STUDIES ON ENCAPSULATION OF PROTOCORM-LIKE-BODIES OF CYMBIDIUM GIGANTEUM WALL. WITH SPECIAL REFERENCE TO STORAGE, GERMINATION AND GROWTH BEHAVIOUR.

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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C E R T I F I C A T E

I certify that the thesis entitled 'Studies on encapsulation of Cymbidium giganteum Wall. plbs with special reference to storage, germination and growth behaviour' submitted by Ms. Shashi Corrie for the degree of Doctor of Philosophy in Botany of the North Eastern Hill University, Shillong, embodies the record of original investigation carried out by her under my Supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D Degree. This work has not been submitted for any degree of any other University.

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I .GENERAL INTRODUCTION : REVIEW OF LITERATURE.

Artificial seed technology involving encapsulation of *in vitro* grown somatic embryos, shoot buds and other plant organs in different gels have attracted considerable interest because of the advantages it offers over the conventional *in vitro* culture system. Coated plant organs provide a potential method to deliver propagules produced from tissue-culture directly to the green house or field. The direct planting of somatic embryos bypasses the *in vitro* plant production steps which require high labour effort and expense. The technique also offers easy transportation of a large number of plants in low bulk and at reduced cost of transportation.

An artificial seed is a novel analog to botanic seed consisting of a somatic embryo surrounded by a protective gel. The protective gel acts as an 'artificial seed coat' protecting the embryo from mechanical damage during storage, shipping, handling and planting. It also acts as an artificial endosperm supplying necessary nutrients to the developing embryo as the natural endosperm do. The capsule also has potential to hold and deliver beneficial adjuvants such as growth promoting bacteria, growth control agents, nematodes, fertilizers, pesticides and antimicrobial agents.

Somatic embryo is similar to zygotic embryo in morphology, physiology and biochemistry. Even the developmental stages in both the cases is same. Under certain conditions, somatic embryos tolerate severe desiccation (Kitto and Janick, 1982), an ability shared only with the zygotic embryos. Finally, in some species such as Brassica napus and Medicago sativa, somatic embryos produce the same embryo specific proteins as do the zygotic embryos (Crouch, 1980; Stuart etal. , 1985). And since through somatic embryogenesis, thousands of embryos can be produced rapidly from a few grams of a tissue, artificial seed system would be a rapid mass propagation method that would maintain the genetic fidelity of the plants. Thus, high volume propagation potential of somatic embryogenesis combined with the formation of artificial seed for low cost delivery would open a new field for clonal propagation.

The application of the technology is thus quite diverse and the ultimate development of dry artificial seed promises to revolutionise several aspects of micropropagation, germplasm storage and even methods of plant breeding in several economically important crop species. Drying artificial seeds would serve as a germplasm storage system which could maintain the propagules in a quiescent state for an extended period of time and would also provide a more efficient use of space and labour in a commercial production system. Artificial dry somatic embryo is easily maintained sterile and can be transplanted internationally pathogen-free. Artificial seed, besides serving as a low-cost, high volume propagation method and a delivery system of elite and improved germplasm of seed crops such as labour-intensive, hand pollinated hybrids, stable and unstable genotypes and genetically engineered plants, would also be a channel for new plant lines produced through biotechnological

advances to be delivered directly to the field. Artificial seed would also be useful in cases where seed fertility is reduced such as wide hybridisation (protoplast fusion, cybrid production, and single gene transfer designed to incorporate diverse genetic material in a unique genotype. It would also be useful for vegetatively propagated plants which do not bear seeds or others which have poor seed set or aborted seeds. It would also serve as an analytical tool for comparative as well as zygotic embryology.

Though somatic embryogenesis was observed in 1958 by Steward et al. (1958), the concept of artificial seed appeared in print only in 1978 (Murashige, 1978). Eversince research on artificial seed has increased significantly, as demonstrated by increased attention from several laboratories (Kitto and Janick, 1982, 1985a, 1985b; Obendorf and Slawinska, 1986, 1987; Redenbaugh et al., 1986, 1988 ; Kim and Janick, 1987; Gray,1987a, 1987b). Two types of artificial seeds have been reported in the literature: desiccated and hydrated. Success has been reported in coating carrot and celery somatic embryo in Polyoxyethylene (Polyox), drying the embryo/Polyox mixture and obtaining survival of the embryo (Kitto and Janick, 1985 b ; Kim and Janick, 1987). Only 3% of coated somatic embryo survived desiccation. Without the coating survival was nil. In another report (Kitto and Janick, 1985 b) improved embryo survival was described with high sucrose, high inoculum density or chilling treatment with or without ABA. Some researchers have focussed on the desiccation of somatic embryo without encapsulation to understand embryogeny better (Obendorf and Slawinska, 1986; Carman et al, 1987; Gray, 1987; Slawinska and Obendorf, 1987).

Redenbaugh et al . (1984, 1986) reported the encapsulation of somatic embryos in hydrated gel, such as sodium alginate to

produce single embryo artificial seed. Several general methods were tested for production of synthetic seeds, of which gelation method was found to be the best. Several water soluble hydrogel / gel combinations were tested for capsule production and embryo survival. Sodium alginate was chosen as a major encapsulation gel because of its gelation properties, ease of handling, non-toxicity and low cost. Alternatively, gelation via a moulding process could be tried where the embryos could be mixed in a temperature dependent gel such as agar and gelled as the temperature was lowered.

Another potential component of artificial seed is a synthetic endosperm. For albuminous seeds, an artificial endosperm providing nutrients and carbon sources is required for optimum germination and conversion (Redenbaugh et al., 1987b). Drew (1979) found that sucrose was a requirement for carrot somatic embryo germination and plant development. But sodium-alginate matrix was found to be very 'leaky' and the nutrient retaining power was nil. Hence various starches, crude alginates and sucrose microcapsules were used as additives to provide controlled release inside the artificial seed. Unrefined crude alginate used to form capsule was the only additive that resulted in an increase in in vivo conversion. Calcium alginate capsules were very sticky and dried out rapidly unless kept in humid environment or coated with a hydrophobic membrane (Redenbaugh et al., 1987 b). One compound, ELVAX 4260, was very hydrophobic and showed a significant impediment to capsule drying and also the coated capsules could be planted using a seed planter (Redenbaugh et al.,1986; Upadhya et al., 1987).

Several workers have used sodium alginate or carrageenan to form artificial seed (Mitra and Chaturvedi, 1972; Yamakawa,

1985; Lutz, 1985; Kamada, 1985; Hama, 1986; Gupta and Durzan, 1986a ; Bapat and Rao, 1988; Mascarenhas, 1988; Fernandes et al., 1992). Mitra and Chaturvedi (1972) reported encapsulation of flower buds of Citrus and reported embryo and complete plant formation from unpollinated ovaries. Kamada (1985) reported 5-10% conversion from alginate encapsulated somatic embryo. Hama (1988) emphasized the incorporation of water absorbing polymeric material and antimicrobial agents in the alginate or carrageenan matrix. Mascarenhas (1988) reported 50% germination of Eucalyptus somatic embryos. The encapsulation of Santalum album somatic embryos in sodium alginate gel and in a composite gel of silica and alginate resulted in 17% and 4-16% germination using sodium carbonate as antimicrobial agents (Fernandes et al.,1992). However, no conversion into plantlets was observed. Jha et al. (1993) encapsulated seeds of Sesbania sesban with sodium alginate entrapped rhizobium and reported effective symbiotic nitrogen fixation. Mathur et al. (1989) successfully encapsulated apical and axial shoot buds of Valeriana wallichi DC in alginate beads and reported 98% and 64% germination in vitro and in vivo conditions. Bapat and Rao (1988) reported encapsulation of somatic embryo of Santalum album in 3% alginate with 16% germination. Encapsulation of mulberry axillary buds in agar and alginate resulted in 80% germination under in vitro conditions using alginate gel and 30% germination using Sisco agar (Bapat et al.,1987). Fujii et al.(1989) reported an increase in in vivo conversion frequency of alfalfa somatic embryo after maturation with abscisic acid, prior to planting. They were able to get 46% conversion in soil under growth chamber conditions and 64% conversion when humidified air was used to prevent soil surface drying under green house conditions. A cold maturation treatment was found to improve the conversion of alfalfa somatic embryo

with an optimum period of 7-10 days at 4 ^{O}C (Redenbaugh et al., 1987).

Besides desiccated and hydrated artificial seed, other delivery system such as fluid drilling, desiccated, non-coated embryos have also been suggested (Drew, 1979; Baker, 1985; Gray et al., 1986). The concept of fluid drilling is to suspend the propagules in a carrier gel supplemented with additives which is then pumped into the soil. Durzan (1980) suggested pelletization of desiccated somatic embryo to form artificial seed using inert compounds like diatomaceous earth, binders etc.

Storage of artificial seed has been found to be a critical parameter. The storage life for synthetic seed was found to be quite short using the alginate protocol for alfalfa (Redenbaugh et al., 1986, 1988). There was a subsequent decrease in conversion after 7 days, whereas, the conversion frequency of non-encapsulated embryo was only slightly diminished over a 49 day period. Gupta and Durzan (1986a, 1987) stored encapsulated somatic embryo of loblolly pine at 4°C for 4 months in the dark. Upon return to the light at 20°C the embryos produced chlorophyll indicating survival. But no conversion was obtained. Mathur et al.(1990) reported that the shelf-life of encapsulated propagules of Selinum candola, Nicotiana tabaccum and Valeriana wallichi was enhanced from 25-30 days to 150-240 days when their cultures were overlaid with mineral oil. Coating of apices and long-term presevation at ultra-low temperature of Solanum sp. was reported by Faber and Dereudre (1991). Bapat and Rao (1987) reported storing encapsulated axillary buds of mulberry at 4°C for 45 days without loss of viability. Bapat et al. (1987) stored encapsulated cell suspension of sandal wood at 4 ^{O}C for 45 days. On transfer to the medium the 5% cell suspensions revived and

produced somatic embryos.

Desiccation of artificial seed could allow a new approach to germplasm storage. If the somatic embryo are dried to moisture content as approximately 10% as occurs in a true seed, the propagation system has the additional advantage of serving as a germplasm storage system which maintains the propagules in a quiescent state for an extended time. Until recently attempts to desiccate somatic embryo (with or without encapsulation) to produce analogs to true seeds has met with limited success (Kitto and Janick, 1985; Obendorf and Slawinska, 1986, 1987; Gray et al., 1987; Kim and Janick, 1989) . It was possible to improve soyabean embryo conversion from 30% to 60% after embryo desiccation at 70% humidity (Obendorf et al.,1987). Gray (1987b) reported that desiccation appeared to release grape somatic embryo from a quiescent state and increase conversion. He reported an increase in conversion (20-28%) for desiccated embryo stored for 7-21 days compared to 5% for non-desiccated embryo. However, no coating method was used. Gray (1987b) also observed that embryo conversion decreased rapidly with storage. Carman et al. (1987) could achieve 59% conversion of wheat somatic embryo after desiccation.

Mc Kersie et al. (1989) induced somatic embryos of alfalfa to acquire desiccation tolerance by treatment with ABA. The embryos were air-dried slowly (over 7 days) or rapidly (over 1 day) to moisture content less than 15% water and remained viable. Dry somatic embryo could be stored with no loss of viabilty for 8 months at room temperature and humidity. Under appropriate conditions, 65% of the somatic embryos survived and germinated (Mc Kersie et al., 1989). Anandarajah and Mc Kersie (1990) reported that the quality of dry somatic embryo could be accomplished by increasing the sucrose concentration of standard

development and maturation medium to 5-6%. High sucrose concentration has been reported to alter maturation and conversion of somatic embryo (Ammirato and Steward, 1971; Drew, 1979).

No reports have appeared so far on the field performance of the regenerants of somatic embryo (with or without encapsulation). Altered environmental conditions due to encapsulation may bring about certain changes in the regeneration. For plant micropropagation and germplasm preservation the inherent stability of cultures are required. However, cultured cells are not genetically and phenotypically stable - polyploidy, aneuploidy and chromosome structural changes in cells in culture under various conditions are extensively described (D'Amato, 1971; Larkin and Scowcroft, 1981; Ogihara, 1981; Mc Coy and Phillips, 1982). Plants regenerated from shoot apex occasionally show variation and yield difference (Bush et al., 1976; Denton et al.,1977; Templeton-Somers and Collins 1985). Even the different agar bands containing different elemental composition is known to induce variations in the culture (Singha et al., 1985). Cytogenetic changes are also known to occur with increasing time in culture (Baybliss, 1980; Mc Coy, 1980; Peloquin, 1981). Besides, growing plants in vitro bring about subtle morphological and physiological changes (Grout and Aston, 1977a, 1977b; Sutter & Langhans, 1979, 1982; Fuchigami et al., 1981; Brainerd & Fuchigami, 1981, 1982; Wetzstein and Somner, 1982). The use of Scanning electron microscope (SEM) for examining the external manifestation of various developmental processes in plants both in vivo is becoming increasingly common and useful (Haydu and Vasil, 1981; Lu and Vasil, 1982; Ozias-Akins, 1982; Botti and Vasil, 1984 ;

Hew and Veltkamp, 1985).

Marked changes occur in the protein constituent of plants at various stages of growth (Coulson and Sim, 1965; Honold et al.1966; Bhatia and Nelson, 1968; Nainawatee et al., 1974; Singh and Singh, 1974; Macko et al., 1976). Different environmental conditions are known to cause minor qualitative changes in the protein constituents of the plants (Koering et al., 1964 ; Key et al., 1981). Similarly, the enzyme pattern of plants have also been shown to be influenced by the physiological and environmental conditions (Bjorn, 1967; Roberts, 1969; Mc Cover et al., 1970; Nainawatee, 1971; Singh, 1973; Singh and Singh, 1974). The role of various enzymes in relation to germination and growth has been focussed by several workers (Scandalios, 1974; Blackman et al ., 1976; Gaspar et al., 1977; Dendsay and Sachar, 1982; Barendse, 1983; Pilet et al.,1984; Miller, 1985). The IAA oxidase has been widely studied in an attempt to understand the regulation of IAA level in plant tissues (Barendse, 1983; Goyal, 1989). Isozymes of peroxidases have been employed as a marker enzyme for a varieties of studies on altered growth and development (Van Huystee and Cairns, 1980, 1982). One reason for this line of investigation is the ability of peroxidase to function also as IAA-oxidase (Sembdner et al.,1980; Chibbar et al., 1984a). The peroxidase activity has been found to be exact reciprocal of IAA production by Vanda seedlings (Alvarez, 1968; Alvarez and King, 1969). The activities of polyphenol oxidase, ascorbic acid oxidase and catalase were greatly increased in infected protocorms of Dactylorhiza purpurea, Rhizoctonia sp. (Blakemanet al., 1976). Stuart and Redenbaugh (1986) found somatic embryo and zygotic embryo of alfalfa to contain 115 storage proteins and in Brassica sp. they found no difference between the zygotic embryo and somatic embryo except in the

banding pattern of five of the isoenzymes.

Orchids have tremendous horticultural and floricultural importance. With their beautiful , long-lasting inflorescence orchids have a special place in the cut-flower industry and hence micropropagation of orchids is a commercial venture with ample opportunities to cater to international market. Though orchids produce many seeds, the seeds are extremely minute lacking endosperm and require mycorrhizal association for germination in nature (Arditti, 1967). As a result less than 5% germinate in their natural environment (Rao, 1967). Moreover, as orchids are highly heterozygous, it is very difficult to obtain large number of uniform plants from seedling population. Further, vegetative propagation yields only a few plants of a clone per year. However, through clonal propagation using tissue-culture techniques, it is possible to obtain a large number of genetically uniform plants, that too, in a much shorter period. Morel (1960, 1964) and Wimber (1963) noted the unique possibility of clonal increase when explanted opical meristems of Cymbidiums produced numerous "bulblets", which, resembled the protocorms found in embryogeny. These "bulblets" termed as protocorm-like-bodies (plbs) when cut and subcultured form a large number of plbs each with the potential to form a plantlet. Thus, a large number of identical plantlets (mericlones) could be produced starting from a single shoot tip measuring less than 1 mm (Morel, 1960, 1972), thus preserving the unique characters of a cultivar.

Besides shoot-tips, reports of leaf-tips (Churchill et al., 1971; Chaturvedi and Sharma, 1986), leaf segments (Wimber, 1965; Arditti, 1977a; Vij and Pathak, 1990; Abdul Karim and Hairani, 1990), immature inflorescence (Intuwong and Sagawa,

1973), basal buds of inflorescence (Intuwong et al. , 1977; Vij, 1986), axillary buds, (Wang, 1988), and , root-tips (Tanaka et al., 1976; Kerbuay, 1984; Sanchez, 1988; Yoneda and Momose,1988; Holters and Zimmer, 1990), flower stalk (Intuwong and Sagawa, 1972) have appeared.

Various media have been formulated to culture diverse tissues and organs . For orchid culture, the medium proposed by Vacin and Went (1949), Knudson (1949), Murashige and Skoog (1962), White (1963) have been widely used (Stoutamire, 1964; Arditti, 1967; Arditti et al., 1972; Nishimura, 1981; Nagashima,1982; Yoneda et al. , 1983; Sharma and Tandon,1988; Kumaria and Tandon, 1991).

Growth-regulators have profound effect on plb-proliferation and seedling growth of orchids (Torikata and Sasa, 1964; Ueda and Torikata, 1969; Fonnesbech, 1972b; Bose and Mukherjee, 1976, Kusumoto, 1978, 1980; Choi et al., 1990). The plbs of Cymbidium proliferated when grown on a medium supplemented with IAA or NAA (Fonnesbech, 1972a, 1972b). Torikata and Sasa (1964) and Choi et al . (1990) observed the growth of seedlings of Cymbidium was most favourable in medium with NAA and BAP. Ueda and Torikata (1969) found addition of NAA in medium promoted shoot formation. Fonnesbech (1972a) reported the inhibitory effect of 2-4-D on plb-formation, whereas Bose and Mukherjee (1976) noticed its beneficial effect. Elongation of leaves (Fonnesbech, 1972), axillary buds (Kusumoto, 1978), plbs (Bose and Mukherjee, 1976) by GA_3 have been reported. Kinetin has been reported to be stimulatory for plb-formation and seedling growth of Cymbidium (Fonnesbech,1972; Kusumoto, 1978). Though mericloning is currently practiced for rapid orchid propagation, this process involves several in vitro plant production and plant raising steps, gradual acclimatisation of plants from culture room to the

green-house and then to the field which requires high labour effort, time and expense. Moreover, the hardening procedure is inadequately standardised and the direct transplanting of in vitro grown plant to the field results in high mortality. An alternative procedure would be to plant plbs directly to the green house or field in the encapsulated form as artificial seed. Planting of aseptically grown plbs into the soil for plantlet regeneration would make this technique economically and practically viable which could be exploited by commercial growers.

The purpose of the present study is to produce synthetic seeds of Cymbidium giganteum, a highly ornamental orchid (Fig.1) with tremendous export potential by encapsulating the plbs in various hydrogels fortified with fungicides, bactericides, growth regulators and essential nutrients component (Fig. 2) and study conversion frequency, storage potential and physiological state. The study has been designed to explore

i) the possibility of direct delivery of 'synthetic seeds'(in vitro grown encapsulated plbs) of C. giganteum to the field, thus economising on time, space and cost of micropropagation, ii) easy transportation of large number of plbs in low bulk with minimum damage and at much reduced cost, iii) the possibility of desiccating the encapsulated plbs to produce dehydrated synthetic seed analogous to true seeds in angiosperms, iv) the production of 'clonal seeds' as orchids are heterozygous. Keeping the above objectives in mind plants with desirable genotypes could be mericloned and resulting plbs encapsulated to get 'clonal seeds'.

II. MICROPROPAGATION AND ESTABLISHMENT OF CYMBIDIUM GIGANTEUM WALL : STANDARDISATION OF MEDIA; ACCLIMATISATION AND TRANSFER TO THE GREEN HOUSE.

Introduction

Orchids constitute the largest and most advanced flowering family with floral complexities beyond belief. Their unmatched ornamental value accounts for their multimillion dollar cut flower industries in several countries. India, claiming 925 species (Jain and Mehrotra, 1984) of the world's 35,000 species (Bose and Bhattacharjee, 1980), posseses fascinating and commercially potential genotypes. However, owing to diverse human activities, excessive collection and lack of suitable steps to conserve and propagate them on a large scale, the rich orchid flora that we boast of is on the decline. Out of India's 925 species, 284 are categorised as endemic, 105 are endangered and 30 are extinct (Hegde, 1986). Cymbidium giganteum Wall., confined to North-east India, has been listed as a rare species (Arora, 1986). Since this species has great commercial value, it is desirable to evolve a suitable method to propagate this plant clonally and rapidly using modern tissue culture techniques. Micropropagation has been a commom practice for orchids ever since Morel (1960, 1964) and Wimber (1963) described the

technique of meristem culture for producing virus-free cymbidiums. Because the genotypes of many choice cultivars are highly heterozygous and the traditional method for propagating orchid by division of offshoot is slow, the meristem culture technique has been useful in mass propagating a large number of orchid species.

Since shoot meristem culture endangers the mother plant, organ culture is fast emerging as a promising avenue for micropropagation of orchids. Reports on use of leaf-tips (Churchill et al., 1971; Chaturvedi and Sharma, 1986), leaf segments (Wimber, 1965; Arditti 1977; Vij and Pathak, 1990; Abdul Karim and Hairani, 1990), axillary buds (Wang, 1988), immature inflorescence (Intuwong and Sagawa, 1973) and basal buds of inflorescence (Singh and Sagawa, 1972; Arditti et al., 1973, Intuwong et al., 1972) and root-tips (Tanaka et al., 1976; Kerbauy, 1984 a,b; Sanchez,1988; Yoneda and Momose, 1988; Holters and Zimmer, 1990) and flower stalk (Intuwong et al., 1972) have appeared.

Tissues differ in their nutritional requirements. For orchid culture various media like basal Knudson (KC, 1946), Vacin and Went (VW, 1949), Murashige and Skoog (MS, 1962), White (W, 1963) have been used (Stoutamire, 1964; Arditti et al., 1973; Fonnesbech, 1972 a,b; Bose and Mukherjee, 1976; Nishimura, 1986; Wang, 1988; Kumaria and Tandon, 1991). Kumaria and Tandon (1991) studied the effect of various media on seed germination and plb development of Dendrobium fimbriatum and found Nitsch medium to be the best for seed germination and MS for seedling growth. Yoneda et al. (1983) observed that VW medium favoured maximum plb- proliferation in Phalaenopsis sp. Choi et al. (1990) found the hyponex medium best for seedlings growth of Cymbidium kanran, C. sinensis, C. faberi and C. ensifolium.

Nagashima (1982) found hyponex medium better than MS for seed germination and plb growth of Cymbidium goeringii and Paphiopedilum insigne.

Growth regulating substances have been widely used for enhancing plb proliferation and seedling growth of orchids. The stimulatory effect of IAA and NAA on plb proliferation, shoot formation and seedling growth of cymbidiums have been emphasized by several workers (Ueda and Torikata, 1969; Fonnesbech, 1972a). Choi et al. (1990) found combination of NAA and BAP to be the best for shoot formation from rhizome and seedling growth for several varieties and F1 hybrids of Cymbidiums. Fonnesbech (1972a,b) reported the inhibitory effect of 2,4-D on plb formation of Cymbidiums, whereas, Bose and Mukherjee (1976) noticed its beneficial effect on plbs of Cymbidium giganteum. GA_3 favoured elongation of leaves, shoot apical meristems, axillary buds and plbs in Cymbidiums (Fonnesbech, 1972a,b; Bose and Mukherjee, 1976; Kusumoto, 1978). Kinetin has been reported to be stimulatory for germination and seedling growth of cymbidiums (Fonnesbech, 1972). In some species, the combined effect of 2,4-D, Kn and BAP favoured plb formation (Kusumoto, 1978). Kusumoto (1980) studied the interform variation in the proliferation and organogenesis of 47 forms of Cymbidium plbs. He found that the effect of growth regulatory substances were different in case of solid and liquid media.

Acclimatisation of in vitro grown plants is necessary because in vitro plants are not adapted for in vivo conditions. The waxy cuticle and stomata on leaves of in vitro grown plants are inadequate or ineffective (Grout and Aston, 1977a; Sutter and Langhans, 1979, 1982; Fuchigami et al., 1981; Wetzstein and Sommer, 1982). A slow and progressive weaning (hardening) is

required for the transfer of *in vitro* plants from the laboratory to the field to acclimatise them to the outside conditions. A gradual reduction in the concentration of nutrients and humidity is essential. Interpolation of liquid culture between *in vitro* culture and *in vivo* pot culture helps to improve rooting system which in turn boosts shoot growth (Chaturvedi and Sinha,1990). Roberts *et al.* (1987) and Conkie (1988) have found the transplanting easier for plantlets grown in sorbarod system.

In case of orchids the correct method of potting is essential for rapid growth and development of the plantlets (Kang, 1979). *Cymbidium*, a renowned epiphytic orchid genus, require special attention during cultivation, especially in providing a substratum similar to its natural habitat. Among the most commonly used materials for potting orchid seedlings are fir and red wood bark, pieces of cork bark, tree fern, osmunda fern root, sphagnum moss, gravel and coconut fibre (Fitsch, 1981). Coconut husk is recommended by orchid growers in Kerala (Abraham and Vatsala, 1981). Many workers have tried different materials and their mixtures for epiphytic orchids (Bose and Bhattacharjee, 1980; Verdonck, 1984; Bhattarcharjee, 1985; Pessosa and Pessosa, 1985). Cymbidiums are voracious feeders hence compost with plenty of organic matter is of great necessity (Pradhan, 1976). Cymbidiums in nature are epiphytic, but, they being sturdy species, could be grown equally well in pots also. Leaf mould, cow-dung diluted in water, chemical fertilizers like Hoagland solutions or MS liquid nutrients diluted ten times could be used for manuring orchids. Orchids like a spray of water unlike other plants which are watered by pouring water.

Fig.1. _Cymbidium giganteum_ Wall. in bloom.

Fig.2. A closer view of the flowers.

Fig. 1

Fig. 2

Materials and Methods

Cymbidium giganteum Wall.,(Fig.1&2) a rare species, confined to North-East India (mainly in Jaintia and Khasi hills) is the experimental material. It is an epiphytic plant but can adapt well on terrestrial condition also. It has short pseudobulbous stem with linear -lanceolate to lanceolate leaves and with sub-erect to pendulous inflorescence.

