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Synchronous Plantlet Formation by Using Banana Extract and *In vitro* Hardening in Orchid, *Dendrobium lituiflorum* Lindl.

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Abstract

The present study was undertaken to investigate the role of banana extract (BE) on synchronous plantlet formation from seeds of *Dendrobium lituiflorum* Lindl. The seedlings formed on modified Knudson C (KC) medium supplemented with 20% (v/v) BE had significantly high fresh and dry weights. Morphologically uniform plantlets with elongated leaves and well developed roots were formed on KC medium with 20% (v/v) BE in comparison to KC medium without BE (control) after 30 d of fourth subculture. The plantlets so formed were subjected to *in vitro* hardening on agar-agar gelled medium, *Luffa* sponge and cocopeat: perlite (9:1) as support matrices, each containing one-half strength KC major salts and were successfully acclimatized under greenhouse conditions. Banana extract helps in synchronous plantlet formation *in vitro* which is not only beneficial for conservation purposes but also to the biotech industries as a large number of uniform plantlets can be obtained for transplantation, thereby, reducing the cost of production.

Keywords: Orchids, Propagation, Support Matrices, Synchrony.

INTRODUCTION

Synchrony in the present report refers to the uniformity in plantlet growth and is exclusively a phenotypic parameter. In other instances, synchrony has been extensively used in cell culture (Sharma, 1999), as in a cell population growing at a logarithmic rate, all cells should be in a homogeneous state in order to analyse the chemical and physical setup at each phase. Synchronized system, essential to study cell cycle related events, is induced by blocking the cell cycle at S (synthetic) or M (mitotic) phases. Several chemicals used in inducing synchrony are colchicine, amphidicolin, hydroxyurea, etc. Sharma (1999) elaborated that synchronization in plant cells is also important for the study of cell cycle related events and to study specific enzyme activity and increase in production of useful metabolites. The success of tissue culture on a commercial scale lies in the fact that a large number of similar sized propagules at a given stage can be produced in a given time period. Asynchrony is considered disadvantageous *in vitro* as it hampers seedling maintenance and transplanting (Thompson *et al.*, 2006). This may further lead to an increase in the cost of production. Synchrony with respect to germination of seeds of the orchid, *Cypripedium calceolus* var. pubescens was also reported by Chu and Mudge (1994) where it referred to the occurrence of majority of protocorms in a treatment at a given developmental stage at approximately same time. Synchrony in other aspects of tissue culture such as secondary somatic embryogenesis has been reported by Mondal *et al.,* (2001) in *Camellia sinensis*. In tissue culture, induction of mass somatic embryogenesis and successful somatic hybrid production can be achieved by synchrony in cell behavior (Sharma, 1999).

Commercial propagation of orchids has many bottlenecks such as low multiplication rate, vitrification, poor rooting and high mortality during acclimatization being the major limitations (Hew, 1994). At this juncture, rapid production of synchronous plantlets *in vitro* with well developed leaves and established root system is extremely desirable as it will provide ample material for acclimatization in the greenhouse and finally to the field conditions. *Dendrobium lituiflorum* Lindl. is an extremely rare and threatened sympodial epiphyte of North-Eastern states of India (Chowdhery, 2001). The ultimate success of tissue culture protocol on a commercial scale depends on the production of plants raised by tissue culture at low cost and with high survival rates (Hazarika, 2006). Mass scale production of plantlets can be achieved by many methods, involving reduction of the cost of medium ingredients and use of a natural additive that leads to faster production of synchronous plantlets, thus, reducing the time period of plantlet formation. Even if low cost *in vitro* propagation can be achieved, the transplantation stage continues to be a major bottleneck in the micropropagation (Hazarika, 2003). After *ex vitro* transfer, these plantlets might easily be impaired by sudden changes in environmental conditions, and need a period of acclimatization to correct the abnormalities (Pospíšilová *et al*., 1999). It is, therefore necessary, that a protocol is devised wherein the *in vitro* grown plantlets can gradually adapt to the new environmental conditions. It is at this juncture that the process of *in vitro* hardening, where the process of acclimatization can begin while the plantlets are still under *in vitro* conditions, becomes important (Hazarika, 2006). For mass propagation of *D. lituiflorum* orchid for conservation, *in vitro* method needs to be devised that would lead to the formation of synchronous plantlets (so that a large number of morphologically uniform plantlets can be obtained at the same time) which can be transplanted to the greenhouse or field conditions on a large scale. BE is responsible for higher percentage germination and rapid progress to advanced stages of development and root formation (Vyas *et al*., 2009). In this communication, we report that besides the above mentioned roles of BE, it plays a significant role in mass scale synchronous plantlet formation which is a prerequisite for a commercial venture. The *in vitro* raised plantlets can be used for commercial purposes only when these can be successfully acclimatized. After *ex vitro* transfer, these plantlets might easily be impaired by sudden changes in environmental conditions, and need a period of acclimatization to correct the abnormalities (Pospíšilová *et al*., 1999). It is, therefore necessary, that a protocol is

