **BTBB**, balloon-type bubble bioreactor; **LED**, light-emitting diode; **NAA**, α-naphthaleneacetic acid; **PGR**, plant growth regulator; **PLB**, protocorm-like body; **TIS**, temporary immersion system; **TDZ**, thidiazuron

the growth and development of *Chrysanthemum* spp. *in vitro* and *ex vitro* was presented for the first time by Nhut (2005). During preliminary tissue culture tests, the presence of a strain of bacterium (*Bacillus*) was observed in some culture vessels (Nhut 2005). Surprisingly, their presence did not negatively affect the growth and development of host plants. Contrarily, they enhanced the development and function of the host plants' root systems. Initial nomination tests could only determine their strain as being *Bacillus. Bacillus* spp. were positively confirmed by Gram and spore formation tests. Investigated bacteria was derived from *Bacillus* spp. colonies randomly present in some culture vessels of strawberry (*Fragaria vesca* L.), African violet (*Saintpaulia ionantha* H. Wendl), and chrysanthemum (*Chrysanthemum* spp.), etc., despite the strict sterilization of these explants with $HgCl_2$ and $CaOCl_2$. Only single, homogenous *Bacillus* spp. colonies isolated from vessels were isolated with a *Bacillus* spp isolating medium containing 10 $g.l^{-1}$ meat extract, 5 $g.l^{-1}$ peptone, 20$g.l^{-1}$ agar with pH maintained at 7.0 ± 0.2. Bacterial suspension was applied to 20-25 ml medium in sterilized Petri dishes. Following isolation, 1 g of *Bacillus* spp. was diluted in 9 ml NaCl 0.5% (sterilized) of which 1 ml of this homogenous suspension was further diluted in another 9 ml NaCl 0.5% (sterilized). The new solution was vortexed. Culture dishes were thereafter incubated at 30°C for 72 hours. These bacteria were applied to the experimental recipient host plants at the same time. *Bacillus* spp. affected the growth and development of *Chrysanthemum* spp. plantlets after 4 weeks of culture. Affected parameters included plant height, root number, and plant fresh weight. *Chrysanthemum* plantlets that were infected with *Bacillus* spp. had significantly higher values in these growth parameters than uninfected, control *Chrysanthemum* plantlets. Moreover, *in vitro* plantlets continued their vigorous growth in the greenhouse 4 weeks after they were transferred to the greenhouse. The height, fresh weight, and survival rate of *Bacillus* spp.-infected *Chrysanthemum* spp. plantlets grown *ex vitro* remained significantly higher than those of uninfected, control *Chrysanthemum* plantlets. Endophytic bacteria may stimulate the host plant growth through any of several possible mechanisms including biological control (Pleban *et al.* 1995), induced systemic resistance to plant pathogens (Benhamou 1996, Hallmann *et al.* 1997), or phytohormone production and improvement of nutrient and water uptake (Pleban *et al.* 1995, Nowak and Lazarovits 1997). In addition to these positive effects on plant growth, once present in culture media, *Bacillus* spp. restrict the development of other pathogens. Naturally, in most of the cases, there are often close mutual relationships between microorganisms and root systems. Once adapted to the presence of microorganisms as of the *in vitro* culture, plantlets may readapt to the natural environment and survive better when they are transplanted to the greenhouse, as is the case of *Bacillus* spp. and *Chrysanthemum* spp.

Fig. 1 *In vitro* flowering of *Rosaceae* sp., *Limonium* sp., *Torenia* sp., and *Gerbera* sp. (from left to right).

3. *IN VITRO* FLOWERING OF ROSE, GERBERA, LIMONIUM, AND TORENIA

Flowering is complex in mechanism, sensitive in environment, meaningful in science and useful in agricultural and horticultural production. The researches on *in vitro* flowering field play theorically- and practically-important roles. The flowering capacity of some *in vitro* ornamental plants was studied by adjusting some factors affecting on those such as sucrose concentration, plant growth regulators, macro and micro inorganic and organic elements in MS (Murashige and Skoog 1962) medium. Furthermore, the effect of time and genetic properties on the flowering ability was also investigated. The results indicated that the key factors affecting *in vitro* flowering vary from species to others. Different kinds of PGRs (plant growth regulators) have different affects on flowering. In rose, torenia (*Torenia fournieri*) and gerbera (*Gerbera jamesonii*) the age of explants revealed considerable factors (**Fig. 1**).

Effect of carbohydrates. Rose shoot formation rate increases correlatively with sugar concentration in culture medium. Carbohydrate is demonstrated to be a crucial factor affecting shoot differentiation which turns into reproductive state. There may be a threshold below which *in vitro* flowering cannot occur. *In vitro* rose flowering cannot be obtained with medium's sucrose concentration below 15 $g.l^{-1}$. Although carbohydrate plays an important role in differentiation enhancing, its single presence is not sufficient for *in vitro* flowering. Early withered flowers observed *in vitro* have shown that further differentiation process requires many other factors.

Effect of plant growth regulators. Most of studies on *in vitro* flowering reported that cytokinins play a crucial role in formation and differentiation of reproductive shoots (Polowick and Sawhey 1991, Hasan and Nobusama 1996, Kostenyuk 1999). Supplementation of BA in culture medium significantly improves the early flower withering in rose shoots than those in PGR-free culture containing sucrose. *In vitro* flowering was also observed in some gerbera varieties cultured in medium supplemented with cytokinins (BA, adenine, coconut water). Contrary results, however, were also obtained in other cases (Franklin *et al.* 2000) in which flowering was inhibited by cytokinins and enhanced by auxins. In *Limonium* spp., flowering rate was high in media with low BA (6-benzyl aminopurine)/NAA (α-naphthaleneacetic acid) ratio. *Limonium* shoots cultured in lowest BA/NAA ratio media are single shoots with larger leaves while those in higher BA/NAA ratio media grow as shoot clusters with small leaves and lower flowering rate. Vu *et al.* (2006) reported that BA and zeatin are more appropriate than TDZ (thidiazuron) in *in vitro* flowering. *Limonium* shoots cultured in media supplemented with BA showed flowering while no flowering phenomenon was observed in media containing TDZ.

Effect of nutrients. Rose flowering cannot be observed in MS basal medium supplemented with TDZ. However, this can be observed when nutrient salt concentration is reduced into a half-strength. Torenia floral buds can be observed uniformly after nine weeks of culture in MS and 1/2 MS media; floral buds in MS medium were bigger and gave higher flowering rate than those in half-strength MS medium. In *Limonium*, higher flowering rate was obtained in those whose leaves were removed before culturing. Nutrient properties might also be different between *Limonium* shoots separated (for subculturing) into single shoots and those separated into clusters, with higher flowering rate from singly-separated shoots.

