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# Development of a cost effective *in vitro* regenerative protocol of *Cymbidium aloifolium* (L.) Sw. using nodal segments as an explants source

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## Abstract

Regenerative competence of *in vitro* sourced nodal explants of *Cymbidium aloifolium* was tested positively. About 90% morphogenetic response was achieved on MS medium supplemented with sucrose (3%) (w/v) and α-naphthalene acetic acid (9 µM) after 18 days of culture initiation. Of the three basal media tested for culture initiation, MS medium supported optimum morphogenetic response in comparison to other two media. The shoot buds converted into rooted plantlets on regeneration medium containing sucrose (3%) and benzyl adenine (3 µM). Amongst the different media at different strengths used, optimum regeneration of plantlets and culture proliferation was achieved on full MS medium where as many as 12 shoot buds formed per subculture while, at lower concentrations of MS medium impaired regeneration. Apart from agar, three other alternative substrata were used in the initiation and regeneration medium with the objective to reduce the production cost. Amongst the different substrata, regeneration on 'polyurethane foam' was at per with the agar gelled medium followed by coconut coir as substratum. The well rooted plantlets were hardened for 3-4 wk with $1/10^{th}$ MS salt solution containing sucrose (1%) and charcoal pieces, brick pieces and chopped mosses as support under normal laboratory conditions before transferring to community potting mix. About 80% of the transplants survived after two months of transfer.

***Key words:*** Alternative explants, alternative substrata, *Cymbidium aloifolium,* nodal segment culture, therapeutic orchid.

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## 1. Introduction

A great deal of heterozygosity is produced in their progeny in orchids owing to its out breeding characteristic. Because of this embryo culture appears to be a disadvantageous proposition in cut-flower industry where pure lines of desired genotypes are preferred. It was Morel who first demonstrated the possibility of using excised shoot-meristem for regenerating complete plant from *Cymbidium in vitro* [1]. Since then, with the refinement of tissue culture techniques, the emphasis has now been shifted towards exploring alternative plant organs for *in vitro* mass multiplication of horticultural and other economically important taxa, whereby the very existence of mother plants are not threatened [2-8]. The regeneration potential of alternative explant sources like shoot apex, foliar explant, aerial roots, inflorescences and nodal meristem has been exploited in a number of orchid species [2, 5-6, 8-10]. Consequently, efforts have been directed towards testing the regeneration potential of a variety of donor organ with a view to identify alternative explants whose excision does not jeopardize the survival of mother plants [11]. There are various factors which can influence the *in vitro* regenerative competence of these explants sources. Development of tissue culture techniques for commercial scale production of orchids have included development of low cost protocols including low cost alternative substrata to agar, as agar is considered to be one of the costliest component [12-15].

*Cymbidium aloifolium* (L.) Sw. is a multipurpose orchid grows as an epiphyte on tree trunk in the primary forests. It produces yellowish flowers with purple mid rib. This species has formed the basis for many hybrids of floricultural value due to its long pendant racemes. Besides horticultural value, the species is of great therapeutic value. It is a source of '*Salep*' used as nutrient and demulcent and its rhizomes are also used as emetic and purgative [16-17]. The aerial roots of *C. aloifolium* are made into paste and plastered rightly on fractured bones [18]. Furthermore, the seeds of *Cymbidium* species are used as oral contraceptive in some parts of Eastern Ghat of India [19]. Moreover, it has

also been observed that several species under this genus have the potential to become effective pesticides and fungicides, which indirectly emphasizes the presence of abundant quantity of defense proteins like lectins in them. The regeneration of this species in natural condition is very poor. During the present investigation we investigated into the role of different factors on *in vitro* morphogenetic response of nodal segments and mass multiplication of *C. aloifolium* and also on the potential use of three different low cost substrata against agar in the initiation and regeneration medium with the objective to reduce the production cost.