devised wherein the *in vitro* grown plantlets can gradually adapt to the new environmental conditions. It is at this juncture that the process of *in vitro* hardening, where the process of acclimatization can begin while the plantlets are still under *in vitro* conditions, becomes important (Hazarika, 2006). The study also compares the suitability of inexpensive support matrices in *in vitro* hardening of the plantlets.

MATERIALS AND METHODS

The capsules of *Dendrobium lituiflorum* Lindl. were procured from Assam, India. Banana extract was prepared according to Vyas *et al*., (2009) by cutting banana into thin circular slices and blending these with distilled water in the ratio of 4:1 (w/v). The modified KC medium (Knudson, 1946) was prepared by adding BE in the required volume to KC medium. BE was used at 1-20% (v/v). Agar–agar (Qualigens, India) and sucrose (Daurala, Meerut, India) were used at 0.8% and 2% (w/v), respectively, in the medium and the pH was adjusted to 5.8. 50 ml medium was dispensed in culture bottle (500 ml) and was autoclaved at 121ºC at 1.06 kg cm^{-2} for 30 min. The cultures, inoculated under aseptic conditions, were then incubated under 12 h photoperiod by cool white fluorescent tubes (30 µmol m^{-2} s^{-1}, Philips, India) at 25±2ºC. The subculture of propagules was carried out after every 30 d on fresh medium of similar composition.

The fresh weight of the propagules was taken directly by removing cultures from the medium and gently separating the agar-agar sticking to these propagules with a soft brush. The cultures whose fresh weight was recorded were then wrapped in Aluminium foil and kept in oven at 60ºC and the weight was periodically recorded after every 24 h and three consecutive readings were taken until the weight stabilized.

The plantlets were subjected to *in vitro* hardening on agar-agar and *Luffa* sponge or cocopeat: perlite (9:1) in liquid medium. Each of these contained one-half strength KC major salts only except $Ca(NO_3)_2.4H_2O$ with each culture bottle containing 25 ml medium. The *Luffa* sponge (derived from dried fruits of *Luffa aegyptica*) was used as a matrix in liquid media (Gangopadhyay *et al*., 2009) was cut transversely. The plantlets were further transferred to the greenhouse in plastic trays containing cocopeat: perlite (9:1) (potting mix) and maintained under controlled conditions of light, 25 ± 2º C and 85-90% relative humidity.

Twelve replicates were carried out for each treatment and the experiments were repeated twice. Fifteen replicates were taken for recording the data on *in vitro* hardening. The data was subjected to univariate analysis of variance and least significant difference (LSD) at $p \leq 0.05$ was applied to test statistical significance by using SPSS, Sigmastat, Chicago, IL, USA.

RESULTS

The seeds inoculated on KC medium supplemented with BE [1-20% (v/v)] germinated and the propagules on BE supplemented media showed early rooting and rapid progress to advanced stages of development leading to rapid seedling formation in comparison to control (Vyas *et al*., 2009). After 30 d of third subculture, the most optimum response was observed on 20% (v/v) BE supplemented KC medium. The plantlets showed significantly higher shoot and root lengths in comparison to control (Fig. 1A). There was no significant difference in the fresh weight of propagules in comparison to control upon incorporation up to 10% (v/v) BE in the KC medium but it was significantly high on KC medium supplemented with 20% (v/v) BE (Fig. 1B). When dry weight was recorded, no significant difference was observed in comparison to control up to 5% (v/v) BE supplemented KC medium. But with further higher percentages of BE [10% and 20% (v/v)], it was significantly higher than the control (Fig. 1B).