Effect of time duration. Flowering rate in early stages from explant incubation was very low and often gave abnormal floral properties (Vu *et al.* 2006). This may be affected by explant ages which were too young to be differentiated. Subcultured shoots (after every 45 days) within a year had higher flowering rate and reduced abnormal phenomena. In torenia 'Pink-Edged White', 2-month-old explants had no floral buds after 12 months of culture. Three-month-old explants, however, showed visible floral buds after 56 days of culture. Three-month-old explants of torenia 'Purple' also has higher flowering rate in all experimental media.

Effect of genotypes. *In vitro* flowering differs significantly from varieties to varieties. In same medium and culture condition, each species/variety shows its typical responses, particularly regarding *in vitro* flowering. These can be found in rose, torenia and gerbera.

4. NOVEL METHODS FOR RECALCITRANT PLANT MICROPROPAGATION: *Paphiopedilum delenatii*

Paphiopedilum is a terrestrial orchid genus, which grows from the Himalayas, Southeast Asia to Papua New Guinea (Teoh 2005). As a highly valuable cut flower and pot plant, *Paphiopedilum* can be traditionally propagated by seeds or "keikis" (Bahasa Indonesian, referred to plantlet derived from mature orchid plants). However, propagation using seeds gives low survival rate. Propagating *Paphiopedilum* via keikis has higher survival rate but a mature slipper orchid plant must take two to three years to form keikis. Therefore, plant cell tissue culture through which a large numbers of plantlets can be obtained within a short period has become an ideal solution for preserving this genus from extinction. The pioneering work to micropropagate *Paphiopedilum* was carried out by Bubeck (1973). Since then, many researchers have attempted to multiply this unique kind of orchids by using various parts of the plant as explants and different methods. Some of them were plantlet regeneration from meristem culture (Bubeck 1973), lateral bud and plantlet induction (Stewart and Button 1975), axillary shoot induction and plantlet regeneration from shoot tips (Huang 1988) and shoot multiplication from seedlings (Huang *et al.* 2001). Recently, plantlets were successfully regenerated through protocorm-like bodies (PLBs) from totipotent calli (Lin *et al.* 2000), and direct shoot bud formation was obtained from *in vitro* leaf explants (Chen *et al.* 2004).

Paphiopedilum delenatii, an endangered species of Vietnam recognized and protected by the Convention on International Trade of Endangered Species of Wild Flora and Fauna (CITES), has been a favourite potted plant for centuries due to its attractive colour and distinctive shapes (**Fig. 2**). However, the increasing market demand, together with the low multiplication rate of conventional propagation methods has endangered the survival of this unique orchid. In addition, success in *Paphiopedilum* culture was relatively limited due to the inadequacy of media, bacterium and/or fungi contamination, easy-to-die explants, and low multiplication rate. All of them made the above procedures remained under laboratory-scale, and desired plantlets cost rather expensive. In what follows, two practical methods for *P. delenatii* propagation via wounding technique in combination with liquid culture and stem node culture were established.

4.1. Wounding technique and liquid culture

The "wounding" technique involves an easy procedure for *P. delenatii* propagation, ensures better shoot survival rate, increases multiplication rates and helps in (i) propagation and preservation of endemic and endangered species, (ii) production a large number of vigorous plantlets in a very short period, and (iii) reducing the plantlets cost (Nhut *et al.* 2005c) (**Fig. 2**).

Fig. 2 Emerging adventitious shoots from wounded *Paphiopedilum delenatii* plantlet (left) and flowering plant (right).

In vitro six-month-old seedlings (2.5-3.0 cm in height) were used as explants for shoot induction via wounding technique. Their roots were carefully removed and the seedling bases were pierced three or four times using a sharp needle, whose diameter was 0.3 mm. These wounded seedlings were then placed onto media-containing flask which contains basal MS medium supplemented with different concentrations of TDZ (0.5, 1.0, or 3.0 $mg.l^{-1}$) or TDZ (0.25, 0.5, 1.0, or 2.5 $mg.l^{-1}$) in combination with 0.5 $mg.l^{-1}$ NAA, 1 $g.l^{-1}$ AC, 20 $g.l^{-1}$ sucrose, and 8 $g.l^{-1}$ agar (for solid media only) for investigating the shoot regeneration on wounded seedlings. A 2 x 2 cm piece of filter paper (OSI, France), which acted as a support keeping the explants from completely submerging in the medium, was also added in the liquid-containing culture vessels. Shoot survival rate and number of shoot per explant were recorded after 12 weeks of culture in 25 ± 2°C, 70-80% relative humidity and a 12-hour photoperiod at 45 $\mu mol.m^{-2}.s^{-1}$ photosynthetic photon density flux, which was provided by fluorescent tubes (Rang Dong, Vietnam).

The highest survival rate was recorded from wounded seedlings cultured on MS solid medium supplemented with 0.5 $mg.l^{-1}$ TDZ, without external auxin, whereas no shoot formation was obtained in control treatments with non-wounded shoots in all kinds of media. An average of 2.3 green and vigorous shoots were obtained on medium containing 0.25 $mg.l^{-1}$ TDZ and 0.5 $mg.l^{-1}$ NAA, suggesting that the wounding step is a prerequisite for shoot formation in seedlings. Wounded cells at the damaged site may be responding to stimulating agents (e.g. certain PGRs) that are present in nutrient media by differentiating into novel organs, such as adventitious shoots. No shoot formation was recorded on non-wounded seedlings in control treatments, showing that intact cells of these seedlings were not affected by the stimulators. In semi-solid, auxin-containing media, the number of newly formed shoots tended to decrease as the concentration of TDZ increased. In contrast, the number of shoots formed in liquid medium increased considerably when TDZ levels were increased. These results suggest that the effects of TDZ on explants depend significantly on the physical properties of the medium, and that the physical state of the medium (liquid or solid) substantially affected shoot formation in wounded seedlings. Not being completely submerged in the culture medium, wounded seedlings can uptake nutrient

components and PGRs easier. This stimulates the differentiation of the affected tissue and results in higher number of shoots per explant. In liquid media, a significantly lower adverse effect of TDZ on plant tissue was observed. The survival rate was relatively high (85%) and did not vary considerably among treatments. Furthermore, the survival rate was lower when TDZ was the sole external PGR than when TDZ was used in combination with auxin. This suggests that the presence of auxin in liquid media may increase the survival rate. However, auxin at high concentration may play an inhibitory role in liquid media since the number of shoots per explant formed in liquid media without auxin was two times greater than in media that contained auxin.

Wounding technique was demonstrated to be efficient for inducing shoot regeneration, and highest numbers of shoot were obtained in a liquid medium that contained 1.0 mg.l^{-1} TDZ. These shoots developed vigorously after three months of subculture on fresh medium. Rooting plantlets could be obtained after three months on rooting medium. Because of their efficiency, the wounding method and liquid culture can be applied for large-scale micropropagation of this and other endangered species.

