Chapter 6

Discussions
6.1 Scope of mechanization and automation in micropropagation

Mechanization and automation are being rigorously worked out by the present day plant tissue culture researchers and engineers worldwide. Problems still requiring solutions for automation are numerous and efforts are on for understanding and improvement of methods to be employed for automation of micropropagation in order to develop more cost effective systems. Once this is achieved, the new automated systems can be fully evaluated in a commercial environment. The present work comprised a step forward for accomplishing this wider objective.

Most of the bioreactors presently available are vertical column type or bubble-type designed for cell culture rather than plant propagation and are unsuitable for generation of hardened plantlets that can survive transfer to ex vitro conditions. Moreover, they are too expensive to be used in a commercial setup. It was, therefore, decided to undertake work on development of an indigenous, chamber type culture vessel/bioreactor with provision to control various physico-chemical parameters and at the same time, ensure ease of operations without compromising asepsis. As a consequence of this work, efficient indigenous models for bioreactor and various accessory components were designed, which have the potential to be cost effective and highly competitive as compared to the expensive models available internationally.

6.2 Achievements with design and technical prowess of the bioreactor system developed as part of current work

A survey for availability of suitable bioreactor for micropropagation of plantlets did not meet any success, as no bioreactor could be found meeting the objectives listed by us (Section 3.1). Keeping these objectives as the guiding principles, a bioreactor was designed and fabricated. Various accessory components were also fabricated to set up a complete system. The bioreactor system was tested for its performance and limitations were overcome before it was used for micropropagation.
All the objectives as described in section 3.1 were achieved. The modular design for the layout of the bioreactor system enabled setup of the system in different configurations, i.e., with different permutations of bioreactors and nutrient medium tanks, as per the experimental requirements. Provision of cone and socket connection mechanism for the tubing allowed flexibility for independent sterilization of the various components of the bioreactor system by either autoclaving or through chemical treatments, followed by their aseptic inter-connection using a portable laminar air flow cabinet. It also enabled addition or removal of various components, while the experiments were underway, without compromising asepsis. The portable laminar air flow cabinet was also a remarkable achievement of this work. It not only facilitated the setting up of the bioreactor system, but also served as the genesis for development of a commercial model, which was later on patented and commercialized. Use of glass was minimal, as all the components were made either of autoclavable plastic or stainless steel, thereby, reducing the risk of breakage. The added advantage of plastics was that the overall weight of the vessels was less, with added convenience in handling despite their large size.

The design of the bioreactor was highly suited for tissue culture, by virtue of ease of operations and the provisions to control the physical environment inside it. The support arm structure provided in it kept the lid in a raised position, thereby, not only saving the limited bench space on the laminar flow, but also enhancing the ease of operations. The low height of the base trough made it convenient to carry out operations involving the plantlets. It had provision for application of nutrient medium either by simple filling through an inlet port in its base trough, or by misting at high pressure through the foggers installed under its roof. This enabled uniform submergence/misting of the plantlets as desired. The provision for complete drainage was useful as a measure to avoid contamination, as nutrient medium stagnating in the bioreactor was more prone to contamination, than in case it was recycled to the collection tank after passing through the in line UV disinfection unit. While, the padding at the interface between the lid and the base trough permitted limited movement of air, the design of the lid at this interface prevented the nutrient medium, when applied in the form of mist, from collection and soaking the padding, and hence, preventing contamination. The rectangular
footprint of the bioreactor enabled most efficient utilization of the culture shelf space, when more than one bioreactor were kept together. The larger size of the bioreactor (45x30x28cm³) meant less tubing and less number of valves to be regulated for culture of a given amount of biomass, and hence enhanced ease of operation. A larger bioreactor size enabled culture of larger number of plantlets at a time (96 to 504 explants depending on their size), as each tray has a total of 63 big and small cubicles and eight trays could be accommodated at a time in the bioreactor.

Tanks also fulfilled all the requirements that were to be served by them. These not only facilitated nutrient medium supply and recycling, but also forced ventilation. The valves fitted to their ports needed to be regulated to bring about all these operations in conjunction with an air-pump. The glass windows provided in the lid and side wall, allowed convenient monitoring of the medium inside them.

The nutrient medium supply and ventilation systems were based on the principle of driving fluids by pneumatic pressure generated by an air-pump. This mechanism was extremely suitable because of its ability to transport fluids at highly variable flow rates, without significant variation in the pressure of supply. This resulted in efficient execution of both, simple filling as well as misting of nutrient medium in the bioreactor, without any breakdowns. The pressurized air in the tank served as the reservoir for supply of air to the bioreactor, in order to bring about forced ventilation. The air flow was monitored through a flow meter and was regulated through a knob. Moreover, forced ventilation was executed automatically, with the pressure switch and the non-return valve playing a crucial role for this. Water lost by evaporation through forced ventilation, was made up by regularly monitoring the total volume of the nutrient medium and adding sterile distilled water to it, whenever necessary.

The macro-filtration unit installed in the nutrient medium supply channel to trap any suspended particles which could clog the foggers of the bioreactor, also performed its function very efficiently. It filtered out all such particles, with a high retention capacity without posing much resistance to the flow of the liquid, and hence facilitated trouble free recycling of the nutrient medium. It did not get clogged even after being used in several experiments.
The designs of the bioreactors, tanks, macro-filtration unit, allowed their dismantling to enable cleaning in the event of any contamination.