After 30 d of fourth subculture, shoot, leaf and root length and root number were significantly higher on 20% (v/v) BE supplemented KC medium in comparison to control (Figs. 1C; 2A). The plantlets on 20% (v/v) BE supplemented KC medium exhibited elongated intense green

leaves and well developed velamenous roots in comparison to control which were with tiny leaves and roots (Fig. 2A). Synchronous growth pattern was observed in cultures on 20% (v/v) BE supplemented KC medium (Fig. 2B-D). The plantlets were morphologically uniform. The shoot and root lengths of these plantlets were nearly similar and these exhibited well formed root system and were produced on a mass scale.

These synchronously growing plantlets were then subjected to *in vitro* hardening on plain agar-agar medium and *Luffa* sponge and cocopeat: perlite as support matrices. The plantlets hardened *in vitro* on *Luffa* sponge as support matrix exhibited elongated intense green leaves with broad lamina and long velamenous and light green roots some of which intertwined with fibres of *Luffa* sponge (Fig. 3A). The plantlets on cocopeat: perlite as support matrix formed long leaves with broad lamina, but many basal leaves senesced (Fig. 3B). The plantlets on agar-agar medium developed a cluster of numerous green and velamenous roots but the leaves had narrow lamina and some of the leaves also turned chlorotic (Fig. 3C). The plantlets immediately before transfer exhibited well formed leaves and roots (Fig. 3D). The number of shoots and roots of the plantlets on 0 d of inoculation on *Luffa* sponge, cocopeat: perlite and agar-agar was approximately similar (Fig. 4A). The maximum increase in the number of shoots of plantlets was observed on *Luffa* sponge as support matrix followed by agar-agar and cocopeat: perlite after 30 d of *in vitro* hardening in comparison to that on 0 d of inoculation (Fig. 4B). The number of roots also increased in plantlets on medium with *Luffa* sponge as support matrix (Fig. 4B). These plantlets also showed maximum shoot and root length (Fig. 4C&D). Some of the roots were smaller than the other roots. So the root length was clubbed into two categories- those lesser than or equal to two cm were referred to as short roots while those above two cm as long roots. The maximum length of short roots was observed in plantlets on *Luffa* sponge followed by those on agar-agar and cocopeat: perlite while that of long roots was observed in plantlets on *Luffa* sponge followed by cocopeat: perlite and agar-agar (Fig. 4D).

A marked feature of leaf senescence was observed during *in vitro* hardening. The plantlets on cocopeat: perlite recorded the maximum percentage of leaf senescence followed by those on agar-agar. The minimum percentage of senescing leaves was observed in plantlets on *Luffa* sponge as support matrix (Fig. 4E). The plantlets with shoots showing pseudobulbs and well developed intense green leaves and long light green roots with velamen, growing in the greenhouse conditions exhibited prolific growth after six weeks of transfer (Fig. 3 E-F).

DISCUSSION

Synchronous formation of plantlets (morphological uniformity) of *Dendrobium lituiflorum* on 20% (v/v) BE supplemented KC medium with elongated intense green leaves and well developed roots was observed after 30 d of fourth subculture. This synchronous growth and development is desirable as it helps in providing a large number of plantlets at a given stage of development after a particular time period. In Dendrobium lituiflorum, BE was not only promotory in causing higher percentage germination, early rooting and faster growth and development in comparison to control (Vyas *et al*., 2009), it was also effective in achieving a large number of plantlets with higher fresh and dry weights indicating increased biomass and exhibiting the same stage of development when it was supplemented at 20% (v/v) to KC medium as reported in this investigation. Sudeep *et al*., (1997) also observed significant increase in shoot length and leaf number of Dendrobium nobile in Vacin and Went medium (Vacin and Went, 1949) supplemented with 10% banana pulp. Pierik *et al*., (1988) observed that although banana homogenate was inhibitory in seed germination of *Paphiopedilum ciliolare* but it promoted the growth of seedlings. The growth promoting effect of banana can be speculatively attributed to any of the following reasons in the present study: (a) buffering of media, (b) chelation of iron rendering it more readily available to the plants, (c) mineral nutrients in concentration and form that is appropriate for the plants and (d) growth substances