Explants

The young off-shoots and new roots of mature Cymbidium giganteum Wall. plants growing in the university botanic garden were collected in the month of May-June . Young shoots (5-6 cm), roots (2-3 cm), rhizome pieces (2-3 cm), leaf tips and leaf bases (1-2 cm) were excised and washed with teepol, a common liquid detergent, (0.05% v/v), scrubbed with a very soft tooth brush and left under running tap water for 1 hr. The young shoots were stripped off outer leaves and the explants treated with 70% ethanol for 30 sec and in 15% sodium hypochlorite solution (0.2-0.3% available chlorine) for 5 min and rinsed repeatedly with sterile distilled water. Shoot tips were then stripped off the leaves and the meristems (1-2 mm) excised under aseptic condition and kept aside. The leaf tips (1 mm), leaf bases(1-2 mm), rhizome (1-2 mm) and root tips of different sizes ranging from 1-4 mm were excised from these explants and 5 explants/flask were placed in MS basal medium (semisolid and liquid) and in MS medium with various hormonal supplementation of IAA + Kn and IAA + BAP. The cultures were incubated at 25 ± 2°C under a photoperiod of 14-hr duration using cool-white fluorescent light of 2000 lux. Three replicates were taken for each explant and the experiment was repeated thrice. The liquid cultures were agitated by keeping the flasks in rotary shaker at 80 rpm. The plbs obtained from

various explants were multiplied by cutting and subculturing in MS liquid medium. For subsequent experiments these plbs derived from various explants were used.

In order to obtain uniform , healthy plbs and seedlings of C. giganteum several media and combinations of growth regulators were tried.

Media

Well developed plbs (3-4 mm in diameter) obtained from various explants and grown in MS basal medium were selected, separated into single plbs, blot dried and placed aseptically on six different nutrient (liquid) media viz. KC, VW, MS, W, Gamborg et al. (B_5, 1968) and Nitsch and Nitsch (NN,1969) contained in flasks. Fresh weight of the randomly selected plbs (5 plbs together) were taken. Five plbs were placed in each flask and agitated in a rotary shaker at 80 rpm. The cultures were incubated at the above mentioned culture room conditions. Ten replicates were taken for each medium and the experiments were repeated thrice.

After 30 days of incubation all the plbs of the flasks were taken out and the plbs were blot dried and weighed. The number of plbs formed were counted. These plbs were separated into single plb and their plb volume determined using the formula given by Stoutamire (1981) for an oblete spheroid ($4/3 \pi a^2 b$), where a and b are minor and major axes, respectively. The well developed mature plbs were also placed in the six different nutrient solid media and seedling growth recorded after 120 days of culture. Seedling growth was quantified in terms of leaf size and number and the vigor of the seedlings measured in terms of fresh weight of the seedlings.

Growth regulators

Well developed plbs (around 3-4 mm in diameter) grown in MS basal medium were placed aseptically in MS medium (semisolid and liquid, 5 plbs in each flask) containing different concentrations (0.1-5.0 mg/l) of growth regulators viz., NAA, GA_3, BAP and 2,4-D separately and in combination of NAA+BAP and IAA+BAP (0.1+0.1 mg/l, 1.0+1.0 mg/l). Fresh weight of the randomly selected plbs was taken (5 plbs together). Ten replicates were taken for each medium and the experiments were repeated thrice. The cultures were incubated at the above mentioned culture room conditions. The various parameters of the plbs and seedling growth were recorded as before.

Acclimatisation and transfer of the seedlings to soil

Around 6-8 months old plantlets (about 6-8 cm high) were used for transfer to the field. These plantlets were taken out gently from the flask with the help of a forcep and then separated from each other and shaken off gently to remove the adhering agar. These were then rinsed in sterile water to remove the last bit of adhering agar, blot dried and placed under the following conditions:-

i. Plantlets were transferred directly to pots containing various potting mixtures.

ii. Plantlets were placed on sterilised polyurethane foam half submerged in MS liquid nutrients (without sucrose) placed either in a 500 ml beaker or in a flask (the foam cut into smaller cubes or a large piece kept at the centre of the flask). The polyurethane foam was washed thoroughly with teepol and water before placing in the beaker/flask. The mouth of the beaker/flask was then covered with aluminium foil and the beaker autoclaved.

After a week of culture, the foil was removed and the plantlets allowed to grow in the culture room for a week before being transferred to pots in the green house.

iii. Plantlets were kept on a tissue paper moistened with a little MS nutrients (without sucrose) kept in a beaker (autoclaved) and the plants allowed to grow for a week under culture room conditions. The plantlets were then transferred to pots in the green house.

iv. The plbs and small plantlets were placed on polyurethane foam half submerged in MS liquid nutrients kept in a flask (all autoclaved). The mouth of the flask closed with a cotton plug and the plantlets incubated under culture room conditions. The plantlets were grown for 6-8 months and then transferred to the pots in the green house. The liquid medium in the flasks was replenished once or twice according to the need during 6-8 months of culture.

Transplantation to pots in the green house

Four different types of potting mixtures-i)soil + sand + cowdung (1:1:1) + brick pieces (bp) + charcoal (c), ii)soil + sand + cowdung (1:1:1) + bp + c + Jal shakti(1g/pot), iii) soil + sand + cowdung (1:1:1) + wood pieces + decaying leaves + bp + c, iv)soil + sand + cowdung(1:1:1) coconut husk +bp + c were used for the transfer of the plantlets. The different categories of plantlets grown in the above mentioned conditions were transferred to each type of potting mixtures in the following way - i) agar grown plants after washing directly to the potting mixture, ii) after growing on polyurethane foam for a month, iii)after growing on tissue paper for a month, and (iv) plb and plantlets grown on the foam for 6-8 months.

Five plantlets of the above categories were transferred to the four different types of potting mixtures and the pots kept in

the glass house maintained at 25°C and 60% RH. Care was taken to keep the pots in a shaded place till such time the plants established themselves. A strict watering regime was followed throughout. At no time was the soil fully clogged with water or fully dry. Watering was done once a week atleast to ensure that water does not become a limiting factor. At fortnightly intervals MS nutrients diluted 10 times were sprayed to the plants. For established plants cowdung diluted in water was also supplied at fortnightly intervals.

Results

Explants

The morphogenetic response of various explants to different hormonal supplementation in the medium is shown in Table 1. The response pattern of shoot tips to different growth regulator treatments is shown in Table 2.

Shoot tips pale green in colour became dark green and enlarged slowly in MS medium supplemented with NAA+BAP combinations of 0.5+1.0, 0.5+0.5 , 1.0+1.0 mg/l and 5.0+0.5 mg/l. The best response was in NAA+BAP, 0.5+1.0 mg/l combination (45% in semi solid and 15% in liquid media) followed by 0.5+0.5 mg/l (30% in semisolid and 25% in liquid, 1.0+1.0 mg/l (25% in semisolid and 10% in liquid and 5.0+0.5 mg/l). The enlarged shoot tips (Fig 3&5) slowly assumed a ball shaped structure resembling the plb derived from the embryo. The shoot tips took around 60-90 days to develop into spherical plbs (Fig. 3). However, in 5.0+0.5 mg/l of NAA+BAP (10% in semisolid) a friable callus was formed on the shoot explants which formed a few plbs in around 30-40 days (Fig. 4). At times these plbs showed proliferation into many plbs (Fig. 6). The shoot tips also showed response in basal medium

Table 1. Effect of growth regulators on the formation of plbs from various explants of C. giganteum.

Treatment (mg/l)	Percentage response									
	Root tips		Shoot tips		Leaf bases		Leaf tips		Rhizome	
	S	L	S	L	S	L	S	L	S	L
MS basal control	-	-	35	15	-	-	-	-	50	60
IAA+Kn										
0.1+0.1	-	-	-	-	-	-	-	-	-	-
0.5+0.1	-	-	-	-	-	-	-	-	-	-
1.0+0.1	-	-	-	-	-	-	-	-	-	-
5.0+0.1	25	10	-	-	-	-	-	-	35	50
1.0+0.5	-	-	-	-	-	-	-	-	-	-
NAA+BAP										
0.1+0.5	-	-	-	-	-	-	-	-	-	-
0.5+0.5	-	-	30	25	-	-	-	-	25	-
1.0+0.5	40	-	-	-	-	-	-	-	-	-
5.0+0.5	20	-	10	-	-	-	-	-	-	-
0.5+0.1	-	-	-	-	-	-	-	-	-	-
0.5+1.0	-	-	45	15	-	-	-	-	-	-
1.0+1.0	-	-	25	10	-	-	-	-	55	75

\- = No response.
S = Semi-solid medium.
L = Liquid medium.

Table 2. Response of shoot-tip of *C. giganteum* to various concentrations of growth regulators in the medium.

Treatment(mg/l)	Response
MS Basal	Shoot-tips (about 35% in semi-solid and 15% in liquid) turned green and proliferated into nodular mass of organised cell resembling callus which eventually formed many plbs. Rest of the explants showed etiolation and death.
NAA + BAP	
0.1 + 0.5	Many of the shoot-tips showed enlargement, but failed to form plbs.
0.5 + 0.5	Shoot-tips became green and enlarged slowly to form 3-5 plbs after around 30-60 days in semi-solid medium only.
1.0 + 0.5	The explant showed elongation (5-6mm), but no plbs formed.
5.0 + 0.5	10% of the shoot-tips formed friable callus which eventually got converted to plbs in around 30-40 days.
0.5 + 0.1	Shoot-tips showed etiolation and death.
0.5 + 1.0	Explant turned green, enlarged and assumed a ball-shaped structure resembling a plb. A period of 60-90 days was required to develop into a spherical plb.
1.0 + 1.0	25% of the shoot-tips in semi- solid and 10% in liquid media became green and enlarged slowly and bifurcated in around 30-40 days to form 4-5 plbs.

Fig.3. Shoot tip showing enlargement and conversion into plb with subsequent formation of leaf primordia in MS+NAA+BAP (0.5+1.0 mg/l)after 60 days of culture.

Fig.4. Friable callus formed on shoot tips in around 30-40 days which eventually converted into many plbs in MS+NAA+BAP (5.0+0.5mg/l).

Fig. 3

Fig. 4

Fig.5. Enlargement of shoot tip with subsequent formation of plbs at the tip in MS+NAA+BAP (0.5+ 0.5mg/l).

Fig.6. Proliferation of plbs on shoot apex cultured in MS+NAA+BAP (1.0+1.0mg/l) after 90 days of culture.

Fig.7. Shoot tip proliferating into nodular mass of cells eventually forming many plbs in MS basal medium.

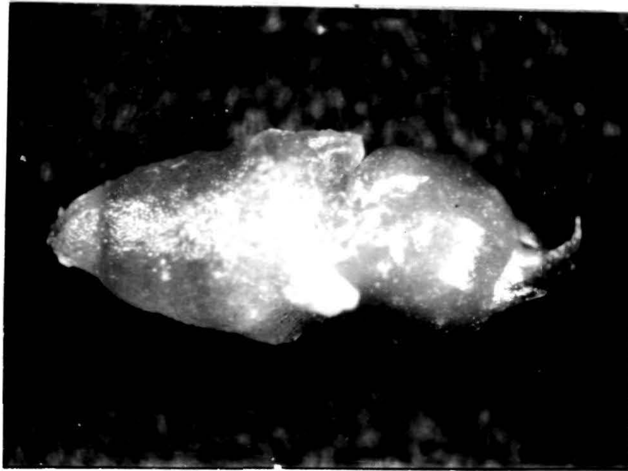

Fig. 5

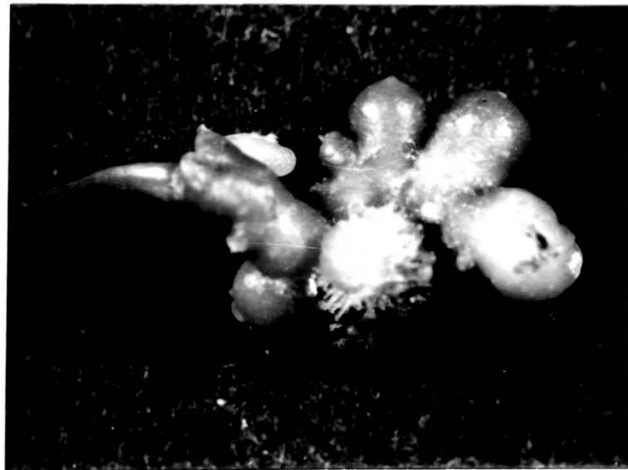

Fig. 6

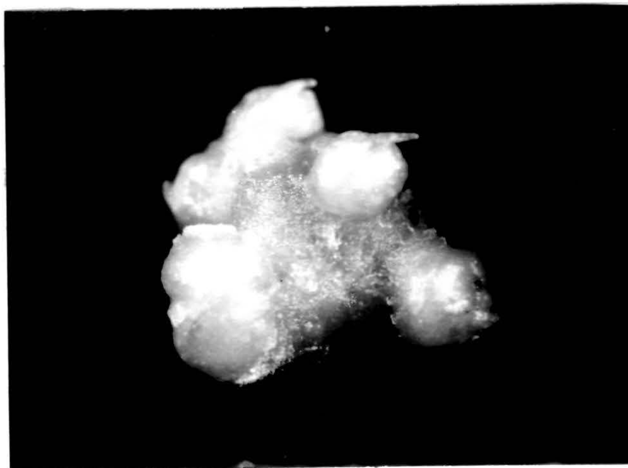

Fig. 7

without any growth regulators (about 35% in semisolid and 15% in liquid) and proliferated into a nodular mass of organised cells resembling a callus which eventually formed many plbs (Fig. 7). All these plbs when separated into a single plb and planted in fresh MS semi-solid medium supplemented with NAA+BAP (1.0 mg/l each) could grow into healthy plantlets. The proliferated plbs after growing to a certain size (2-3 mm) formed apical buds which grew into a short leaf bearing stem. The shoot tips showed more response in semi-solid than in liquid medium.

Root tips exhibited varying responses depending on the size of the roots taken and also on the growth regulators used in the medium (Tables 1&3). The root tips of around 3 mm cultured on both semi-solid and liquid media supplemented with both NAA and BAP in combination (1.0 mg/l each) showed slight swelling at the tip but it remained at this stage without any differentiation. Around 30% of the root tips of this size showed this type of response, the rest of the root tips showing gradual yellowing and browning and ultimate death. However, the root tips of 2 mm size became dark green in colour and showed further elongation into thinner dark green tips that penetrated the medium and remained at this stage but no shoot bud or plb differentiation occurred and the tips remained at this stage for an unlimited period. 40% of the root tips of around 1-2 mm size when given a slight incision at the tip showed slight brownish callussing with a few plbs at the periphery. This response occurred only in semi-solid medium containing 1.0+0.5 mg/l of NAA+BAP. 20% of the root tips of around 1 mm size showed slight swelling at the tip and green globular plbs (2-4) were formed on the tips in around 3 months of culture in the medium containing NAA+BAP (5.0+0.5 mg/l). In IAA+Kn (5.0+0.1 mg/l) containing

Table 3. Response of root-tips of *C.giganteum* to various concentrations of growth regulators in the medium.

Treatment (mg/l)	Response
MS Basal (semi-solid)	No response. Root-tips initially light green in colour turned yellow and brown and ultimately dried up.
IAA + Kn	
0.1 + 0.1	No response. 20% root-tips showed slight greening and enlargement but failed to differentiate.
0.5 + 0.1	No response.
1.0 + 0.1	No response.
5.0 + 0.1	About 25% of the root-tips (1-2mm size) in semi-medium and 10% in liquid medium showed enlargement at the tip to form a round globular structure which finally differentiated into 3-4 plbs after 30-40 days.
1.0 + 0.5	No response.
NAA + BAP	
0.1 + 0.5	No response.
0.5 + 0.5	No response.
1.0 + 0.5	40% of the root-tips of about 1-2mm size in semi-solid medium showed light brown callusing at the tip which formed 3-4 plbs at the periphery.
5.0 + 0.5	20% of the root-tips of 1mm size showed enlargement at the tip and green globular plbs (2-4) were formed in around 90 days of culture.
0.5 + 0.1	No response.
0.5 + 1.0	No response.
1.0 + 1.0	30% of root-tips of 3 mm size in semi- solid, and, 20% in liquid media showed slight swelling at the tip, but failed to differentiate. Rest of the root-tips of 3mm size showed loss of green pigmentation, yellowing, browning and ultimate death. Root-tips of size 2mm developed chlorophyll became dark green and showed elongation. It remained at this stage without differentiation.

medium the root tips of 1-2 mm size showed enlargement and finally formed 1-4 plbs (Table 3) at the tip in around 30-40 days of transfer (Fig. 8). These plbs eventually were converted into healthy shoot and root (Figs.9&10).

Various morpho-responses were exhibited by rhizome pieces too (Table 1&4). 50-60% of the rhizome in MS basal semi-solid and liquid media showed further elongation producing plbs at regular intervals (Fig.11). These plbs eventually grew into shoots with roots below. In NAA+BAP (1.0 mg/l each) the rhizome pieces branched out like a ginger producing plbs at the tips. This type of response was more in liquid (75%) than in semi-solid (55%)medium. Frequent clusters of plbs arising in the axil of the root (25%) was observed in 0.5+0.5 mg/l of NAA+BAP. At times the rhizome pieces showed light greenish callussing at the cut ends with a few plbs arising out of the callus. This response was observed in IAA+Kn combination of 5.0+0.1 mg/l (35% in semi- solid and 50% in liquid).

Unique responses. A fascinating response was observed in some flasks with NAA+BAP combination of 0.5+1.0 mg/l (Fig.12). An aerial branch resembling a miniature inflorescence with small greenish globular plbs - like structure with brownish apex (resembling an unopened bud) arising at the axil of brownish bract-like structures was seen. These structures did not develop further and showed ultimate browning up and death.

Some intact roots coming from the axil of the shoot after growing for some time produced a cluster of plbs at the tips (Figs.13&14). Some of the plbs from these clusters eventually rooted and produced shoots above.

Media

The effects of various media on plb development and

Table 4 Response of rhizomes to various concentrations of growth regulators in the medium.

Treatment (mg/l)	Response
MS basal	About 50-60% of the rhizome of 2-3 mm size in both solid and liquid media showed further elongation producing plbs at regular intervals. These plbs eventually grew into shoots with roots below.
IAA + Kn	
0.1 + 0.1	Failed to show any response. Etiolation and browning took place.
0.5 + 0.1	No response.
1.0 + 0.1	No response.
5.0 + 0.1	Rhizome showed light greenish callussing at the cut ends with few plbs arising out of the callus (35% in semisolid and 50% in liquid).
1.0 + 0.5	No response.
NAA + BAP	
0.1 + 0.5	No response.
0.5 + 0.5	Rhizome elongated producing roots and shoots at regular intervals, and about 25% of the rhizomes produced clusters of plbs at the axil of root and rhizome.
1.0 + 0.5	No response.
5.0 + 0.5	No response.
0.5 + 0.1	No response.
0.5 + 1.0	The rhizomes showed elongation and produced plbs at regular intervals. An aerial branch resembling a miniature inflorescence with small brown globular structures (like an unopened bud) in the axil of a small pointed bract-like structure was formed. This structure did not develop further but showed browning up and ultimate death.
1.0 + 1.0	The rhizome branched out like ginger producing plbs at the tips. 75% of the rhizomes showed this type of response in MS liquid and 55% in solid media.

Fig.8. Enlarged root tips showing plb formation at the tip in MS+IAA+KN (5.0+0.1mg/l).

Fig.9 & 10. Plantlet formation from plb formed at the root tips after 60-90 days of culture.

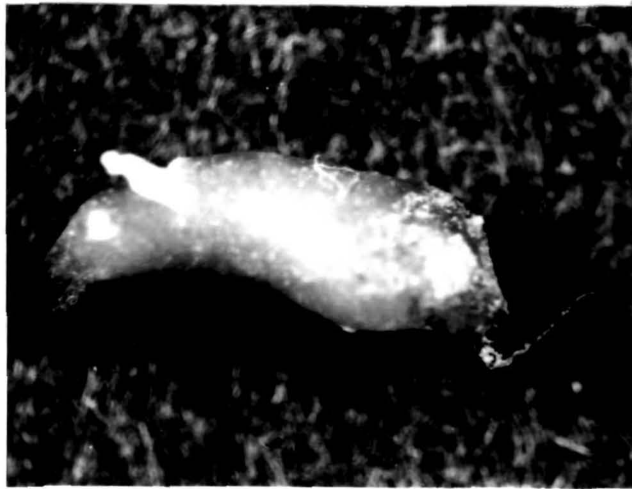

Fig. 8

Fig. 9

Fig. 10

Fig.11. Rhizome showing further growth with plbs coming out at regular intervals in MS+NAA+BAP (0.5+1mg/l) after 120 days of culture.

Fig.12. Inflorescence like structures coming from the basal of the shoot in MS+NAA+BAP (0.5+1 mg/l) after 120 days of culture.

Fig. 11

Fig.12

Fig 13. Plb formation from the intact root-tips.

Fig 14. Plantlet formation from the plb borne by the intact root.

Fig. 13

Fig. 14

proliferation and seedling growth of Cymbidium giganteum are summarized in Table 5. The composition of various media are shown in Table 6. The best plb proliferation ocurred in MS as observed in the percentage increase in plb number (84.85) followed by B_5(80.00), VW (79.16), NN (79.16), KC (76.19) and W (70.58). Percentage increase in plb volume after 30 days of culture in the respective media were 38.68 in MS, 25.39 in B_5, 24.73 in NN, 1.8 in KC and 2.09 in W. The plb vigor as shown by % increase in weight were also higher in MS followed by NN, B_5, VW, KC and W.

Seedling growth was best in MS medium as observed in the fresh weight, leaf size and overall development of the seedlings after 120 days of culture in the media. The leaves were healthy, dark green in MS and B_5 compared to the rest of the media. However, root development was better in W and NN compared to other media in terms of number and size of the roots.

The best chlorophyll development was in MS and B_5. In rest of the media the seedlings though showed good growth chlorophyll development was poor and the leaves were yellowish green in colour. The average number of leaves in KC and W was 2 compared to the rest of the media where average number of leaves was 4. Vigor as measured in terms of fresh weight was best in MS, followed by B_5, VW, NN, KC and W.

Growth regulators

The effects of various growth regulators on plb development and seedling growth of C .giganteum are summarised in Table 7.

IAA+NAA

NAA and IAA at low concentration (0.1-1.0 mg/l) were stimulatory for plb development and plb proliferation. Plb development was better in 0.5 mg/l of IAA and 1.0 mg/l of NAA added separately into medium . At these concentrations individual

Table 5. Plb development and seedling growth of *C.giganteum* in different media .

Media (basal)	Plb development after 30 days			Seedling characteristics after 120 days of culture						
	%Inc.in weight (g)	%Inc.in number	%inc.in plb volume (mm^3)	Fresh weight (g)	Leaf No.	Leaf Size (cm)	Root No.	Root Size (cm)	Colour	Development
MS	79.34	84.85	38.68	0.24	4	2.14	2	1.5	Green	Good
NN	73.83	79.16	24.73	0.20	4	1.20	3	1.8	Yellow green	Poor
B_5	77.71	80.00	25.39	0.22	4	1.20	2	1.2	Green	Good
KC	73.83	76.19	01.80	0.19	2	1.60	2	1.2	Yellow green	Good
VW	70.98	79.16	12.50	0.21	4	1.80	2	1.1	Yellow green	Good
W	66.82	70.58	02.09	0.18	2	1.50	3	1.8	Yellow green	Poor

Data for increase in weight and number recorded for 5 plbs each. Ten replicates were taken for eac medium and the experiments were repeated twice.

Table 6. Composition of media used.

Constituents	Media (amount in mg/l)					
	W	MS	B_5	NN	VW	KC
Inorganic						
NH_4NO_3	-	1650	-	720	-	-
KNO_3	80	1900	2527.5	950	525	180
$CaCl_2.2H_2O$	-	440	150	-	-	-
$CaCl_2$	-	-	-	166	-	-
$MgSO_4.7H_2O$	750	370	246.5	185	250	250
KH_2PO_4	-	170	-	68	250	150
$(NH_4)_2SO_4$	-	-	134	-	500	100
$Ca(NO_3).4H_2O$	300	-	-	-	-	200
$Ca_3(PO_4)_2$	-	-	-	-	-	-
Na_2SO_4	200	-	-	-	-	-
NaH_2PO_4,H_2O	19	-	150	-	-	-
KCl	65	-	-	-	-	-
KI	0.75	0.83	0.75	-	-	0.80
H_3BO_3	1.5	6.2	3	10	-	6.2
$MnSO_4.4H_2O$	5	22.3	-	25	-	-
$MnSO_4.H_2O$	-	-	10	-	6.8	0.075
$ZnSO_4.7H_2O$	3	8.6	2	10	-	-
$ZnCl_2$	-	-	-	-	-	3.9
$Na_2MoO_4.2H_2O$	-	0.25	0.25	0.25	-	0.25
$CuSO_4.5H_2O$	0.01	0.025	0.025	0.025	-	0.025
$CoCl_2.6H_2O$	-	0.025	0.025	-	-	-
$Fe_2(SO_4)_3$	2.5	-	-	-	-	-
$FeSO_4.7H_2O$	-	27.8	-	27.8	21	25
$Na_2EDTA.2H_2O$	-	37.3	-	37.3	157	74.6
Sequestrene 330 Fe	-	-	28	-	-	-
Organic						
Riboflavin	-	-	-	-	-	0.30
Inositol	-	100	100	100	-	-
Nicotinic acid	0.05	0.5	1	5	-	-
Pyridoxine HCl	0.01	0.5	1	0.5	-	0.30
Thiamine HCl	0.01	0.1	10	0.5	-	0.30
Glycine	3	2	-	2	-	-
Folic acid	-	-	-	0.5	-	-
Biotin	-	-	-	0.05	-	-
Sucrose	2%	3%	2%	2%	2%	2%

plbs were larger as shown by the higher percentage increase in plb volume. Plb proliferation was also favoured at 0.1 mg/l of IAA. Seedlings showed increased vigor at 0.1-1.0 mg/l of NAA and IAA (Figs.15 &18) added separately as shown by the fresh weight of the individual seedlings. The leaf and root sizesin NAA and IAA treatments were higher than control (Fig. 16) as also the root number. The roots were healthy and vigorous. This would have contributed more to their fresh weight. Leaves were dark green and the overall development was very good. However, NAA was better than IAA for seedling growth,though root development was better in the latter. IAA and NAA at higher concentrations were inhibitory.

