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Augmented shelf-life and regeneration competence of activated charcoal (AC) supplemented synthetic seeds in *Cymbidium pendulum* (Roxb.) Sw.

Anju Pehwal[1], Suraj Prakash Vij, Promila Pathak, and Lucky K. Attri [2]

Orchid Laboratory, Department of Botany, Panjab University,Chandigarh-160014, India

Abstract

A protocol for their long term storage at low temperature has been developed using synthetic seeds technology in *Cymbidium pendulum* (Roxb.)Sw. in present piece of work. This species is known as an important ornamental and medicinal orchid. Protocorm Like Bodies (PLBs) were used as propagules for encapsulation. They were raised on Basal M medium [1], in addition with inorganic plant growth regulators such as [Indole-3-acetic acid (IAA); Indole-3-butyric acid (IBA) and Naphthalene acetic acid (NAA); 6-Benzyl amino purine (BAP); Kinetin (KN) at 1mg/l concentrations. Different combinations were compared for their efficacy in supporting large scale production of elite propagules for encapsulation. Among these NAA proved to be the best as it supported 99.5% of asymbiotic seed germination in to largest PLBs (2.2mm) with highest chlorophyll content at 2.15µg/mg. PLBs were encapsulated in 3% sodium alginate and di-hydrated salt calcium chloride (100mM). Resultant synthetic seeds were observed for their viability after different period of storage at 25^{0}C and 4^{0}C. Role of AC if added to nutrient matrix in extended storage of synthetic seeds with better conversion at mass scale has been the main focus of the study. Interestingly, AC supplemented synthetic seeds could be stored for 480 days with 10.5% conversion and showed fairly good regeneration or production of secondary PLBs.

Keywords: Activated charcoal (AC); Orchid; Plant growth regulators, PLBs Synthetic seeds

INTRODUCTION

Possibility of encapsulating somatic embryos / regenerants in a nutritive gel was suggested in late seventies [2], and the technique was developed subsequently [3] for the purpose. Synthetic seed technology has been implicated to some of the plant species. Storage of synthetic seeds always has been a challenge to make this conservation strategy practically viable, as they are associated with numeral problems like lack of oxygen inside the matrix, production of phenolics/secondary plant metabolites as result of continued metabolism of encased propagules. In the present investigation a successful effort has been made to store synthetic seeds in *Cymbidium pendulum* for long through addition of AC (Activated charcoal inside the matrix). It made the synthetic seeds porous to some extent hence solved he problem oxygen supply, provided enough empty space inside the storage container is available and synthetic seeds are not overcrowded. Supplementation of AC also regulated the release of phenolics from encased PLBs they were adsorbed by AC. *Cymbidiums* comprise a commercially important taxon of orchids and occupy the leading status as cut flowers. They fetch highest price per flower in the international market [4]. *Cymbidium pendulum* is an epiphytic species of highly floriferous plants, distributed in South Asia (Burma, China, India, Malaysia). The species with attractive foliage and pendant racemes forms an important garden plant of the tropical and sub-tropical regions. The plant is said to be emetic and purgative[5]. Unregulated commercial collection and unregulated habitat destruction have detrimentally affected the size and frequency of its natural populations. *In vitro* propagation is one of the best options to meet the urgent need of conservation and high rate multiplication of this important orchid species. In the present investigation encapsulated PLBs were better used for *in vitro* propagation than naked ones. Propagules were raised using Asymbiotic seed germination technique, for this full strength M medium supplemented with different growth adjuvants was used, elite propagules were encapsulated in Calcium alginate matrix. Selection of propagules (PLBs) was based upon the size, chlorophyll content and rate of morphogenesis i.e. development of root and leaf primordia.

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*Corresponding Author
Anju Pehwal
Orchid Laboratory, Department of Botany, Panjab University,Chandigarh-160014, India

Email: anjupehwalo2@yahoo.co.in

MATERIAL AND METHODS

Asymbiotic seed germination and propagules (PLBs) production

Asymbiotic seed germination was observed on M et al., 1976 medium supplemented with auxins (IAA, IBA, NAA) and cytokinins (BAP, KN) at 1mg/l in the absence or presence of AC. Seeds sourced from the undehisced green capsules (harvested at 36 WAP) were inoculated on M medium. The sterilized capsules were then split open longitudinally with a sterilized blade to scoop out the immature seeds, under aseptic conditions. Germination percentage was checked by taking out the seeds with sterilized blade under sterilized condition and preparing temporary slides in dilute glycerin. The orchid cultures were stored at the 25±2^{0}C for 16 hr photoperiod under cool white fluorescent lamps (Philips TLD, 39 W) at 150 µmol/m^2/ s.