(Arditti, 1968) and needs to be confirmed with further experimentation. Banana contains IAA (Khalifah, 1966b), GA_7 and GA_x (Khalifah, 1966a) and zeatin, zeatin riboside and 2-iP (Van Staden and Stewart, 1975). The growth-enhancing effect of banana might occur due to the individual action of any of these substances or a synergistic effect of two or more of them (Arditti, 1968). Synchronous plantlet formation is advantageous as it not only reduces the time frame for complete plantlet formation (which is advantageous for commercial growers), it also eases the task of transplanting as a large number of plantlets can be removed from the culture vessel without an additional step of subculture for the remaining smaller plantlets (Vyas, 2010). In the present study, a large number of uniform plantlets with well developed root system and shoot growth can be transferred for *in vitro* hardening and finally to the greenhouse conditions which will be beneficial for the conservation of this threatened orchid. Synchronous rapid plantlet regeneration, thus, have a major impact at a commercial level as an efficient time saving strategy.

Synchrony in secondary somatic embryogenesis was also achieved by Mondal *et al*., (2001) in *Camellia sinensis* by using nitrate salts of potassium (9.39 mM) and ammonium (10.3 mM) with potassium sulphate (1.5 mM) and also on MS medium containing the above mentioned salts with BAP (8.88 µM), IBA (0.98 µM) and glutamine (10 mM). Our report differs from previous studies as in this case a natural additive (BE) has been used as a supplement to KC medium for the study instead of synthetic plant growth regulators. Banana grows in tropical areas which form the native habitat of this orchid, reduces the input cost considerably at a commercial scale (Nayar Gopalan, 1962; Vyas, 2010).

The plantlets growing in the medium enriched with minerals of the basal medium (here, KC), natural additives and sucrose depend mainly on the exogenous supply of nutrients for their growth and development. In the present study, the plantlets of *Dendrobium lituiflorum* were subjected to *in vitro* hardening by using various support matrices (*Luffa* sponge and cocopeat: perlite) in culture vessels and on agar-agar medium without sucrose containing one-half strength of KC major salts. Sucrose was eliminated from the medium in the present study for two reasons, firstly, although it promotes growth, but it also depresses photosynthesis and reduces *in vitro* hardening (Deng and Donnelly, 1993) and secondly, to minimize contamination (see De Faria *et al*., 2004). Besides, the use of growth-regulating substances, vitamins and other organic substances can be minimized because some of these will be produced endogenously in sufficient quantities by the plantlets growing autotrophically (Kozai, 1991). This view is also supported by Deb and Imchen (2010) who reported the use of $1/10^{th}$ liquid MS basal medium without sucrose for the *in vitro* hardening of three orchid species, *Arachnis labrosa*, *Cleisostoma racemiferum* and *Malaxis khasiana* on support matrices such as charcoal pieces, brick chips, mosses and decayed wood. Therefore, in the present study, only minimal amount of nutrients were provided to the plantlets.

Senescence of leaves was observed in the cultures hardened *in vitro* on the two support matrices, viz., *Luffa* sponge and cocopeat: perlite and agar-agar gelled medium. Pic *et al*., (2002), while working on pea, speculated that acceleration of leaf senescence is an adaptation in plants subjected to water shortage, firstly, as it reduces the water demand accumulated over the whole plant cycle and secondly, as it allows recycling of scarce resources to the reproductive sinks. Merrien *et al*., (1981) emphasized that it is hastened by water or nitrogen deficits. In the present findings, since plantlets on the support matrices viz., cocopeat: perlite, *Luffa* sponge and agar-agar medium (containing liquid KC medium without sucrose with one-half strength major salts) showed senescing leaves, it was possible that both water and nitrogen deficits were causing it. However, the percentage of senescing leaves varied in the support matrices. If nitrogen deficit was presumed to be the major factor, the percentage senescence would have been nearly same in plantlets in all the three treatments as same amount of KC salts were present under all the three conditions. Therefore, possibly water deficit was the main reason for the leaf senescence which was the highest in the plantlets on cocopeat: perlite which was drier than agar-agar gelled medium and *Luffa* sponge