The sterile drain developed with a new design enabled convenient sampling of the nutrient medium, without the need for its repeated sterilization, during use. It functioned efficiently, and did not allow any back spread of contaminating microbes.

The bioreactor could be used for culture of plantlets under temporary immersion or misting. Having provision for forced ventilation, the bioreactor system could also be used for photoautotrophic culture. The environmental conditions created in this scaled-up vessel (with forced ventilation) facilitated acclimatization in vitro.

### 6.3 Evaluation of the bioreactor system presently developed vis-à-vis comparable systems developed earlier

The acoustic window mist reactors (AWMRs) and the micropropagation chamber systems designed for culture of plantlets under temporary immersion as described in section 2.2 may be considered to be comparable to the system developed and tested in the present study. In the AWMRs since an air flow was needed to carry the nutrient mist throughout the bioreactor volume, the possibility of the medium loss and contamination was increased due to the mist entrainment through the gas exit tube. In order to prevent their desiccation, the explants to be inoculated needed to be kept in semi-solid medium prior to inoculation in reactor or box. In the bioreactor developed in the present study, the mechanism of misting being a spray system, mist entrainment was minimal and therefore, the problems associated to it were also avoided. Inoculation of the plantlets was more convenient as they were first placed in trays in small sterile boxes, and later these trays were quickly kept in the bioreactor, without exposing the plantlets to desiccation.

AWMRs had a capacity for only 32 nodes/clusters per bioreactor. Sealing and clamping of the lid to the base was required to prevent opening and free movement of air between the outside and inside environment. It was essential to maintain the liquid up to certain level above the transducer which generated ultrasound to bring about mist generation. Scaling up had not been very
successful due to difficulties in uniform mist and gas distribution, and because of its complicated design. Similar problem was experienced in the micropropagation chamber with forced ventilation developed by Zobayed et al. (1999) and Zobayed et al. (2000). The bioreactors (40.5L capacity) developed in the present study were relatively simpler and each had a capacity for holding 96 to 504 nodes/clusters depending on their size. Their explant holding trays were especially designed to accommodate explants of different sizes. The nozzles installed in it for misting and aeration were capable of uniformly distributing the mist, and generating enough turbulence, resulting in uniform growth of all the plantlets inspite of the larger size of the bioreactors. The complementary design of the lid and the base of the bioreactor, with a pad at the interface resulted in efficient cutting off of free movement of air between the inner and outer environments. Besides, the automatic locking mechanism ensured firm closing of the lid, without the risk of accidental opening. Scaling up was also not a problem, and in the present study three bioreactors were conveniently operated in parallel, and technically it offered feasibility to scale-up further. Thus it is easy to operate and automate the system.

One general problem with any type of acoustic window mist bioreactor was that the plantlets started showing browning and necrosis after 15-20 days of culture, most likely due to accumulation of certain substances beyond toxic concentration, as a result of evaporative concentration (Chun et al., 2003; Woo et al., 1996). In the bioreactor system developed presently, misting of nutrient medium in the form of sprays resulted in repeated rinsing of the entire explants, thereby, avoiding accumulation of any substances to toxic levels and the plantlets could be cultured for upto 45 days without any signs of adverse effects.

6.4 Standardization of various culture parameters for efficient growth and propagation of Lilium plants in bioreactor system

After standardizing the various technical parameters for the bioreactor system, work was initiated to evaluate its efficacy for micropropagation. For this, Lilium plants were selected for experimentation, because of their economic importance and easy availability in the form of already maintained cultures. Before their culture in the bioreactor system, certain culture parameters were standardized.
6.4.1 Strength of MS medium

Various studies (Kim et al., 1997; Jones, 2000) suggest that medium with lower concentrations of salts should be used for hydroponic culture of plantlets for better growth and for lowering the cost of production. In the present study, experiments were conducted using MS medium with its salt concentration lowered to half and quarter and the results were compared with the plantlets grown on medium with full strength. The results however, indicated that the overall growth of the Lilium plantlets was adversely affected upon culture on medium with lower MS salt concentrations for all the growth parameters observed, i.e., total leaf area (TLA), fresh weight (FW), total bulb volume (TBV), decreased with decrease in the salt concentration. Similar results were obtained by Lim et al. (1998b), who reported that in vitro regeneration and growth of bulblets from bulb scale segment in Lilium longiflorum sp. increased when grown on a medium containing 1.5 X the standard concentration of MS salts. Better bulb and root development in medium with 2x MS salt concentration was also reported in Lilium cv. Enchantment (Marinangeli and Curvetto, 1997) and in Lilium oriental Hybrid 'Casa Blanca' (Woo et al., 2001). Thus, application of MS salts at full strength was found to be important for Lilium propagation in bioreactors.

6.4.2 Supporting material

It was found that the growth of the plantlets was much better in liquid media as compared to agar solidified medium, or medium with other supporting material such as sand, glass beads and cotton. Similar results have also been observed earlier, for instance, Jimenez et al. (1999) reported more uniform growth and tuber formation in potato using temporary immersion than when supporting substances were used. In their work, temporary immersion resulted in contact of medium with all parts of the plant for short periods, thus the tuber induction was more uniform among axillary buds, resulting in more tuber formation.