4.2. Stem elongation and stem node culture of *P. delenatii*

As mentioned earlier, clonally-propagated orchids via the division of plants is a very low process and it's rather difficult to multiply monopodial species like *Paphiopedilum* by such method. It was, thus considered necessary to develop a method of vegetative propagation which can produce a large number of plants from single individual. In the present research, by determining the optimal intensity of fluorescent light and red LEDs (light-emitting diodes) for *P. delenatii*'s stem elongation and the suitable medium for shoot regeneration, an innovative protocol for micropropagating *P. delenatii* through shoots from elongated plantlet-derived nodal segments was reported (Nhut *et al*. 2005d).

Fig. 3 Stem elongation of *P. delenatii*'s shoots (left) and stem node-derived shoots (right).

In vitro plantlets 1.5 cm in height were placed excessively in MS medium containing 2.0 mg.l^{-1} BA, 0.5 mg.l^{-1} NAA, 1 g.l^{-1} AC, coconut water (20%, v/v), 30 g.l^{-1} sucrose and 9 g.l^{-1} agar (modified MS medium) and incubated in darkness or under different intensity of light of fluorescent tube (FL) and red LEDs. The greatest plant height was recorded in plantlets incubated in darkness after four weeks culture, followed by the plant height of those exposed to 30 µmol.m^{-2}.s^{-1} of red LEDs, 30, 45, and 60 µmol.m^{-2}.s^{-1} of FL. The plant height of *P. delenatii* shoots was highest in the dark but plantlets were yellow and not very vigorous. When *P. delenatii* shoots were incubated under low intensity of light (30 µmol.m^{-2}.s^{-1} of FL and red LEDs), their shoots were elongated, and distances among leaves were widened along the stem. Furthermore, it had been reported that red LEDs affect stem elongation and leaf expansion (Hoenecke *et al.* 1992) and chlorophyll synthesis (Tripathy and Brown 1995). Nhut (2002) also reported that plantlets' leaves of *Eucalyptus*, *Phalaenopsis* and *Cymbidium* elongated under red LEDs. The elongated plantlets of *P. delenatii* in this study also had a thin stems and normal growth as shown in former experiments of Nhut (2002). In this case, 30 µmol.m^{-2}.s^{-1} of FL and red LEDs seemed to be optimal for growth of *P. delenatii* stem and its photosynthetic activity. These intensities of light together with excessive crowding in culture caused internode elongation of shoots, resulting in their development into rhizome-like structure that was atypical in *Paphiopedilum*.

The elongated plantlets (8 to 10 cm) obtained from this experiment were used as explants for the following step in which they were collected, roots and leaves were carefully removed for exposing nodes. These stems were then segmented into nodal discs. The isolated nodal segments were cultured on nutrient medium (modified MS medium) containing PGRs including BA, TDZ, zeatin for investigating their effects on shoot formation of nodal segments. Of the cytokinins (BA, TDZ and zeatin) used for inducing the shoot formation on elongated-stem-derived nodal segments, TDZ is potentially the most effective cytokinin for shoot proliferation of this recalcitrant species. Highest shoot formation rate (75%) was obtained on medium supplemented with 2.0 mg.l^{-1} TDZ. However, bigger and more greenish shoots were observed on modified MS medium containing 1.5 mg.l^{-1} TDZ. The shoot size varied among treatments: the biggest on medium containing 2.0 mg.l^{-1} TDZ, the smallest on medium supplemented with 0.5 mg.l^{-1} TDZ (**Fig. 3**). TDZ was realized to be effective for morphogenesis induction such as shoot regeneration and proliferation of several orchids (Chen and Piluek 1995, Nayak *et al.* 1997). However, Huang *et al.* (2001) reported that TDZ inhibits shoot proliferation of *Paphiopedilum*. In the research of Nhut *et al.* (2005), TDZ in combination with NAA were found to be effective in adventitious shoot induction at high concentration, low concentration of TDZ was not appropriate for the formation of *P. delenatii* shoots. For further investigation on rooting ability of shoot derived from nodal segment, three-month shoots were subcultured on rooting media to induce root formation. Root primordia were firstly observed after two weeks of culture, rooting plantlets were ready to be transferred to greenhouse after three months. Moreover, it was also observed that regenerated plantlets derived from stem node were uniform and vigorous.

Though this method have been successfully applied for propagating of many orchids including *Dendrobium*, *Cymbidium*, *Phalaenopsis*, there have not yet any reports on the effect of intensities of fluorescent tubes and LEDs on *P. delenatii* elongation and micropropagation via stem node culture. Nhut *et al.* (2005) demonstrated the effectiveness of the method as it can produce a large number of plantlets with lower cost in a shorter period. Due to its superior features to conventional methods, stem node culture of *P. delenatii* might pave the way for commercial production and conservation of this valuable slipper orchid.

5. SYNSEED IN PROPAGATION AND PRESERVATION OF SOME VALUABLE ORNAMENTAL PLANTS

Artificial seeds, termed synseeds, was first mentioned by Murashige (1978), but the concepts of synseeds have been emerging for over 30 years. Initially, several methods of encapsulation with a number of hydrogel coatings were examined for producing synseeds, namely gelation, complex conservation, and interfacial polymerisation (Redenbaugh *et al.* 1987). To date, encapsulation methodologies have been met with success in only a few non-orchid angiosperms (Datta *et al.* 1999). Until now, synseed production by encapsulating PLBs has been achieved in a few orchids such as *Dendrobium wardianum* (Sharma *et al.* 1992), *Spatholottis plicata* (Singh 1991), *Geodorum densiflorum* (Lam) Schltr. (Datta *et al.* 1999), *Oncidium* and *Cattleya* (Saiprasad and Polisetty 2003).

Nhut *et al.* (2005b) have investigated the direct germination of these synseeds – with an external protective coating made of chitosan solution – as well as the *ex vitro* performance of the *in vitro*-derived plantlets on a non-sterilized fern root substratum. The authors reported the production of artificial seeds (synseeds) through the encapsulation of PLBs of *Cymbidium* spp. Three-month-old PLBs were encapsulated in a sodium alginate solution at different concentrations. Thereafter, synseeds were germinated on different *in vitro* culture systems. In addition to the investigation of direct germination of these synseeds – with an external protective coating made of chitosan solution – the *ex vitro* performance of the *in vitro*-derived plantlets on a non-sterilized fern root substrate was also tested. Survival ability of these synseeds cultured *in vitro* was 100% and the regeneration capacity was also high. The synseeds did not show a reduction in survival rate after storage in sucrose-free liquid medium for one year. Synseeds showed 35.5% survival rate and 45.8% shoot formation when directly transferred to non-sterilized after coating with chitosan solution. The plantlets derived from these synseeds had a survival rate of 100% after six months in greenhouse.