GA_3

Plb proliferation was much lower than in NAA treatment but higher than the control. GA_3 at 1.0 mg/l produced larger dark green plbs with larger average plb volume. At higher concentration (5.0 mg/l) GA_3 inhibited plb proliferation and development. Seedling growth was also higher at 0.5 and 1.0 mg/l of GA_3. Leaf size was higher at 1.0 mg/l of GA_3than control (Fig.17). However, higher concentration (5.0 mg/l) was slightly inhibitory for seedling growth especially for the root .

2,4-D

2,4-D at 0.5 mg/l and 1.0 mg/l concentrations was slightly stimulatory for plb proliferation and development. The average size of the plbs was larger as shown by the percentage increase in average plb volume.

Seedling growth was slightly stimulated at 0.5 and 1.0 mg/l of 2,4-D but the root size was much less as compared to control (Fig. 19).

Table 7. Plb development and seedling growth of <u>C. giganteum</u> in MS medium supplemented with various growth regulators.

Treatment	Conc. mg/l	Plb development after 30 days: % weight increase	% increase in number	% increase in plb vol.	Seedling characteristics after 120 days of culture: Fresh wt. (g)	Leaf No.	Leaf Size (cm)	Root No.	Root Size (cm)	Colour	Development
Control		79.34	84.85	38.68	0.24	4	2.1	2	1.5	Green	Good
NAA	0.1	87.53	89.48	82.00	0.38	4	3.4	3	2.2	D.green	Good
	0.5	87.69	90.00	82.97	0.42	4	3.7	3	2.2	,,	Good
	1.0	88.01	91.66	83.33	0.48	4	3.8	3	2.3	,,	V.good
	5.0	82.22	83.25	42.85	0.26	4	2.8	3	2.1	Green	V.good
IAA	0.1	85.04	91.52	72.41	0.38	4	3.1	2	2.8	Green	Good
	0.5	86.06	90.74	84.31	0.35	4	2.8	2	2.9	,,	,,
	1.0	82.12	90.00	78.37	0.35	4	2.7	2	2.9	,,	,,
	5.0	81.71	83.87	38.46	0.22	4	2.1	2	2.8	,,	,,
GA_3	0.1	84.18	86.11	84.90	0.28	4	2.9	2	1.8	,,	,,
	0.5	83.50	86.11	87.52	0.33	4	3.1	2	1.9	,,	,,
	1.0	82.31	87.80	87.87	0.35	4	3.3	2	2.0	,,	,,
	5.0	80.36	83.33	74.19	0.26	3	2.3	2	1.3	,,	,,
BAP	0.1	85.77	89.48	78.94	0.28	3	1.9	2	1.9	,,	,,
	0.5	86.99	90.00	82.60	0.29	3	1.8	2	1.8	,,	,,
	1.0	87.04	90.56	84.00	0.36	3	1.8	2	1.8	,,	,,
	5.0	85.60	87.50	82.22	0.31	3	1.4	2	1.4	,,	,,
2,4-D	0.1	85.77	84.15	84.90	0.26	3	1.9	2	1.4	,,	,,
	0.5	85.77	85.71	86.20	0.28	3	2.1	2	1.3	,,	,,
	1.0	83.15	82.14	89.04	0.31	3	2.4	2	1.3	,,	,,
	5.0	81.60	78.26	78.94	0.23	3	1.9	2	1.2	,,	,,
NAA+BAP	0.1+0.1	87.69	90.90	85.71	0.44	4	3.8	3	2.3	D.green	V.good
	1.0+1.0	88.14	92.06	89.18	0.49	4	3.9	3	2.9	D.green	V.good.
IAA+KN	0.1+0.1	86.14	91.80	87.87	0.39	4	3.1	3	2.9	Green	V.good
	1.0+1.0	86.08	87.50	87.69	0.40	4	3.4	3	2.9	Green	V.good

Fig.15-18.Two year old *C.gigganteum* plantlets.

15,in MS+NAA (1 mg/l)
16,in MS basal
17,in MS+Ga_3(0.5mg/l)
18,in MS+IAA (1mg/l)

Fig. 15

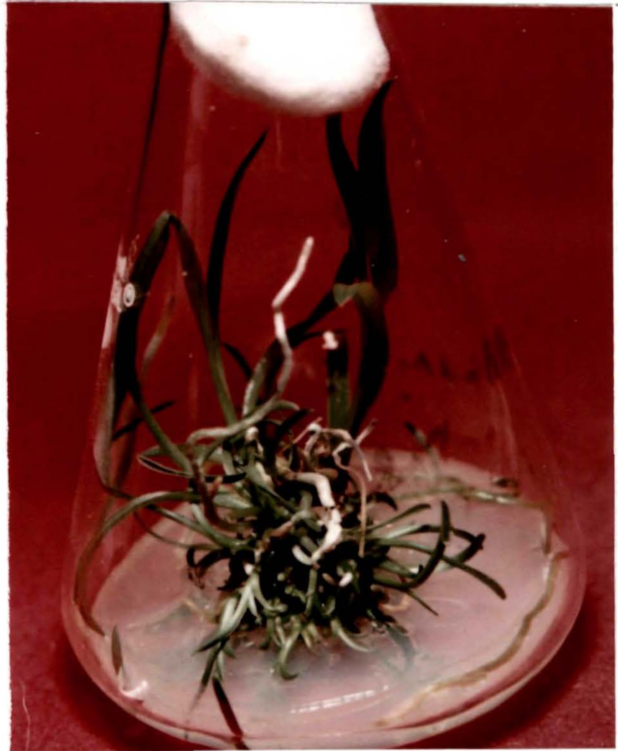

Fig. 16

Fig. 17

Fig. 18

BAP

All the concentrations of BAP especially 1.0 mg/l stimulated profuse plb proliferationn and higher plb volume. A large number of healthy plantlets were formed . Seedlings had higher fresh weight but possesed smaller sized leaves (Fig.20).

NAA+BAP

NAA in conjunction with BAP (0.1+0.1 and 1.0+1.0 mg/l) was better than NAA alone for plb development and seedling growth. The combination of 1.0+1.0 mg/l of NAA+BAP exhibited the best response results (Fig.21). Seedling growth was much higher than any of the hormones at this concentration.

IAA+Kn

Plb development and seedling growth was stimulated at both 0.1+0.1 (Fig.22) and 1.0+1.0 mg/l of IAA+Kn. However, plb volume and seedling fresh weight was higher than IAA alone.

Acclimatisation and transfer to the pots

Table 8. shows the growth performance of the transplanted plantlets after 3 months of transfer to the potting mixtures. Since the 6-8 months old plantlets were big enough and had well developed roots, all the plantlets survived with proper care. Therefore, there was 100% survival of plants. The plantlets showed difference in the growth rates, vigour and extent of wilting.

The transfer of plantlets directly to the potting mixture resulted in wilting of the plants for a few days. But, this wilting was not fatal, and all the plants could establish themselves in the potting mixture. Potting mixture with Jal-shakti could retain moisture in the soil for a longer period of time. The frequency of watering was therefore reduced. The wilting tendency of the plants was overcome to some extent only by watering the plants regularly. The plantlets grown on

Fig.19-22. Two year old *C.Giganteum* *plantlets* .
19, in MS+2,4-D (0.5mg/l)
20, in MS+BAP (1mg/l)
21, in MS+NAA+BAP (1+1mg/l)
22, in MS+IAA+Kn (0.1+0.1mg/l).

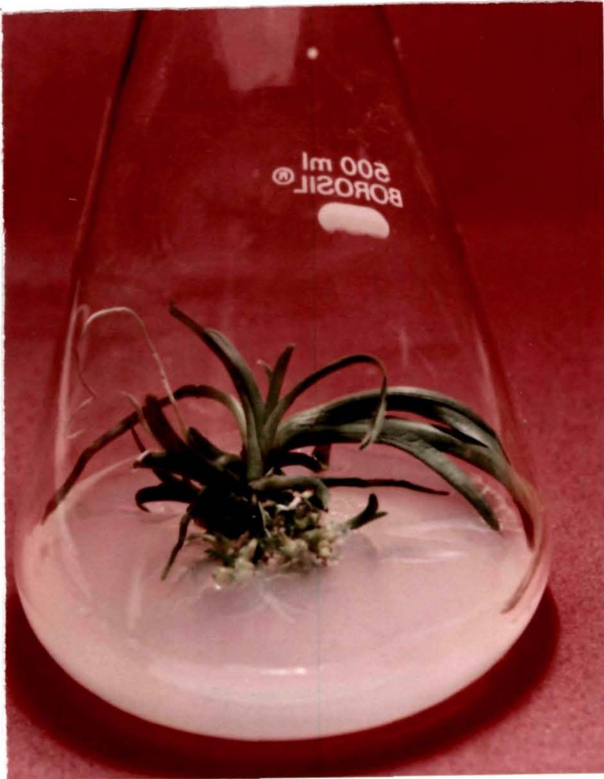

Fig. 19

Fig. 20

Fig. 21

Fig. 22

Table 8. Growth and height of the plantlets after three months of transplantation in various substrata.

Source of Plantlets	Potting media			
	I	II	III	IV
Grown on agar	+ + (10-12 cm)	+ + (10-12 cm)	+ + (12-13 cm)	+ + (10-12cm)
Grown on polyurethane foam for 1 month	+ + + (15-16cm)	+ + + (15-16 cm)	+ + + (16-18cm)	+ + + (15-16cm)
Grown on tissue paper	+ + (13 -14cm)	+ + (12- 14cm)	+ + (14- 15cm)	+ + (14- 15 cm)
Grown on polyeurethane foam for 6-8 months	+ + + (15-16cm)	+ + + (15-16cm)	+ + + (10-18cm)	+ + + (14-16cm)

I. soil +sand + cow-dung (1:1:1)+ brick pieces + charcoal

II. soil+ sand + cow-dung (1:1:1)+ jal shakti(1g)+ brick pieces+charcoal

III. soil+ sand + cow-dung + wood + bark pieces + decaying leaves + brick pieces+ charcoal

IV. soil+ sand + coconut husk + coconut shell+ brick pieces + charcoal

+ Poor growth
++ Good growth
+++ Very good growth

polyurethane foam for a month had better growth and showed less wilting (Fig.24). The growing of plants on the foam initiated the development of a large number of roots and the plants established readily in pots (Fig 25), and possesed healthy, larger leaves. When the plantlets grown on foam for 6-8 months (Fig.23) were transferred to potting-mixture it showed better growth and established readily.

Though all the potting mixtures were good, the one with decayed leaves, wood pieces and bark seemed best. The plants in this potting mixture reached a height of 16-18 cm after three months of transfer (Fig.25). Even plantlets initially grown on tissue-paper and agar showed better growth when transferred to this potting mixture (Fig.27). The use of plenty of cowdung in the potting media was highly beneficial for seedling growth (Fig.26). The use of foam,coconut husk and shell in potting mixture was also found beneficial in retaining moisture of the soil.

Discussion

Explants

Though several reports of meristem culture of many species of cymbidiums have appeared (Morel ,1960 ,1964; Wimber, 1965; Ueda and Torikata, 1979; Kusumoto,1980; Wang, 1988) the detailed work on C. giganteum has not been undertaken.

The shoot tips showed inconsistency in their morphogenetic responses to growth regulators requirements. The shoot tips showed morphogenetic response in MS basal medium and in MS medium supplemented with either equal amounts of auxins and cytokinins or higher concentration of auxin to cytokinin. However at one instance higher concentration of cytokinin was required

Fig.23-24. Acclimation of *in vitro* raised plantlets.
23, plantlets grown on polyurethane foam for 6-8 month.
24, plantlets grown on polyurethane foam for 1 months.

Fig. 23

Fig. 24

Fig. 25-27. Establishment of *in vitro* raised plantlets on pots.

25,plantlets grown on foam transferred to pots with soil+ sand+ cowdung+brick pieces+charcoal + wood pieces+pieces+decaying leaves.

26,plantlets grown on foam transferred to pots with soil+sand+plenty of cowdung+ brick pieces+charcoal.

27,plantlets grown on tissue paper transferred to pots with sand+ soil+ cowdung+ brick pieces charcoal+ wood pieces+decaying leaves.

Fig. 25

Fig. 26

Fig. 27

for plb formation. This difference in response probably indicates the various physiological states of each shoot tips (and hence of the donor plant) each with their own endogenous levels of growth regulators. The response therefore does not entirely depend on the exogenous supply only.The explants responded more in semi-solid than in liquid medium, though both contained the same amount of growth regulators. Kusumoto (1980) also observed that the effects of growth regulating substances were different between liquid and semi-solid media when the same amount of medium was added.

Morel (1960, 1964) and Wimber (1963) have reported plb formation in basal medium without the use of growth regulators. In the present study also the shoot tips could be induced to form plbs in basal medium only. The different growth regulators had either inhibiting effect or slight promotory effect depending upon the growth regulator used and their concentrations.

Mitra (1971) working on _Arundina bambusifolia_ Lindl. found better response with longer shoot tips (6-8mm) than shorter (1-2 mm) ones. Plb formation is not restricted to the apical dome of the meristem alone (Morel,1960, 1964; Wimber,1963). Sections away from the apex also develop into plbs. But in the present experiment shorter apices of 1-2 mm were found to be the best. Apices larger than this failed to respond. A delicate interplay of growth regulators was observed for plb initiation from the shoot apices. A slight variation resulted in either inhibition or promotion of plb formation.

Root tips showed diverse responses according to the size of the roots taken and also to the growth regulators used in the media. In the present study the root tips of 1-2 mm size were best for plb formation. Longer roots (2-4 mm) showed swelling and elongation but did not produce plbs. This observation is not in

conformity with Holters and Zimmer (1990) who found that in many species of orchids the formation of plbs depended on the length of the root-the longer isolated roots producing more plbs.

Root tips of 1-2 mm responded to higher concentration of auxin in combination with very low concentration of cytokinin. This is not in agreement with Sanchez (1988) who found that NAA and BAP stimulated plb-formation only if present in low concentration and in a ratio of 1:1. When IAA was more, proliferation of apical callus and differentiation of fascicle root tips were observed. On a medium with lower concentration of these hormones, normal roots were differentiated. In the present study at lower concentration of NAA+BAP (when NAA was more than BAP), root-tips of 1-2 mm size in semi-solid media, showed light brown callusing at the tip and formed a few plbs at the periphery. At higher concentration of NAA+BAP (when NAA was more than BAP), root-tips showed enlargement at the tip and formed green globular plbs.

Many of the root-tips showed enlargement, greening and elongation but were incapable of differentiation. This probably indicates some specific requirements for differentiation which is dependent not only on exogenous supply, but perhaps on the endogenous level also.

Chaturvedi and Sharma (1986) observed greening and enlargement of the root-tips of Rynchostylis retusa before plb-differentiation at the cut ends. A similar response was observed in C. giganteum, when root-tips of around 1mm size were cultured. However, the plbs were formed at the tips and not at the cut ends. Brown callusing was observed when the root-tips were given a slight incision at the tip.

Some roots showed remarkable extension of their tips.

Yoneda and Momose (1987) observed a similar response in Phalaenopsis root-tip culture; the extended roots showing plb-formation below the root cap. However, no plbs were formed in the extended roots in the present study.

Rhizomes, however, required varied concentration of growth regulators for plb and shoot formation. Higher concentration of IAA to Kn, or, NAA to BAP, or, equal concentration of NAA+BAP was required. This observation is not in conformity with the observation of Ueda and Torikata (1969) who found that rhizome tips of Cymbidium goeringii formed shoots only after addition of 10.0 mg/l of Kn. The requirement of BAP and Kn in the formation of plbs from root-tips and rhizome has been emphasized by Holters and Zimmer (1990). Rhizome pieces of about 2-3 mm size were found to be the best for further growth and plb-formation.

One of the most interesting findings in the present study was the observation of inflorescence-like structures coming from the axil of the shoot. These branches were always upright and hence did not look like abnormal development of roots. Another interesting finding was the formation of plbs in the intact root-tips.

The plbs obtained from various explants when cut and sub-cultured in liquid medium proliferated into numerous plbs with subsequent regeneration into plantlets. Since vegetative propagation is slow, labour-intensive, and time-consuming, this method of micropropagation could be utilised for large-scale propagation of this beautiful ornamental plant. Further, researchers on orchid propagation have emphasized use of root tips, leaf-bases, and inflorescence buds as explants for plb-induction. This method reduces the damage to the mother plant which could sometimes have to be sacrificed as in case of shoot tips of cymbidiums.

Media

The compostion of the various media differ from each other mainly in the quantity of various salts and ions (Table 6). This may account for the differences in growth behaviour of the plbs and seedlings of C. giganteum .

Ammonium nitrate is the best source of nitrogen (Burgeff, 1932). Withner (1951) emphasized the incorporation of both NH_4^+ and NO_3^- ions in the medium for better results. The requirements of the two ions in the medium have been observed in Cattleya cabiata (Raghavan and Torrey, 1964), Bleittia striata (Ichihasi and Yamashita, 1977), Vanda species (Mathews and Rao,1980). Ammonium nitrate present in the two media (MS and NN) is considered to be the most suitable source of nitrogen for orchid seedlings (Arditti and Ernst,1984). VW and KC media contain ammonium sulphate and calcium nitrate which did not support good germination and development of plbs of Dendrobium fimbriatum var oculatum HK.f (Kumaria and Tandon, 1991).

The N_2 content it is very high in MS (60.03) as compared to B_5 (27.03), and NN (27.40), whereas it is very low in W (3.33) medium. Calcium ions are more in MS and NN than in others. W medium, one of the earliest plant tissue culture medium, includes all the necessary nutrients, however, qualitively the inorganic nutrients are inadequate for good seedling growth (Murashige and Skoog, 1962). Besides nutrients the nutritional requirements of the seedlings also greatly depend on the physiological condition of the plbs (Yam and Weatherhead, 1988).

The better growth of plbs and seedlings in the present experiment can be explained by the very high N_2 content of MS medium in the form of ammonium nitrate which is considered to be the best for plb growth and development (Burgeff, 1932). VW and

KC media besides containing nitrogen source in the form of ammonium sulphate and calcium nitrate do not contain sufficient amounts of nutrients and vitamins .

As regards vitamins and amino-acids MS, NN and B_5 contain inositol which is absent in W, VW, and KC media. Thiamine-HCl has been proved to be an essential ingredient present in all the media except in VW.

Healthy growth of orchid plbs in medium containing balanced supply of organic and inorganic nutrients has been reported (Arditti and Ernst, 1984). The present study reveals that the plbs of C. giganteum require a rich medium containing higher concentration of nutrients and vitamins for both plb development and seedling growth.

Growth regulators

Growth substances are being widely used in agriculture and horticulture to enhance seedling growth - orchids are no exception. Seed germination, seedling growth are all influenced by the type and amount of growth substances used. Out of the various growth regulators / combinations of growth regulators used in the present experiment, NAA+BAP combination of 1.0 +1.0 mg/l was found to be the best for proliferation and development of plbs and growth of seedlings. NAA at 0.1-1.0mg/l was also found to be highly stimulatory for overall development of C. giganteum plbs. Other hormones used had, but very slight stimulatory influence. Higher concentrations of all growth regulators were either inhibitory or had no effect and results were comparable to the control. Fonnesbech (1972a,b) reported that IAA had no effect alone but NAA resulted in optimum fresh weight at 10.0 mg/l and the plbs of Cymbidium were vigorous but lighter green than usual. He found that higher concentrations of NAA inhibited chlorophyll synthesis. Better plb proliferation

was obtained in interspecific Vanda hybrids, when NAA was used along with other additives (Mathews and Rao,1980). NAA stimulated seedlings growth in Cymbidium madidum (Strauss and Raisinger, 1976). It has been found to be stimulatory for germination and seedling growth of cymbidiums (Fonnesbech, 1972a,b). Kusomoto (1978) observed that root development was effectively promoted by adding NAA to the basal medium, producing occasional aberrant roots at higher NAA concentrations. Shoot formation was favoured at lower concentration of 0.1mg/l of NAA. In the present experiment, plb proliferation was favoured at 0.1-1.0 mg/l NAA and IAA added separately in the medium. Leaves were dark green, healthy and sizes of leaf and root were bigger. Like Fonnesbech's (1972a,b) observation C. giganteum plbs and seedlings showed enhanced chlorophyll formation at lower concentration of NAA (0.1-1.0 mg/l). The root formation was also favoured at this concentration of NAA. IAA was only slightly stimulatory for plb development and seedling growth compared to NAA. Higher concentrations of IAA and NAA were not suitable for seedling growth as compared to lower concentration. Kinetin has been found to stimulate germination and seedling growth of cymbidiums (Fonnesbech, 1972a,b). Further, BAP at lower concentrations promoted callus formation and increased fresh weight of plbs of cymbidiums. Ueda and Torikata (1969) found that addition of 10.0 mg/l of Kn led most samples of rhizome tips to form shoots in C. goeringii.. In the present experiment, BAP stimulated profuse plb proliferation at all concentrations, more so at 1.0 mg/l. Seedlings had higher fresh weight but showed stunted growth with smaller leaves and shorter roots.

The interacting effects of cytokinins and auxins on shoot/ root balance in orchids is well documented (Kusumoto, 1978; Ueda

and Torikata, 1968, 1969, 1979; Fonnesbech, 1972a,b). Fonnesbech (1972) reported that combination of Kn and NAA resulted in maximum fresh weight of cymbidium plbs. Prasad and Mitra (1975) noted a high percentage of germination and good plb growth of C. mastersii in the treatment of IAA and Kn. Ueda and Torikata (1969) found low concentration of NAA in combination with 10 mg/l Kn to promote shoot formation in the rhizome tips of C. goeringii. Higher concentrations of NAA tend to counteract the stimulatory effect of Kn on the shoot formation. Choi et al. (1990) observed that the growth of C. kanran was most favourable in medium containing 1.0 mg/l of NAA and 3.0 mg/l of BAP and that of C. goeringii with 1.0 mg/l each of NAA+BAP. On the other hand, seedlings of C. sinensis, C. faberi and C. ensifolium were well grown in the medium containing 2.0 mg/l of NAA and 1.0 mg/l of BAP in combination. Multiplication of rhizome of these species was highly stimulated in the medium containing 2.0 mg/l of NAA and 0.2 mg/l of Kn. Shooting from rhizome and growth of seedlings of the six varieties of cymbidiums and seven F1 hybrids were good in medium with 2 mg/l NAA and 1 mg/l BAP.

In the present experiment, combination of NAA+BAP at 1 mg/l each was found to be highly stimulatory for plb formation and development, and seedling growth. Seedlings were vigorous, dark green and possesed a rich healthy look. NAA used singly also had stimulatory effect but the interaction of these two hormones when used together had the most beneficial effect, followed by lower concentration of IAA+BAP (0.1+0.1 mg/l).

GA_3 promoted shoot and leaf growth of Cymbidium plbs (Fonnesbech, 1972a,b). Bose and Mukherjee (1976) found that GA_3 at 1.0 or 2.0 mg/l induced formation of a number of plantlets in C. mastersii. Plant height was also enhanced by GA_3 . Shoot formation was improved by addition of GA_3 in the medium

containing NAA+BAP (Ueda and Torikata, 1969). In the present experiment GA_3 favoured the development of the plbs as shown by *longer, dark green plbs with larger average plb volume.* The effect of 2,4-D on plb-formation and development was variable in different orchid species. Fonnesbech (1972a) reported the inhibitory effect of 2,4-D on plb formation of cymbidiums, whereas Bose and Mukherjee (1976) noticed its beneficial effect on plbs of C. giganteum. In the latter case addition of 2,4-D to the medium containing NAA+BAP stimulated shoot formation from rhizome. In the present experiment, 2,4-D at 0.1 mg/l was stimulatory for plb proliferation and development, and seedling growth. Plb volume was much larger at 0.1 mg/l of 2,4-D as compared to other hormones. However, the growth of the roots were inhibited by 2,4-D. Growth hormones have pronounced effect on germination, plb formation, proliferation and development and seedling growth - each species showing differential response to the nature and amount of various hormones. Knowledge about the exact hormonal requirements of each species is useful for large scale multiplication of the species.

The transfer of agar grown plants directly to the soil was accompanied by wilting of the plants for a few days which could not be completely overcome by watering. The wilting may be due to the excessive water loss from the leaves of these plants exceeding absorption by the roots. Grout and Aston (1977a), Fuchigami et al.(1981), Brainerd and Fuchigami (1981, 1982) have indicated that waxy cuticle and stomata of leaves of in vitro grown plants are inadequate or inoperative. Hence such leaves are not able to reduce water loss in the in vivo environment. The photosynthetic ability of the in vitro plants are also reduced since they are grown in the high sucrose and salt medium (Grout

and Aston, 1977a; Wezstein and Somner, 1982). For this reason, the _in vitro_ plants have to be slowly acclimatised to cope up with the _in vivo_ condition by a series of steps involving a gradual reduction in the concentration of sucrose, nutrient salt and relative humidity in the culture environment. These steps could reduce the transplantation shock, prevent wilting mortality of the _in vitro_ grown plantlets. The transfer of plantlets to foam and tissue paper in a beaker containg autoclaved 1/2 MS nutrients under the culture room conditions was tried with a view to reduce transplantation shock and it was successful to some extent as such plants showed less wilting compared to the plants transferred directly to the pots.

The use of cheaply and easily available foam proved to be an ideal substratum to grow plantlets in the culture room before transfer because a large number of roots were formed when the plantlets were grown on foam. The initiation of large number of roots could be due to the non-availability of the nutrients directly to plantlets as in agar and hence there was the need to produce more roots. The large number of young actively growing roots might have been the reason for the better establishment of the plants in the potting mixture. The high vigour of such plants in the potting mixture could be attributed to the faster absorption and utilization of nutrients by the numerous roots that were present on the foam grown plantlets. The large number of roots that formed on the foam grown plantlets did not penetrate the sponge instead gradually surrounded the foam or just remained on the surface, remained active and absorbed nutrients from the surface.