## 2. Material and Methods

***2.1. Explants source*****:** Seedlings were raised by culturing immature embryos from green pod age of 9 months after pollination (MAP) on MS medium containing sucrose (2%) (w/v), NAA and BA (3 and 6 μM respectively in combination). Within 6-7 wk of culture, the germinated embryos formed protocorm-like bodies (PLBs). The PLBs differentiated into plantlets on MS medium containing sucrose (3%) and BA (3 μM) [20]. Elongated shoots of about 8-10 wk old were harvested from the regeneration medium. The leaves were removed from the shoots and the nodal segments were used as explants for the present study.

***2.2. Processing of alternative substrata***: A part from agar as gelling agent, three other types of substrata, viz., betel nut coir, coconut coir and polyurethane foam (foam) were used as alternative to agar for initiation of culture, regeneration and mass multiplication of plantlets. 'Foam' was collected from the local market at Nagaland, India which is generally used for preparation of mattresses. While betel-nut coir, coconut-coir were extracted from the dried fruits and chopped into small pieces according to our requirements. All the three substrata were soaked with 'Extran' (a commercial laboratory detergent, make: Merck, India) (1:100 ratio) (v/v) for about two hr followed by washing under running tap water till water ran clean. The substrata were air dried and stored till used. The dried betel-nut coir and coconut coir were chopped into small pieces (~0.5 cm size), while the foam was cut into disk (according to the culture vials). All the substrata were then autoclaved at1.05 Kg $cm^{-2}$ pressure and 121$^o$C for one hr before putting them in the culture vials.

***2.3. Media for culture initiation***: In the present study, three different basal media were studied for culture initiation from nodal segments viz. MS [21], Mitra *et al* [22] and Knudson 'C' [23]. The basal media were fortified with different levels sucrose (0-4%) (w/v), casein-hydrolysate (0.1$gl^{-1}$) (CH) and different quality and quantity of plant growth regulators (PGRs) like α-naphthalene acetic acid (NAA) and benzyl adenine (BA) (0-9 μM) either singly or in combination. Difco-bacto agar (0.8%) was used as gelling agent and the pH of the media was adjusted to 5.6 using 0.1 N NaOH and 0.1 N HCl. About 15 ml of medium was dispensed in each borosilicate test tube (size: 25x150 mm, make: Borosil, India) and cotton plugged before autoclaving at 1.05 kg $cm^{-2}$ pressure and at 121$^o$C for 20 min. For alternative substrata, liquid media were dispensed in the culture vials and the pre-processed substrata were added before autoclaving the media.

***2.4. Initiation of culture***: The nodal segments from the axenic sourced plants were cultured on different basal media containing different adjuncts. The nodal explants were cultured on agar gelled media, and media with other substrata. The newly sprouted shoots/buds, PLBs were separated and cultured on optimum initiation medium and maintained for another two passages for further differentiation.

***2.5. Regeneration of plantlets and mass multiplication*****:** The young plantlets and well developed PLBs with the first set of leaflets (advanced stage PLBs) were separated from the initiation media and cultured on regeneration media. For regeneration of plantlets three different basal media (MS, Mitra *et al*, Knudson 'C') at different strength were used. The basal media were enriched with sucrose (3%) (w/v), CH (0.1 $gl^{-1}$) and different PGRs like NAA, BA, KN, (0-9 μM) either singly or in combination. The resulted multiple shoot buds/plantlets were separated from the regeneration and cultured on fresh regeneration medium for further multiplication. For regeneration of plantlets, the shoot buds were also cultured on different substrata like agar gelled medium, media with 'foam', 'betel-nut coir' and 'coconut coir' as substrata alternative to agar.

***2.6. Experimental design***: A completely randomized experimental design was performed. For each treatment at least 20 explants were maintained and all the experiments were repeated at least thrice. The cultures were maintained at 25±2$^o$C under cool white fluorescent light at 40 μmol $m^{-2}$ $s^{-1}$ and 12/12 hr each (light/dark) photo period. All the cultures were sub-cultured at 4-5 wk interval unless mentioned otherwise. *In vitro* response was evaluated based on the percentage of explants responded and number of propagules formed in the culture after specific period of time (as stated in the table) and data was expressed as the mean of replicates ± standard error.