6.4.3 Treatment with growth retardants

The preliminary experiments involving culture of Lilium plantlets in bioreactor using liquid medium resulted in plantlets that took much longer to resume active growth upon ex vitro transfer as compared to the plantlets raised in jars with agar.
solidified medium (Fig. 5.27 A and B). It was observed that though the plantlets were uniform in size, and possessed long uniform leaves, the bulblet development remained poor (Fig 5.28E). Starch accumulation and dry weight content were also low (Table 5.7). Therefore, there was a need to incorporate means or manipulate the culture conditions in order to improve the quality of the plantlets raised in bioreactors. Earlier also, Takahashi et al. (1992) reported that *Lilium* bulblets cultured in liquid medium had an increased water content, and for this reason, their rotting occurred easily upon *ex vitro* transfer. To solve this problem, they tried to decrease the water content by addition of sorbitol in the medium and found that lowering of osmotic pressure below 7.5 atm favoured lowering the water content of the bulblets, which became increasingly resistant to rotting.

Botcher et al. (1988) described that plantlets cultured in liquid medium tend to have higher FW and a low dry weight, relative to non-hyperhydrated plantlets. These were reported to have bigger intercellular spaces filled with water. Growth retardants have been widely used by various researchers in order to overcome these drawbacks associated with liquid cultures and to generate robust plantlets (Hazarika et al., 2002; Soon et al., 1998). Therefore, experiments were conducted for culture of *Lilium* plantlets on media containing growth retardants, paclobutrazol and ancymidol at various concentrations.

It was found that application of growth retardants at optimum concentrations facilitated development of plantlets with higher bulb to leaf ratio, i.e., whereas on one hand, larger nodular bulbs were formed with higher starch content, on the other hand, the size of the leaves got reduced, although their rate of proliferation remained unaffected. Larger sized bulblets had a higher chance of leaf emergence after transplantation (Lian et al., 2003a). Similar reduction in shoot growth was earlier observed in apple upon treatment with PBZ (Quinlan and Richardson, 1984; Steffens et al., 1985). At optimum growth retardant concentrations, numerous normal roots were also formed, without application of any other growth regulator, whereas at higher concentrations of growth retardants, an unusual thickening of roots was observed. Further, application of growth retardants also led to significant increase in the contents of dry weight, chlorophyll and cuticular wax. All these improved features conferred robustness to the plantlets, ensuring their
survival *ex vitro*. Smith *et al.* (1990) reported that paclobutrazol (0.5 - 4 mg/l) in the medium resulted in reduced stomatal apertures, increased epicuticular wax, shortened stems and thickened roots, reduction in wilting after *ex vitro* transfer, and also increased chlorophyll concentration per unit area of leaf, hence, facilitating development of hardy plants.

A comprehensive index based on the measurements of all the growth parameters, was calculated as a measure of quality of the plantlets. This index correlated well with the actual *ex vitro* survival data (Fig. 5.19). On the basis of this information, it was observed that PBZ could be used singly at low concentrations for culturing *Lilium*, without adversely affecting their growth rates, and incorporation of PBZ at 1 mg/l in the liquid medium, was determined to be the best suited for optimum culture of *Lilium* plantlets in the bioreactor system.

Anatomical studies of the leaves did not reveal any significant variations among various treatments, except when PBZ treatment was given along with BAP at higher concentrations (2.5 mg/l and above), that deformations in the normal structure of the leaves was observed (Fig. 5.22).

The use of growth retardants have been shown to prevent shoot elongation, and requirement for an additional culture period on medium free of the growth retardant, thereby increasing the cost and labour associated with plant production (Lorenzo *et al.*, 1998; Ziv, 1992; Ziv and Shemesh, 1996). Upon transfer of growth retardant treated plantlets to growth retardant-free medium, the after-effects were not evident for optimal growth retardant concentrations (0.5 mg/l and 1 mg/l), but for higher concentrations (2.5 mg/l and 5 mg/l), number of roots per bulb, number of bulbs, TBV and starch content were higher. Chlorophyll content was however, higher in all the plantlets precultured on medium with growth retardants (Fig. 5.23B). Thus, it was found that treatment with growth retardants did not have any significant after-effects for optimal concentrations (0.5, 1.0 mg/l), and those observed for concentrations higher than these were not of inhibitory nature.

### 6.5 Outcome of measures taken for contamination control

Since, occurrence of contaminations was always anticipated; strategies were adapted to have effective safeguards.
6.5.1 Lowering of pH

Leifert et al. (1994b) reported that lowering of pH of the nutrient medium was inhibitory to the growth of the contaminants. Therefore, in an experiment study was carried out on tolerance of plantlets to lowering of medium pH. Though it was observed that the initial pH did not have any significant impact upon the growth of the plantlets, any conclusions can not be drawn from this result which would fit a bioreactor system. Reason being that in the experiments conducted herewith, only 15ml medium was used per plantlet in test tubes, and this quantity being too little, its pH was rapidly changed and stabilized to a uniform value due to the buffering action by the plantlets themselves. However, this may not hold true for bioreactors, where the amount of medium per plantlet is much higher. Nevertheless, it could be inferred that pH of the medium could be safely lowered to 4.5 for culture of the Lilium plantlets as a step towards avoiding occurrence of contaminations, without having any adverse effects.