Chlorophyll estimation

The spectrophotometric method of chlorophyll content determination was based on the Harbone [6] method

Encapsulation of PLBs and storage of synthetic seeds

Highly chlorophyllous Protocorm-like bodies (PLBs) of uniform size (3x6mm) at the stage with root and shoot primordia were used as propagules for synthetic seed formation. The best quality synthetic seeds were prepared through hydrogel encapsulation technique [3] by using 3% of the sodium alginate (CDH) and 100mM of di- hydrated calcium chloride. Their complexation (ion-exchange reaction between sodium alginate and calcium chloride) was allowed for 30 min. Nutrient matrix was provided with NAA, (1mg/l) in absence (Fig. 5) or presence of Activated Charcoal (AC, Fig. 6). For avoiding the chances of contamination, antimicrobial agents; bavistin (0.05%), streptomycin (0.02%), and mercuric chloride (0.01%) were added in artificial endosperm. As a carbohydrate source $20gl^{-l}$ sucrose was added. Prior to autoclaving, pH was adjusted between 5.7-5.8. All the solutions were autoclaved for 20 min at 121˚C temperature and 1.1kg/cm. Propagules with 2µl sodium alginate were dropped in conical flask containing 100ml of $CaCl_2.2H_2O$ (100mM) solution kept on magnetic stirrer. After the dispensing was over, beads were left in solution to complete the complexation reaction for 30 min. Beads were washed with autoclaved distilled water at least 3 times. Resultant synthetic seeds were subsequently used for testing their viability in terms of conversion on agarised M medium at different time intervals of their storage at 25°C and 4°C.Conversion is the process of breaking of synthetic matrix by growing propagules inside and development of full grown plantlet with leaf and roots. They were stored in autoclavable petri- plates (Hi-media), Flasks, test tubes and glass petri-plates (Borosil) were also used for the storage experiments. All the glassware and autoclavable petri-plates were sealed with parafilm. Plantlets obtained after the conversion were hardened by sequential cutting down of different elements of nutrient supply under controlled conditions. They were finally transferred on epiphytic mix (Fig. 9). These plantlets were successfully established in greenhouse at high frequency.

Stastical analysis

The experiments consisted of four replicates (test tubes) in case asymbiotic seeds germination experiments and for synthetic seeds experiments four Petri plates with 10 synthetic seeds in each. Means were analyzed through the Independent Samples' T-test in the selection of the best PLB size for encapsulation, with level of significance set at 0.05. Means in the storage experiments were analyzed through the one-way analysis of variance (ANOVA) and differentiated with Tukey's test, with the level of significance taken at 0.05.

RESULTS AND DISCUSSION

Asymbiotic seed germination and Protocorm-like bodies (PLBs) production

Presently, PLBs were raised using asymbiotic germination of orchid seed technique. The orchid seeds are microscopic and produced in large numbers (Fig. 1). They comprise an undifferentiated embryo suspended within a transparent seed coat [7], and contain lipidaceous food reserves, which occur as discrete inclusions within the embryonic cells [8].When inoculated o nutrient media swelling of this embryo is considered as the first sign of germination (Fig. 2) and this swollen embryo is known as spherule which break the seed coat and take a top shaped structure known as protocorms. These protocorms further multiply to produce secondary protocorms (Fig. 3) known as PLBs (Protocorm Like Bodies). Germination frequency was invariably enhanced by inorganic growth adjuvants. Auxins supplemented medium proved better than that supplied with cytokinins (Table. 1). Amongst auxins NAA supported maximum germination as well as multiplication of PLBs into secondary PLBs or daughter PLBs. PLBs thus produced were of largest size, and they had maximum chlorophyll content and at the best differentiation stage at the time of harvesting for encapsulation purposes. NAA promoted synchronized development of highly proliferative and high quality PLBs in accord with its similar efficacy in a number of orchids [9,10]. Asymbiotic seed germination technique satisfies the need of large supply of propagules.