(all containing one-half strength KC major salts without sucrose). The above results clearly point towards the efficacy of *Luffa* sponge as support matrix for the *in vitro* hardening of Dendrobium lituiflorum. The roots received ample space to grow and entangled the fibrous network of the *Luffa* sponge which aided in plantlet growth. Gangopadhyay *et al*., (2004, 2005) successfully micro-propagated *Philodendron* and pineapple by using *Luffa* sponge as a matrix instead of agar-agar. Gangopadhyay *et al*., (2004) reported that for the *in vitro* propagation of *Philodendron*, *Luffa* sponge was better than coir. The plantlets of *Dendrobium lituiflorum* exhibited successful survival in the greenhouse conditions after six weeks of transfer.

CONCLUSIONS

For conservation of threatened orchids, synchronous plantlet formation is important as these can then be transferred to the greenhouse and ultimately to the field conditions. Secondly, synchronous plantlet formation is a big boost to the biotech industries where a large number of plantlets at a particular stage of development at a given time period are required. Besides, incorporation of banana extract, an inexpensive and readily available natural additive, can bring down the cost of production considerably without compromising with the quality of plantlets produced. *In vitro* hardening by using inexpensive support matrices also serves as a cost effective measure aiding in successful acclimatization of these plantlets produced at a mass scale *in vitro* (Vyas, 2010).