6.5.2 Application of catechins

Catechins which have been reported to possess antimicrobial properties (Hara et al., 1999; Kakuda et al., 1994; Shahidi, 1999; Vijaya and Ananthan, 1996), could not exercise any control upon the contaminants that appeared in the bioreactor system in the present study. Earlier, Hara et al. (1999) had reported that catechins were not active against lactic acid fermenting bacteria. This prompted us to examine the contaminated cultures, which smelled similar to lactic acid fermentation. In view of this, further studies with catechins were suspended.

6.5.3 Application of sodium hypochlorite

Plant tissue culture has been carried out with sodium hypochlorite incorporated in nutrient medium for exercising a varied degree of control upon occurrence of contaminations (Kohmura et al., 1999; Yanagawa et al., 1995; Yanagawa et al., 2000). In the present study, sodium hypochlorite at a strength equivalent to 150ppm to 200ppm of active chlorine was found to be effective in substantially controlling contaminations, without having any adverse effects upon the growth of Lilium plantlets. Therefore, sodium hypochlorite was routinely incorporated in the nutrient medium in the bioreactor, as a step towards minimizing contaminations.
6.5.4 Application of antibiotics

Experimentation with various antibiotics did not yield any success in controlling contaminations. Moreover, antibiotics would be too expensive to be used for control of contaminations in commercial micropropagation. Studies conducted earlier to control microbial contamination could not find any viable solutions (Leifert et al., 1991a, 1991b, 1994b). Scortichini and Chiariotti (1987) attempted control of contamination in *Nectarine* (cv. May Fair) using six different antibiotics of which only streptomycin sulphate (30mg/l) had any curative effect. Bastiaens et al. (1983) assessed eight antibiotic compounds with different modes of action for antibacterial and phytotoxic effects in tissue cultures of *Cordyline terminalis* and *Ficus benjamina*. None of the compounds completely eliminated bacterial contamination, and moreover, fungal development was stimulated by the suppression of bacteria.

6.5.5 Application of Plant Preservative Mix (PPM™)

Plant Preservative Mix (PPM™), has been reported to be useful in reducing the occurrence of contaminations (Babaoglu and Yorgancilar, 2000). However, the two concentrations, 0.74ml/l and 1.33ml/l which were tested in the present study, it failed to control the occurrence of contamination. Considering its cost, which was $1/ml, it certainly could not have been an economical means of controlling contaminations, and therefore studies at concentrations higher than the once in the present study were abandoned.

6.6 Studies in bioreactor

6.6.1 Culture in different types of vessels

The manually fabricated culture vessel (bioreactor) designed for use in scale up of micropropagation was found to be superior to other vessels which were equipped with similar features (means of nutrient medium misting/simple filling; aeration; drainage). Its features included: higher capacity for holding plantlets for culture; better bench space utilization; more uniform mist distribution; less tubing requirements (Larger size of bioreactors ⇒ Less number of bioreactors for culture of a given amount of biomass ⇒ Less tubing for medium supply and drainage ⇒
Easier management). Therefore, only these vessels were used for all further studies.

6.6.2 Suitability of mode of application of nutrient medium

Plantlets were cultured with two different modes of application of the nutrient medium, viz., misting and temporary immersion through simple filling. Temporary immersion turned out to be a poor competitor against misting for culture of *Lilium* plantlets. Whereas, starch content, dry weight content and wax content were insignificantly higher in the plantlets raised in temporary immersion culture (Table 5.8), the increase in FW and the rate of proliferation (1.11 bulbs per cluster), were significantly higher in case of plantlets raised in mist culture. The added advantage of misting was that only a little volume of nutrient medium (approx. 300ml) was required as compared to the volume (3000 ml) required for temporary immersion, and consequently, the operation cycle was also shortened, each time the medium was supplied and recycled. The advantages of mist culture far outweighed those of temporary immersion culture, and therefore, it became the method of choice for micropropagation of *Lilium* plantlets in bioreactor. Similarly, higher biomass production has been achieved earlier also by using misting in micropropagation of *Dianthus caryophyllus* (Correl and Weathers, 1998), potato (Hao et al., 1998), *Musa*, *Cordyline* and *Nephrylepsis* (Weathers et al., 1988), and in hairy root cultures of *Artemisia annua* (Chun-Zhao et al., 1998; Kim et al., 2001, 2003) and *Atropa belladonna* (Wyslouzil et al., 2000). Most widely accepted reasoning for better growth of plantlets under mist culture is that high surface area of the mist droplets results in high gaseous exchange, and that regular misting brings about replenishment of various nutrients necessary for growth.

6.6.3 Photoautotrophy vs. photomixotrophy

Several studies (Fujiwara and Kozai, 1994; Kozai and Smith, 1994; Lian et al., 2003b) have indicated that photoautotrophy is useful because of various advantages it offers in terms of development of normal plantlets and possibility for use of larger vessels due to lowered risk of contaminations. Keeping this in view, in the present study, experiment was conducted to compare the influence of photoautotrophic and photomixotrophic culture of *Lilium* plantlets. It was observed that the best growth occurred when the *Lilium* plantlets were cultured
photomixotrophically, under forced ventilation, as compared to photoautotrophic culture with forced ventilation, or photomixotrophic culture without forced ventilation. Not only growth and proliferation were higher under photomixotrophic culture, as indicated by their higher TLA, FW and number of bulblets per unit weight; but also, their robustness was better as indicated by higher chlorophyll content, higher bulb volume with higher starch content (Table 5.9). Differences between their cuticular wax and dry weight contents were insignificant. Difference between their rates of photosynthesis was also insignificant, clearly indicating that it was not adversely affected by the presence of sucrose in the nutrient medium. It may be inferred here that the surplus growth in the former case is the result of additive effects of growth supported by autotrophy as well as by sucrose uptake.