A remarkable phenomenon was observed, that is when sodium alginate concentration was 30 $g.l^{-1}$, most of the synseeds produced multiple shoots and roots, but a 50 $g.l^{-1}$ sodium alginate concentration resulted in the production of single shoot and root per synseed. Both multiple and single shoots and roots were observed at concentrations in the middle of 40 and 50 $g.l^{-1}$. The number of new PLBs per synseed decreases correlatively with an increase in sodium alginate concentration, proving that shoot and root formation may be affected by sodium alginate concentration (Nhut *et al.* 2005). A 4% sodium alginate solution was shown to be most suitable for *Geodorum densiflorum* PLB synseeds (Datta *et al.* 1999), 3% for sissoo (Chand and Singh 2004), cassava (Danso and Ford-Lloyd 2003) and chrysanthemum (Halmagyi *et al*. 2004), and 2% for sweet potato (Hirai and Sakai 2003). *Cymbidium* PLBs were demonstrated to have good vitality for synseed production. Chitosan-coated synseeds showed a relatively high survival rate and regeneration after one month when germinated directly *ex vitro* although some of them died due to contamination.

It is expected to have further study on appropriate chitosan concentration for each species/variety to protect synseeds from fungal contamination and on suitable carbohydrate concentration for the conversion from synseeds into vigorous plantlets. In *Cymbidium*, appropriate sodium alginate concentration was 40 $g.l^{-1}$ as seed diameter was 5 mm. Optimal seed development was recorded on MS basal medium supplemented with 15% (v/v) coconut water, 30 $g.l^{-1}$ sucrose, 1 $g.l^{-1}$ AC, 0.5 $g.l^{-1}$ NAA, 8 $g.l^{-1}$ agar. Suitable substratum for seed regeneration in liquid medium (MS basal medium supplemented with 30 $g.l^{-1}$ sucrose, 1 $g.l^{-1}$ AC, 0.5 $mg.l^{-1}$ NAA) was cotton wool (98.6%). Chitosan-encapsulating concentration of 10 g l^{-1} was shown to give lowest, fungus-contaminated seed (27.3%) in nursery (**Fig. 4**). In case of direct acclimatization, lowest fungus contamination rate (0%) and highest survival rate (90%) of alginate capsule seeds were recorded in those without mannitol. Generally, encapsulation with two layers of alginate was shown to be superior to encapsulation by alginate and chitosan (Nhut *et al.* 2005b).

Fig. 4 Synseeds of *Cymbidium* sp. as preservation (left) and *ex vitro* germination of directly-sown synseeds (right).

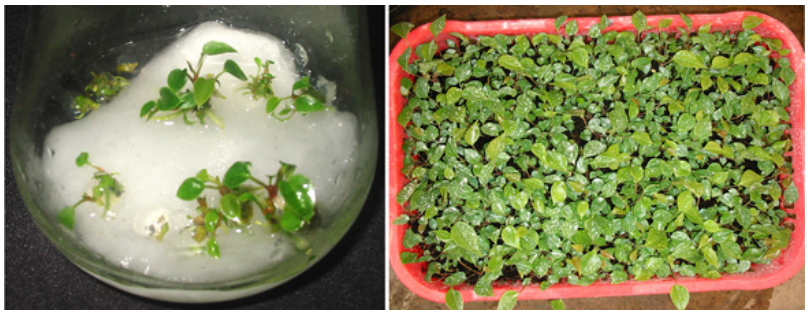

Fig. 5 Germination of *Anthurium* spp. synseeds (left) and synseed-derived plantlets (right).

Synseeds can also be produced from many other plant materials. Nhut *et al.* (2006) have applied callogenesis and somatic embryogenesis from leaf explants in micropropagation and synseed production of some anthurium cultivars (*Anthurium* spp.). These processes were shown to be significantly affected by genotypes.

Anthurium, a perennial herbaceous plant, is an economically important genus of the Araceae because of their colourful attractive, luxurious flowers and exotic foliage. Propagation methods of this flower have been developed so far. Reports showed that leaf pieces were the best explants for inducing callus and somatic embryo, but genotypes affected this process deeply. In this study, leaf explants of ten cultivars of *Anthurium andreanum* including: 'Carnaval', 'Neon', 'Choco', 'Sonate', 'Midori', 'Pistache', 'Tropical', 'Safari', 'Arizona' and 'Cancan' was used as the materials for propagation. Genotype played an important role during the most critical step in *Anthurium* micropropagation, 'Pistache' on 1/2 MS medium with 0.44 $g.l^{-1}$ $CaCl_2.2H_2O$ and 0.37 $g.l^{-1}$ $MgSO_4.7H_2O$ added with 1 $mg.l^{-1}$ BA, 0.08 $mg.l^{-1}$ 2,4-D, 30 $g.l^{-1}$ glucose, 8 $g.l^{-1}$ agar, and adjusted to pH 6.0 had the highest callus induction ratio (65.1%) but 'Carnaval' and 'Cancan' showed no response. Shoots were obtained from cultivars with different potential of shoot regeneration on 1/2 MS supplemented with 20 $g.l^{-1}$ glucose, 1 $mg.l^{-1}$ BA, 8 $g.l^{-1}$ agar, and adjusted to pH 6.0. The average number of shoots per explant in 'Tropical' and 'Sonate' was higher than others, but shoots with best quality were obtained in 'Neon' and 'Arizona'. All shoots consistently formed roots after 30 days on 1/4 MS medium supplemented with 30 $g.l^{-1}$ glucose, 8$g.l^{-1}$ agar, 1 $g.l^{-1}$ activated charcoal, and adjusted to pH 6.2, and plantlets developed well after being transferred to the nursery. The propagation process took nine months to complete.

Anthurium micropropagation by leaf explants culture was characterized by the use of different media at different stages of morphogenesis. Of the various media tested, including NH_4NO_3 concentration and the presence or absence of different auxins and cytokinins, NH_4NO_3 level had the most significant effect on callus and shoot formation, a low level being best for regeneration in all the genotypes investigated. The most

difficult step in this propagation process was callus induction from leaf explants due to the restricted dedifferentiation of mature tissues, depending strongly on genotype. *Anthurium* 'Pistache' could best induce calli on 1/2 MS medium supplemented with 30 g.l^{-1} glucose, 1 mg.l^{-1} BA, 0.08 mg.l^{-1} 2,4-D, 8 g.l^{-1} agar and adjusted to pH 6.0 (65.1% explants induced calli after 100 days of culture), whereas 'Carnaval' and 'Cancan' could not. Genotype affected shoot regeneration slightly but did not affect root formation. These calli and somatic embryos have been widely used for synseed production, indicating that the production of anthurium artificial seed is a method of propagation and preservation of great promise (**Fig. 5**).