***2.7. Hardening of plantlets and transplantation in community potting mix (CPM)***: About 6-7 cm long well rooted plantlets (with 3-4 roots) were taken out from the regeneration medium and used for *in vitro* hardening. For the purpose the rooted plantlets were cultured on 1/10$^{th}$ MS liquid medium supplemented with sucrose (1%) freed from any PGRs. In the culture vials small charcoal pieces, brick pieces and chopped mosses (at 1:1 ratio) was used as substrata. The cultures were maintained for 3-4 wk in normal laboratory condition before transferring to perforated plastic pots with potting mixture containing charcoal, brick pieces, coconut husks, decayed wood and sand (at 1:1 ratio) and topped with a layer of moss and covered with holed transparent poly bag. The potted plants were irrigated at regular interval for about two months before transferring to the wild.

## 3. Results and Discussion

Wimber successfully developed PLBs from the foliar explants of *Cymbidium*s, which opened up an effective route for *in vitro* propagation of orchids using alternative explants other than seeds [24]. In the present study the immature embryos from 9 MAP old green pods were initially germinated on MS medium containing sucrose (2%), NAA and BA (3 and 6 µM respectively in combination). The germinated embryos formed protocorm-like bodies (PLBs) within 6-7 wk of culture which subsequently converted into plantlets on MS medium containing sucrose (3%) and BA (3 µM) (Fig. 1a). Elongated shoots of about 8-10 wk old were harvested from the regeneration medium and leaves were removed from the plantlets before nodal segments were cut and used for culture initiation (Fig. 1b).

***3.1. Initiation of culture from nodal explants***: Within 7 days of culture sprouting of shoot buds/PLBs from the nodal region were initiated (Fig. 1c) and within 15 days of culture distinct shoot buds formed. The orchids of different species exhibit a preferential requirement to specific nutrient media for culture initiation, regeneration of plantlets. The requirements also vary with the source of explants and their physiological status [2-3, 5, 25]. In the present study, amongst the three basal media tested, optimum morphogenetic response was achieved on MS medium whereas on other two media morphogenetic response was delayed and fewer shoot buds were formed (Table 1).

As many as 5 shoot buds/PLBs developed at a single node on MS medium supplemented with sucrose (3%) and NAA (9 µM) after 18 days of culture (Table 2). About 90% explants responded positively with the sprouting of shoot buds/PLBs from nodal regions under optimum conditions. In the present study NAA was found to be superior over BA for morphogenetic response. But in *Phalaenopsis* BA (2 $mgl^{-1}$) and NAA (0.5 $mgl^{-1}$) in combination was found to be optimum for breaking of axillary buds and formation of multiple shoot buds [26] and incorporation of NAA was found to be promotory. Tisserat and Jones [27] and Roy and Banerjee [28] also observed that an appropriate combination of NAA and BA stimulate multiple shoot buds formation. But, Arditti and Ernst [11] reported that the addition of NAA reduced the induction and regeneration. The PLBs and shoot buds formed from the cultured nodal explants of *C. aloifolium* were maintained for another two passages for further differentiation and proliferation on optimum growth conditions before they are transferred on regeneration medium.

Amongst the different substrata used for initiation of culture, better culture initiation was achieved on medium containing foam as substratum apart from agar. While other two substrata did not support healthy morphogenetic response and cultures degenerated subsequently.

***3.2. Regeneration and mass multiplication***: The propagules from the initiation media were maintained on three different media, viz., Knudson 'C', Mitra *et al* and MS media with different growth adjuncts. In the preliminary study, it was observed that MS medium supported better regeneration and multiplication of culture compared to other two media (Table 3). Further experiments were conducted to study the effect of different strengths of MS medium on regeneration of plantlets and culture proliferation. It was found that full strength MS medium supported the formation of maximum shoot buds and well rooted healthy plantlets. It was observed that at lower strengths ($1/4^{th}$, ½ and $3/4^{th}$ strengths) of MS medium plant growth was stunted accompanied by fewer new shoot buds formation. Optimum plant height (~6 cm) as well as maximal shoot buds formation (12 shoot buds) were achieved on full MS medium (Table 4, Fig. 1 d).