Similar observations have been reported by Kubota et al. (2001) in tomato and Vyas and Purohit (2003) in Wrightia tomentosa. They reported that whereas, photomixotrophic conditions produced the greatest dry weight and the longest shoots; photoautotrophic conditions produced the highest net photosynthetic rate. Ticha et al. (1998) also reported that plant growth, dry matter accumulation and TLA were higher under photomixotrophic than photoautotrophic conditions. Not only biomass formation, but photosynthesis was also positively affected by exogenous sucrose application. Photomixotrophic growth of plantlets has been reported to have prevented the occurrence of photoinhibitory symptoms (Ticha et al., 1998). In the present study, the culture of Lilium plantlets involves accumulation of nutrient reserves in the form of starch in the bulbs. For this, indeed the presence of sucrose in the nutrient medium is very important, whereas, application of forced ventilation promotes the ability for photosynthesis and regulation of transpiration in the plantlets. Together, they have an additive effect in generating robust plants that can show better survival upon ex vitro transfer.

Though various researchers have reported better growth of plantlets in photoautotrophic conditions, many others have stressed upon the importance of sugar for development of hardy plants that can survive well upon ex vitro transfer (Fowler, 1983; Hazarika, 2003; Merrilon et al., 1984). Grout and Millan (1985) had highlighted the nutrient function of persistent leaves when raised on media with higher sucrose concentration. Wainwright and Scrace (1989) found that maximum values for shoot height, fresh and dry weight of Potentilla fruticosa and Ficus lyrata...
Discussions were obtained in vivo when previously conditioned with 2 or 4% sucrose. Capellades et al. (1991) reported that size and number of starch granules increased with level of sucrose in the culture medium. Kawarabayashi, (1993) used 4% sucrose for bulblet differentiation in *Lilium japonicum*. Misra and Datta (2001) had even used 9% sucrose in nutrient medium and found it suitable for bulb development in *Lilium*. Paek et al. (2001) also reported better bulb formation in the presence of higher sucrose concentrations. Sugars were reported to have an osmotic role (Takayama and Misawa, 1979), as a source of energy and carbon in inducing shoot regeneration (Bieleski, 1993; Brown et al., 1979; Kumar et al., 2002) and as a precursor in metabolic activity (Moalem-Beno et al., 1997). Lim et al. (1998a) also reported that maximal bulblet growth in *Lilium* plants was achieved when grown in medium with 9% sucrose. The present study reaffirms that presence of sucrose in the medium is important for better development of hardy *Lilium* plants.

6.6.4 Culture of plantlets in bioreactor along with paclobutrazol treatment

The quality of the plantlets raised in bioreactor during the preliminary experimentation was not satisfactory, due to abnormalities associated with liquid culture. Therefore, experiments were conducted in jars with growth retardants incorporated in the nutrient medium, to counteract these problems, and PBZ (1mg/l) was found to be the most effective for generation of hardy plantlets without having any adverse effects on their growth and development, and therefore, it was incorporated in medium for culture of *Lilium* plantlets in bioreactors. The results were compared with plantlets raised in (Control: C1) bioreactor with medium devoid of growth retardant (Table 5.10). Incorporation of PBZ in the medium for culture in bioreactor brought about tremendous improvement in the quality of the plantlets, without affecting the quantity of production. For plantlets raised in bioreactor, the increases in number of bulbs (without PBZ: 142.7%; with PBZ: 415%), TBV (without PBZ: 187.42%; with PBZ: 811%), starch content (without PBZ: 3.09%w/w; with PBZ: 6.12%w/w), chlorophyll content (without PBZ: 6.14mg/g; with PBZ: 8.37mg/g), wax content (without PBZ: 0.642g/m²; with PBZ: 1.231g/m²), dry weight content (without PBZ: 6.08% of FW; with PBZ: 11.3% of FW) were much higher for the plantlets raised with paclobutrazol treatment in
the bioreactor. All these characteristics influence successful transfer of plantlets to 
*ex vitro* environment (Hazarika, 2003). Such plantlets showed better survival 
percentage and an early resumption of growth, without any special acclimatization 
treatment. Apart from these qualitative aspects, the quantitative aspect i.e., the 
total FW (without PBZ: 418.94%; with PBZ: 710%) was also high for plantlets 
cultured in bioreactor in the presence of growth retardants. Also Konstas and 
Kintzios (2003) in cucumber, and Chen and Ziv (2001) in *Narcissus* plantlets, 
reported increase in FW and size in response to growth retardants.