6. ADVANCE CULTURE SYSTEMS: AERATION, TEMPORARY IMMERSION, LIQUID CULTURE

Control of culture conditions such as light (quality, intensity, duration), temperature, gaseous components (CO_2, O_2, C_2H_4), relative humidity and medium additives has increasingly attracted attention of researchers and plant producers worldwide. In conventional micropropagation, CO_2 concentration decreases in the culture vessel during the photoperiod (Kozai 1991), resulting in reduced photosynthetic ability. Since CO_2 gas concentration in the vessel is different from the external CO_2 concentration and the number of air exchanges per hour in the vessel, CO_2 can be supplied by using CO_2-permeable films, in combination with or without CO_2 enrichment to the culture room (Jeong *et al.* 1996). Improved aeration can relieve negative effects of *in vitro* culture. Herbaceous and woody plants propagated in conventional micropropagation system have altered water relations: in *Gypsophyla*, carnation and other ornamental hyperhydricity is often observed (Majada *et al.* 2001).

Nhut *et al.* (2004) have developed a new culture system for the micropropagation as well as quality improvement of *Gypsophyla paniculata* plantlets. Ventilated vessels had their caps perforated with a 5-mm drill, and the holes were sealed with microporous filters (Miliseal®). Shoots were cultured in vessels with either a) one or two aeration holes, b) in non-aerated Magenta vessels (MV) or c) non-aerated jam vessels (JV) for one month and then transferred to greenhouse. Fresh weight and plant height of *Gypshophyla* plantlets cultured in aerated vessel systems increased significantly and their subsequent growth and development were improved considerably in the greenhouse Among several experimental systems, two-aerated-hole Magenta vessel system and one-aerated-hole jam vessel (commercially-available small plastic box) are best suited for *Gypsophyla* micropropagation and subsequent growth as compared to the others. Ventilation has a positive impact on the growth and development of *in vitro Gypsophyla* plantlets in Magenta vessel. However, the system using Magenta vessels may not be widely applied in developing country due to its high cost while jam vessel (normally used as food containers) can be utilized instead. Aerated micropropagation holds more advantages than conventional methodology. Using systems with air exchange can increase the quality of *Gypsophyla* plantlets, which, have higher photosynthetic ability and stronger root formation when cultured in these systems. As a result, their growth and development are better than those cultured in other systems after transfer to greenhouse. These results can be found in both shoot, leaf growth and development, and root growth as well.

Strawberry (*Fragaria vesca*) was also used as a model plant for aeration culture on media with different sucrose concentrations in glass bottle (GB, 500 ml), jam vessel (JV, 600 ml) and the large-scale culture system – rectangular plastic vessel (RPV, 3600 ml). Generally, fresh weight, plant height, root length of strawberry plantlets cultured in aerated vessel systems increased significantly and their subsequent growth and development were improved considerably in the greenhouse. Among these systems, two aerated holes with JV and RPV are best suited for micropropagation and subsequent growth as compared to the other systems (**Fig. 6**).

Fig. 6 Micropore™ 3M (left) and strawberry (*Fragaria vesca* L.) shoots grown in RPV aerated with Micropore™ 3M (right) (modified from Nhut *et al.* 2004).

6.1. Liquid culture system and its application in mass micropropagation of jewel orchid (*Anoectochilus formosanus* Hayata) – a valuable medicinal plant

Recently, liquid culture has been considered as an alternative approach to plant micropropagation. Embryogenesis and organogenesis have been performed using large-scale liquid culture system with high automatic level (Ziv 1995). Due to homogenous liquid medium, explants can uptake nutrients at constant concentration in any position on/in the medium. Besides, some modern techniques including shaken culture and bioreactor have been also utilized to upgrade this system. Cell suspension, somatic embryos, bulblets, corms, microtubers or shoot have been cultured in liquid suspension in bioreactors. However, it is showed that some shoots which do not immerge in the water are still seriously vitrificated (Ziv 1986, Ziv *et al.* 1987).

Fig. 7 Jewel orchid shoots grown in liquid medium (left) and plantlets derived from liquid culture system (right).

Anoectochilus formosanus Hayata (Orchidaceae), commonly knows as "Jewel Orchids" due to their attractive foliage, is an important ethnopharmaceutical herb of China and belongs to a group of terrestrial orchids. *A. formosanus* is regarded as "the King of Medicines" by aborigines in China because of its diverse pharmaceutical effects, such as liver protection, cancer prevention, blood sugar reducing for diabetes, and the treatment of cardiovascular diseases, etc.

Nhut *et al.* (2006a) has developed a method for mass propagation of *A. formosanus* by using nodal explant. Nodal explants (1-3 cm in length) had been sterilized by 0.1% mercuric chloride ($HgCl_2$), cultured on solid MS medium supplemented with BA, coconut water. BA, kinetin and TDZ were supplemented to MS medium separately or in combination with NAA at different concentrations to investigate their effects on shoot regeneration. Optimum shoot formation rate (9,4 shoots per explant) was recorded in liquid MS medium supplemented with TDZ after 60 days of culture (**Fig. 7**). Vigorous plantlets were washed with tap water, planted into pots containing peat moss, and transferred to nursery. Survival rate of the plants over 90% was recorded after one month. The liquid culture system can be applied for further researches on medical production and large-scale propagation of *A. formosanus* and some other orchids.

6.2. Application of balloon-type bubble bioreactor system and initial establishment of temporary immersion bioreactor system for African violet (*Saintpaulia ionantha* H. Wendl.) shoot multiplication culture

Bioreactors for micropropagation cover a wide range of size (0.5-10000 L) and complexity (jelly jar to modified microbial fermenter) (Curtis 2005, Paek *et al.* 2005). Large-scale plant production through cell tissue and embryo cultures using bioreactors is promising for industrial plant production. Bioreactors are usually described as self-contained, sterile environments which capitalize on liquid nutrient or liquid air/ inflow and outflow systems, designed for intensive culture and affording maximal opportunity for monitoring and control over microenvironmental conditions (agitation, aeration, temperature, dissolved oxygen, pH, etc.) (Paek *et al.* 2005).

So far, many research activities have been reported on automation in micropropagation; recently, many liquid media systems for tissue culture have been proposed in this regard. High production costs generally limit the commercial use of *in vitro* micropropagation. Using liquid media is considered to be the ideal solution for automation and reducing production costs. However, the use of liquid media can be responsible for other problems such as asphyxia, hyperhydricity and the need for more complex equipment (Etienne and Berthouly 2002). Several methods have been proposed to avoid these problems, one being the twin flasks system or temporary immersion bioreactor/system (TIB/TIS), which allows temporary immersion of the explants (Escalona *et al.* 1999, Roels *et al.* 2005).