ACKNOWLEDGEMENTS

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Figures

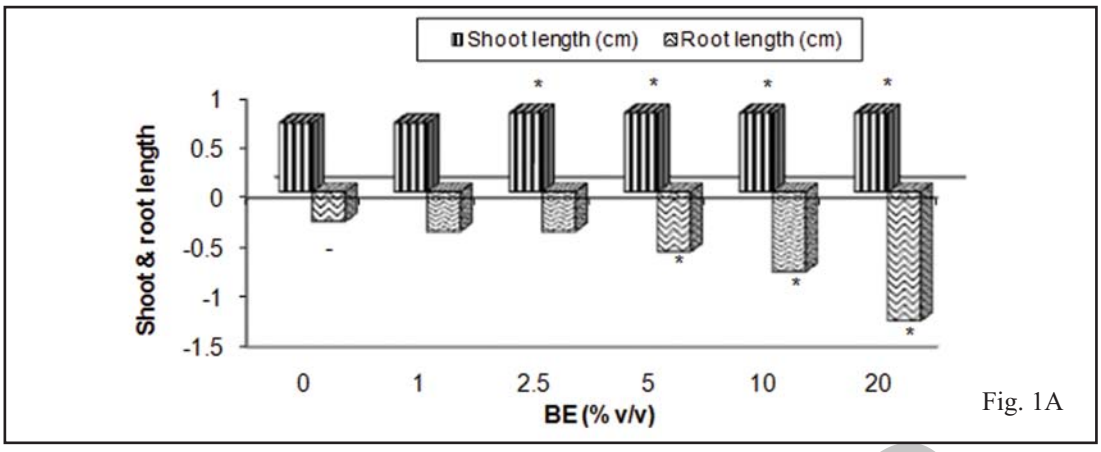

Fig. 1A

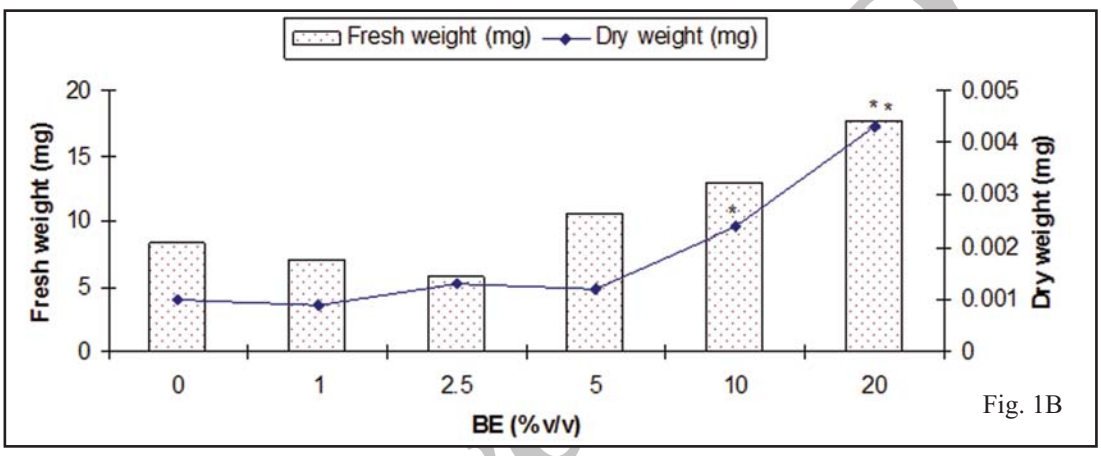

Fig. 1B

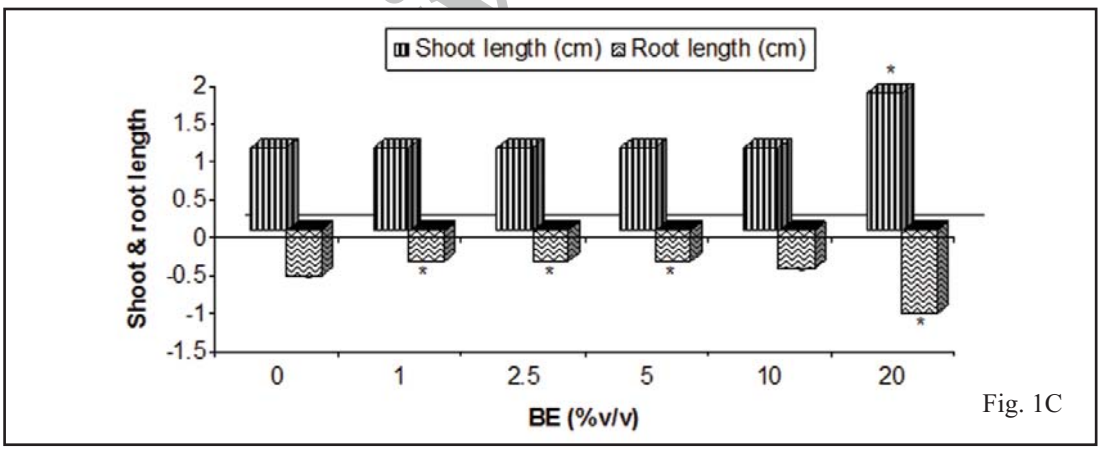

Fig. 1C

Fig. 1. Effect of BE on growth and fresh and dry weights of plantlets of *Dendrobium lituiflorum.*

A. The plantlets growing on 20% (v/v) BE supplemented KC medium show maximum growth in comparison to control after 30 d of third subculture.
B. The fresh and dry weights of plantlets on 20% (v/v) BE supplemented KC medium are significantly higher than those on control.
C. The cultures on KC medium supplemented with 20% (v/v) BE show maximum growth in comparison to control and other treatments after 30 d of fourth subculture.
($\bar{X}$ = 12 replicates, *, L.S.D., $p<0.05$)