As compared to control (plantlets raised in jars containing medium with PBZ : 
Control - C3), the dry weight partitioning in the plantlets raised in the bioreactor, 
was more inclined towards the bulb formation, which is the most desirable trait in 
order to ensure better survival and early resumption of growth upon *ex vitro* 
transfer. A review by Hazarika (2003) mentioned of several reports stating that 
treatments with paclobutrazol resulted in a shift in the partitioning of assimilates 
from the leaves to the storage organs and roots. Haruki *et al.* (1998) emphasized 
the significance of good bulb formation with high nutrient reserve for *ex vitro* 
 survival of *Lilium* plants. Similarly, Wang *et al.* (1999) reported that paclobutrazol 
treated plantlets showed better bulb formation, resulting in greater percentage of 
 survival *ex vitro*. In the present study, a lower average bulb size in spite of higher 
TBV, in the plantlets raised in the bioreactor with growth retardant treatment, 
indicated a higher rate of proliferation, as compared to the controls (C3 and C4; 
Plantlets raised in jars in liquid and agar medium respectively, with 1mg/l PBZ 
each) (Fig. 5.38). Upon *ex vitro* transfer, though the survival percentage of 
plantlets with larger bulbs was better, total number of surviving plants obtained per 
unit biomass weight was higher for plantlets having smaller sized bulbs. Therefore, 
the biomass generated in the bioreactor, having higher proportion of smaller sized 
bulblets gave rise to a larger number of surviving plants per unit FW, upon *ex vitro* 
transfer.

6.6.5 Comparative study of plantlets cultured under different 
conditions: Stomatal density and area

The stomatal density in the leaves of the plantlets in bioreactor with forced 
ventilation was less than the plantlets cultured in air-tight jars, and more identical
to the acclimatized plantlets from the green house. These results were coherent with the earlier observations of Majada et al., (2001). They conducted an electron micrographic study of leaves of carnation plantlets grown in air-tight and ventilated containers and found that, as the number of air exchanges per hour increased, the stomatal density decreased and the values obtained were similar to those of acclimatized plants, maintained at higher air exchange. Similar results had been described earlier also for other species like *Liquidambar styraciflua* (Wetzstein and Sommer, 1983); apple (Blanke and Belcher, 1989). Further, it was observed that the size of the stomata was larger in plantlets cultured in ventilated environment in the bioreactors, as compared to the ones cultured in air-tight jars. Earlier also such differences have been highlighted by Donnelly and Vidaver (1984), Blanke and Belcher (1989) and Capellades et al. (1990). Zobayed et al. (2000b) upon working on *Ipomea batata* observed that, the leaf area under stomata and the ratio of their length to width was 23% and 1.6 in forced ventilation, and 30% and 1.2 in air-tight vessels, respectively. The low value of length to width ratio of the stomata in the latter indicated that the stomata were widely open. They also observed that the stomata of the plantlets cultured in air-tight vessels did not close upon desiccation stress, whereas stomata of the plantlets cultured in ventilated vessels responded well and closed, upon *ex vitro* transfer. Whereas, the former wilted quickly due to excessive transpirational loss of water, the functional stomata in the latter prevented their wilting and desiccation. Similar results were also observed by Brainerd and Fuchigami (1981, 1982) in plum and Majada et al. (2001) in *Dianthus caryophyllus*. In the present study, it was also observed that the degree of stomatal opening was more in the leaves of the plantlets which were cultured in air-tight jars (0.62) as compared to bioreactor with forced ventilation (0.50) (Fig. 5.48). This further explains the suitability of the bioreactor raised plantlets for *ex vitro* transfer.

### 6.6.6 Comparative study of plantlets cultured under different conditions: Chlorophyll content and rate of photosynthesis

The chlorophyll content was found to be higher in plantlets cultured in bioreactor with forced ventilation (8.69mg/l), than in the plantlets raised in air-tight jars (6.10mg/l) (Fig. 5.49). Likewise, the rate of photosynthesis was also found to be higher in plants cultured in bioreactor with forced ventilation (4.9 µmol CO₂m⁻²s⁻¹),
than in the plantlets raised in air-tight jars (2.91 μmol CO₂ m⁻² s⁻¹) (Fig. 5.47). Quite evidently the two characteristics were directly related, and this could be a direct consequence of forced ventilation. Higher photosynthetic ability of plantlets raised in bioreactors, also contributed to their higher survival and earlier resumption of active growth ex vitro. It is well known that plantlets grown under conventional photomixotrophic conditions have a low photosynthetic ability associated with low activities of photosynthetic enzymes such as Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase) and abnormal chlorophyll fluorescence responses (Desjardins et al., 1995). The enhanced net photosynthetic rates of Eucalyptus plantlets in vitro increased the survival percentage ex vitro (Kirdmanee et al., 1995).

6.6.7 Comparative study of plantlets cultured under different conditions: Epicuticular wax content and contact angle of water

Zobayed et al. (2000b) reported that wax deposition was greater in plantlets cultured under forced ventilation, than those cultured in air-tight vessels. In the present studies also, a similar result was observed. The cuticular wax content in plantlets cultured in air-tight jars with liquid medium was 0.89 g/m², whereas it increased to 1.03 g/m² in plantlets cultured in bioreactors with forced ventilation (Table 5.13). Earlier, an electron microscope study by Majada et al. (2001) revealed that the leaf surfaces of in vitro cultured plantlets (in air-tight vessels) remained similar to those of acclimatized plantlets, but had less wax deposition on the surface of leaves.

Further, in the present study, it was revealed that the plantlets raised under mist culture had low wax content of 0.64 g/m² as compared to 1.03 g/m² in temporary immersion culture (Table 5.13). This drastic lowering of the wax content under mist culture could be a direct consequence of the washing action of repeated misting of the nutrient medium, and because of high availability of water in the aerial environment. The wax content of ex vitro plants was also measured and found to be 3.85 g/m².