Different quality and quantity of PGRs marked a pronounced effect and elicit different responses in the seedling development. Inclusion of PGRs in the regeneration medium was obligatory for successful regeneration of plantlets and mass multiplication. Of the different PGRs used in the regeneration media, a lone treatment of BA (3 µM) supported optimum regeneration and mass multiplication (Table 5). It was observed that NAA used singly did not support healthy regeneration and in most of the cases plantlets either etiolated or fewer shoot buds formed. While both BA and KN singly supported better regeneration and culture proliferation and BA was found to be superior over KN.

***3.3. Effects of different alternative substrata on regeneration and mass multiplication***: The goal of the present study was also to screen some low cost substrata as alternative to agar for use in plant tissue culture so that the production costs could be reduced substantially. The hunt for the cheap substratum could be that which is/are of very low cost. In the past some efforts have been put into to use different substrata as alternative to agar, viz., agarose [29], gelrite [30], guar gum [31], xanthan gum [32] with reasonable success as substitute of agar. However, these are not expected to find universal acceptance for various reasons. In the present study besides agar gelled medium, betel-nut coir, coconut coir and foam were successfully used as alternative to agar for regeneration and culture differentiation. It was observed that the initial response was better on agar gelled medium as cultures establish on this medium faster compared to other substrata. However, once cultures establish themselves on the alternative substrata especially on foam and coconut coir, they exhibited healthier growth and rapid culture proliferation compared to cultures on agar gelled medium (Fig. 1 d, e, f) (Fig. 2). There were as many as 12 shoot buds developed on agar gelled medium against 10 shoot buds on foam (Fig. 2).

***3.4. Hardening of plantlets and transplantation in CPM:*** The hardening of *in vitro* raised plantlets is essential for better survival and successful establishment. Losses of micropropagated plants after transferring to nature are attributed to low humidity, high levels of light and non-sterile condition of the field environment [13, 33]. Conventionally the tissue culture raised plants are hardened by transferring on a low nutrient medium having low organic carbon sources and maintained at high light intensity. Different matrix or substrates with manipulation in salt solution were employed for hardening of different *in*

**Table 1:** Effect of basal nutrient media on *in vitro* morphogenetic of nodal explants of *C. aloifolium**

| Basal media | Time for Initiation of response (days) (±SE)** | Type of Response |
|---|---|---|
| **MS** | 15 ±2.0 | Multiple shoot buds/PLBs formation. Plantlets were healthy with 2-3 roots. |
| **Mitra *et al*** | 20 ±3.0 | Fewer shoot buds formation. Plantlets were moderately healthy but root formation was poor |
| **Knudson 'C'** | 35 ±2.0 | Very few shoot buds formed, but failed to differentiate well and degenerated subsequently |

* On media containing sucrose (3%) (w/v), CH (0.1 $gl^{-1}$), and NAA (9 µM); ** Standard error
Data represents the mean of three replicates.

**Table 2:** Effects of PGRs on morphogenetic response of nodal explants of *C. aloifoium* from *in vitro* source#

| PGR Conc. (µM) NAA | BA | % response (±SE)* | Time for culture initiation (days) | Mean No. of shoot/ PLBs formed/Node* |
|---|---|---|---|---|
| Control | | 60 (0.50)$^{d}$ | 5.5 | 1.2 (0.2)$^{d}$ |
| 3 | 0 | 70 (1.50)$^{c}$ | 13.2 | 2.4 (0.2)$^{c}$ |
| 6 | 0 | 80 (1.50)$^{b}$ | 13.3 | 5.1 (0.1)$^{a}$ |
| ***9*** | ***0*** | ***90 (1.00)***$^{a}$ | ***18.5*** | ***5.2* (0.2)**$^{a}$ |
| 0 | 3 | 50 (0.50)$^{e}$ | 15.2 | 1.3 (0.1)$^{d}$ |
| 0 | 6 | 88 (1.25)$^{b}$ | 18.6 | 3.4 (0.3)$^{b}$ |
| 0 | 9 | 90 (1.50)$^{a}$ | 14.3 | 4.2 (0.2)$^{a}$ |
| 3 | 3 | 55 (2.00)$^{d}$ | 13.2 | 1.2 (0.2)$^{d}$ |
| 3 | 6 | 60 (1.00)$^{d}$ | 18.5 | 3.3 (0.2)$^{b}$ |
| 3 | 9 | 58 (1.50)$^{d}$ | 14.3 | 2.1 (0.1)$^{c}$ |
| 6 | 3 | 72 (1.00)$^{c}$ | 15.4 | 2.4 (0.3)$^{c}$ |
| 6 | 6 | 48 (0.50)$^{e}$ | 21.3 | 2.2 (0.1)$^{c}$ |
| 6 | 9 | 80 (1.00)$^{b}$ | 18.5 | 2.5 (0.3)$^{c}$ |
| 9 | 3 | 60 (2.00)$^{d}$ | 18.6 | 2.3 (0.2)$^{c}$ |
| 9 | 6 | 30 (1.00)$^{f}$ | 20.4 | 1.5 (0.1)$^{d}$ |
| 9 | 9 | 30 (1.00)$^{f}$ | 15.2 | 1.4 (0.2)$^{d}$ |