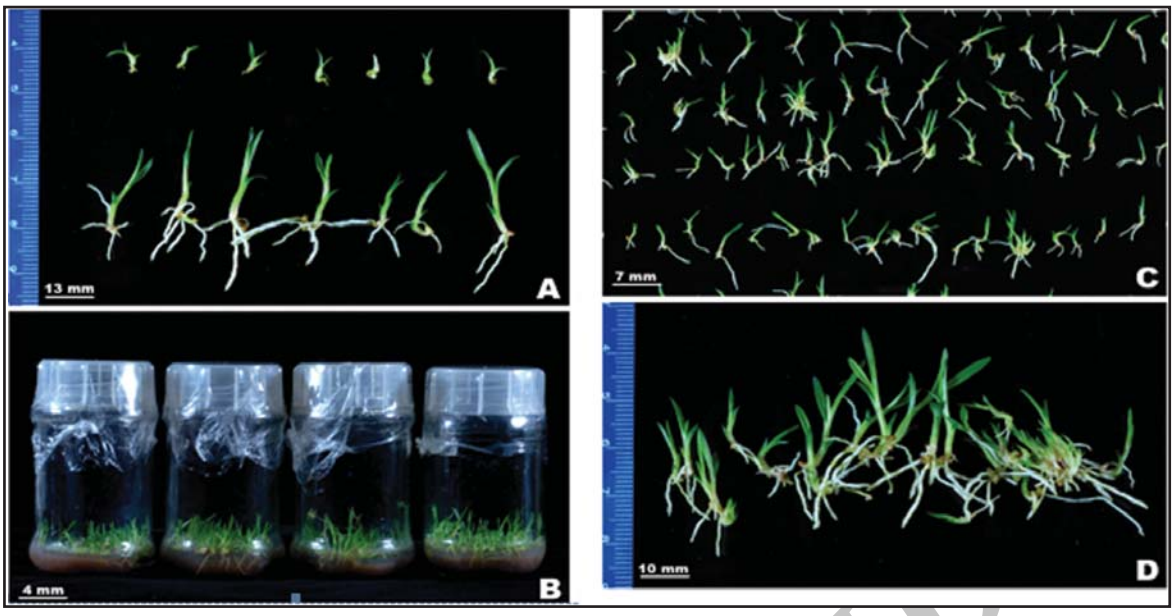

Fig. 2. Effect of BE on growth of plantlets on KC medium after 30 d of fourth subculture.
A. The cultures growing on 20% (v/v) BE supplemented KC medium (lower row) show significant shoot and root growth in comparison to control (upper row).
B. Synchronous mass scale production of culture on KC medium with 20% (v/v) BE.
C&D. Synchronously developed plantlets on KC medium supplemented with 20% (v/v) BE taken out of culture bottles to show shoot and root growth.

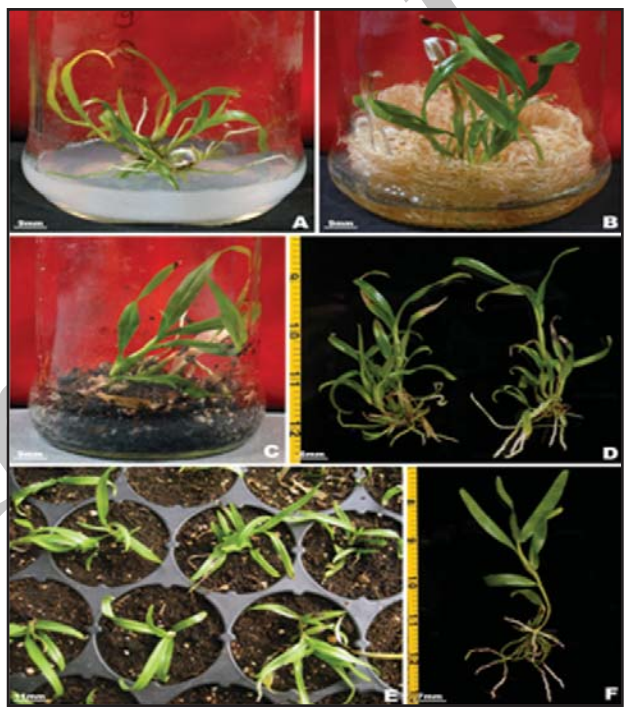

Fig. 3. *In vitro* hardening and acclimatization of plantlets of Dendrobium lituiflorum.
A. Plantlets on plain agar-agar medium showing a few chlorotic leaves.
B. Plantlets on Luffa sponge showing well expanded leaves growing prolifically.
C. Plantlets on cocopeat: perlite (9:1) showing senesced leaves.
D. Prolifically growing plantlets after in vitro hardening immediately before transfer to the greenhouse conditions.
E. Plantlets growing vigorously in the greenhouse on potting mix [cocopeat : perlite (9:1)] after six weeks of transfer.
F. Plantlets taken out of potting mix showing well expanded intense green leaves and light green long roots with velamen.