For further understanding, wettability of the leaf surfaces was studied by measuring the contact angle of the meniscus of a 10μl distilled water droplet as described in section 4.5.7.
Leaf surface wetness may have both negative and positive consequences as summarized by Pandey and Nagar (2003). The negative effects comprised facilitation of pathogen infection (Evans et al., 1992), pollutant deposition and foliar nutrient leaching (Massman et al., 1994; Cape, 1996), and reduction of gas exchange (Ishibashi and Terashima, 1995; Nobel, 1991). On the other hand, retention of water droplets on individual leaves and throughout the leaf canopy may lead to enhanced water use efficiency by reducing transpiration (Smith and Mclean, 1989).

Contact angle measurement is a very sensitive indicator for wettability of a surface and has been applied in various biological areas, since the degree of surface wetting by water gives information on the characteristics of the outermost layer of the interface between the liquid water and the atmosphere (Holmes-Farley and Whitesides, 1987).

The contact angle differed substantially between the plantlets raised under mist culture (BR-mist) and the ones raised by temporary immersion (BR-TIS), the angle being just 51.2 ± 14.2° in the former as compared to 97.6 ± 7.6° in the latter. In case of plantlets raised in BR-mist, the difference between contact angles for upper (45.1 ± 16.8°) and lower (57.4 ± 11.6°) surfaces, and a higher overall variation of the contact angle from the mean value (standard deviation of ±14°), support the possibility of washing away of waxes, as different leaves and their different surfaces are exposed to different levels of liquid medium precipitation, leading to this variation. The contact angle in case of ex vitro plantlets was identical to that of BR-TIS plantlets, inspite of their having over two fold higher wax content (1.03g/m² in BR-TIS plantlets; 2.60g/m² in ex vitro plants). This indicates that a minimum critical thickness of wax layer was required to avoid wetting, and further deposition of waxes did not contribute further in this aspect.

6.6.8 In vitro performance of plantlets raised in bioreactor with paclobutrazol

The plantlets raised in bioreactor (Ex-BR) with forced ventilation and in the presence of paclobutrazol, upon transfer to agar solidified medium with composition as in section 4.1 (without growth retardants), exhibited growth performance comparable to the control plantlets (Plantlets already being cultured
in jars on agar solidified medium with composition as in section 4.1). Though slightly lower, the growth parameters: TLA, FW, and TBV of the Ex-BR plantlets were not significantly different from the control plantlets (Fig. 5.50). The minor negative difference in overall growth of the former could be attributed to the change in phase of the medium from liquid to solid, which might have exerted a temporary stress upon them. Thus, the plantlets raised in bioreactor under controlled conditions as described above were suitable for further in vitro culture as well.

6.6.9 Ex vitro performance of plantlets raised in bioreactor with paclobutrazol treatment as compared to those cultured in other conditions

Upon ex vitro transplantation, per gram of biomass yield, 0.89 surviving plants were obtained in case of C4 control plantlets (cultured on agar solidified medium in jars), as against 1.04 plants in case of C3 control plantlets (cultured on liquid medium in jars), but almost all their leaves wilted soon after transplantation and some of these were unable to recover. On the other hand, 1.50 surviving plants were obtained per gram of biomass yield in case of the plantlets transplanted from the bioreactor (Table 5.12), and most of the leaves showed recovery. In contrast the conventionally cultured (without forced ventilation and without growth retardant treatment) Lilium plantlets could not tolerate the freezing winter conditions upon ex vitro transfer and showed a high degree of mortality (Fig. 5.51). The best performance of the plantlets raised in the bioreactor was due to a combined effect of growth retardant treatment, forced ventilation and application of nutrient medium in the form of mist. All these favoured development of quite normal and hardy plants as described in section 6.4.3 (Role of growth retardants), 6.6.2 (Mode of application of nutrient medium) and 6.6.10 (Significance of forced ventilation).

6.6.10 Role of forced ventilation

The relative humidity in the conventional air-tight vessels is known to be around 95% - 98% (Zobayed et al., 2000a). However, in the bioreactor studied here, it was around 70%, at a time when the ambient humidity was 32% approximately.

In conventional air-tight vessels, whereas the CO2 concentration decreases with time, the ethylene concentration increases correspondingly (Zobayed et al.,
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2000a; Zobayed et al., 2001). These two factors are well taken care of by forced ventilation. Forced ventilation is also important to replenish the oxygen demand in the bioreactor, especially during the dark period. Takahashi et al. (1992) reported that the bulblet formation was prepone and showed an increase owing to a suppression of callus formation with increasing DO of the liquid medium, and moreover, the bulblets formed were larger. Konstas and Kintzios (2003) reported increase in biomass production in bioreactor cultures of cucumber, as a result of increased aeration.