The use of foam in tissue culture for culturing plantlets could be numerous. They are readily available, non-toxic and can be autoclaved and reused. Foam can conveniently replace the use

of filter paper bridges and sorbarod system as used by Short et al.(1987) and Conkie (1988) for growing cultures in liquid medium which is expensive and can not be reused. The transfer of plantlets grown on foam was easy compared to agar grown plantlets with minimum damage to the roots and washing of the roots avoided. The growth of the plantlets after transfer to the soil was rapid and needed no acclimatisation steps. Further, foam proved to be an inexpensive substratum for growing in vitro plantlets and as a replacement for filter paper bridges, shaker and agar. The culture of orchid plbs and plantlets on foam placed in liquid culture medium produced higher rates of growth than agar solidified medium. The plantlets could be easily transferred undisturbed to the fresh liquid medium or the medium could be easily replenished or the composition of the medium changed (if necessary) without having to set up new cultures. Further, polyurethane foam can be reused economising on tissue culture methods. Coconut husks were very good in retaining moisture and frequency of watering was considerably reduced. Small pieces of foam in the potting media was found better than coconut husks in retaining moisture as excessive moisture retention could possibly lead to root decay and root diseases which could be avoided using foam as subsitute for water retention. Using water absorbing polymer was another better way of retaining moisture in the soil. The correct method of potting is essential for rapid growth and development of orchids as emphasised by Kang (1979). The direct transfer of plantlets to community pots resulted in slight retardation in growth of the plants. The plants of 6-8 cm height grew upto 10-13 cm only whereas plantlets grown on foam for a month and 6-8 months showed high vigor and reached upto 18 cm height in potting media

containing cowdung + wood+ bark+ decaying leaves and a slightly lower height (15-16 cm) in other potting mixtures. So growing plantlets on foam was an ideal intermediate step for the establishment of plants in the soil. The proper drainage of water is a must and hence important measures were taken to ensure proper drainage of water. The earthern pots had holes at the bottom and the pots had charcoal and broken pot pieces filled to 2/3 of the pots. This also ensured proper aeration so that roots do not get suffocated. Pieces of charcoal and moss were used as top dressing to prevent loss of moisture and protect the young seedlings from direct exposure to sunlight. Cymbidiums are voracious feeders and hence a compost with plenty of organic matter is of great necessity (Pradhan, 1976). Hence to ensure regular supply of nutrients MS salts diluted ten times were given to the plants just after transplantation and then cowdung diluted in water later.

III. OPTIMISATION OF ENCAPSULATING MATRIX FOR MAXIMUM CONVERSION OF PLBS.

Introduction

Artificial seed is an analog to a natural seed and the encapsulating matrix is expected to play the role of a seed coat, endosperm and cotyledons. So, in providing an appropriate matrix for somatic embryos diverse factors have to be considered. The encapsulating matrix should be pliable enough to cushion and protect the embryos/plbs, allow germination, supply nourishment, and, yet be sufficiently rigid to allow for rough handling of the capsules (during manufacture, transportation and planting). The matrix should make up the major part of the somatic embryo /plbs. It should be able to contain and deliver sufficient nutrients, growth and developmental control agents, and other biological or chemical components necessary for the embryo to plant development. Ideally, the capsules should contain plant growth promoting micro-organisms and agricultural chemicals specifically chosen for cultivar and environmental conditions. The encapsulation should allow for the formation of single-embryo capsules. Furthermore, the encapsulated somatic embryo should be handled and planted using the existing seed planting equipment to facilitate acceptance at the farm level.

Two systems of encapsulation have appeared in literature (i) coating with polyoxyethylene (Kitto and Janick, 1982, 1985), and (ii) hydrated hydrogels (Walgate, 1985; Redenbaugh, 1986).

Redenbaugh (1986) tested four encapsulation methods for production of synthetic seeds :Two types of gelation, complex coecervation, and, interfacial polymerisation. Alfalfa somatic embryos survived only when encapsulated using one of the two gelation methods (i) gel complexing via a dropping procedure, or (ii) moulding via reduction in temperature .They tested a number of gels for embryo survival using ion-exchange method. Somatic embryos were mixed with an uncomplexed gel solution and dropped into the complexing electrolytic solution. Embryo survival was achieved for six of the eleven gels that formed capsules. Of these, embryo to plant conversion occurred using alginate, alginate with gelatin, and, carrageenan with locust bean gum. The embryos were not hardy using other gels.

Alginate has a variety of properties that make it amenable to somatic embryo encapsulation. Sodium-alginate solubilizes at room temperature (25°C), does not require heat to produce a gel, and gels upon contact with relatively non-toxic divalent metal salts. The divalent metal salts cause complexation by forming ionic bonds between carboxylic acid groups on the guluronic acid molecules of the alginate polymers to form metal alginate gels. The hardiness of the capsule is a function of the glucoronic-mannuronic acid ratio, the cation and the complexing time.

The nutrient retaining power of alginate was found to be very poor. To overcome this problem two approaches were used (i) addition of complex carbohydrates to the gel capsule to slow leaching, and, (ii) micro-encapsulation of nutrients to provide

their controlled release inside the synthetic seeds.

Various starches, crude alginates, and sucrose microcapsules (produced via two methods, solvent evaporation and complex coecervation) were used as additives. Unrefined crude alginates used to form the capsules was the only additive that resulted in an increase in *in vivo* conversion (Redenbaugh,1986).

To overcome capsule desiccation and stickiness several coating procedures were tested. One compound, Elvax 4260 (Dupont) was very hydrophobic and showed significant impediment to capsule drying and degree of tackiness was reduced such that the coated capsules could be planted using a Stanhay seed planter (Redenbaugh *et al*, 1986; Upadhyaya *et al.* , 1987).

Redenbaugh *et al*. (1986, 1988) reported conversion frequency as high as 86% (*in vitro*) for alfalfa seeds. Several workers have used Redenbaugh's technique for encapsulating somatic embryo/ axillary buds using sodium alginate gel and obtained *in vitro* and *in vivo* conversion (Hama, 1986; Gupta and Durzan,1987; Bapat and Rao 1988; Bapat *et al.* 1987; Gupta and Durzan,1987; Mascarenhas,1988; Fernandes *et al*, 1992).

Kitto and Janick (1985a,b) and Kim and Janick (1987) coated carrot somatic embryos in polyoxyethylene gel, drying the embryo/polyox mixture and obtaining the survival of embryos. But, they reported only 3% conversion. Embryo survival was increased with high sucrose, high inoculum density, or chilling treatment with or without ABA.

In the present study the utility of various gels has been assessed, singly, or, in combination using various complexing agents, and the conversion frequency of orchid plbs to plantlets evaluated. New methods of encapsulation also have been tested. A simpler method of micro-encapsulation and coating procedure has been used. Also, a simple process for faster production of a

large number of beads has been tried.

Materials and Methods

A number of gels were tested using the two gelation method for encapsulating the plbs.

A. Bead formation by dropping procedure

a. Sodium alginate beads - Various concentrations (2-8%) of sodium alginate (100 ml each) were prepared by adding sodium alginate in water with constant stirring to avoid lump formation. Different concentrations (25-150 mM) of complexing agents (100 ml each of $CaCl_2.2H_2O$, $Ca(OH)_2$, $Ca(NO_3)_2.2H_2O$, $FeCl_2.6H_2O$, $ZnSO_4.H_2O$, $Al(NO_3)_2.H_2O$) were prepared separately in water . Beads were formed by dropping sodium alginate solution in various concentration of each of the complexing agents with the help of a microspoon and complexation allowed to take place for 20-40 minutes. The quality of beads viz. shape and size, firmness, ease of handling were noted for each concentration of sodium alginate and that of complexing agents. The quality of beads as a function of time was also noted. The concentration of sodium alginate and the complexing agents in which the best beads formed were selected for the subsequent experiment.

100 ml of sodium alginate solution and complexing agents were prepared separately in MS liquid medium and the solutions autoclaved for 15 min at 121°C after adjusting the pH to 5.8+ 0.1. The plbs (90 days old, size 3-4 mm in size) were mixed in sodium alginate solution and dropped in the complexing solution and the gel complexation allowed to take place for the appropriate duration and the beads strained, washed in sterile water and plated out in MS medium and the plb conversion into

plantlets studied. Ten beads were placed in each flask (5 replicates) and the whole experiment repeated thrice. The cultures were maintained at 25+2°C at 60-70% relative humidity under 14 hr photoperiod using cool white flourescent light (2000 lux).

b. Sodium alginate + silica beads.

Solutions of 4% sodium alginate and 2% silica were prepared separately and mixed in 1:1, 1:2, 1:3 proportion. This composite gel was dropped in various concentrations of $Ca(NO_3)_2$ solution and the best bead formation observed after a period of 30 min. The concentration at which the best beads formed was selected and plbs encapsulated.

Sodium alginate and silica solutions were then subsequently prepared in MS liquid solution , mixed in the required proportion and autoclaved. Plbs were mixed with the solution. The beads were prepared by dropping the plbs with the solution in 100 mM $Ca(NO_3)_2$ solution. The beads were strained , washed in sterile water and plated out.

c. Sodium alginate+gelatine beads. 4% sodium alginate and 2% gelatin solutions were prepared and mixed in 1:1, 1:2, 1:3 proportion and the concentration at which best beads formed was selected. Subsequently, the solutions were prepared in MS liquid medium, mixed in the right proportion and the plbs encapsulated in the above mentioned way. The beads were plated out in the medium for the conversion studies.

d.Carboxy-methyl cellulose. 2-8% carboxy-methyl cellulose solution and copper sulphate solution (25-100 mM) were prepared separately in water. Carboxy methyl cellulose solution was dropped in copper sulphate solution and the bead formation observed. Since no proper beads could be formed, carboxy- methyl

cellulose solution was not used subsequently for plb encapsulation.

e. Sodium alginate + potato starch. A 4% solution of potato starch was added to 4% sodium alginate solution in 1:1, 1:2,and 1:3 proportion and complexed in $Ca(NO_3)_2$ solution. The best concentration was selected for plb encapsulation. The encapsulated plbs were plated out in the MS medium for conversion studies.

e. Sodium alginate + Jal-shakti. 0.2 g of Jal-shakti was dissolved in 4% sodium alginate solution and the formation of beads observed in Ca $(NO_3)_2$ solution. Since the composite gel formed beads the gel solution was freshly prepared in MS liquid nutrients and the plbs encapsulated in the above mentioned way and plated out for conversion studies.

B. Gels tested via moulding process.

a. Agar- 3-5% agar solutions were prepared in MS liquid medium and autoclaved. The autoclaved agar was then allowed to cool down and at around 45°C when the agar starts solidifying the plbs were mixed with it and the mixture allowed to solidify. The block of agar with the trapped plbs were cut into small cubes and transferred to the medium for conversion studies.

b. Agarose - A solution of 0.3-0.7% agarose was prepared in MS liquid medium and autoclaved at 120°C for 20 minutes. The plbs were suspended in agarose at 35°C and the mixture allowed to solidify. The block of agarose with trapped plbs were cut into square cubes. The agarose cubes were then transferred to the appropriate medium.

c.Starch -12% hydrolysed potato starch solution was made by dissolving in MS liquid nutrients medium and heated with constant stirring and the solution formed was subsequently

filtered and autoclaved. When the solution was sufficiently cool plbs were mixed with the solution and allowed to cool further when it solidifies into a relatively rigid gel. The gel was cut into small cubes and plated out in the medium.

Coating of the alginate capsule

The following substances were tried for coating the beads.

1. **Glutaraldehyde** - 10 ml of glutaraldehyde was poured into the petridish containing alginate beads and the solution allowed to form a thin film over the beads. The excess liquid was drained off and the beads allowed to dry off. A thin film is formed over the beads.

2. **Dry gelatin capsule** - 2-3 alginate beads with the plbs were placed in the capsule along with Jal- Shakti powder and the two halves of the capsule closed . Before placing the capsule in the liquid medium a few holes were made in the capsule either at the centre or at the ends with the help of a clean needle.

Micro-capsule encapsulation - 10 g of sucrose was slowly dropped in 5 ml of hot safflower oil in a beaker and heated over a slow fire. The sugar melts and caramellizes. The carramelised solution was quickly dropped in a oiled paper/petridish dropwise to obtain small drops of carramelised sugar. These small micro capsules were mixed with sodium alginate solution along with the plbs and dropped in the complexing solution to form beads. The beads were plated out in the medium and the plbs conversion studied.

Capsule production - A faster method of capsule production was tried since the process of synthetic seed production by dropping procedure is time consuming and inefficient for large scale production. The plbs mixed in alginates were quickly poured over a metal sheet with a large number of holes, the holes large

enough to allow plbs to pass through. The alginate drops falling into the calcium nitrate solution formed beads.

Results

An assessment of various concentrations of sodium alginate (2-8% w/v) and Ca $(NO_3)_2$ $2H_2O$ (25-150 mM) for the formation of beads is presented in Table 9. A 4-6% solution of sodium alginate upon complexation with 70-100 mM solution gave excellent round *beads with an ion- exchange duration of 30 min (Table 10). The* diameter of the beads ranged between 4-9 mm (Fig 28). Lower concentrations of sodium alginate (2-3%) resulted in the formation of very fragile ill formed (flattened beads and beads with trailing ends) beads whereas concentration higher than 6% proved to be too viscous for the free flow of the dispersed explants. Lower concentrations of $Ca(NO_3)_2$ on the other hand, not only adversely affected the bead quality but also prolonged complexation time. So in the subsequent experiments the plbs were encapsulated in 4% sodium alginate (prepared in liquid MS nutrients) in 70-100 mM $Ca(NO_3)_2$ solution (also made in liquid MS nutrients) and the gel complexation allowed to take place for 30 min. The beads showed 100% conversion frequency (Fig.29).

The experiment was repeated with other complexing agents using 4% Na-alginate. The quality of beads, best concentration of complexing agents,time of incubation are presented in Table 11. The best beads were obtained with 4% Na-alginate in 70-100 mM Al $(NO_3)3$ H_2O as complexing agents for 20 min. The beads were firm, round and transparent, and could be picked up with forceps. The beads prepared using $Ca(NO_3)2$ $.2H_2O$ and $CaCl_2$ $2H_2O$ followed next with firmer beads, but, in $FeCl_36H_2O$ the beads were soft and *fragile even after an incubation period of 40 min. No conversion* was obtained using $ZnSO_4$ as complexing agent.

Table 9. Effect of different concentrations of Na-alginate and $Ca(NO_3)_2$ on bead formation.

$Ca(NO_3)_2$ mM	Na-alginate(% w/v) 2	3	4	5	6	7	8
25	+	+	+	+	+	+	+
50	+	+	++	++	++	+	+
75	+	+	+++	+++	+++	+	+
100	+	++	+++	+++	+++	+	+
125	+	++	+++	+++	+++	+	+
150	+	++	+++	+++	+++	+	+

Quality of beads with reference to shape, size and firmness, indicated as follows:

+ Flat shapeless mass with trailing ends.

++ Round, not firm, fragile.

+++ Round, firm beads.

Table 10. Effect of incubation time and complexing agents on quality of beads.

Incubation time (min)	Quality of beads					
	Calcium nitrate	Calcium hydroxide	Calcium chloride	Ferric chloride	Zinc sulphate	Aluminium nitrate
5	+	+	+	+	+	+
10	+	+	+	+	+	+
20	++	++	++	+	++	++
30	+++	+++	++	+	+++	+++
40	+++	+++	+++	++	+++	+++

+ Soft, fragile, breaks when handled with forceps.

++ Relatively firm, breaks when handled with forceps.

+++ Firm and round.

Table 11. Gels tested for synthetic seed production based on dropping method of encapsulation.

Gels	Best conc. (% w/v)	Complexing agents	Best conc. (mM)	Time (min)	Capsule formed	Plb survival	Percentage conversion (*in vitro*)
1. Na-alginate	4-5	$CaCl_2, 2H_2O$	75-100	30	Yes	Yes	100
		$Ca(NO_3)_2.2H_2O$	100-125	30	Yes	Yes	100
		$Al(NO_3)_2.H_2O$	70-100	30	Yes	Yes	100
		$ZnSO_4.7H_2O$	70-100	30	Yes	No	0
		$FeCl_3.6H_2O$	50-100	30	Yes	No	80
		$Ca(OH)_2$	100-125	40	Yes	No	100
2. NA+SI	4+2	$CaCl_2, 2H_2O$	100-125	30	Yes	No	100
3. NA + GE	4+2	$CaCl_2, 2H_2O$	75-100	30	Yes	No	100
4. CMC	8	$CuSO_4$	75-100	40	No	No	0
5. NA+PS	4+4	$Ca(NO_3)_2$	75-100	30	Yes	Yes	100
6. NA +JS	4+0.2	$Ca(NO_3)_2$	75-100	30	Yes	Yes	100

Gels tested for synthetic seed production based on moulding process.

Gel	Conc (%)	Time (min)	Plb survival	Percentage survival (*in vitro*)
1. Agar	0.5-9	30	Yes	100
2. Agarose	0.5-0.7	20	Yes	100
3. Starch	12	40	Yes	80

NA = Sodium alginate
SI = Silica gel
GE = gelatin
CMC = Carboxy methyl cellulose
PS = Potato starch
JS = Jal shakti

Fig 28. *C. giganteum* plbs encapsulated in calcium alginate beads.

Fig 29. Conversion of encapsulated plbs into plantlets.

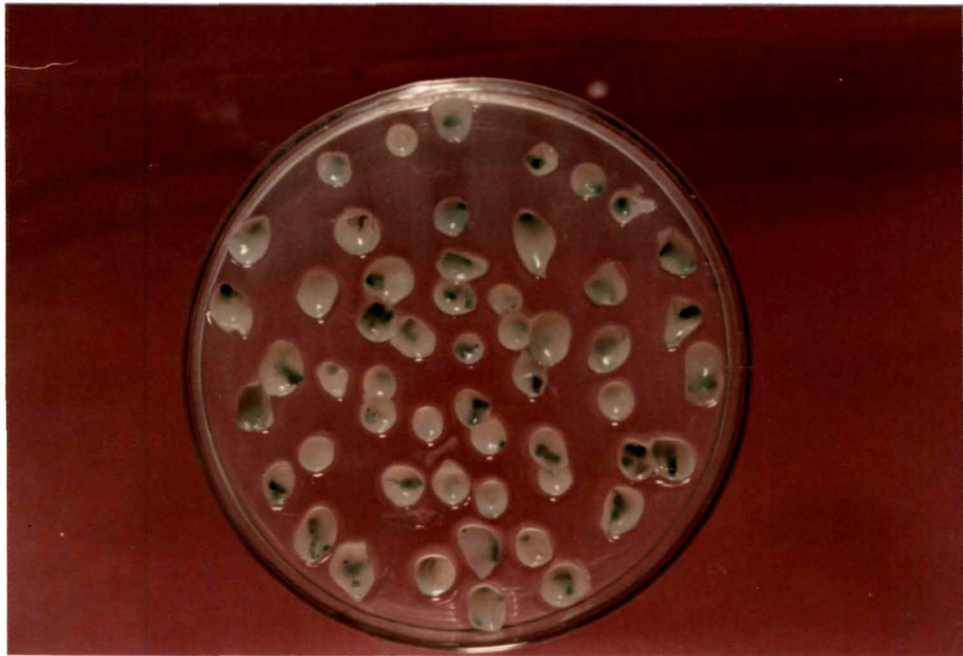

Fig. 28

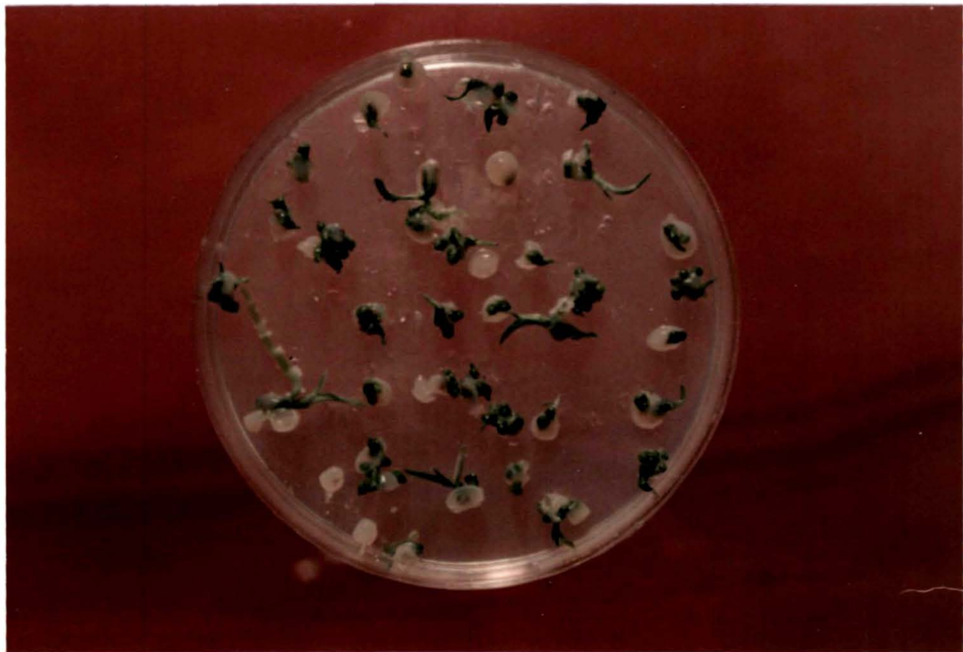

Fig. 29a

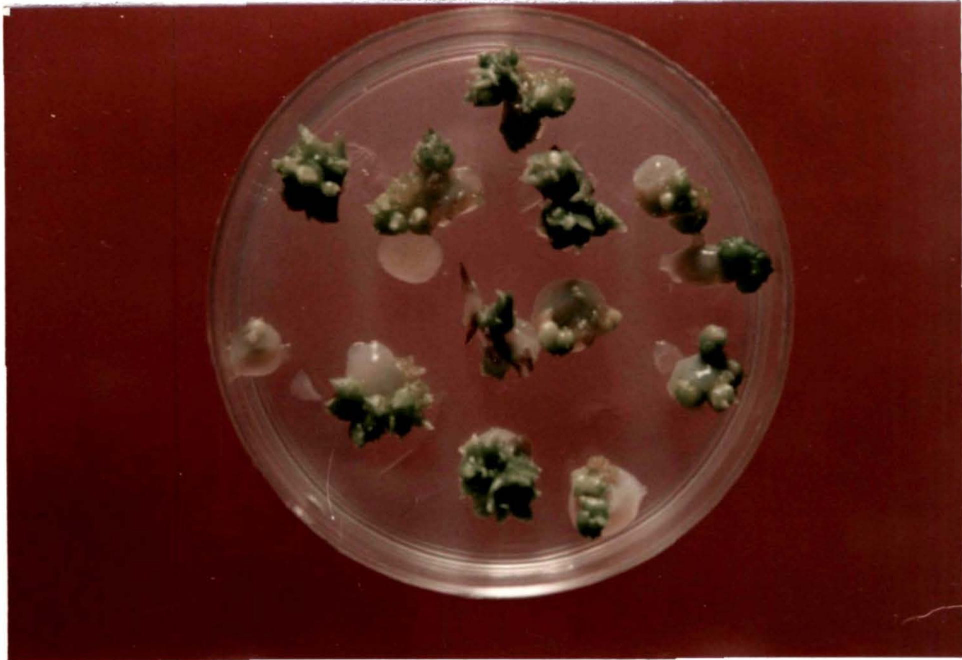

Fig. 29 b

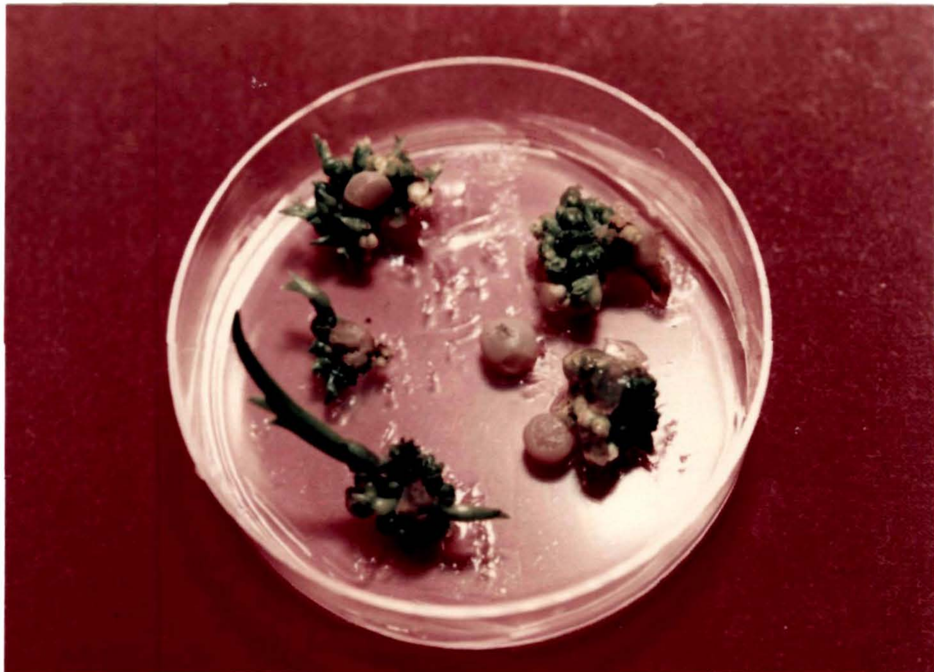

Fig. 29 c

The quality of beads also depended upon the time of incubation in the electrolyte (Table 10). Short incubation (5-10 min) resulted in very fragile beads. The quality of beads was better as the incubation period increased. Using $Ca(NO_3)_2$, an incubation time of 30 min or more was required, but for $Al(NO_3)_3$ incubation time of 20 min was enough to get firm beads. Na-alginate (4%) and silica gel solution in 1:1 ratio resulted in firm beads in 100 mM calcium nitrate or calcium chloride solutions. There was 100% conversion, but the plbs took longer time to come out of the capsule. Apparently the tough coating impeded the emergence of the shoot apex from the capsule. 4% Na-alginate and 2% gelatin solution in 1:1 proportion was found to be the best, but this composite bead was more sticky than the Na-alginate beads.