Table 1. Effect of the plant growth adjuncts added to M *et al*., 1976 medium on asymbiotic germination of zygotic seeds and on the quality of PLBs

Plant growth Regulators ($1mgl^{-1}$)	Conversion (%) after two weeks	Number of PLBs formed after		Size of PLBs (mm)	Total chlorophyll content (µg mg^{-1} of PLBs)	Time taken in weeks for the development	
		One month	Two months			First Shoot primordi	First Root primordia
Control (No PGR)	50±0.5^{a}	35±1.6^{a}	50±2.2^{f}	1±0.8^{a}	0.05±0.2^{a}	8±0.1^{c}	7±0.01^{c}
IAA	95±1.7^{b}	55±1.7^{c}	61.5±1.1^{d}	1..5±0.1^{c}	1.17±0.1^{c}	7±0.3^{b}	5±0.2^{a}
IBA	86±0.5^{a}	82.7±92.7^{d}	70.75±0.4^{e}	1.8±0.6^{b}	1.19±0.4^{c}	8±01^{c}	7±0.5^{c}
NAA	**99.5±0.5^{a}**	**92.75±1.1^{a}**	**92.5±1.0^{a}**	**2. 2±0.2^{a}**	**2.15±0.1^{d}**	**4±0.5^{a}**	**5±0.2^{b}**
KN	72.5±1.1^{d}	81.5±1.2^{a}	70.75±0.4^{c}	1. 2±0.4^{d}	0.9 ±0.8^{b}	8±0.4^{c}	9±0.4^{d}
BAP	82.7±1.1^{c}	73.0^{b}	75.25±1.8^{b}	1. 9±0.5^{b}	0.8±0.5^{b}	4±0.9^{a}	6±0.3^{b}

Values are presented as means ± SE. Different letters within a column indicate significant differences at $p < 0.05$ according to analysis of variance (ANOVA).

Table 2. Effect of encapsulation, storage period and temperature on viability of the Protocorm like bodies (PLBs) *Cymbidium pendulum*

Storage Temperature	25^{0}C			4^{0}C		
Propagules(Plbs)	Nake	Encapsulated		Nak	Encapsulated	
Storage period (days)		AC Depleted	AC Supplemented		AC Depleted	AC supplemented
0	100±1.1^a	100±0.2^a	100±0.5^a	100±0.	100±0.25^a	100±0.2^0
20	21.25±2.1	100±0.1^a	100±0.2^a	36.25±	100±0.2^a	100±0.5^a
40	11.25±2.1	100±0.4^a	100±0.1^a	20.5±0	100±0.8^a	100±0.^{a}
60		93.75±1.1	99.75±1.5^a		97±0.2^b	100±20^a
80		93.25±1.5	98.5±0.5^a		95±0.3^b	100±0.4^a
100		88.25±0.4	97.5±0.2^a		91.25±0.12	100±0.5^a
120		85±0.6^{bc}	96.5±1.7^a		86.25±1.1^c	100±0.8^a
140		82.5±0.1^{bc}	95.25±0.3^{ab}		85.25±0.2^c	100±0.4^a
160		80.5±1.1^{cd}	90.5±0.5^{ab}		81.25±0.6^c	100±0.25^a
180		72.5±0.14	87.5±0.2^{ab}		76.75±0.4^d	97.5±0.25^{ab}
200		58.5±0.9^e	85±0.2^{bc}		70.75±0.1^d	95±0.2^{ab}
220		50.5±0.2^e	80.5±3^{bc}		61.25±1.1^c	90±0.2^{ab}
240		39±1.4^f	69.25±1.4^c		55±1.75^f	87.5±0.4^{bc}
260		13.75±1.2	61.75±0.1^c		41.25±1.2^g	80±0.5b^c
280			51.25±0.1^d		22.5±0.4^h	76.25±.4^c
300			50.75±2.5^d		15±0.2^i	75±0.4^c
320			19.5±2.5^e			73.75±0.5^{cd}
340			5±0.25^f			71.25±0.2^{cd}
360			2.5±0.3^f			68.5±0.5^d
380						60.25±0.5^d
400						58.25±0.2^{de}
420						56±0.2d^e
440						36.75±0.8^e
460						15.25±2^{fg}
480						10.5±0.2^1
500						

Values are presented as means ± SE. Different letters within a column indicate significant differences at $p < 0.05$ according to analysis of variance (ANOVA).

Table 3. Effect of encapsulation and presence of AC (Activated Charcoal), on *in vitro* morphogenesis of PLBs sown on basal M medium after 40 days of storage at 4^{0}C and observed for 4 weeks days.