Nhut *et al.* (2006b) has conducted several researches with objectives to determine shoot propagating abilities of two types of bioreactor, the balloon-type bubble bioreactor (BTBB) and TIB, and establish the appropriate immersion length and aeration condition for shoots cultured in TISs in African violet micropropagation (**Fig. 8**). The temporary immersion of the explants was achieved filling the bottle containing the explants with the medium transferred through a silicone tube from the second bottle, in which an overpressure was created by an aquarium air pump. The stationary liquid and agarized substrates were compared as controls. In TIS, the shoot multiplication rate was very high, the leaves were green and showed the typical homogeneous morphology, the best result was obtained with an immersion of ten minutes for every 12 hours, hyperhydricity was decreased compared to controls and BTBB systems. BTBB gave high multiplication results but abnormal in shoot morphology. The static liquid culture condition was absolutely inappropriate for African violet propagation. In all experiments, shoots were multiplied on MS medium supplemented with 30 $g.l^{-1}$ sucrose, 0.2 $mg.l^{-1}$ BA and 0.1 $mg.l^{-1}$ NAA at pH 5.8. Data were collected and analyzed after six weeks of culture. These results are basis for further studies on optimization of bioreactor culture systems for plant tissue, organ and cell culture. With some additional modifications, the system may be used for African violet micropropagation in large scale towards product cost reduction and system automation.

Fig. 8 African violet shoots in temporary immersion (two left vessels) and permanent immersion (right) culture systems.

7. DIRECT SOMATIC EMBRYOGENESIS AND PROTOCORM-LIKE BODY FORMATION CONTROL OF *LILIUM* SPP.

To date, there have been a few reports on somatic embryogenesis of *L. longiflorum*. Somatic embryogenesis was shown to occur from bulb scales in different genotypes of *Lilium* hybrids (Haensch 1996) or by suspending the stigma- or pedicel-derived callus of *L. longiflorum* in medium containing 2 µM dicamba (Tribulato *et al*. 1997). Lately, somatic embryogenesis was achieved directly from pseudo-bulblet thin cell layers of *L. longiflorum* (Nhut *et al*. 2002). This research was conducted with culturing callus of *L. longiflorum* in liquid media system only to simplify the procedure inducing the formation of high qualitative and quantitative somatic embryos.

7.1. Liquid culture for indirect somatic embryogenesis in *Lilium longiflorum*

Calli of *L. longiflorum* obtained from transverse thin cell layers (0.8-1.0 mm in thickness) of pseudo-bulblets in MS medium supplemented with 1.0 $mg.l^{-1}$ NAA and 0.2 $mg.l^{-1}$ TDZ as reported by Nhut *et al*. (2002) were used.

These calli were cultured in liquid or solidified medium to examine the effect of physical state of media on somatic embryogenesis. One clump of callus (around 0.5 gram in weight and 11 mm in size) was transferred to each 250 ml Erlenmeyer flask containing different volumes (10, 20, 30 or 40 ml) of liquid MS media supplemented with NAA, TDZ and sucrose. Afterwards, these flasks were cultured in the static condition. These flasks were then incubated in either light or dark condition to investigate the effect of light on somatic embryogenesis. Growth rate of calli were recorded according to net fresh weight after 45 days of culture.

Masses of proembryogenic calli grew in liquid medium more slowly than in solid medium, moreover, in 40 ml medium, there was no developmental manifestation due to the loss of oxygen during long-termed submerged in static liquid medium limited the aerobic respiration. According to Kessel and Carr (1972), somatic embryogenesis was induced efficiently once oxygen levels decreased. The results of these experiments were agreed with the former since there was a different embryogenic ability of calli cultured in liquid from solid culture. Calli cultured in solid medium grew faster than being cultured in liquid medium. Calli culturing in liquid media have nodular surface which bearing numbers of globular structures around 1 mm in diameter, whereas the surface of calli culturing in solid medium was smooth, as observed under stereo microscopic. When being transferred to hormone-free MS medium added 30 $g.l^{-1}$ sucrose, 1.0 $g.l^{-1}$ AC and 8.0 $g.l^{-1}$ agar, the expression of somatic embryos from these calli could be seen through their maturation from globular to cotyledonous stages and their subsequent germination and conversion into whole plantlets.

In the first 10 days, numerous globular embryos (1 mm) radiated outward, and somatic embryos at heart- and torpedo-staged (2-3 mm) were appeared in the next 20 days. Dicotyledonous embryos (3-4 mm) but not scutellar-staged embryo developed. Germinated embryos gave rise to plantlets with full-developed shoot and root when cultures were kept in light condition, showed their possession of a bipolar organization with a shoot and root axis, while in complete darkness, bulbils along with roots were formed prior to shoot forming whole plantlets. About 78% of the embryos developed into plantlets with normal radicle and plumule.

Embryogenic calli culturing in solid media formed much less somatic embryos rather than in liquid media. Especially, 20 ml liquid medium were shown to be optimum condition for somatic embryogenesis induction than any other volumes since the number of somatic embryos in this medium was highest. Culture environment also controlled somatic embryogenesis when darkness was proved to be the better condition during the induction phase. Embryogenesis was possible in light or in dark condition, but in complete darkness somatic embryos were better induced, embryogenic potential was 123.3 in darkness rather than 61.21 in light culture in 10 ml volume MS medium. Through culturing in static liquid containing 1.0 $mg.l^{-1}$ NAA and 0.2 $mg.l^{-1}$ TDZ, calli were induced efficiently to give rise to numerous somatic embryos appeared on the outer periphery of the embryogenic calli on media devoid of plant growth regulators. The above results are shown to be completely different from the previous study by Tribulato *et al*. (1997) in which somatic embryos were obtained from cell suspension cultures established by suspending the friable callus of *L. longiflorum* in liquid medium containing 2 µm dicamba.

Of the methods used for clonal propagation, somatic embryogenesis is potentially the most important, as it is capable of providing a larger number of plants in shorter period of time than organogenic approaches. Although the phenomenon has been reported in numerous species, there is as yet no commercial production of plantlets by this method (Thorpe 1993). There have not yet any reports on the facts and figures of regenerants of *L. longiflorum* through somatic embryogenesis pathway. More than 98% of 1,500 somatic embryo-derived plantlets adapted well to *ex vitro* condition and thrived in nursery. Regenerated plants were uniform and did not differ phenotypically from parental plants. Moreover, it was of interest that plants originated from somatic embryos were shown to better survive and develop normally as compared to plants obtained via organogenesis from bulb scale. Somatic embryogenesis in *L. longiflorum* was successfully attained, in which different morphological stages and germination of somatic embryos were recorded. Its high ability to converse without any special treatments is advantageous for the propagation purpose. Through this research, a perfect system of commercial production of *L. longiflorum* by embryogenic pathway was achieved and put into practice. Moreover, it is important that somatic embryogenesis in this research is the basal experiment to supply valuable target tissue for genetic manipulation, cell selection and many other practical applications of this ornamental species through somatic embryos as well.