Alginate (4%) and starch (4%) in 1:1 proportion gave firmer beads and the dehydration of the capsule was not as rapid as alginate. Besides starch could serve as a slow energy releasing compound compared to sucrose which is depleted very fast. 5-9 % agar and 0.5-0.7 % agarose formed gels of reasonable firmness.

Besides starch, caramellized sucrose was added in the alginate capsule. The sucrose from the micro-capsule were released slowly and extended over a period of 7-10 days. However, when the beads were placed in the liquid medium, the release of sucrose was rapid and within 2-3 days the caramellized sucrose dissolved and leached out in the medium. When the micro-capsules were incorporated in the Jal-shakti + Na-alginate gel the leakage of sucrose from the micro-capsules was very slow and sustained release of sucrose continued throughout the 10-day period. This sustained release of sucrose from this alginate + water absorbing

polymer beads is promising for direct field planting of orchid plbs.

Glutaraldehyde solution was tested on alginate capsule to prevent capsule desiccation. This coating was able to form a membrane around the alginate capsule . However, only the dry gelatin capsule showed significant impediment to capsule drying. Glutaraldehyde did not prevent drying nor did it reduce the degree of tackiness. When placed in water or liquid, the dry gelatin capsule burst, owing to the tremendous pressure developed due to absorption of water by the polymer. On bursting, the plbs were liberated well cushioned in the gel (Fig.30 & 32). The plbs could regenerate plantlets in the gel, and the frequency of watering was reduced considerably.

The new approach attempted for faster production of beads was found to be quite efficient and a large number of beads could be formed in a short time, eventhough a large amount of alginate solution was wasted as some of the alginate drops passed through the holes without the plbs.

Discussion

Out of the various gels and combination of gels tried in the present study, Na-alginate + Jal shakti, Na-alginate + starch appear promising for in vivo planting. Jal-shakti could retain moisture for a prolonged period thereby preventing the desiccation of the capsule and the plbs. The rapid water loss and leaching of the capsule was one of the major problems with alginate capsules. Starch incorporated in Na-alginate matrix could serve as a slow energy releasing compound. Fujii et al. (1989) using 3% hydrolysed potato starch incorporated in the alginate capsule did not observe any significant increase in

Fig 30-32.An outer coating for alginate beads.

30, Plbs in alginate capsule.

31, capsule pierced with holes before planting.

32, a burst capsule with plb exposed well- cushioned in the gel.

Fig. 30

Fig. 31

Fig. 32

conversion frequencies of alfalfa somatic embryos. In the present study, it was found that _in vitro_ plb-conversion was as good as calcium alginate capsule. Further research is needed to determine the release rates of substances added to the capsule to understand the role of nutrients in the capsule for synthetic seed conversion. Using crude alginate may be beneficial, as the carbohydrates and proteins present in it may aid in plb-conversion besides its cheap cost. Redenbaugh (1986) using crude alginate reported higher somatic embryo conversion.

Composite beads of Na-alginate+silica, Na-alginate+gelatin, beads formed capsules of sufficient integrity for handling and were mild enough to allow plb-germination, at frequencies equal to those of calcium-alginate encapsulated plbs. However, in Na-alginate + silica beads, the plbs took considerable time for emergence from the capsule and Na-alginate + gelatin beads were tacky to handle. Na-alginate + jal Shakti beads were bigger (1-1.2 cm in diameter) but fragile. However, the _in vitro_ conversion was good. Carboxy-methyl cellulose did not form proper beads and hence were not considered fit for further experimentation.

Out of the various complexing agents tried, best beads (firm and round) were formed in aluminium hydroxide with 100% conversion into plantlets. However, the regenerants did not show much vigour and were yellowish green in colour, indicating inhibition of chlorophyll synthesis. Conversion freqency in gels formed via moulding procedure was high, but, singulation of the plbs could not be achieved . The slab has to be cut into a number of pieces, as it contains a number of plbs. This fragmentation process is time-consuming, and, might at times injure the plbs. These gels were, therefore, not tried for _in vivo_ conversion.

Redenbaugh's technique (1986) of encapsulating somatic embryos in hydrogels is superior to Kitto and Janick's (1982)

process. Using the former technique singulation of the embryo can be achieved for species which needs precision planting.

Redenbaugh (1986) allowed sodium alginate to drop from a separatory funnel into the complexing solution. As the sodium alginate drop formed at the tip of the funnel a somatic embryo was inserted. The use of micro-spoon was more convenient than separating funnel. The Cymbidium plbs readily germinated and emerged from the alginate capsule. The alginate gel was found non-inhibitory for well-developed orchid plbs. However, the capsules inhibited germination and conversion of small, under-developed plbs, hence, only bigger mature plbs were used.

Alfalfa and celery somatic embryos encapsulated in Na-alginate germinated to form complete plants. In vivo conversion frequency for synthetic seeds ranged from 45-64% for alfalfa to 60-90% for celery. Kamada (1985) reported 10% conversion for alginate encapsulated carrot somatic embryos. Gupta and Durzan (1986a, 1987) reported the storing ability of alginate-encapsulated loblolly pine and Norway Spruce somatic embryos, but were unable to obtain conversion. Mascarenhas (1988) reported encapsulation of Eucalyptus somatic embryos and indicated 50% germination. In this study the conversion frequency for encapsulated orchid plbs were very high (100%) compared to all the earlier reports. Bapat and Rao (1987) reported 50% in vitro conversion frequency for both agar and alginate encapsulated axillary buds of mulberry. Bapat et al.(1988) observed 10-16% in vitro conversion for alginate encapsulated somatic embryos of sandal wood. A very high conversion of 94% in vitro was reported for encapsulated axillary and apical buds of Valeriana wallichi(Mathur et al., 1989). Fernandes et al. (1992) observed 17% conversion for somatic embryo of sandal wood encapsulated in

sodium alginate and 4.7% for embryos encapsulated in composite beads.

The alginate concentration required to form capsules firm enough for handling, depends on the source of alginate. Redenbaugh (1986) found 2% refined alginate to be the best for somatic embryo encapsulation. In their earlier experiments, they used BDH alginate and found 3.2% to be ideal. BDH alginate is produced from Laminaria hyperborea fronds which have a high M:G (mannuronic:guloronic acid) ratio. Harder gels are produced from brown algae that have a low M:G ratio. Na-alginate used in the present study was in the crude form (Wilson Company, Bombay), and, perhaps contains less of glucoronic acid (high M:G ratio). Hence, higher concentrations was required for bead formation. With lower concentrations, very fragile beads were formed. 6% solution became too viscous for the free flow of the plbs.

Alginate capsules dry out rapidly when exposed to air. Fernandes et al. (1992) reported drying of the beads before somatic embryo could develop into plantlets and thus this was the major hurdle for in vivo germination of somatic embryo. In the present experiment, glutaraldehyde coating was sticky and difficult to handle. The chemical may also be toxic for the plbs. The alginate beads and water absorbing polymer in the gelatin capsule looked ideal for synthetic seed production. The capsule is non-tacky, easy to handle, and, can be planted individually. The capsule is air-tight to prevent dessication of the beads. However, the plbs lost viability after 10-15 days, and, were dessicated. If this loss of moisture could be prevented, the gelatin capsule could be ideal for the production of synthetic seeds.

Recently, Jha et al. (1993) reported encapsulation of Sesbania seeds carrier entrapped rhizobia, within gelatin

capsule, and, reported effective symbiosis. Gelatin is a pure protein, and is known to be a good source for slow release of nitrogen. Infact gelatin is treated as a home-made compost fertilizer.

Encapsulating micro-capsules of caramellized sugar inside the capsule showed considerable utility in slow release of sugar. The technique could prove useful for in vivo conversion.

The simple method of dropping plbs and alginate solution from a sheet with a large number of holes big enough to allow plbs to pass through might prove useful for large scale production of synthetic seeds, as dropping the embryo/plbs using a spoon /funnel /pipette is a very slow process.

IV. EFFECT OF VARIOUS ADJUVANTS IN THE MATRIX ON THE CONVERSION OF PLBS: TRANSFER TO DIFFERENT SUBSTRATA.

Introduction

The encapsulated embryo is expected to germinate drawing upon the available nutrients provided in the matrix. In a true seed, i.e. zygotic embryo draws nutrients from the endosperm and cotyledons and has its own inherent mechanisms to protect the embryo. The encapsulating matrix thus has to be provided with suitable nutrients and additives to nourish and protect the encapsulated embryos as well to enable them to germinate under non-sterile environment. Besides nutrients and growth regulating substances the matrix could be made to hold and deliver beneficial adjuvants such as growth promoting bacteria, nematodes, fertilizer and pesticides, anti-microbial agents etc. A few reports have appeared on incorporation of various adjuvants like ABA, fungicide, bactericide, water absorbing polymer in the matrix and their effects on embryo germination and growth (Baker, 1985; Hama, 1986; Mathur et al., 1989; Fernandes et al., 1992). Hama (1986) reported using an anti-microbial solutions and a water absorbing polymeric material besides hydrogel to encase the adventitious embryo. The antibacterial solution used were copper hydroxyquinoline with penicillin, streptomycin or zineb

with cloramphenicol. The polymeric material used was either polyacrylate or saponified starch/ acrylonitrile graft polymer. Mathur et al. (1989) used rose bengal and bavistin as antimicrobial agents in the alginate matrix. Baker (1985) used sucrose and hormones in the fluid gel. ABA as a growth retardant has been used in encapsulating matrix (Kitto and Janick, 1985a,b). Fernandes et al. (1992) used fungicides bavistin, dithane M-45, and food preservative sodium carbonate in the alginate matrix and in a composite matrix of alginate and silica. Though several experiments have been successfully conducted for regeneration of plantlets from encapsulated somatic embryos under in vitro conditions, very few soil conversion experiemnts have been reported using 'artificial seeds' (Kamada, 1985; Baker, 1985; Redenbaugh, 1986; Nishimura, 1986; Fujii et al., 1989).

Jal Shakti, a water absorptive polymeric material based on starch, a product of National Chemical Laboratory, Pune, India, has the unique property of absorbing hundreds of times of its weight of water and making it available to the plants. It is non-toxic, biodegradable, which when mixed with soil or any synthetic growing media, increases both the water retention capacity and aeration thus improving the soil quality. The plants therefore have a healthy root system. In the area of seed coating, Jal Shakti helps faster germination, earlier emergence and greater yield.

The use of sorbarod system in plant micro-propagation is now well established. They are non-toxic to plant cells and have a high absorbancy-rapidly absorbing their own volume of liquid. Sorbarod system are composed of cylindrical rods of absorbent polymers.

In the present experiment, various adjuvants (fungicide, bactericide and water absorbing polymers) were incorporated in

the matrix and their effect observed on the conversion of plbs into plantlets. The emerging plbs were transferred to various substrata and their performance observed.

Material and Methods

Well developed plbs (3-4 mm) of Cymbidium giganteum Wall. were used as the experimental material. The selected plbs (5 plbs in each flasks) were placed aseptically in MS basal medium (both solid and liquid) containing different concentrations of (0.1-0.3 mg/l) of bactericides soframycin and rosebengal and fungicides dithane (2-6 mg/l) and their effects observed on plb proliferation, plb development (in liquid medium) and seedling growth (in solid medium). Plb characteristics were analysed after 30 days of culture and seedling characteristics after 120 days as described earlier in chapter II.

Solutions of 100 mM calcium nitrate and 4% Na-alginate were prepared separately in liquid MS medium (without sucrose) containing 1mg/l each of NAA and BAP. Bactericde soframycin ranging in concentrations from 0.1-0.3 mg/l and fungicide dithane (2-6 mg/l) were added separately in Na-alginate and Ca-nitrate solution. pH of the solution was adjusted to 5.8 before autoclaving at 106 kg/cm^2 for 20 min.

Well developed, 90-day old plbs, about 3-4 mm in size were obtained from various explants of the in vitro grown seedlings, mixed in Na-alginate solution and dropped in Ca-nitrate solution with the help of a small sterlised spoon and gel-complexation was allowed to take place for 30 min. The beads were then sieved and plated on (i) MS medium containing 1 mg/l each of NAA and BAP (ii) Sterile sand (0. 3 mm size), and, (iii) Sterile soil mix (a mixture of sand, garden soil and cow-dung in 1:1:1 ratio).

Ten beads were placed in each flask/pot. Five replicates were used for each substratum, and the experiemnt was repeated twice. The controls comprised of non-encapsulated plbs placed in MS medium with 1 mg/l each of NAA + BAP, sterile sand and soil mixture. The encapsulated and non-encapsulated plbs placed in plastic pots containing sterile sand and soil mixture were supplied with 1/2 MS nutrient salt solution containing anti-microbial agents at an interval of 2 days for 2 weeks. The pots were covered with polyurethane bags to maintain high humidity,and water was sprayed twice daily. The pots were kept under culture room conditions as described before for 2 weeks and subsequently transferred to glass-house maintained at 20-25°C and 60-70% RH. The development of plbs were divided into six different stages (Table 12).

Besides the above mentioned three substrata, substrata like sorbarod (sterlised), sponge (sterlised polyurethane foam) under <u>in vitro</u> conditions and Jal Shakti + sand in the ratio 1:6 and Jal Shakti and soil in the ratio of 1:6 were also used. Ten beads (five replicates) were placed on sorbarod and sponge and kept under <u>in vitro</u> conditions. Beads were also placed in Jal Shakti + sand and Jal Shakti + soil and placed under <u>in vitro</u> conditions for 2 weeks, and then transferred to green-house. The pots were covered with polyurethane bags and supply of nutrients and water was followed as mentioned above. The surviving plants of both culture room and green-house (after about 8-12 months of plb-growth) were transferred to community pots and their growth parameters studied.

Results

The effects of bactericides and fungicides on plb development and seedling characteristics is shown in Table 13.

Bactericides, soframycin and rosebengal and fungicides dithane had no adverse effect on plb development and seedling growth. Plb volume was slightly higher than control in 0.3 mg/l of soframycin. Increase in weight of plbs and seedlings were better in 4 mg/l of dithane. Rosebengal did not show appreciable effect on plb proliferation and growth.

Plbs mixed in 4% Na-alginate and dropped in 100 mM calcium nitrate solution resulted in firm, round beads of about 0.6-0.9cm diameter (Fig.33). These beads mixed with various adjuvants were placed in different substrata for growth and development studied.

Under _in vivo_ conditions, the encapsulated plbs required an average of 20-25 days to emerge out of the beads and plantlet of two leaf stage (about 10 mm in size) were produced within a period of 55 days (Table 12). Sometimes profuse multiplication of plbs from the bead was also observed. Such proliferating plbs assumed a globular ball-like shape. The non-encapsulated plbs when sown in MS medium germinated in 10-15 days and within 30-35 days plantlets of two-leaf stage were produced. Some of the non-encapsulated plbs proliferated into numerous protocorms too. The conversion frequency under _in vitro_ conditions for both encapsulated and non-encapsulated plbs were 100%. The seedlings from encapsulated plbs reached stage IV and V earlier than the non-encapsulated plbs. Plbs of around 3-4 mm size readily germinated (Fig.34) and emerged from the capsule whereas those plbs below 2 mm size either failed to grow or required very long time for emerging out of the capsule (Fig.36). Hence, only big and mature plbs were selected for further studies.

Under _in vivo_ conditions, the non-encapsulated plbs when grown in sterile sand or sterile soil mixture did not germinate. The beads with MS nutrients and sucrose showed severe fungal and

Table 12. Development of plantlets from non-encapsulated and encapsulated plbs on different substrata.

		Development stages					
		I	II	III	IV	V	VI
		Days required for development					
Nutrient medium	N	10-15	20	35	80	90	110
	E	20-25	30-35	55	70	85	90
Sterile sand	N	Nil	Nil	Nil	Nil	Nil	Nil
	E	8-10	25	60	75	90	120
Sterile soil mixture	N	Nil	Nil	Nil	Nil	Nil	Nil
	E	8-10	25	70	80	110	130

N, non-encapsulated plbs; E, encapsulated plbs; Nil, no conversion

Stages I. Plbs showing pointed vegetative apex.

II. Plbs with leaf initials (6.0-8.0 mm).

III. Two-leaf stage with root initials (8.0 mm-1.0 cm).

IV. Three-leaf stage with rhizoids (2.0-2.5 cm).

V. Four-leaf stage (2.5-3.0 cm).

VI. Four-leaf stage with well developed leaves (3.0-3.5 cm).

Table 13. Effect of fungicides and bactericides on conversion of encapsulated and non-encapsulated plbs under *in vivo* and *in vitro* conditions.

Fungicides/bactercides (mg/l)		% Conversion			
		Media		Sand	Soil mixture
		N	E	E*	E*
Control (without antimicrobial agents)		100	100	44 + 0.37	20 + 0.42
Soframycin	0.1	100	100	54 + 0.34	35 + 0.32
	0.2	100	100	48 + 0.27	40 + 0.40
	0.3	100	100	60 + 0.35	48 + 0.35
Rose bengal	0.1	100	100	58 + 0.51	42 + 0.43
	0.2	100	100	42 + 0.48	26 + 0.44
	0.3	100	100	40 + 0.45	28 + 0.42
Dithane	2.0	100	100	64 + 0.34	46 + 0.23
	4.0	100	100	80 + 0.37	46 + 0.45
	6.0	100	100	56 + 0.40	44 + 0.37
Dithane(4.0) + Rose bengal(0.1) + Soframycin(5.0)		100	100	88 + 0.12	64 + 0.16

+, SE

*, non-encapsulated explants failed to survive upon *in vivo* planting

Fig 33. An enlarged view of the alginate beads with plbs inside.
Fig 34. Plbs emerging through the beads.

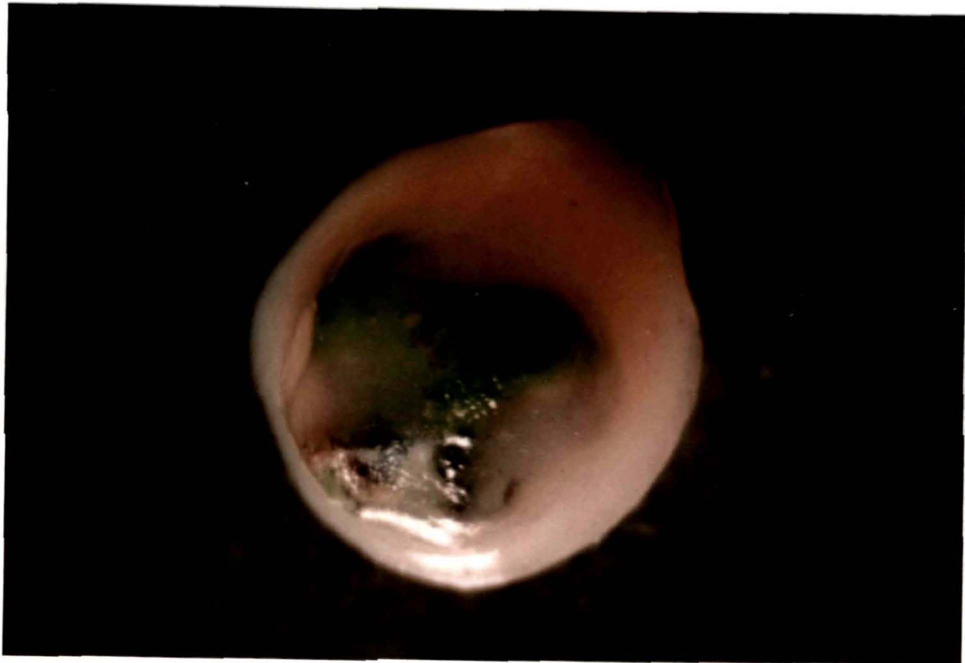

Fig. 33

Fig. 34

bacterial contamination when placed in sterile sand and soil mixture and did not form plantlets. When sucrose was excluded from the medium the conversion frequency was 44% in sand and 20% in sterile soil mixture (Table 13). However, when antimicrobial agents were added in the matrix there was a marked increase in the conversion frequency. The multiplication rate of non-encapsulated plbs was not affected by incorporation of antimicrobial agents in the medium. When all the three antimicrobial compounds were added together in the matrix there was significant increase in conversion frequency to 88% in sand and 64% in soil. The capsules placed in sand and soil mixture gradually shrunk and the growing plbs came out of the capsules in 8-10 days.

Addition of bactericide soframycin, considerably changed the conversion frequency in sand and soil. Highest conversion frequency in sand and soil was obtained when soframycin was added at concentration of 0.3 mg/l. No effect was observed on plb formation and seedling growth, when soframycin was added to the medium.

Addition of rosebengal in the matrix increased the conversion frequency slightly as compared to the control. Rosebengal at 0.1 mg/l was most effective. Dithane at concentration 4 mg/l was found to be optimum for plb conversion both in sand and soil. Since 0.3 mg/l soframycin, 0.1 mg/l rosebengal and 4 mg/l of dithane separately were found to be the best for plb conversion, a combination of all these were incorporated in the matrix. Conversion frequency of 88% in sand (Fig.35) and 64% in soil (Fig. 37) was obtained at this concentration. This was higher when compared to adding any bactericide and fungicide alone. Hence this combination was added in all the subsequent experiments.

Fig 35. conversion of encapsulated plbs in sand.

Fig. 35

Fig 36. Effect of the size of encapsulated plbs on conversion frequency in MS+NAA+BAP (1 mg/l each) after 35 days of transfer.

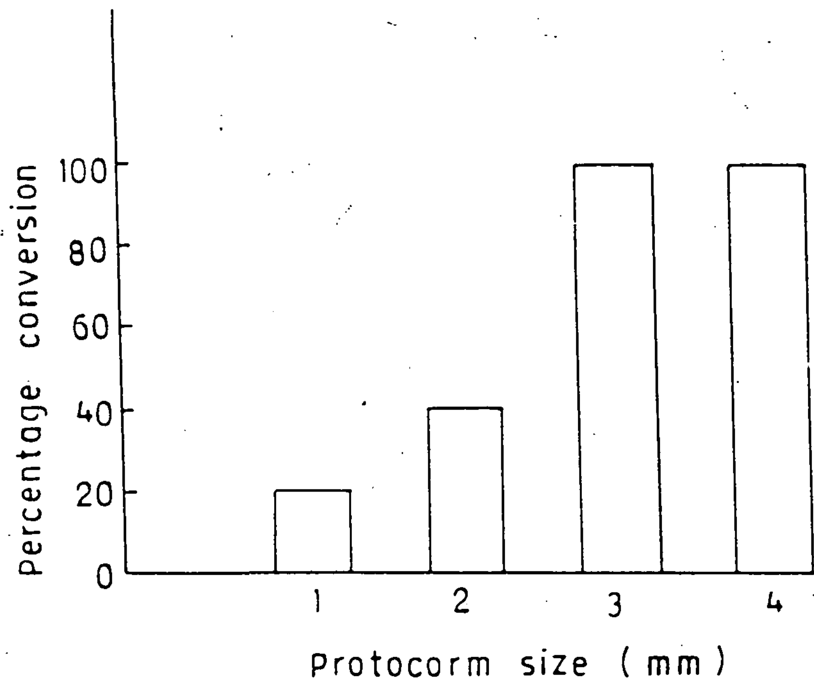

Fig. 36

Fig 37. conversion frequency of non-encapsulated and encapsulated plbs in nutrient medium, sterile sand and sterile soil mixture after 55 days of transfer.

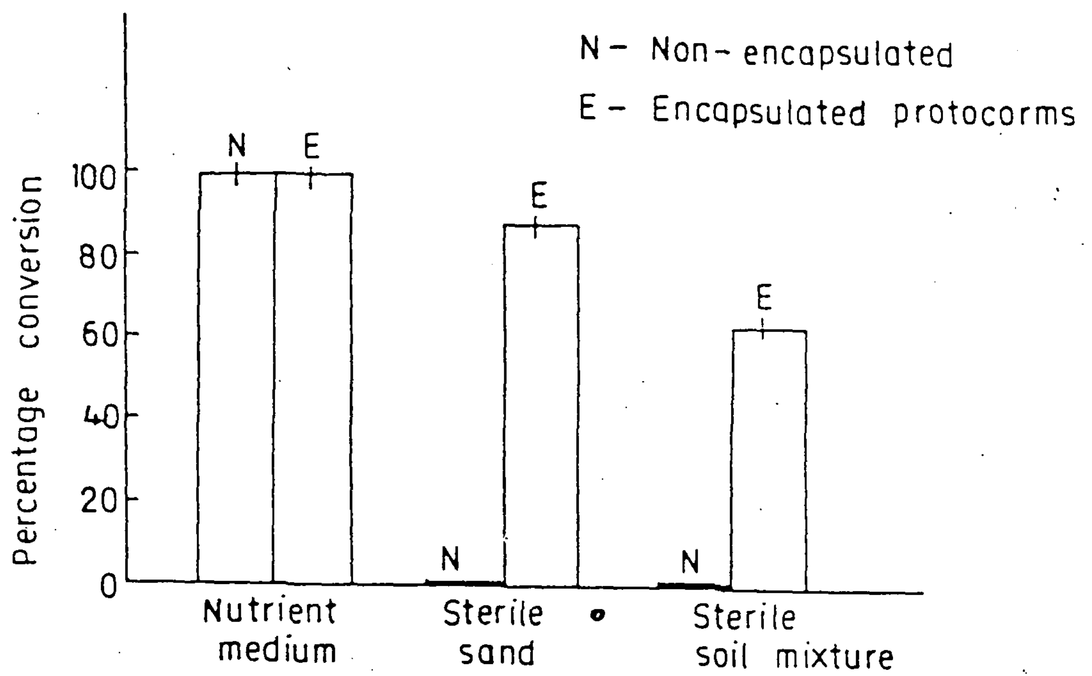

Fig. 37

The capsules placed in sand and soil mixture gradually shrunk and the growing plb came out of the capsule in 8- 10 days and attained three leaf stage (stage IV) in 75 days in sand and 80 days in soil mixture.

Polyurethane foam and sorbarod as substrata for plating beads under culture room conditions

Besides nutrient medium, sterlised polyurethane foam submerged in MS liquid nutrients proved to be good for both non-encapsulated and encapsulated plbs. The plantlets emerging from these plbs showed larger number of roots, and the plantlets could establish themselves better in the soil indicating tremendous potentiality of this foam as substrata for growing orchid plbs.