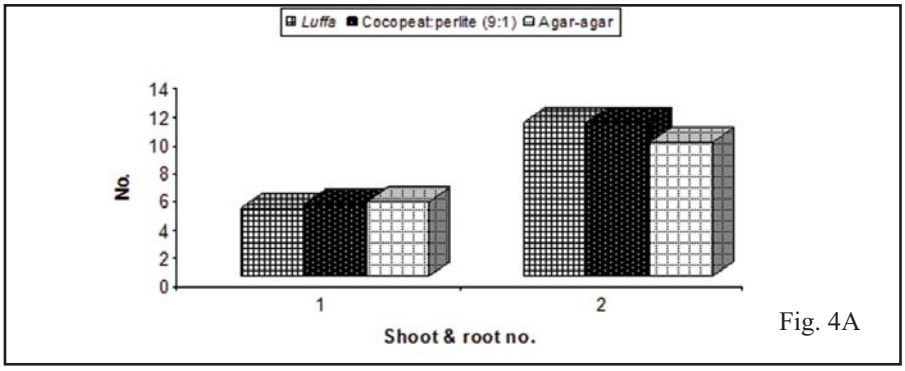

Fig. 4A

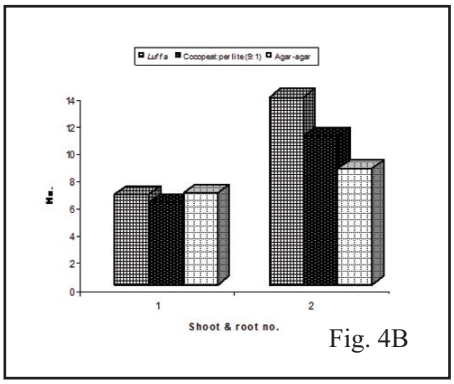

Fig. 4B

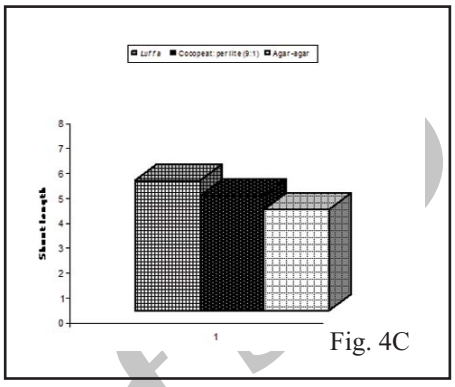

Fig. 4C

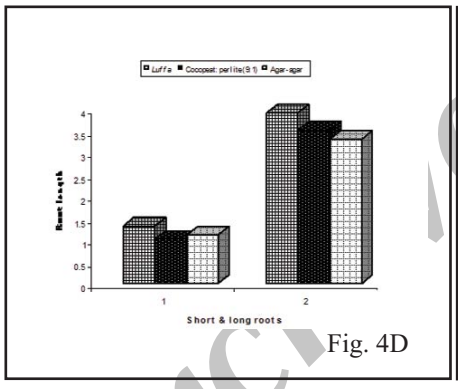

Fig. 4D

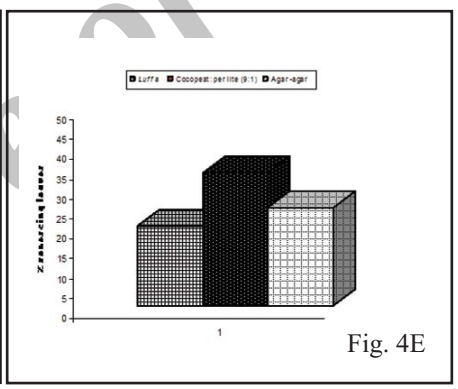

Fig. 4E

Fig. 4. Effect of various support matrices on in vitro hardening of plantlets of *Dendrobium lituiflorum* on liquid KC medium (one-half strength major salts only) after 30 d.

A. The number of shoots and roots on zero day of inoculation on various support matrices viz., Luffa sponge, cocopeat: perlite (9:1) and agar-agar medium.
B. Among the three matrices, maximum increase in the number of shoots and roots is observed on *Luffa* sponge as support matrix (c*f* A). After that the maximum number of shoots are on cocopeat: perlite (9:1) and roots on agar-agar medium, respectively.
C. The shoot length is higher in cultures on *Luffa* sponge followed by cocopeat: perlite and agar-agar medium.
D. The length of both short and long roots is maximum on *Luffa* sponge.
E. The percentage of senescing leaves is maximum in plantlets on cocopeat: perlite followed by agar-agar and minimum in plantlets on *Luffa* sponge.