7.2. Rapid propagation of lily (*Lilium* spp.) via protocorm-like bodies

Many *in vitro* culture systems have been applied in order to propagate and increase quality of lily plantlets. PLB-like structures were observed through culturing slice of bulb scale (1-1.5 mm in thickness). These propagules were proven to be PLBs, a term used in *in vitro* propagation of orchids (**Fig. 9**). Highest number of PLBs (23 PLBs/explant) were formed on MS basal medium supplemented with 0.5 $mg.l^{-1}$ TDZ and 0.2 $mg.l^{-1}$ NAA. PLBs were cut vertically and subcultured every 6 weeks. Under effects of different plant growth regulators, PLBs were differentiated successfully into different organs (**Fig. 9**). MS medium containing 0.2 $mg.l^{-1}$ TDZ was most suitable for maintenance and proliferation of PLBs (16 PLBs/explant), whereas medium added 1.0 $mg.l^{-1}$ BA was shown to be optimal for shoot formation with highest multiplication coefficient (11 shoots/explant). PLBs cultured in medium supplemented with 0.1 $mg.l^{-1}$ zeatin gave better proliferation than those in medium added 0.1 $mg.l^{-1}$ kinetin, 2,082.3 and 1,760.0 mg, respectively. Regenerated plantlets were obtained from vertically-cut PLBs (6 plantlets/explant) on MS basal medium supplemented with 1.0 $g.l^{-1}$ AC and 30 $g.l^{-1}$ sucrose. Using this protocol, 598 x 32^6 plantlets were obtained from one bulb scale in one year. In addition to propagation purpose, lily PLBs can be considered a novel model for morphogenesis and transgenic studies (Nhut *et al.* 2006c).

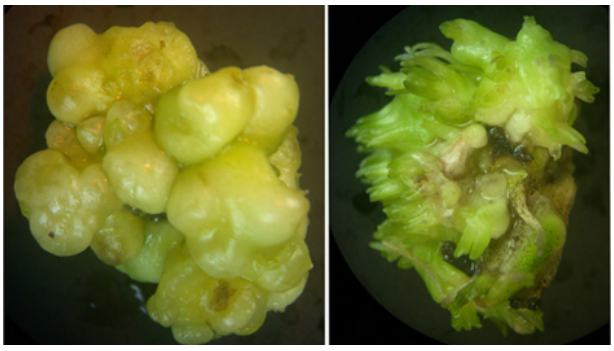

Fig. 9 Lily PLBs (left) and PLB-derived shoots (right).

8. MICROPONICS FOR ROOTING IMPROVEMENT

Conventional *in vitro* culture methods have successfully produced disease-free plant production for the last decades but some limitations still remain. Alternative techniques for propagation have been studied, including autotrophic tissue culture systems (Fujirawa *et al.* 1983) and microponics. In autotrophic culture systems, plantlets are grown in large vessels whose atmosphere contains both carbon dioxide and oxygen with appropriate concentration; relative humidity and nutrient components are also controlled. However, internal environment in these systems also need to be sterile and controlling the culture conditions has increased the production cost. Whereas, several preferable features were approved in microponic systems which is the combination of micropropagation and hydroponics, making a novel technique in plant tissue culture. In the microponic system, nutrients are supplied in solution in a small-scale.

Hahn *et al*. (1998) reported that chrysanthemum plantlets in microponic culture system grew more rapidly and vigorously compared to those in conventional *in vitro* system. The requirement of complicated culture steps was not necessary. In addition, labour and time could be saved because primary culture, multiplication, shoot formation and rooting steps are carried out continuously. Microponic system does not require completely sterile conditions. However, plantlets (or cuttings) should be placed under normally "clean" conditions. This facilitates several kinds of opening propagation system, promoting automation in plant tissue culture and decreasing production cost.

8.1. Application of microponics for propagation and flower quality improvement of gloxinia (*Sinningia* sp.)

Sinningia sp. (Gesneriaceae) is rhizomatous plant species, native to Brazil and commonly known as gloxinia. Gloxinia is favoured and used for decoration. Although it can be rooted by normal propagation, root formation usually requires long time and gives low survival rate when acclimatized in greenhouse. As reported by Nhut *et al.* (2006d), microponics was applied for gloxinia rapid multiplication. Cuttings were treated with 500 ppm NAA, cultured on MS medium, half-strength MS medium, 1/5 MS, 1/10 MS and water. The highest root regeneration rate was obtained in water due to low nutrient requirement for root initiation. Cuttings treated with 500 ppm IBA (indole butyric acid) showed more rapid growth and development than those with 500 ppm NAA or 1,000 ppm NAA. IBA was found to be more effective than NAA. In microponics, optimal shoot cutting formation and growth were recorded in water and treated with 500 ppm IBA (**Fig. 10**).

Fig. 10 Gloxinia shoots in microponic culture system.

These results can be applied for large-scale production of gloxinia and foundation for further studies on microponics and hydroponics of gloxinia as well as other ornamental plant.

8.2. Another economically-important floral crop micropropagated with microponic technology: chrysanthemum

Chrysanthemum is one of the most valuable flowers, which is offering a great profit for growers. Conventional meristem culture methods have successfully produced disease-free chrysanthemum for the last decades. Shoots originated from these methods, however, have to undergo rooting periods on semi-solid media (containing agar). Then normally, these plantlets are subsequently transferred into soil. This may lead to disease contamination and reduce quality in next generations, especially product "clean" level. Thus, a great number of plantlets derived from *in vitro* culture are always required.

Fig. 11 Chrysanthemum root formation in microponic culture system (**a**) and plantlets in nursery tray (**b**).

In order to satisfy the strict standards of clean level which market demands, as well as to overcome some defects of conventional propagation methods, Nhut *et al.* (2006e) studied microponic system of chrysanthemum in which cuttings can grow more rapidly and vigorously. In addition, labour and time could be saved because rooting period and acclimatization can be ignored, and liquid nutrient media can be controlled.

In microponic system, NAA concentrations and interval for cutting pre-treatment before planting into microponic system, and determined the concentration of 500 ppm and pre-treatment interval of 20 minutes as the optimal conditions were investigated. Further studies on microponic system for chrysanthemum and other floral crops are to be carried out for large-scale disease-free flower production (**Fig. 11**).

9. *IN VITRO* HYDROPONICS AS A NOVEL MEANS FOR HYGROPHILOUS PLANT PROPAGATION

9.1. *In vitro* hydroponics: a novel method for propagation of potato (*Solanum tuberosum* L.), lily (*Lilium* sp.) and cymbidium (*Cymbidium* sp.)