Sorbarod system too proved to be a good substratum for plb development and seedling growth though plb-proliferation was less as compared to the control (non-encapsulated and encapsulated plbs in the media).

Jal Shakti with sand and soil as substrata for the plbs under *in vivo* conditions

Jal Shakti with sand in the ratio 1:6 and with soil mixture in the ratio 1:6 were better substrata for encapsulated plbs, than sand or soil mixture alone, as it retained moisture better due to the moisture absorbing properties of the polymer (Jal Shakti).

Discussion

Bactericide and fungicide, in particular did not affect plb proliferation and growth. No reports have appeared on the effect of such chemicals on orchid culture. Mathur *et al*.(1991)

reported the use of fungicides bavistin and bactericides rosebengal in the matrix of encapsulated axillary shoots of Valeriana wallichi but did not show any adverse effect of these chemicals on the encapsulated explants and indicated 64% in vivo conversion. Baker (1985) using sucrose and hormone in the fluid gel was able to plant somatic embryos of carrot in the green house and obtained 4% embryo survival for 7 days but further development and plant conversion was not possible because of embryo desiccation and death. Hama (1986), using antibacterial solution and water absorbing material reported no adverse affect of these materials on the germination of the embryo and the embryo broke the coating within approximately 10 days and showed normal growth thereafter. Kitto and Janick (1985a,b) improved embryo survival using ABA in the matrix. Fernandes et al.(1992) using 25 mg/l of sodium carbonate in the matrix obtained 17% (in vitro) germination for sandal wood somatic embryo with alginate matrix and 4.16% germination using composite matrix. Using sodium carbonate in the matrix the beads did not show contamination even after 12 months under in vivo conditions. Somatic embryos did not germinate when the matrix was supplemented with any of the fungicides bavistin, dithane, carbendazin but there was no contamination of the beads. The concentration of bavistin and dithane used by them was very high (10-100 mg/l). The concentrations used in the present experiment is very low (2-6 mg/l) which did not visibly harm the orchid plbs. Moreover, Fernandes et al.(1992) reported no plantlet formation because of drying of the capsule which did not happen in the present case as the plbs grew into healthy plantlets. Inclusion of antimicrobial agents had increased the conversion frequency in sand and soil mixture. The highest conversion for alfalfa was 20% in soil as reported by Redenbaugh, (1986). Fujii

et al.(1989) were able to get 48% conversion of alfalfa synthetic seeds in soil under growth chamber conditions. The embryos were planted in a commercial soil potting mix under non-sterile conditions in a manner similar to zygotic seeds. Under green house conditions, they were able to get 64% conversion when humidified air was used to prevent soil surface drying. Nishimura (1986) produced plants from artificial seeds planted on vermiculite under non-sterile, high humidity and aseptic conditions. In the present experiment, the conversion frequency could be increased by incorporating fungicide and bactericide. The combination of rosebengal, soframycin and dithane was found to be most effective than using any of these alone.

The 100% conversion frequency and better growth at stage IV and V of encapsulated plbs under in vitro conditions indicate that encapsulation is advantageous for the growth of the plbs. The initial lag (55 days) in plb conversion to reach the stage III from the encapsulated plbs compared with the non-encasulated ones (30-35 days) may be accounted for by the time required for the adjustment of plbs to the new environment and also for the time taken during the emergence of developing plbs through the beads. The reason for higher growth rate of encapsulated plbs may probably be due to the availability of abundant nutrients and growth regulators in the immediate cell surroundings.

The ability of encapsulated plbs to establish plantlets in the soil (conversion frequency 88% in sand and 64% in soil mixture) shows great promise for direct field planting of tissue-culture grown plbs. Non-encapsulated plbs failed to germinate in soil despite proper growth conditions and supply of nutrients. The smaller size encapsulated plbs showed poor conversion frequency which could be due to immature nature of developing

plbs (which could not withstand encapsulation). This limitation was overcome by taking larger, well-developed plbs of 3-4 mm diameter. In the present study, polyurethane foam was found to be an ideal and inexpensive substratum for growing the beads under *in vitro* conditions. As reported in Chapter I the plantlets cultured on polyurethane foam placed in liquid culture showed better growth with higher vigour than on agar-solidified medium. The non-encapsulated and encapsulated plbs too showed better growth, larger development of roots and better establishment in the soil.

The use of sorbarods in tissue culture have also been discussed in Chapter I. The encapsulated plbs placed on sorbarod could grow normally as in agar. Like polyurethane foam they had well developed roots and the plantlets could be easily transferred to the soil with minimum damage to the roots, with washing of the roots being avoided.

With further research and improvements, encapsulated plbs could revolutionize the propagation system for orchids. This technique has made it possible to place aseptically grown plbs directly in the soil for plantlet regeneration. The by-passing of the *in vitro* plant production and acclimation step makes this technique economically and practically viable, which could be exploited by commercial growers. Further, a large number of encapsulated plbs could be easily transported in low bulk with minimum damage and at a much reduced cost.

V. STORAGE OF SYNTHETIC SEEDS USING GROWTH RETARDANTS, OSMOTIC INHIBITOR AND DEHYDRATION APPROACHES.

Introduction

Storing germplasm in tissue culture is useful for maintaining clones. Storing shoot-tips culture guarantee elimination of viruses, and are ideal for germplasm exchange. Alternatively, artificial seed,which are clonal propagules of the donor plants could allow storage of a unique genotype for prolonged periods of time. However, if artificial seed could be dried to moisture content typically found in true seeds (approximately 10 %) then it could have the advantage of serving as a germplasm storage system which maintains the propagules in a quiescent state for extended periods of time. It would also provide a more efficient usage of space and labour in a commercial production system.

Germplasm in cultures are stored under reduced conditions to avoid frequent subculturing. Germplasm can be stored in cultures at non- freezing, low temperature (1-9 °C). At these temperatures the ageing of the plant materials is slowed down but not completely stopped. Consequently, sub-culture of the plant material is necessary though only very frequently. An alternative often used with temperature reduction is the

application of hormone (ABA) or osmotic inhibitor like sucrose, hexitol like sorbitol, mannitol (Morel, 1975; Mullins and Schlegel, 1976; Lundergan and Janick, 1979; Kartha et al, 1981 Mix, 1981). Germplasm have also been stored under ultra-low temperatures (Kartha et al, 1981; Withers, 1986, Fabre and Dereudre, 1990). Mineral overlay for tissue conservation have also been tried (Caplin, 1959; Mathur et al., 1991).

However, the storage life for synthetic seed was found to be quite short using the current alginate protocol for alfalfa (Redenbaugh, 1986). When stored at 4°C there was a decrease in subsequent conversion after 7 days, while the conversion frequency of naked embryos was only slightly diminished over a 49-day period.

Attempts to dessicate somatic embryo (with or without encapsulation) to produce analogs to true seeds have met with limited success. Obendorf, (1986) was able to improve soyabean embryo conversion from 30% to 60% after embryo dessication at 70% humidity (Obendorf and Slawinska, 1986). Gray (1987b) found that desiccation appeared to release grape somatic embryos from a quiescent state and increased conversion (20-28% for desiccated embryos stored for 7-21 days and 5% for non-desiccated embryos). However, no coating method was used. He also observed that embryo conversion decreased rapidly with storage.

Senaratna et al. (1989a,b) observed that the application of ABA to cotyledonary stage of somatic embryo imposed quiescence and simultaneously induced the expression of desiccation tolerance. Once the embryos are at the cotyledonary stage of development, heat shock, cold stress, osmotic stress and nutrient deprivation can also be employed to induce similar responses (McKersie et al., 1989, Senaratna et al., 1989), which appear to act by stimulating the synthesis of ABA within the embryo itself.

Subsequently, somatic embryo can be dried to less than 15% moisture and stored for prolonged periods of time, similar to true seed. Fujii _et al_ (1989) reported increase in conversion frequency of alfalfa somatic embryo maturation with ABA, prior to planting. They were able to get 48% (6% without maturation treatment) conversion in soil under growth chamber conditions. Under green-house conditions, they were able to get 64% conversion when humidified air was used to prevent soil surface drying. Alternative to ABA- high sucrose concentration has been reported to alter maturation, prevent precocious germination and conversion of carrot embryos (Ammirato and Steward, 1971; Drew, 1979), as well as conversion and vigour of desiccated alfalfa somatic embryo when applied with ABA (Anandarajah and McKersie, 1990a,b). Increasing sucrose concentration in both media by 3-6% almost doubled the vigour without substantially reducing the quality of somatic embryo. McKersie _et al._ (1988) could store dry somatic embryo with no loss of viability for 8 months at room temperature and humidity. The present experiment is an attempt to store encapsulated plbs of an orchid _Cymbidium gigantium_ using various approaches like cold temperature, growth and osmotic inhibitors and dehydration. An attempt is also made to desiccate the non-encapsulated and encapsulated plbs and to see their storage potential after desiccation.

Materials and Methods

Incorporation of ABA and Sucrose in the medium .

Well developed, 90-day old plbs (2-3mm in diameter obtained from various explants of the _in vitro_ grown seedlings described in chapter II) grown in MS basal medium were selected separated into single plbs, blot-dried and placed aseptically

on MS medium (5 plbs in each flask, both semi-solid and liquid) containing different concentrations of sucrose (2-8%) and ABA (0.5-2.0 mg/l) and their effects on plb development and seedling growth studied. Plb development, weight, number were analysed after 30 days of incubation and seedling characteristics after 120 days. Sucrose (8 %) and ABA (1mg/l) were found to be the best for plb development and increase in fresh weight but considerably inhibited plb proliferation and seedling growth and hence these two concentrations were incorporated together in the medium to see their additive effect on plb development and seedling growth. The development of the seedlings were categorised into different stages and the time taken to reach these stages was noted.

Cold storage

Well developed plbs (3-4 mm) were selected, separated from each other, and blot dried. Some of them were encapsulated in 4% Na- alginate according to the technique of Redenbaugh et al.(1986) as described earlier. Beads of about 6mm diameter containing 1 plb were selected for the experiment. The beads were washed in sterile water and plated out in petriplates containing (i) a few drops of MS liquid nutrients with or without ABA + sucrose, (ii) MS solid nutrients with or without ABA + sucrose. 12 replicates of 10 beads each were taken for each treatment. Non- encapsulated plbs (12 petriplates of 10 beads each) for each treatment comprised the control. Six petriplates each of non-encapsulated and encapsulated plbs were kept in the fridge at 4°C and the other set kept at room temperature. Plbs were also stored in the above manner after desiccation.

Desiccation

Plbs grown in MS with and without ABA + sucrose after 30 days were taken for desiccation. Both non-encapsulated and encapsulated plbs were rapidly surface dried on filter paper and then dehydrated under sterile flow at culture room temperature and humidity. The moisture content of 10 beads and plbs were determined using 5 replicates of 10 beads/plbs each weighed each hr to determine their dry weight. The other 5 replicates of plbs (both naked and encapsulated) were plated out in the MS medium after desiccation, hourly, to determine their survival rate. Three hrs of desiccation treatment was found to be optimum for plb survival. Hence after 3 hr of desiccation, 12 replicates of 10 beads (non-encapsulated and encapsulated) were taken. 6 replicates were placed in the fridge and the other 6 at the room temperature.

Each replicate was taken out regularly at 5, 30, 60, 90 and 120 days interval (6 transfers), plated out in the MS medium contained in the flasks. Their survival and growth behaviour was observed. Survival was assessed from three independent experiments. The time taken by the plbs to produce shoot apex and to reach different stages were noted for both naked and encapsulated plbs.

Results

Effect of ABA and sucrose on plb-development and seedling growth

Sucrose at 4% and 6% was ideal for plb development and seedling growth (Table 14). However, plb proliferation was poor in 6% and 8% but the plbs were of bigger size with higher fresh weight and the seedlings had slower but healthy and rich growth. 10% sucrose was not good for plb development and resulted in retarded growth and loss of chlorophyll and ultimate death. In 8%

Table 14. Plb proliferation and seedling growth as affected by sucrose and ABA in the medium.

Treatment	Plb development			Seedling growth					
	Avg. inc. in wt. (g)	Avg. inc. in no.	Avg. plb vol. (mm^3)	Avg. F.W (g)	Leaf No.	Leaf Size (cm)	Root No.	Root Size (cm)	Dev.
Control (MS + 3% sucrose)	1.46	28	13.7	0.24	4	2.14	2	1.5	Good.
Sucrose (%)									
2	1.44	28	48.0	0.25	3	2.80	2	1.9	,,
4	1.46	55	64.0	0.28	3	2.90	2		,,
6	1.48	18	65.0	0.39	3	1.82	2	1.1	,,
7	1.48	12	72.0	0.39	2	1.30	2	0.6	Slow
ABA (mg/l)									
0.1	1.45	10	72.0	0.32	2	1.5	2	0.8	Slow
0.5	1.20	13	72.0	0.32	2	1.5	2	0.9	,,
1.0	1.45	10	100	0.38	2	1.2	2	0.6	,,
2.0	1.25	10	72.0	0.26	2	1.0	2	0.6	,,
ABA(1.0)									
+sucrose(8%)	1.46	12	72.0	0.37	2	1.2	2	0.6	,,

Avg.inc. : Average increase; F.W.: Fresh weight; Dev.:Development.

sucrose containing medium the plbs took 55 days to reach stage I compared to MS with 2% sucrose where stage I was reached in 10-15 days (Table 15). And hence the growth was considerably deferred by 40-45 days.

Plb proliferation was considerably affected by all concentrations of ABA (Table 14a). However, plb volume was more at all the concentrations being the highest at 1 mg/l of ABA. The growth of the seedlings was considerably delayed and after 120 days of culture the seedlings were at the two leaf stage only (Table 15). The plb volume in this treatment increased showing more fresh weight than plb proliferation (Table 14). Plbs took around 55 days to produce shoot apex compared to MS without ABA where shoot apex was produced in 10-15 days only. Thus ABA at 1mg/l could considerably defer growth of the plbs into seedlings without affecting the fresh weight.

Both sucrose 8% and ABA 1 mg/l were found to be most suitable for plb development and fresh weight but considerably inhibited plb proliferation and seedling growth (Table 14). These two concentrations were tried together in the medium to see their additive effect on plb development but the combination of ABA and sucrose gave no additive effect was observed in all the five replicates but there was continued inhibition of plb-proliferation and seedling growth hence both ABA (1mg/l) and sucrose (8%) were chosen for our subsequent work on plb storage before and after desiccation.

Experiments in petriplates with a few drops of nutrients

The plbs (naked and encapsulated) kept at room temperature and at 4°C were taken out regularly at an interval of 5, 30, 60, 90, and 120 days and placed in fresh MS medium in a flask and the rate of survival noted for each type of plbs (Table 16). The survival rate declined gradually for both naked and

Table 15. Development of seedlings from non-encapsulated and encapsulated plbs in various treatments of ABA and sucrose at room temperature and at 4°C.

Treatments		I	II	III	IV	V	VI
		Days required for development					
At room temperature							
MS	N	10-15	20	35	80	90	110
	E	20-25	30-35	55	70	85	90
ABA	N	55	90	120			
(1 mg/l)	E	60	90	120			
Sucrose	N	55	90	120			
(8%)	E	60	90	120			
ABA+Sucrose	N	55	95	120			
(1mg/l+8%)	E	60	90	120			
At 4°C							
	N	45	80	115			
MS	E	55	90	120			
ABA	N	60	95	125			
(1mg/l)	E	65	95	130			
Sucrose	N	60	90	120			
(8%)	E	65	95	125			
ABA+Sucrose	N	65	95	130			
(1mg/l+8%)	E	70	105	135			

N. non-encapsulated. E. encapsulated.

Stages I. Protocorms showing pointed vegetative apex (4-5mm);
II. Protocorms with leaf initials (6-8mm);
III. Two leaf stage with root initials (8mm-1cm);
IV. Three leaf stage with rhizoids (2.0-2.5cm);
V. Four leaf stage (2.5-3.0cm); and
VI. Four leaf stage with well developed leaves (3.0-3.5cm).

encapsulated plbs after 30 days of observation. The decline in survival rate was observed even after 5 days of storage at both room temperature and at 4°C. Though compared to the non-encapsulated plbs, the encapsulated plbs showed consistent high rate of survival throughout the 30 days of storage in both the media with or without ABA+sucrose. However , none of the plbs (naked and encapsulated) survived beyond 60 days. When plbs were plated out after 30 days of storage at room temperature the naked plbs showed 20% survival compared to encapsulated ones which showed 60% survival in ABA+sucrose containing medium. On the other hand, in media without ABA+sucrose, non-encapsulated showed no survival and encapsulated one showed 20% survival. Thus both non-encapsulated and encapsulated plbs showed higher survival rate in medium with ABA+sucrose than in medium without it.

At 4°C naked plbs showed 28% survival and encapsulated plbs 40% survival after 30 days of storage in the medium with ABA + sucrose.In the medium without ABA + sucrose, non-encapsulated showed no survival and encapsulated plbs still exhibited 20% viability. However , none of the plbs survived beyond 60 days in petriplates with limited amount of liquid nutrients. Addition of a few drops of liquid nutrients at the end of 30 days did not alter the survival rate of plbs.

In nutrient solid medium

At regular intervals the plbs were taken out and placed in fresh medium in a flask. At room temperature non-encapsulated and encapsulated plbs showed 100% survival (Table 16). However, at 4°C non-encapsulated plbs showed decline in the survival rate even after 5 days of storage and showed 60% survival in MS + ABA+ sucrose and 40% in MS without ABA+ sucrose at the end of 30 days.

Table 16. Survival percentage of non-encapsulated and encapsulated plbs stored for 120 days at room temperature and at 4°C.

Treatment	Duration of storage in days	At room temperature				At 4°C			
		Without ABA +Sucrose.		With ABA +Sucrose		Without ABA +Sucrose		With ABA +Sucrose	
		N	E	N	E	N	E	N	E
A.	5	80	88	80	92	72	72	70	76
	30	-	20	20	60	-	20	28	40
	60	-	-	-	-	-	-	-	-
	90	-	-	-	-	-	-	-	-
	120	-	-	-	-	-	-	-	-
B.	5	100	100	100	100	65	100	80	100
	30	,,	,,	,,	,,	12	40	15	60
	60	,,	,,	,,	,,	,,	,,	,,	,,
	90	,,	,,	,,	,,	,,	,,	,,	,,
	120	,,	,,	,,	,,	,,	,,	,,	,,

A. Plbs kept in a few drops of nutrients.
B. Plbs kept on nutrient solid medium.
N. Non-encapsulated plbs.
E. Encapsulated plbs.
-. No survival.

Compared to non-encapsulated plbs encapsulated plbs showed consistent high rate of survival and at the end of 30 days the survival rate of non-encapsulated plbs reduced drastically to 15% in MS with ABA and sucrose at 4°C and 12% without ABA+sucrose.

At room temperature the non-encapsulated plbs kept in MS medium took around 35 days to reach stage III (three leaf stage) and encapsulated plbs took 55 days (Table 15). PLbs cultured in MS with ABA and sucrose showed considerable growth inhibition and reached stage III only at the end of 120 days (non-encapsulated) and 125 days (encapsulated). Even the plbs kept at 4°C showed considerable growth inhibition and reached stage III only at the end of 130 (non-encapsulated) and 135 days (encapsulated). The low temperature reduced the growth of plbs as also ABA. The growth inhibition by ABA at 4°C was slightly more compared to its influence at room temperature. Even at room temperature the plbs kept in ABA +sucrose did not show much proliferation (Table 14). This growth inhibition of plbs stored at 4°C and grown in medium with ABA + Sucrose were temporary and the plbs showed normal growth as soon as they were placed in the fresh medium.

Desiccation

Table 17 shows the moisture content (% of fresh weight) of the non-encapsulated and encapsulated beads (kept in MS with ABA + sucrose for 30 days) dehydrated for 4 hrs. When dried for 4 hrs the water content of non-encapsulated plbs dropped to 45% of fresh weight and encapsulated plbs to 33% of fresh weight. After 4 hr of desiccation the survival % of non-encapsulated plbs was nil whereas encapsulated plbs still showed 85% survival. Since the encapsulated plbs showed decline in per cent survival after desiccation of 4 hrs, only 3 hrs of

Table 17. Water content (% of fresh weight) and percentage survival of treated non-encapsulated and encapsulated plbs after hourly dehydration.

Duration of dehydration (hrs)		Initial weight	Final weight	% moisture content	Percentage survival
1	N	1.6	1.4	87	100
	E	5.1	4.0	78	100
2	N	1.4	1.1	68	80
	E	4.0	3.1	43	100
3	N	1.1	0.9	59	50
	E	3.1	2.2	43	100
4	N	0.9	0.7	45	0
	E	2.2	1.7	33	85

N. non-encapsulated. E. encapsulated.

desiccation was done in the subsequent experiment. For the whole beads, a moisture content of about 43% of fresh weight seemed to be the optimum. Lower moisture content resulted in gradual decline in the survival rate. The survival percentage of 3 hr dehydrated non-encapsulated and encapsulated plbs stored for a duration of 120 days at room temperature and at 4°C are given in Table 18. The desiccated plbs (nonencapsulated) could not be stored in this condition as such. It had to be rehydrated straightaway by placing them either on filter paper soaked in water or liquid/solid medium. Even the encapsulated plbs stored after desiccation lost water rapidly when stored at room temperature and lost viability within a day. The orchid plbs can thus be categorised into desiccation intolerant species. Non-encapsulated plbs showed 50% survival after 5 days and 20% survival after 30 days of storage at room temperature. Three hours of desiccation did not affect the survival rate of encapsulated plb and showed 100% survival, both after 5 and 30 days of storage at room temperature. There was a decline in the survival of encapsulated plbs stored at 4°C on the 30th day compared to storage at room temperature. Non-encapsulated plb showed 10% survival after 30 days of storage and 0% after 60 days of storage at 4°C. So desiccation greatly harmed the non-encapsulated plbs but did not seem to affect much the encapsulated plbs.

Discussion

The present experiment using different concentrations with of sucrose and ABA revealed that ABA at 1mg/l and sucrose 8% considerably delayed plb proliferation and growth but allowed plb development by inducing the increase in fresh weight and hence these concentrations of sucrose and ABA were chosen for

Table 18. Survival percentage of plbs stored after desiccation for 3 hrs at room temperature and at 4°C.

Duration of storage	Room temperature		At 4°C	
	N	E	N	E
5	50	100	40	100
30	20	100	10	60
60	,,	,,	,,	,,
90	,,	,,	,,	,,
120	,,	,,	,,	,,

N. Non-encapsulated.

E. Encapsulated.+

subsequent work on plbs storage before and after desiccation. Infact these two concentration of ABA and sucrose individually or in combination in the medium delayed plb and seedling growth as seen by the fact that at the end of 120 days the seedlings had reached stage III (two leaf stage) only.

The low but consistent survival of encapsulated cold stored plbs emphasize the potential of calcium alginate as a coating compound which seems to provide protection perhaps preventing lethal embryo desiccation. Although calcium alginate coating consistently enabled plbs to withstand cold-temperature treatment and desiccation, the survival was improved by ABA+sucrose treatment. ABA + sucrose had 4 effects - i) reduction in the number of plbs, ii) enhancing maturation by increase in fresh weight, iii) inhibition of growth, iv) increased survival after cold treatment and desiccation.

Reduction in the number of plbs by ABA treatment is in agreement with the work of Fujimura and Komamine (1975), Kamada and Harada (1979, 1981) who reported reduction in somatic embryo number with increasing ABA concentration. They also observed retardation in embryo development but not embryo initiation. Kitto and Janick (1985a,b) reported production of fewer embryos with ABA treatment in comparision to those not treated, a greater number of those embryos survived polyox encapsulation. This enhancement of survivability was inferred from the hardening effect attributed to ABA (Walton, 1980; Wong and Sussex 1980 ; Kinf, 1982). ABA regulates many activities late in embryogeny associated with maturation. An increased endogenous ABA level is associated with reduction in precocious germination of the embryo produced in vivo (Santos and Yamaguchi, 1979; Robichaud et al., 1980) and in vitro (Ammirato, 1974; Kamada and Harada, 1981) which suggests that ABA induces embryo quiescence. ABA-

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X 1210 OPH 8 SHILLONG 13 31

FOR+ PROIFESSOR HC
ALRYA B/106
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VIVA VOICLE ELXA
MINLATIONON 6 TH OCLTOBLER 1995 CONFIRMED AAAKINDLYALTTEND AAACANDIDA
TE SHASHI CORRIE AAA GRATEFUL CONF+CONTROLLER EXAMINALTION

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treated embryos , while quiescent, continue to synthesize specific proteins associated with embryonic growth and seed maturation (Crouch and Sussex, 1981) . Increase survival of encapsulated ABA treated carrot embryos may be due to imposition of a developmental arrest (quiescence) during which time embryos mature and develop desiccation tolerance. Ammirato (1974) observed restriction of cell expansion preventing premature cell expansion and precocious germination by ABA in carrot cells. ABA when added at concentration that did not totally inhibit growth and development could selectively inhibit certain aspects of somatic embryo development (Ammirato, 1974).

During maturation, embryos produced in vivo acquire a resistance to desiccation (Abrol and Mc Ilrath, 1966) that has been associated with high endogenous ABA levels. Long (1979) has also shown immature zygotic embryos of Phaseolus desiccation resistance during exposure to ABA. Fujii et al. (1990) used ABA in the maturation medium to prevent precocious germination of somatic embryos of alfalfa and reported increased embryo conversion with ABA treatment. They also reported accumulation of storage reserve levels on exogenous application of ABA. Mc Kersie (1989) reported inhibition of precocious germination and increased seedling vigour. The inclusion of ABA in the medium with 6% sucrose further improved the embryo quality .

High sucrose concentrations have been reported to alter maturation, conversion and vigour of desiccated alfalfa somatic embryo when applied in conjunction with ABA (Anandarajah and Mc Kersie, 1990). Ammirato and Steward (1971) reported inhibition of precocious germination of somatic embryos of carrot and parshnip by raising the osmotic concentration of the culture medium by elevating the sucrose level. Sucrose plays two important roles

in vitro as a carbon source and as an osmotic agent. Increased osmotic stress has been associated with cell plasmolysis (Loveys*et al*. 1975), cell growth inhibition (Wong and Sussex, 1980), and elevation of endogenous ABA level (Loveys *et al*., 1975; Wong *et al.*, 1980). Exposure of embryo to high sucrose concentration has been linked to maturation (Drew, 1979), inhibition of precocious germination (Ammirato and Steward, 1971) and acquisition of desiccation tolerance (Norstog, 1966). With sucrose treatment, Anandarajah and Mc Kersie(1990) found somatic embryo of alfalfa tolerant of drying without exposure to ABA. Combination of sucrose and ABA further improved the quality of embryo. The high sucrose concentration in the media may have increased the osmolarity which may have evoked endogenous ABA synthesis, thus mimicking the *in vivo* osmotic environment of developing zygotic embryo in controlling embryo development and maturation (Moriss *et al.*, 1988).