Morphogenetic events per PLB	No. of Secondary PLBs produced	Shoot number	Leaf number	Root length(cm	Root diameter(mm)
Naked PLBs	0.2±0.2^a	0.4±0.1^a	0.25±0.5^a	0.21±0.2^a	0.22±1.4^a
AC depleted encapsulated PLBs	0.4±1.2^b	0.12±0.6^b	0.86±0.6^b	0.55±0.5^b	0.29±0.1^b
AC supplemented encapsulated PLBs	0.6±0.1^c	0.18±0.8^c	0.95±0.5^c	0.75±1.5^c	1.9±2.2^c

Values are presented as means ± SE. Different letters within a column indicate significant differences at p <0.05 according to analysis of variance (ANOVA).

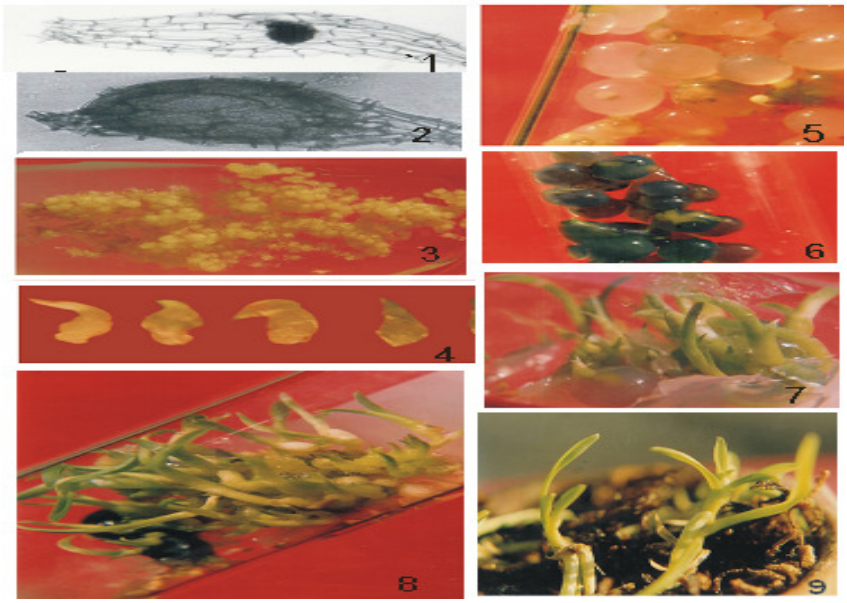
Encapsulation

Highly chlorophyllous protocorms like bodies (PLBs) of uniform size (3x6mm, Fig. 4) were used as propagules for synthetic seed formation. Synthetic seeds were prepared by encapsulating the PLBs in 3% of the sodium alginate (CDH) and 100mM of calcium chloride and their complexation was allowed for 30 min. Alginate is one of the widely used materials for encapsulation. This acidic polysaccharide is obtained from brown algae and is composed of 1-4 linked B-D mannuronic acid and α- L- glucuronic acid residues with varying proportions and a sequential arrangement [11,12].

Storage

Encapsulation proved suitable strategy for the storage of PLBs. Synthetic seeds stored at 4^{0}C maintained their viability for longer as compared to those stored at 25^{0}C, moreover