Majada et al. (2001) while studying plantlets cultured in conventional manner in air-tight vessels, observed that while the most abnormal plants could not survive, others could revert and follow normal development patterns. Hyperhydrated plantlets or plants grown in air-tight in vitro environments, when transferred to ex vitro conditions are more susceptible to desiccation. Additional misting may be used to reduce losses but it enhances the risk of fungal attacks. However, ventilated plants reach an intermediate phenotype between air-tight and acclimatized ones and, this allows acclimatization with negligible stress, indicating that after transplanting to the soil, the degree of survival was directly related to the hydric relationship established in the culture vessels during the proliferation phase (Majada et al., 1997). It was also observed in their work that upon subjecting to desiccation, plantlets grown in air-tight culture vessels had a lower degree of stomatal closing than the ones grown in ventilated vessels. Similar results were also obtained by Marin et al. (1988) and Sutter (1988). Zobayed et al. (2000b) studied the rate of loss of water from detached leaves, and found that while the leaves from plantlets cultured in air-tight vessels lost 50% of their water content in 45 minutes, the ones from plantlets cultured in ventilated vessels lost only 17% of their water content in the same duration. Therefore, irreversible tissue damage occurs much sooner in the former than in the latter. Majada et al. (2001) observed the leaf cuticle after removal of the wax with chloroform, and found that cuticle of the plantlets cultured in air-tight vessels exhibited deformations in contrast to the ones cultured in ventilated vessels, which kept their normal shapes. Correll and Weathers (2001) reported that the stomata on plantlets grown in conditions of high light or low humidity had smaller apertures and appeared sunken when compared to stomata from plantlets grown in low light and high relative humidity.
The observations of the present study are in agreement with these earlier reports, and it was found that if the proliferation stage proceeds in ventilated culture vessels, the characteristics of the plantlets produced are better than those obtained in air-tight culture vessels, as confirmed by their higher survival percentage during ex vitro transfer.

So far all the objectives as mentioned earlier for the bioreactor system have been achieved. A biphasic bioreactor system, with medium recirculation, whereby, the plantlets can be exposed to liquid or gaseous phase, as per experimental requirements, has been developed. The present study demonstrates the possibility of efficient aseptic culture of plantlets in the bioreactor, and also confirms its technological completeness in meeting all the requirements. The bioreactor system developed and tested herewith, has a number of benefits over conventional small vessels, and other bioreactors. Forced ventilation and mode of application of the nutrient medium, resulted in enhanced growth and net photosynthetic rate. This together with incorporation of growth retardant, especially paclobutrazol in the medium played an important role in improving the characteristics of the plantlets. The growth of the plantlets in the bioreactor was uniform, the percentage of dry mass was high; and the bulbs were compact and nodular, with dense nutrient reserve, an important characteristic of a 'quality plant' capable of displaying better survival upon ex vitro transfer. The photosynthetic ability of the plantlets was also better. The plantlets survived well when transferred to ex vitro conditions even without any specialized acclimatization. Using this system, it may be possible in future to increase the amount of produce per worker, hence reducing the costs involved in producing micropropagated plantlets; however, pilot scale studies still need to be done.

Further, survival percentage of the plantlets cultured in bioreactor after ex vitro transfer could be better in reality, because during the experimental production, the process of taking all the observations of various growth parameters, imposed stress upon the plantlets in terms of physical damages and unusually long gap between harvesting and actual transfer to the polytunnel.
6.7 Possibilities for further development of the bioreactor system

6.7.1 Incorporation of additional CO₂ supply

A CO₂ supply system has been widely used to promote autotrophic micropropagation at various scales of propagation (Dang and Donnelly, 1993; Desjardins et al., 1987; Desjardins et al., 1988; Hahn and Paek, 2001; Hayashi et al., 1995; Jeong et al., 1995; Kozai et al., 1999; Nguyen et al., 1999; Nguyen et al., 2001; Seko and Nishimura, 1997; and Zobayed et al., 2000a). Additional CO₂ supply has also been used for propagation of Lilium plantlets (Lian et al., 2003b), and the same can easily be incorporated into the system developed herewith, in order to promote photoautotrophic growth.

6.7.2 Incorporation of automation to various extents

The manually operated valves can be replaced with electronically operated ones. All these valves, the switches for operating the pumps along with level sensors installed in the tanks can be regulated through pro-logic controllers (PLC), that can be programmed to operate the bioreactor system as per requirements and bring about medium application, drainage, and forced ventilation/CO₂ supply.

6.7.3 Further improvement in the design of the bioreactor and the culture practice

There is a scope for further development in the design of the bioreactor to improve its suitability for commercial production. The misting channel can be redesigned and the height of the bioreactor can be reduced. The overhangs of the lid need to be redesigned and the cotton pad can be replaced with a more suitable matrix with the requisite properties.

Detailed studies on nutrient dynamics can be carried out for plantlets to be propagated in the bioreactor, in order to have a better understanding on this aspect. Subsequently, the composition of the nutrient medium can be improved and managed in real time to facilitate faster growth of superior quality plants.

Though photoautotrophic culture of the Lilium plantlets was carried out in the bioreactor system, it may be possible to achieve better results by increasing the photosynthetic photon flux density (PPFD) along with CO₂ enrichment and forced
ventilation. Lian et al. (2003) reported successful photoautotrophic culture of *Lilium* culture and good bulblet formation upon CO$_2$ enrichment. Similarly, Zobayed et al. (2001) reported superior growth during micropropagation of potato upon CO$_2$ enrichment.

Monitoring parameters in a single bioreactor would give information about all the bioreactors connected in parallel, as all are maintained under identical conditions. Hence, a feedback system can be setup for automated control of environmental features such as relative humidity, CO$_2$ concentration inside the bioreactors.