In the present experiment the conversion of plbs was delayed significantly at sucrose concentration above 6%, the maximum delay occured in the medium containing 8% sucrose. At the end of 120 days, the seedlings reached stage III only. Plbs grown in 8% sucrose exhibited high embryo fresh weight, improved survival after drying, high plant conversion and enhanced vigor.

Non-encapsulated and encapsulated plbs kept in a petriplate with a few drops of nutrients failed to survive beyond 60 days even though encapsulated plbs showed consistent high survival rate than non-encapsulated plbs. The possible reasons could be depletion of nutrients at an extremely fast rate, over-crowding (10 beads in a small Laxbro petriplate) and hence depletion of O_2 , CO_2 too quickly or their short supply , or due to toxicity due to accumulation of waste products. The plbs

gradually lost chlorophyll became brown, the beads got discolored and ultimately died. The viability was more with ABA+sucrose in the medium. With further research and findings of the limiting factors under such conditions, the technique of storing artificial seeds with limited nutrients would definitely be a better approach to storing artificial seeds. Thus treatment of plbs by exposure to high sucrose and ABA increased survival of plbs after encapsulation relative to non-treated plbs. These findings are in line with the findings of Kitto and Janick (1985), who found that in comparison to untreated, a greater percentage of treated embryos survived polyox encapsulation . The physiological basis for this enhancement of survivability can be inferred from the hardening effect attributed to ABA.

In the present experiment, encapsulated plbs (pretreated with ABA + sucrose for 30 days) could survive drying test more than untreated. In many respects plbs cannot be compared with the somatic embryos as embryo in case of orchidaceae is nothing more than an undifferentiated mass of cells, without nutritive tissue (endosperm), and no cotyledons, plumule, radicle can be seen. Hence an undifferentiated mass of parenchymatous cells, possibly cannot be made to acquire desiccation tolerance. However, ABA + sucrose treatment could definitely alter the survival percentage.

Keeping the plbs at 4°C without ABA + sucrose also showed considerable arrest in growth of the plbs, similar to that shown by ABA + sucrose treatment. However, there was drastic decrease in survivability in medium without ABA+sucrose than with ABA+ sucrose.

Keeping the plant tissue at 4°C may mimmic some effects of cold hardening that plants aquire outdoors as temperatures decrease in the winter. The first measurable event occurring

during cold acclimatization are cessation of growth (Kacpers-Palaez, 1978), accumulation of carbohydrates (Yelenosky, 1979) and reduction of moisture content (Huneret al., 1981). Associated with the reduction of intracellular water is an increased tolerance of protoplasm to dehydration (Chen and Gusta, 1978). Cold hardening is associated with ABA in some species. Phaseolus vulgaris plants exposed to 5°C had increased ABA levels (Eamus and Wilson, 1983). The morphological appearance of alfalfa seedlings was similar either when cold hardened or ABA treated (Rikin et al., 1975). in vitro storage of plants at low temperature has been reported for several fruit trees and herbage plants . For example grape plants have been stored for over 15 years at 9°C by yearly transfer to fresh medium (Morel, 1975). Medicago sativa has been stored for 15-18 months with 94-95% viability (Cheyne and Dale, 1980), Malus domestica for 12 months with 100% viability (Lundergan and Janick, 1979). Besides mineral overlay for tissue conservation has been tried (Caplin, 1959; Mathur et al 1991). Mathur et al (1991) reported that the shelf life of encapsulated propagules of Selinum candola, Nicotiana tabacum and Valeriana wallichi was enhanced from 25-30 days to 150-240 days, when the cultures were overlaid with mineral oil. The rate of growth and morphogenesis in cultures covered with mineral oil was greatly reduced as compared to the control.

Encapsulated plbs grew after rehydration indicating that the growth retarding effect of the hardening treatments was revesible. Plbs within the gel survived storage in darkness for as long as 30 days at 4°C after which there was no change in survival percentage as all the plantlets had established themselves.

Desiccation of the plbs served no purpose as there was no

enhancement in the survival rate after desiccation. Orthodox seeds remain viable over long periods if both moisture and storage temperature are reduced (Roberts, 1973). More research is needed to define the storage characteristics of orchid plbs and to determine the precise relationship between viability and time limits of the system. The high survival of coated plbs clearly indicate that the new process of coating and dehydration can be used for preservation of plbs at 4°C which can subsequently regenerate into plantlets. This process is easy , presents some advantages as regards micropropagation and allows the establishment of germplasm bank.

The successful encapsulation and storage of orchid plbs as that of somatic embryo has a number of implications for the seed industry. Additional studies would be required to better define hardening treatments, coatings, storage conditions and determination of applicability to a range of germination environment.

VI. COMPARISION OF PHENOTYPIC CHARACTERS IN THE REGENERANTS FROM ENCAPSULATED AND NON-ENCAPSULATED PLBS.

Introduction

For the purpose of germplasm preservation and plant micropropagation the inherent stability of the cultures are required. However, cultured cells are not usually genetically stable. Polyploidy, aneuploidy and chromosome structure changes, single gene changes in cells in cultures under various conditions are extensively described (D'Amato, 1978; Larkin and Scowcroft, 1981; Edallo et al, 1981; Barbier and Dulieu, 1981; McCoy and Phillips, 1982). Cultures over prolonged periods are known to cause changes in karyotypic structures (Baybliss,1980). Variation generated by the use of tissue-culture cycle has been termed somaclonal variation by Larkin and Scrocroft (1981). Plants regenerated from shoot apex cultures occasionally include variants (Bush et al., 1976) Templeton-Somers and Collins (1985) reported yield difference in clonally propagated sweet potato, even the explant origin was a significant factor in yield.

Subtle morphological, physiological and anatomical changes are known to occur in plants grown in cultures (Grout and Aston, 1977a ; Sutter and Langhans, 1979 , 1982 ; Fuchigami et al.,

1981; Brainerd and Fuchigami , 1981 , 1982 ; Wetzstein and Sommer L, 1982 ; Wardle and Short, 1983; Lee _et al_,1985; Cappellades _et al_.,1990).

The use of scanning electron microscope (SEM) for examining the external manifestation of various developmental processes in plants, both _in vivo_ and _in vitro_, is becoming increasingly common and useful. Its ability to look directly at the external features of a specimen with great depth of field at high magnification and high resolution has made the SEM a valuable tool in the study of morphogenesis in a variety of materials and species of Gramineae (Haydu and Vasil , 1981; Lu _et al_., 1982; Botti and Vasil, 1984).

The chromosome number in Indian Orchids vary from 2n = 20 - 164 with nearly 65% of species showing 2n = 38 / 40 or 42 (Vij and Shekhar , 1986). 2n number of _Cymbidium giganteum_ has been established to be 40 (Mehra and Kashyap, 1978; Mehra and Sehgal, 1980 ; Vij _et al_, 1983).

The altered environmental conditions for orchid plbs due to encapsulation especially the various antimicrobial agents used in the matrix might bring about variations in the morphological and genetical constituents in the regenerants. So, the present study was carried out to determine any variations in the regenerants of encapsulated plbs.

Materials and Methods

Various stages of development were defined and the time taken to reach these stages by the regenerants of non-encapsulated and encapsulated plbs (kept at room temperature and at 4°C) were recorded and compared (details given in chapter IV and V).

The leaf-size , leaf number , root size and root number and

fresh weight of the 120-days old seedlings (four leaf stage with well developed leaves) derived from encapsulated and non-encapsulated plbs were measured and compared. The regenerants of encapsulated and non-encapsulated plbs stored at 4°C for 30 days were also analysed.

For SEM studies of leaf surface, mainly with regard to stomatal structure, the leaves from 120-day old seedlings were fixed in 3% glutaraldehyde made in 0.2M cacodylatic buffer for 4 *hrs, and, then washed thoroughly in 0.1 M buffer. The material* was then dehydrated in a series of various concentrations of acetone starting from 30%, 50%, 70%, 80%, 90% and 95%. The material was kept for 15 min in each of the concentrations and finally in dry acetone for 1 hr. The material was then kept for freeze drying (critical point drying) for an hour and then coated with thin conductive metal film (gold) to prevent charging artefact when the electric film hits the specimen under high accelerating voltage. The metal film offers a high secondary electron emission co-efficient and also stabilizes the specimen mechanically. The specimen was then bombarded with the electron film and the surface characters studied on the screen mainly with regard to the ultra-structure of the stomata and root surface.

Results

There was no significant difference between the regenerants of naked and encapsulated plbs (at stage III and IV) as regards to the morphological characters(Fig 38 & 39). Even the regenerants from 30 days stored plbs were morphologically similar and did not show much difference (Table 19). However they showed significant difference in the days required to reach different stages showing different rate of growth (details given in chapter

Table 19. Morphological parameters of regenerants of non-encapsulated plbs (kept at room temperature and at 4°C) at four leaf stage .

Treatment		Fresh weight (g)	Leaf No.	Leaf size (cm)	Root No.	Root size (cm)	Colour
RT	N	0.44	3.0	3.8	3.0	2.3	D. green
	E	0.42	3.1	4.0	2.8	2.0	"
4°C	N	0.39	2.9	4.0	2.0	2.0	Green
	E	0.35	2.6	4.0	1.8	2.0	Green

RT, Room temperature. N, non-encapsulated.
E, encapsulated

Fig. 38 . Regenerants of encapsulated plb after two years of culture.

Fig. 39. Regenerants of non-encapsulated plb after two years of culture.

Fig. 38

Fig. 39

IV).

SEM studies did not reveal any significant difference in the structure of the stomata in the leaves of the regenerants from encapsulated and non-encapsulated plbs. All the stomatas were of open type in the leaves of in vitro plants whereas both open and closed stomatas were present in the leaves of in vivo plants (Fig 40 & 41). The root surface of both regenerants from encapsulated and non-encapsulated did not show any difference.The unicellular root hairs were as numerous in both.

The root-tip squash showed no difference in the chromosome number of both regenerants from both encapsulated and non-encapsulated plbs. The cells at the metaphase stage showed 2n = 40 in both the cases.

Discussion

The seedlings obtained from encapsulated and non-encapsulated plbs (both stored and non-stored) did not differ morphologically. However, they showed differences in the rate of growth as shown by the days needed by these plbs to reach different stages.

The significant finding of the SEM study of the structure of stomata showed permanently open stomatal aperture in the leaves of in vitro grown plantlets as compared to the in vivo plantlets. This observation is in line with the observation of Brainerd and Fuchigami (1982) and Capellades et al. (1990) who reported the presence of non-closing stomata of in vitro raised plants. The impaired stomatal mechanisms has been attributed to the abnormal orientation of micro-fibrils and high deposition of callose in guard cells (Wardle and Short,1983) as well as high levels of Na^+ in guard cells, which may interfere with the movement of K^+ (Wardle et al., 1981).

Fig. 40. Scanning electron microscope view of leaf surface of *in vitro* grown plantlet (x 2200).

Fig. 41. Scanning electron microscope view of leaf surface of *in vivo* grown plantlet (x 860).

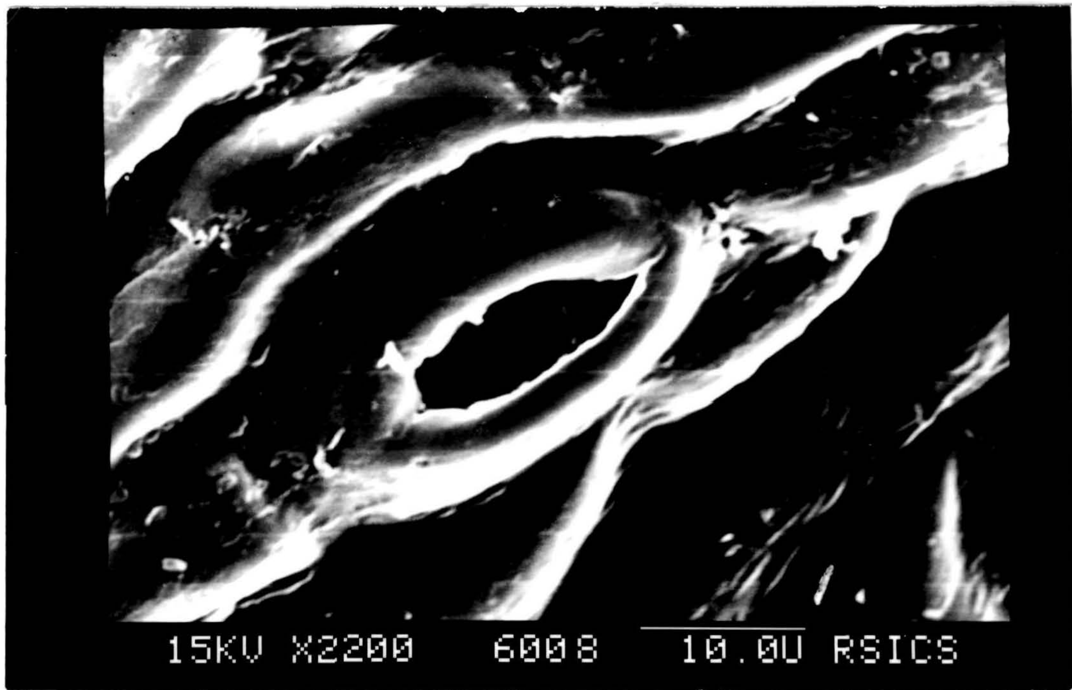


Fig. 40

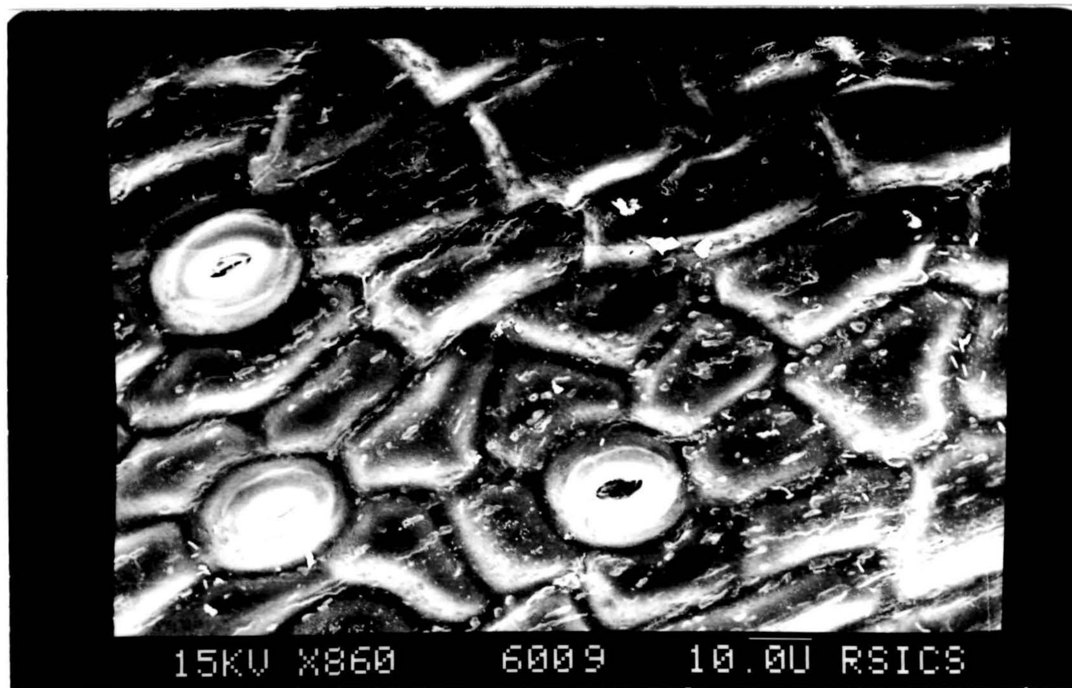


Fig. 41

The root tip squash in regenerants of encapsulated and non-encapsulated plbs (both stored and non-stored) showed no difference in the chromosome number. The cells at metaphase stage showed 2n = 40 in both the regenerants which is the normal chromosome number for C.giganteum as reported in the literature (Sharma and Chatterji, 1966; Mehra and Sehgal, 1980; Vij et al., 1983). Various chromosomal abnormality as reported by several workers (D'Amato, 1971; Larkin and Scowcroft, 1981) were not observerd in the present investigation showing thereby that encapsulation and also the various antimicrobial agents incorporated in the capsule are not harmful for the plbs and does not bring about any variations in the regenerants. Mathur et al. (1989) reported no adverse effect on the regenerant using rosebengal and bavistin in the encapsulating matrix for in vivo planting of axillary buds of Valeriana wallichi. Daubeny et al. (1976) pretreated the strawberry plants with fungicides before storing but found no ill-effects of fungicides treatment on the regenerants. Even the regenerants of plbs stored at 4°C for 30 days showed no aberrant features. Thus the regenerants of encapsulated plbs were phenotypically and genotypicaly similar and did not show any changes in mitotic chromosome number, and morphological characteristics.

VII. EVALUATION AND PARTIAL CHARACTERISATION OF TOTAL SOLUBLE PROTEINS, ACTIVITIES OF PEROXIDASE AND IAA OXIDASE AT VARIOUS STAGES OF GROWTH IN THE REGENERANTS FROM ENCAPSULATED AND NON-ENCAPSULATED PLBS.

Introduction

It has been well established that marked changes occur in the protein constituents of plants at various stages of growth (Coulson and Sim, 1965; Macko et al., 1967; Nainawatee et al., 1974). It has also been found that the plants grown under different environmental conditions show minor qualitative changes in protein (Koering et al., 1964). Protein synthesis pattern has also been shown to change dramatically when seedlings are subjected to different stresses (Key et al.,1981).

The role of enzymes in relation to germination and growth has been focussed by several workers (Scandalios, 1974 ; Blakeman et al., 1976; Gaspar et al., 1977, Dendsay and Sachar 1982; Barendse, 1983; Pilet et al.,1984; Miller, 1985). Studies have also been made of the multiple molecular forms and differential electrophoretic mobilities of enzyme components at various stages of growth (Honold et al., 1966, Macko et al.,1967; Bhatia and Nelson, 1968; Singh and Singh, 1974). The enzyme and isoenzyme pattern of plants have also been known to be influenced by the

physiological and environmental conditions (Bjorn, 1967; Roberts, 1969; McCown et al., 1970; Nainawatee, 1971; Singh and Singh, 1974; Goyal, 1989). Goyal (1989) reported an increase in IAA oxidase activity following a period of water stress.

The IAA oxidase/ peroxidase has been widely studied in an attempt to understand the regulation of IAA level in plant tissues. The oxidation of IAA means inactivation of the hormone, and therefore, the control of IAA oxidase and/or peroxidase activities, that may have repercursions for IAA synthesis and degradation. Thus, it may be involved in plant growth response to auxins (Barendse, 1983).

Isozymes have been employed as effective markers particularly in studies on differentiation and in altered plant growth and development (Scandalios, 1974, 1977; Van Huystee and Cairns, 1980, 1982). For several years peroxidases have been considered to be growth regulators because of their action on ethylene levels (Gaspar et al.,1985) or on auxin levels (Srivastava and Van Huystee, 1973). There is a hypothesis that the activity of peroxidase and the growth of plant cells are inversely related (Sembdner et al.,1980; Van Huystee and Cairns, 1980). The peroxidase bound to the cell wall play a key role in its hardening and in processes associated with plant growth (Taiz, 1984).

The peroxidase activity has been found to be the exact reciprocal of IAA production in Vanda seedlings (Alvarez, 1968; Alvarez and King,1969). Dendsay (1989) has also found the increase in peroxidase activity by two to three-fold in mung cotyledons as a result of exogenous application of IAA. Blakeman et al. (1976) reported the increased oxidative enzyme activity in the infected protocorms of Dactylorhiza purpurea and Cymbidium

hybrid by an endophytic mycorrhizal fungus, Rhizoctonia sp. Kumaria et al (1990) studied the effect of growth regulators on activities of oxidative enzymes in the germinating protocorms of C. giganteum.

Though extensive work has been done on isozyme pattern of oxidative enzyme during the growth in various plants very few are concerned with orchids. The present investigation was undertaken to observe the effects of encapsulation on plb growth using protein profiles and isoenzymes as markers.

Material and Methods

The qualitative assay of peroxidase and IAA oxidase and total protein content and electrophoretic studies were carried out at various stages of development from the regenerants of encapsulated and non-encapsulated plbs in the following manner. The growth and development of the seedlings were divided into six stages as described in chapter IV.

Protein estimation

Fresh samples (1g) were chopped and macerated in a mortar and pestle with hot 80% ethanol and centrifuged at 2,000 rpm for 20 mins. The supernatant was discarded and the pellet suspended in 5 ml of 5% TCA in ice-bath for 15 mins. The pellet was re-extracted in absolute ethanol and twice with hot ethanol and twice with hot ethanol-ether mixture everytime discarding the supernatant after centrifugation. The pellet contained protein which was estimated following the method of Lowry et al.(1951).

The protein sample was treated with 1 ml of 1N NaoH at 100°C for 4-5 mins. Then 5 ml of alkaline copper reagent was added to it and the mixture was allowed to stand at room temperature for 10 mins. Then 5 ml of Folin-Cio-calteu reagent

was added rapidly and mixed immediately. After 30 mins the absorbance was measured at 750 nM in a colorimeter using bovine serum albumin as a standard.

Tissue extraction: One gram each of the above mentioned categories of tissues (regenerants of non-encapsulated and encapsulated plbs at different stages) were homogenized separately in 10 ml of pre-chilled phosphate buffer (0.2M, pH 6.0). The homogenates were squeezed through double-layered cheese-cloth and centrifuged at 10,000 rpm for 20 min at -5°C in the refrigerated centrifuge. The supernatants were used for subsequent analytical studies of enzymes.

(i)**Peroxidase** Peroxidase activity was assayed with o-dianisidine as hydrogen donor at 460 nM (following the method of Worthington enzyme manual,1972) at 25°C . To a 1 cm cuvette, 2.7 ml of 0.2 M phosphate buffer (pH 6.0), 0.1 ml of H_2O_2 (0.3%) and 0.1 ml of enzyme were added and the cuvette inverted immediately to mix the contents. Absorbance was calibrated at zero time and 0.1 ml of 0.1% dianisidine (in methanol) was added. The change in absorbance at 0, 30 and 60 s was recorded. The enzyme activity is expressed as change in absorbance per min per mg protein .

(ii) **IAA oxidase**- The activity of this enzyme was measured by the method of Tandon and Arya (1982). In a test tube 1 ml of 1mM 2,4-dichlorophenol (DCP), 1ml of 1mM $MnCl_2$, 1.5 ml of phosphate buffer (pH.6-0.2M), 0.5 ml of enzyme extract and lastly 1 ml of IAA (1mM) was added. The mixture was incubated at 37°C in a shaking water bath. After 1hr, 2 ml of Salowski reagent (1ml of 0.5 M ferric chloride in 50 ml of 35% perchloric acid) was added to each tube to terminate the reaction and following 1 hr wait, the absorbance of the mixture was measured at 530 nm. The amount of IAA destroyed was calculated from a standard curve of IAA.

The enzyme activity is expressed as mg IAA destroyed per g fresh weight of tissue per hr at 37°C and also per mg protein.

Gel-electrophoresis- The same enzyme extract was used for electrophoretic analysis. The polyacrylamide gel were prepared following the formulation given by Davies (1964). Each batch of gels were prepared by mixing one part of stock solution A (composed of 48 ml 1N HCl, 36.6 g TRIS, 0.23 ml. TEMED made upto 100 ml in distilled water), one part of stock B (composed of 28.0 g acrylamide, 0.735 g bis acrylamide made upto 100 ml in distilled water) and two parts of distilled water and two parts of stock solution C (composed of ammonium per sulphate solution 0.14 gm/100 ml freshly prepared). Tris-glycerine buffer (pH 8.3) was used as an electrolyte.

The gel tubes were mounted vertically in a polymerisation stand having their lower end closed into mounted tubes. Few drops of water was carefully layered on top of each gel solution. The gels were then allowed to polymerise at room temperature for about 40-60 mins. After removing water from the top of the gels, the tubes were placed in electrophoretic chamber in such a way that the lower ends of the tubes were dipped in the buffer of the lower chamber. The operating buffer was then poured in the upper chamber in volume which was sufficient to dip the tubes completely. The samples were mixed with a few drops of freshly prepared 40% sucrose solution and a few drops of bromophenol blue (0.1%) and applied (0.2 ml each in each tube) on the surface of the gels in the tubes. Initially (8-9 mins) a constant current of 4 mA per tube was applied for 2 hr at room temperature. This allowed the tracking dye to reach the bottom of each gel and resulted in the optimal resolution of bands.

After electrophoresis, the gels were taken out from the

tubes by squirting water from a syringe between gel and glass wall. The staining was achieved by keeping the gels for 20-30 mins in 1% w/v solution of amido black in 7% w/v acetic acid. The gels were then mechanically destained at 40°C with a solution of 7% acetic acid and stored in distilled water.

For the staining of peroxidases, hydrogen peroxide (3.0%) was used as a substrate and benzidine as stain. Benzidine solution was prepared by mixing 1 gm benzidine powder in 25% acetic acid. The H_2O_2 and benzidine solution was mixed in the ratio of 1:1 and poured on the gels submerging them. The isozymes of peroxidases appeared in blue coloured bands. The bands were categorised as dense, medium and light depending on the intensity of the colour and the gel patterns were compared visually.

Results

Protein- Estimation of total soluble proteins at different stages of growth showed a progressive increase in the regenerants of both encapsulated and non-encapsulated plbs from stage I to stage V (Table 20). However, the protein content of regenerants of encapsulatd plbs was always slightly lower than the non-encapsulated plbs at all the stages of their development. The protein bands obtained by electrophoretic separation showed 5 bands of different width and intensity at all the stages of development. The bands were of lighter colour at the initial stages and darker at the later stages (Figs.42-45).