conversion percentage after same period of storage reported to be higher in those stored at 4^{0}C(Table. 2). Supplementation of nutrient matrix with activated charcoal improved (AC) the efficacy of synthetic seed technology. AC depleted synthetic seeds showed viability till 260 days of storage at 25^{0}C the use of AC in matrix enhanced the viability up to 320 days. At 4^{0}C synthetic seeds were stored till 300 days without using AC (Fig. 7) whereas supplementation of the matrix with AC Activated charcoal extended the storage period up to 480 days (Fig. 8). Storage of somatic embryos or vegetative propagules using an alginate encapsulation protocol has been attempted only in few species with various degrees of success, at low temperature; the synseeds often leak under storage and loose viability [13, 14]. A similar decline in the viability of synseeds is already reported [15, 16]. Poor conversion of synthetic seeds on storing them at 25^{0}C may be due to desiccation of the matrix and similar Desiccation of the synseeds was observed, when they were stored at 25°C as already reported [17]. Activated charcoal supplemented synthetic seeds were less leaky and plantlets recovered after germination of such synthetic seeds were healthier and showed better conversion (Table. 3). At 25^{0}Ctemperature, synseeds germinated precociously i.e. before they were sown on sowing substrata while, storage of synseeds at 4°C, solved the problems of precocious germination as well as their desiccation during their storage. AC enriched synseeds, showed prolonged viability at all the tested temperatures and the conversion frequency of the synseeds stored at different duration of storage was also higher in comparison to synseeds which were devoid of AC in the nutrient matrix. During conversion secondary PLBs also produced at higher rate in synthetic seeds stored at 4^{0}C and supplemented with AC so, their regeneration was higher. Long term storage of plants *in vitro* without regular subcultures allows one to rationalize production of nuclear stocks and maintain gene collection [18]. Only 13% conversion of synthetic seeds was recorded at 27°C after 40 days [19]. At 10°C after 20 days, 21.5% and after 40 days, 15.0% of conversion was recorded. Encapsulated zygotic embryos were stored for 10 days with 60% conversion frequency at 4°C and 30% conversion after 20 days when stored at 2°C [20]. Strawberry and raspberry shoot tips in alginate beads containing a medium without any growth regulators converted at 90-100% after 3 months in storage at 4°C [13]. Synthetic seeds containing encapsulated PLBs after storage at 4°C for 120 days showed 80% viability but their storage at room temperature showed only 44% viability[15]; the germination potential decreased gradually with increase in storage time. Germination percentage of encapsulated tiny shoots of pineapple (*Ananas cosmosus* L. Mern. cv Queen) was retained up to 20% after 45 days of storage at 4˚C and lost viability completely after 90 days[21]. Viability of encapsulated protocorms up to was recorded at 70% when stored for 180 days at 4°C [22]. It was suggested that activated charcoal helps in the adsorption and desorption to controlled release of the nutrients in the production of synseeds in Banana cultivars basrai [23]. AC has also been used in the culture media to improve growth and promote morphogenesis in a wide variety of species including orchids. Positive effect of darkening the media with charcoal on the growth of *Cymbidium Paphiopedilum* and *Phalaenopsis* plantlets under controlled conditions [23, 24, 25, 26].



Figs .(1-9). Synthetic seeds formation in *Cymbidium*(Roxb.)Sw. 1. Microscopic seed, 2. Swollen embryo showing germination, 3. Multiplying propagules(PLBs) 4. Selected PLBs, 5. Synthetic seeds without activated charcoal(AC) 6. AC supplemented synthetic seeds 7. Germination AC depleted synthetic seeds after storage for 160 days 8. AC supplemented synthetic seeds germinating after storage for 400 days at 40C ,9. Plantlets regenerated from

CONCLUSION

Mitra (1986) medium supplemented with NAA at 1 mgl^{-1}proved best medium for healthy propagules production at

fast rate for uninterrupted supply.The use of Activated charcoal significantly increased the period for which viability could be retained by encapsulated PLBs and this increment was more pronounced at 4^0C storage. It helped to tackle the problem of desiccation and precocious germination of synthetic seeds during storage. It also adsorbed all the harmful secondary plant metabolites and made synthetic seeds little porous thus helped in respiration of encased PLBs.

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REFERENCES

[1] Mitra, G. C., R. N. Prasad, and A. R. Chowdhury .1976. Inorganic salts and differentiation of protocorms in seed callus of an orchid and correlated changes in its amino acid content. *Indian J. Exp. Biol.* 14: 350-51.

[2] Murashige, T. 1978. The impact of plant tissue culture on agriculture. In: TA Thorpe, editor. Frontiers of Plant Tissue Culture. University of Calgary, Calgary, Alberta: International Association of Plant Tissue Culture. pp. 15-26.

[3] Redenbaugh, K., B.D. Pwasch, J.W. Nichol, P.R. Viss, K.A.Walker.1986. Somatic seeds: Encapsulation of asexual plants embryos. *Bio. Technol.* 4: 797-801.

[4] Upadhya and Das. 2001. Cymbidiums at North- Eastern Himalayas. In: Pathak P, Sehgal S N, Shekher N, Sharma M, Sood A, editors. Orchids; Science and Commerce. Dehradun: Mahendra Pal Singh Publ. pp. 459-484.

[5] Claius, J.F. 1936. Medicinal and poisonous orchids of India. *J. Bombay Nat. Hist. Soc.* 38: 791-799.

[6] Harbone, J.B .1973. Photochemical Methods. Chapmann and Hall Ltd., London, pp. 49-188.