Several advantages of culture time and space of *ex vitro* hydroponic system was applied to some hygrophilous plants including cymbidium (*Cymbidium* sp.), lily (*Lilium* sp.) and potato (*Solanum tuberosum* L.) for *in vitro* culture as a novel method, named *in vitro* hydroponic by Nhut *et al.* (2006f). In this system, plantlets were cultured by many floors overlapping each other in the plastic capped box in the *in vitro* aseptic condition, and capillarity of nutritional liquid was taken to each floor by a capillary rope. Therefore, the problem of plantlets being flooded completely by nutritional liquid was solved. And at the same time, effect of multiplication was higher because place culture was economized. Moreover, simpleness and cheapness of components of this system made it economically-attracted.

Plastic boxes (Dai Dong Tien, Viet Nam), 11.5 cm in diameter and 7.5 cm in height; nylon ring with dimension: 36 x 0.05 x 1.5 cm; carrier was form by cutting around the cap of the plastic box and was 11.4 cm in diameter; cotton (Bach Tuyet, Vietnam) 11.4 cm in diameter and 2 mm in thickness was used as substratum; cotton was also used as capillary rope. Following, the *in vitro* hydroponic used for microtuber potato production from stem node was established: pouring the liquid medium in the plastic box; disposing the nylon ring at box bottom; placing the carrier on the nylon ring, placing the substratum on the carrier, putting the explants (the stem node bringing dormant buds) on the substratum (15

explants/substratum), pouring liquid medium to supply immediately to explants, completing similarly the second and third floors, capping the plastic box and sealing the chink between cap and box with a layer of food wrap.

The result showed that this system provided quality of microtuber equivalent or higher towards conventional system, especially in the first floor with higher humid, better capillary capacity, and less light-illuminated (higher lighting intensity would lead to bud rather than microtuber formation). High microtuber germination rate (90-96%) in the greenhouse was recorded, indicating high commercial potential of this system.

Nhut *et al.* (2006f) also established an *in vitro* hydroponic system for lily bulb production from bulb-scales similar to potato but only one floor with cotton subtract was applied. Sucrose concentration was studied and results show that 90 $g.l^{-1}$ concentration resulted in the most lily bulbs.

Some modifications could also be applied, in which the carrier and the supporter were brought together and made the two symmetric holes with 0.5 cm diameter near the edge of them, putting the capillary rope through the two holes of them to have a complex with there components: the carrier, the substratum and the capillary rope. The nylon rings were disposed at box bottom; the complex was then put on the nylon ring, followed by putting the explants on the surface of the substratum, pouring the liquid medium into the plastic box with volumes depend on the kinds of experiments and capping the plastic box and seal the chink between cap and box with a layer food wrap.

The number of PLBs formed from one initial PLB in this system was nearly double the conventional system. The time for cymbidium *in vitro* plantlet formation from PLBs was three months in this system. This showed clearly the advantage of a liquid culture system and commercial potentiality of this method with a higher survival rate and better growth. Cymbidium *in vitro* plantlets regenerated from *in vitro* hydroponics-derived PLBs showed normal growth and development. Normal growth and development of potato, lily and cymbidium have been recorded in these systems (**Fig. 12**), promising a wide application of *in vitro* hydroponics to plant micropropagation.

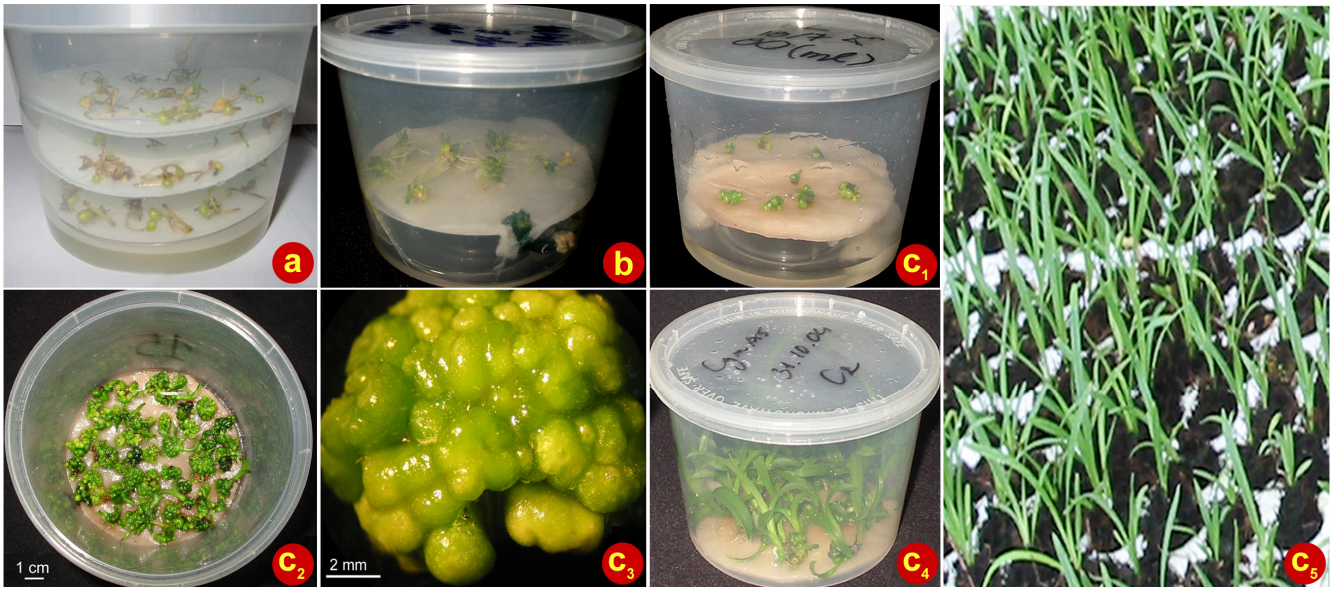

Fig. 12 *In vitro* hydroponic systems and their products: (**a**) *in vitro* hydroponic system for potato microtuber production; (**b**) *in vitro* hydroponic system for lily bulb production; (c) *in vitro* hydroponic system for multiplication of cymbidium PLBs (c_1-c_2), cymbidium PLBs was taken under microscope (c_3); cymbidium *in vitro* plantlets in liquid culture system combining with cotton supporter (c_4); cymbidium *in vitro* plantlets in nursery (c_5).

10. CONCLUDING REMARKS

The above-mentioned techniques and systems are novel applications in *in vitro* plant sciences, towards basic and applied research for new knowledge in plant physiology and genetics, culture system research, micropropagation and disease-free plant production. These results should be considered as a foundation for further studies in new trends of plant science and system engineering, which is expected to greatly and increasingly benefit the developing plant industry worldwide.

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