Peroxidase- The assay of peroxidase activity at different stages of development in the regenerants of encapsulated and non-encapsulated plbs revealed a considerable variation at different stages of development (Table 21). A higher peroxidase activity was recorded at stage I which decreased gradually at later stages

Table 20. Total Protein estimation at various stages of development in the regenerants of encapsulated and non-encapsulated plbs.

Stages of development		mg/g fresh weight
I	N	12.994
	E	11.151
II	N	14.095
	E	11.471
III	N	14.683
	E	12.883
IV	N	14.757
	E	12.907
V	N	14.712
	E	13.405

N. non-encapsulated. E. encapsulated.

Stages. I. Protocorms showing pointed vegetative apex (4-5mm).
II. Protocorms with leaf initials (6-8mm).
III. Two leaf stage with root initials (8mm-1cm).
IV. Three leaf stage with rhizoids (2.0-2.5cm).
V. Four leaf stage (2.5-3.0cm).

Table 21. Peroxidase activity at various stages of development in the regenerants of encapsulated and non-encapsulated plbs.

Developmental stages		Change in absorbance /min/g fresh weight	Change in absorbance /mg protein.
I	N	29.10	2.24
	E	30.10	2.69
II	N	26.30	1.86
	E	27.90	2.43
III	N	16.10	1.40
	E	18.90	1.46
IV	N	15.30	1.03
	E	17.00	1.31
V	N	7.40	0.50
	E	7.00	0.52

N. non-encapsulated. E. encapsulated.

Stages. I. Protocorms showing pointed vegetative apex (4-5mm).
II. Protocorms with leaf initials (6-8mm).
III. Two leaf stage with root initials (8mm-1cm).
IV. Three leaf stage with rhizoids (2.0-2.5cm).
V. Four leaf stage (2.5-3.0cm).

Fig. 42-45. Protein profile in the regenerants of

- non-encapsulated plbs at stage I (Fig. 42),
- encapsulated plbs at stage V (Fig. 43),
- non-encapsulated at stage I (Fig.44),and
- encapsulated plbs at stage V (Fig.45).

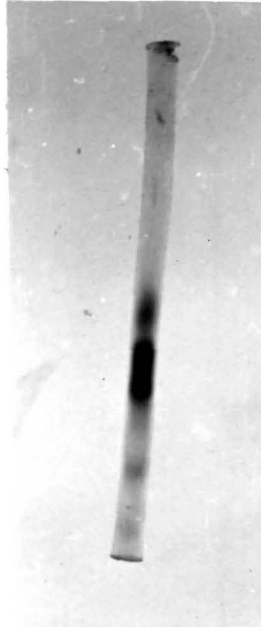


Fig. 42

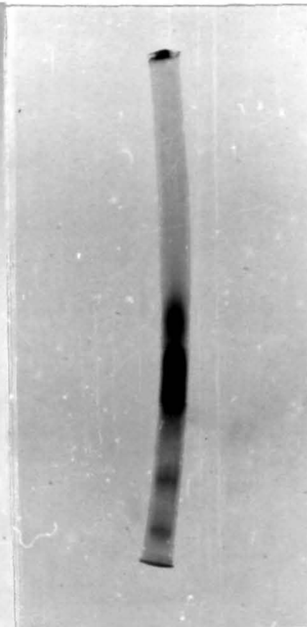


Fig. 43

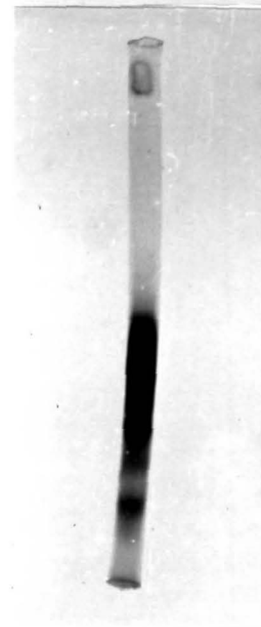


Fig. 44

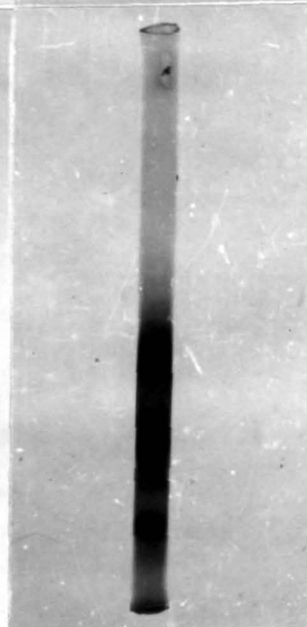


Fig. 45

of development in both the regenerants of encapsulated and non-encapsulated plbs. The regenerants of encapsulated plbs always showed higher activity than regenrants of non-encapsulated plbs. The peroxidase activity decreased quite dramatically at stage V. The peroxidase bands obtained by electrophoretic separation showed a decrease in the number of bands from 5-1 (Fig 46-49) which however differed in their intensity showing higher intensity at lower stages than later stages. There was not much difference in the regenerants of encapsulated and non-encapsulated with regard to the peroxidase bands. The peroxidase activity per unit protein also showed a pattern similar to its activity based on fresh weight.

IAA oxidase- The IAA oxidase showed a progressive decline in activity from plb stage to well developed seedling stage in the regenerants of both encapsulated and non-encapsulated plbs, the activity being more in the regenerants of encapsulated plbs than non-encapsulated plbs (Table 22). However, on unit protein basis the IAA oxidase activity gave a different picture - the non-encapsulated regenerants had higher activity than encapsulated regenerants.

Discussion

Comparison of total proteins and protein profiles of the extract obtained at different stages of development from encapsulated and non-encapsulated plbs did not show any significant difference except the regenerants of encapsulated plbs showed slightly lower total protein content than regenerants of non-encapsulated plbs at all the stages of development. This minor quantitative change in the protein content in the regenerants might be due to the effects of

Table 22. IAA oxidase activity at various stages of development in the regenerants of encapsulated and non-encapsulated plbs.

Developmental stages		IAA oxidised /g fresh weight (μm)	IAA oxidised /mg protein (μm)
I	N	26.208	2.356
	E	28.362	2.183
II	N	25.804	2.240
	E	27.552	1.950
III	N	24.680	1.915
	E	25.632	1.745
IV	N	23.876	1.849
	E	24.014	1.627
V	N	21.883	1.632
	E	23.523	1.597

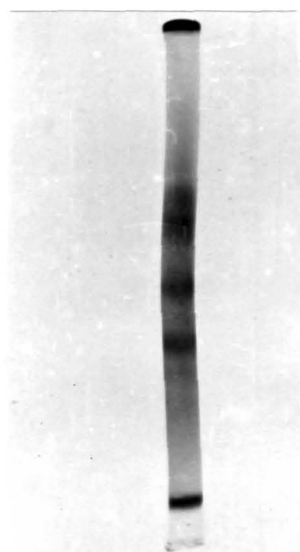
N. non-encapsulated. E. encapsulated.

Stages. I. Protocorms with pointed vegetative apex (4-5mm).
II. Protocorms with leaf initials (6-8mm).
III. Two leaf stage with root initials (8mm-1cm).
IV. Three leaf stage with rhizoids (2.0-2.5cm).
V. Four leaf stage (2.5-3.0cm).

Fig 46-49. Peroxidase bands in the regenerants of

- non-encapsulated plbs at stage I (Fig.46),
- encapsulated plbs at stage V (Fig.47),
- non-encapsulated plbs at stage I (Fig.48),and
- encapsulated plbs at stage V. (Fig.49).

Fig. 46



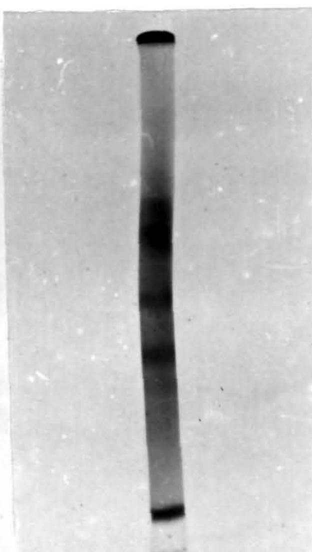
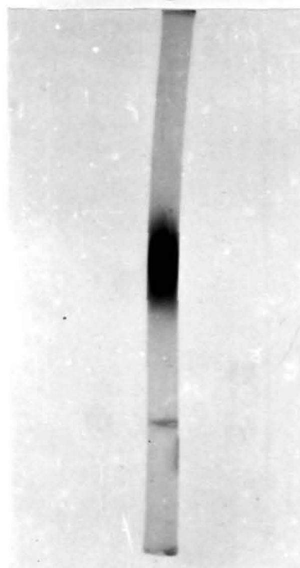


Fig. 47

Fig. 48



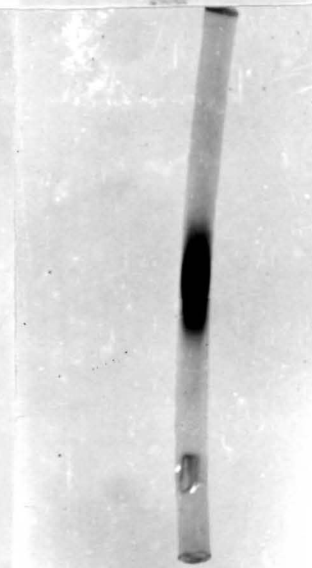


Fig. 49

encapsulation which slightly retards the growth activity especially at the initial stages of development. Other probable reason could be due to initial lag in differentiation of encapsulated plbs and so the encapsulated plbs were naturally at a lower stage of growth than non-encapsulated plbs. The difference in protein content was not much at stage IV showing the ability of the regenerants of encapsulated plbs to cope up with the initial lag in their development. Koering et al. (1974) obtained minor qualitative changes in the proteins in the plants grown under different environmental conditions. The slight difference in protein content between the regenerants of encapsulated and non-encapsulated plbs in the present experiment might also be due to the different environmental conditions (encapsulation) that they are subjected to.

Coulson and Sim (1965), Macko et al. (1967) and Nainawatee et al.(1974) have reported electrophoretic patterns of soluble proteins of wheat at different stages of growth and they observed a progressive increase in the number and intensity of the bands which is in confirmity with the present observations.

The gradual decrease in the peroxidase activity at various stages of development in the regenerants of encapsulated and non-encapsulated plbs in the present study is in agreement with the general hypothesis that the activity of peroxidase and growth of the plant cells are inversely related (Sembdneret al.,1980; Van Huystee and Cairns,1980). Chibbar et al. (1984) also found an inverse relationship between growth and peroxidase activity of two carrot cell lines. Peroxidase has the ability to act as an IAA oxidase thereby controlling the levels of IAA in plants and hence plays an important role in the development of plants (Sembdner et al., 1980; Chibbar and Van Huystee, 1984). In the

present experiment, the higher peroxidase activity at all the stages of development of encapsulated plbs more so immediately after encapsulation indicates the inherent mechanism to slow down the growth by increasing the activity of peroxidase to oxidise IAA. Alvarez (1968), and, Alvarez and King (1969) have found peroxidase activity to be an exact reciprocal of IAA production by Vanda seedlings. The slow growth rate observed immediately after encapsulation however, was not seen at the later stages and the regenerants of encapsulated plbs showed equal vigor in growth and development as regenerants of non-encapsulated plbs. The slow growth (and hence higher peroxidase activity) observed at the initial stages could probably be due to the effects of encapsulation, the plbs taking longer time to adjust to the altered new environment. The new environment created for encapsulated plbs would have altered the enzymatic levels especially the peroxidase leading thereby to degradation of IAA causing suspension in growth at the initial stages. As observed in chapter II, the encapsulated plbs took longer period to reach stage III (55 days) compared to the non-encapsulated regenerants (30-35 days) showing slower growth rate. Though this slight deviation in peroxidase activity was observed in the initial stages between regenerants of non-encapsulated and encapsulated plbs. However, the difference was not much and both showed decline in peroxidase activity as the growth progressed.

With growth, there was a decrease in number of bands showing decrease in the amount of isozymic fraction of peroxidase. Similarly there occurred a decline in the intensity of the bands showing decrease in the amount of these enzymes. This observation is in confirmity with the observation of Sembdner et al, 1980 in contrary to the observation of Singh and Singh (1974) who observed progressive increase in the isozymic

pattern with the increase in the start of germination and also the isozymic pattern was adversely affected when the plants were sown at low temperature (4°C).

An inverse relationship was observed between IAA oxidase and the growth of the plants. The IAA oxidase activity showed a progressive decline from stage I to well developed seedlings stage (stage V) for the regenerants of both encapsulated and non-encapsulated plbs, the activity being more in the regenerants of encapsulated plbs than non-encapsulated plbs at all the stages. This slight deviation in IAA oxidase activity in the regenerants of encapsulated plbs may again be accounted for by the effect of altered new environment the plbs are subjected to during encapsulation.

The decline in IAA oxidase activity from stage I to stage IV is in confirmity with the observation of Barendse (1983) who found an inverse relationship between IAA oxidase avtivity and growth. Goyal (1989) found an increased IAA oxidase activity in etiolated seedlings of winged bean following a period of water stress. The IAA oxidase activity in the regenerants of encapsulated plbs were slightly higher especially at the initial stages which may be due to a sort of stress created by the new environment leading thereby to degradation of IAA produced in the plants. The decrease in peroxidase activity at the later stages may be an indication of reduced IAA oxidase and hence increased IAA level which is needed for the growth.

VI

Explants from various parts of mature plants of *Cymbidium giganteum* Wall. like shoot tips, root tips, rhizome pieces, leaf tips, and leaf bases were excised and placed in MS medium (both semi-solid and liquid), supplemented with various hormones (NAA, BAP, IAA, Kn) in combination , to induce the formation of protocorm-like-bodies (plbs). Shoot tips showed various morphogenetic responses in MS medium supplemented with various concentrations of NAA and BAP. The best response was in 0.5 + 1.0 mg/l of NAA + BAP (45% in semi-solid and 15% in liquid medium), when the shoot tips enlarged and assumed a ball shaped structure resembling a protocorm. A period of 60-90 days was required to develop into a spherical plb. At times the plb showed proliferation into many plbs. However, in 5.0 + 0.5 mg/l of NAA + BAP (10% in solid) a friable callus was formed on the shoot explant, which formed a few plbs in around 30-40 days. The shoot tips also showed response in MS basal medium (35% in semi-solid and 15% in liquid), when the shoot tips proliferated into a nodular mass of organised cells resembling a callus which eventually formed many plbs.

Root tips exhibited various responses depending upon the size and the growth regulators used in the medium; the best being in IAA and Kn combination of 5.0 + 0.1 mg/l (25% in semi-solid

and 10% in liquid medium), when the root tips showed enlargement at the tip and finally differentiated into 3-4 plbs after 30-40 days. In NAA+BAP combination of 1.0 + 0.5 mg/l 40% of the root tips of about 1-2 mm size in semi-solid medium showed light brown callusing at the tip which formed 3-4 plbs at the periphery. Root tips of 1mm size showed enlargement at the tip and green globular plbs (2-4) were formed at the tip in around 90 days of culture. Rhizome also showed various responses. About 50-60% of the rhizomes of size 2-3 mm in both semi-solid and liquid basal media showed further elongation producing plbs at regular intervals. In IAA+Kn combination of 5.0 + 0.1 mg/l (35% in semi-soild and 50% in liquid) the rhizome pieces showed light greenish callusing at the cut ends with a few plbs arising out of the callus. In 0.5 + 0.5 mg/l of NAA + BAP, the rhizomes elongated producing roots and shoots at regular intervals and about 25% of the rhizomes produced clusters of plbs at the axil of the root and the rhizome. In NAA + BAP combination of 1.0 + 1.0 mg/l, the rhizomes branched out like ginger, producing plbs at the tips (75% of the rhizomes showed this type of response in MS liquid and 55% in solid medium).

Some of the unique responses were the emergence of inflorescence-like structures in NAA+BAP combination of 0.5+1.0 mg/l and formation of cluster of plbs at the intact root tips. Leaf tips and leaf bases failed to show any response in the present study.

Different media viz., Vacin and Went (VW), MS, Nitsch and Nitsch (NN), Gamborg (B_5), White(W), and Knudson C (KC) were used to obtain optimum plb-development and seedling growth. Plb development was quanitified in terms of increase in number, fresh weight, and volume. Seedling growth was quantified in terms

of leaf length, leaf number, root length, root number and colour, and overall development of the seedlings. MS medium rich in nutrients was found to be the best for overall plb-development and seedling growth followed by B_5, NN, KC, VW and W.

Various growth regulators like NAA, IAA, GA3, 2,4-D, BAP were incorporated in the MS medium individually ranging in concentration from 0.1 - 5.0 mg/l, and in combination of 0.1 + 0.1 mg/l and 1.0 + 1.0 mg/l of NAA + BAP and 0.1 + 0.1 mg/l and 1.0 + 1.0 mg/l of IAA + Kn. The combination of NAA + BAP (1.0 mg/l each) was found to be the best for both plb development and seedling growth.

Various acclimatization steps were followed to transfer the in vitro grown plantlets to the green-house. Growing plantlets (6-8 months old) on foam were found to be the best, as the plantlets showed better growth and less wilting. The growing of the plants on foam intiated the development of a large numebr of roots and the plants established readily in pots and possessed healthy and larger leaves.

Four potting mixtures were tried. Out of this, the one with soil + sand + cowdung (1:1:1) + brick pieces + charcoal + decayed leaves + wood pieces + bark seemed to be the best. The plants in this potting mixture reached a height of 16-18 cm after 3 months of transfer. Even plantlets initially grown on tissue paper and agar solidified medium showed better growth when transferred to this potting mixture. Use of plenty of cow-dung in the potting media was highly beneficial for seedling growth. The use of foam, coconut husk and shell, and Jal shakti in the potting mixture was beneficial in retaining moisture.

A number of gels were tried using two of the gelation methods : (i) Gel complexation via a dropping procedure,and, (ii) moulding via reduction in temperature, for bead formation.

Alginate beads and a variety of composite beads were formed by the mechanism of ionotropic gelation by dropping various concentrations of sodium alginate and composite gels into a solution containing divalent or multivalent counter ions, and the quality of beads as regards to shape, firmness and ease of handling ascertained. The corresponding conversion frequency of plbs were also studied under in vitro conditions. A 4-6% solution of sodium alginate upon complexation with 70-100 mM ionic solution resulted in excellent round beads (6-9 mm in diameter) with an ion-exchange duration of 30 min. The best beads were obtained with 4% sodium alginate in 70-100 mM aluminium nitrate as complexing agent, but then regenerants had retarded growth. The composite beads of sodium alginate (4%) and silica gel solution in 1:1 ratio resulted in firm beads, but the plbs took longer time to come out of the beads. The composite beads of 4% sodium alginate and 2% gelatin solution in 1:1 proportion was found to be more sticky than the sodium alginate beads. Out of the various gels and combinations of gels used, beads with sodium alginate and Jal shakti looked promising for in vivo planting as Jal shakti retains moisture for a longer period thereby preventing the dessication of the capsule and the plbs. To mimic the natural endosperm of a true seed complex carbohydrates like hydrolysed potato starch was added to the gel capsule to reduce leaching. Carbohydrates were also micro-encapsulated to provide their controlled release inside the synthetic seeds. Besides starch, carramellized sucrose was added in the alginate capsule. The sucrose from the micro-capsule were released slowly and extended over a period of 7-10 days. When the micro-capsules were incorporated in the Jal shakti + sodium alginate gel, the leakage of the sucrose from the micro-capsules was slow and sustained

release was observed throughout the 10-day period. To prevent rapid desiccation of the alginate capsule, coating with a number of substances were carried out to form a thin membrane over the capsule. Glutaraldehyde solution was able to form a membrane around the alginate capsule however, only gelation capsule showed significant impediment to capsule drying. 2-3 alginate beads with the plbs placed in the capsule with Jal shakti powder burst when placed in water due to the tremendous pressure developed on absorption of water by the polymer; thus liberating the plbs well-cushioned in the gel. The alginate bead and water absorbing polymer in the gelatin capsule looked ideal for synthetic seed production. The capsule is non-tacky, easy to handle and can be planted individually. As dropping the embryo/plbs using a spoon/funnel/pipette is a very slow process, the simple method of dropping plbs and alginate solution from a sheet with a large number of holes big enough to allow plbs to pass through might prove useful for large scale production of synthetic seeds.

The beads with MS nutrients and sucrose showed severe fungal and bacterial contamination when placed in sterile sand and soil and did not form plantlets. Various antimicrobial agents (bactericide, soframycin, rosebengal and fungicide dithane) were added in the matrix in various concentrations, either singly or in combination. Before adding this to the matrix, their effects on plb-development and seedling characteristics were ascertained. They apparently had no appreciable effect on plb proliferation and growth. The beads were plated out in various substrates like MS medium, sand, and soil and their conversion frequency studied. The conversion frequency of encapsulated plbs without antimicrobial agents were 100%, 44% and 20% in medium, sand, and soil, respectively. The optimum concentrations of rosebengal, soframycin, and dithane for plb conversion were 0.1, 0.5, and 4

mg/l, respectively. When all the three compounds were added together in the matrix, there was significant increase in conversion frequency to 88% in sand and 64% in soil. Besides nutrient medium, sterilised polyurethane foam submerged in MS liquid nutrients and sorbarod system proved to be good substrata for growing the plbs. Jal shakti with soil mixture in the ratio 1:6 was a better substratum for encapsulated plbs than sand or soil mixture alone, as it retained moisture better due to the moisture absorbing qualities of the polymer. Under in vivo conditions, the naked plbs when grown in sterile sand or soil mixture did not germinate.

Storage potential of non-encapsulated and encapsulated plbs were studied using various approaches like low temperature, growth retardants, osmotic inhibitors and dehydration treatment. Sucrose 8% and ABA (1 mg/l) individuallly or in combination in the medium were found to be highly suitable for plb-development and increase in fresh weight , but considerably delayed plb-proliferation and seedling growth. The inhibition in growth was indicated by plbs taking longer time to produce shoot apex (around 55 days) in medium containing ABA (1 mg/l) and sucrose (8%) as compared to MS without ABA + sucrose, where shoot apex was produced in 10-15 days. At the end of 120-125 days the seedlings reached two-leaf stage (stage III), as compared to plbs cultured in MS medium without ABA + sucrose where the plbs reached four-leaf stage (stage VI). Even the plbs stored at 4°C showed considerable growth inhibition and reached stage III only at the end of 130-135 days. The low temperature reduced the growth rate as also ABA + sucrose. The growth inhibition by ABA was more pronounced when the plbs were cultured at 4°C than at room temperature.

The encapsulated and non-encapsulated plbs were plated out in : (i) a few drops of MS liquid nutrients with and without ABA + sucrose and,

(ii) MS semi-solid medium with and without ABA + Sucrose. Encapsulated and non-encapsulated plbs kept in a petriplate with a few drops of nutrients failed to survive beyond 60 days.Even though encapsulated plbs showed consistent high survival rate than the non-encapsulated plbs, the viability was more with ABA + sucrose in the medium. The plbs cultured in nutrient semi-solid media showed 100% survival at room temperature. However, at 4°C there was drastic reduction in survival rate (the encapsulated plbs in MS with sucrose + ABA showing higher survival than MS without ABA + Sucrose).

Three hours of dessication was found to be optimum for plb survival. After 3 hrs of dessication the moisture content of encapsulated plbs dropped to 43% of fresh weight and showed 100% survival and that of non-encapsulated plbs dropped to 59% of fresh weight and showed 50% survival. The non-encapsulated dessicated plbs could not be stored in this condition as such and they had to be rehydrated straightaway. Even the encapsulated plbs stored after desiccation lost water rapidly when stored at room temperature and lost viability within a day.

Morphological studies were carried out to see the differences in the regenerants of encapsulated and non-encapsulated plbs. Analysis of phenotypic characters like leaf number, leaf size, root number and root size of 120-day old seedlings of the regenerants of both encapsulated and non-encapsulated plbs did not show significant differences. Even the regenerants from stored plbs (stored at 4°C for 30 days) did not exhibit any difference.

Various stage of development were defined and the time

taken to reach these stages by the encapsulated and non-encapsulated plbs stored at room temperature and 4°C were recorded and compared. The regenerants showed significant difference in the days required to reach different stages, indicating different rates of growth. Under in vivo conditions the encapsulated plbs required an average of 20-25 days to emerge out of the beads and plantlets of two-leaf stage were reached within 55 days. The non-encapsulated plbs when plated out on MS medium emerged out in 10-15 days and within 35 days plantlets of two-leaf stage were reached. The plantlets from encapsulated plbs reached stage IV and stage V (four-leaf stage with well developed leaves) in the medium earlier than the non-encapsulated plbs. Scanning electron microscope (SEM) studies were carried out to find out any ultra-structural differences in stomata of the regenerants of encapsulated and non-encapsulated plbs, but, no significant differences were observed. However, stomatas were of open type in the leaves of in vitro plants, whereas in case of in vivo plants it was both open and closed type. Cytological studies were carried out to see any abnormalities in chromosome numbers, but the root tip squash showed no difference in the chromosome number. The cells at metaphase stage showed 2n=40 in both cases.

Investigation was done to observe any effect of encapsulation on plb growth using protein profile and isozymes as markers. Estimation of total soluble protein at different stages of growth showed a progressive increase in the regenerants of both encapsulated and non-encapsulated plbs from stage I to stage V. However, the protein content of regenerants of encapsulated plbs was always slightly lower than the non-encapsulated plbs at all stages of development. The protein bands obtained by electrophoretic separation showed five bands of different widths

and intensity at all stages of development. Lighter bands were obtained at Stage I and darker bands at later stages.

The assay of peroxidase at different stages of development showed higher activity at stage I and decreased gradually at later stages of development. The regenerants of encapsulated plbs always showed higher activity than regenerants of non-encapsulated plbs. The peroxidase activity decreased quite dramatically at stage V.

The IAA oxidase also showed a progressive decline in the activity from plb stage to well developed seedling stage in the regenerants of encapsulated and non-encapsulated plbs, the activity being more in the regenerants of encapsulated than non-encapsulated plbs. However, on unit protein basis the IAA oxidase activity gave a different picture, the non-encapsulated regenerants had higher activity than encapsulated regenerants.

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