[7] Arditti, J. 1967. Factors affecting the germination of orchid seeds. *Bot. Rev.* 33: 1-97.

[8] Poddubanaya-Arnoldi, A. and N. V. Zinger. 1961. Application of histochemical technique to study of embryonic processes in some orchids. *Recent Advances in Botany***.** 8: 711-714.

[9] Miyoshi, K. and M. Mii. 1998. Stimulatory effects of sodium and calcium hypochlorite, pre-chilling, and cytokinins on the germination of *Cyperipedium macranthos* seed *in vitro*. *Physiol Plant.* 102: 481-486.

[10] Pathak, P. 1989. Asymbiotic Germination and Clonal Propagation of Some Important and Endangered Orchids of India Using Tissue Culture Techniques. Ph. D. Thesis, Panjab University, Chandigarh, India.

[11] Ara, H. U. Jaiswal, V.S. Jaiswal .1999. Germination and plantlet regeneration from encapsulated somatic embryos of mango (*Mangifera indica* L.*). Plant Cell Rep.* 19: 166-170.

[12] Basu, A.K. and B. Rao. 2006. Synthetic seed: A demanding technology in plant propagation and conservation. In: Trimurthy PL, editor. Plant Propagation and Biotechnology. Rajasthan India: Pointer Publishers. pp. 214–251.

[13] Lisek, A, and T. Orlokowska .2003. *In vitro* storage of strawberry and raspberry in calcium alginate beads at 4°C. *Plant Cell Tiss Org Cult.* 75:1-6.

[14] Nieves, N., Y. Zambrano, R. Tapia, M. Cid. D. Pina, and R. Castillo. 2003. Field performance of sugarcane plants obtained from artificial seed derived sugarcane plants. *Plant Cell Tiss. Org. Cult.* 75(3): 279-282.

[15] Datta, K., B. Kanjilal, D. D. Sarker .1999. Artificial seed technology: Development of a protocol in *Geodorum densiflorum* (Lam.) Schltr.- An Endangered orchid. *Curr Sci.* 76(8): 1142-1145.

[16] Vij, S. P., P. Kaur, and A. Gupta. 2001. Synseeds and their utility in orchids: *Dendrobium densiflorum* Lindl. *Phytomorphology.* 51(2): 159-165

[17] Janerio, L.V., A. Balester, A. M. Vietez .1997. *In vitro* response of encapsulated somatic embryos of *Cammelia japonica*. *Plant Cell Tiss Org Cult* 51: 119-125.

[18] Lisek, A. and T. Orlikowska .2004. *In vitro* storage of strawberry and raspberry in calcium –alginate beads at 4ºC. *Plant Cell Tiss. Org. Cult.* 78: 167-72.

[19] Malabadi, R.B. and J. Van Staden .2005. Storability and germination of sodium alginate encapsulated somatic embryos derived from the vegetative shoot apices of mature *Pinus patula* trees. *Plant Cell Tiss. Org. Cult.* 82: 259-265.

[20] Sparg, S. G., N. N. Jones, and J. Van Staeden. 2002. Artificial seed from *Pinus patula* somatic embryos. *S. Afr. J. Bot.* 68: 234-238.

[21] Soneji, J., P. Rao, and M. Mahatre .2002. Germination of synthetic seeds in pineapple (*Ananas comosus* L. Merr.). Plant Cell Rep. 20(10): 891-894.

[22] Singh, F. 1991. Encapsulation of *Spathoglottis plicata* protocorms. Lindleyana. 6:61-63

[23] Ganapathi, T. R., V. A. Bapat, and P. S. Rao.1992. Propagation of banana through encapsulated shoot tips. *Plant Cell Rep.* 11: 571-575.

[24] Werkmeister, P. 1971. Light induction of geotropism and the control of proliferation and growth of *Cymbidium* in tissue culture. *Bot. Gaz.* 132: 346-350.

[25] Ernst, R. 1974. The use of activated charcoal in asymbiotic seedling culture of *Paphiopedilum*. Am. *Orchid Soc. Bull.* 43:35-38.

[26] Ernst, R. 1975. Studies in asymbiotic culture of orchids. Am Orchid Soc Bull. 44:12-18.

[27] Ernst, R. 1976. Charcoal or glass wool in symbiotic culture of orchids. In: Proc. 8th World Orchid Conference. pp. 379-383. Frankfurt.