# On MS medium containing sucrose (3%), * Standard error (Values followed by the same *letters* are not significantly different from each other), Data represents the mean of three replicates.

**Table 3:** Effects of different basal media on regeneration of plantlets of *C. aloifolium**

| Basal media | No. of shoot/PLBs formed/explants(±SE)* | Time taken for 1st leaf (days) (±SE)* | Response** |
|---|---|---|---|
| | 1.5 (0.2) | 35.5 (2.5) | Elongation of shoot bud but degenerated subsequently |
| | 3.4 (0.3) | 16.3 (1.7) | Plantlets healthy but stunted in growth with fewer leaves and roots |
| | 12.1 (0.2) | 21.5 (1.5) | Healthy plantlets with 3-4 roots and multiple shoot buds and PLBs |

* Standard error; ** Media containing sucrose (3%) (w/v), BA (3 µM), Data represents the mean of three replicates.

**Table 4**: Effects of different strengths of MS medium for PLBs differentiation, plant regeneration and mass multiplication of *C. aloifolium*

| **Strength of MS medium** | **Avg. plant height (cm.)*** | **No. of shoot buds formed/subculture** | **Type of response**** |
|---|---|---|---|
| - - | Culture degenerated | | |
| 1.0 (0.2) 2 | Plantlets stunted in growth with few small leaves | | |
| 2.0 (0.2) 4 | Plantlets etiolated but with small leaves | | |
| 3.5 (0.1) 5 | Plantlets etiolated with well developed leaves and roots | | |
| 6.0 (0.2) 12 | Well rooted healthy plantlets | | |

* Standard error
** Media containing sucrose (3%) (w/v) and BA (3µM).
Data represents the mean of three replicates.

**Table 5:** Effects of different PGRs on plant regeneration and mass multiplication of *C. aloifolium**

| **PGRs Conc. (µM)#** | | | **No. of shoot/PLBs formed/explants (±SE)**** | **Days taken for 1st leaf** | **1st root** |
|---|---|---|---|---|---|
| **NAA** | **BA** | | | **KN** | **formation** |
| Control | | | | | 1 86 |
| 3 | 0 | 0 | 4.1 (0.2)$^{d}$ | 13 | 38 |
| 6 | 0 | 0 | 4.2 (0.2)$^{d}$ | 38 | 86 |
| 9 | 0 | 0 | 2.0 (0.2)e | 38 | 86 |
| ***0*** | ***3*** | ***0*** | ***12.4 (0.3)***$^{a}$ | ***21*** | ***21*** |
| 0 | 6 | 0 | 8.2 (0.5)$^{c}$ | 25 | 86 |
| 0 | 9 | 0 | 7.4 (0.3)$^{c}$ | 25 | 86 |
| 0 | 0 | 3 | 3.5 (0.5)$^{e}$ | 21 | 21 |
| 0 | 0 | 6 | 3.1 (0.5)$^{e}$ | 10 | 51 |
| 0 | 0 | 9 | 3.0 (0.1)$^{e}$ | 13 | 165 |
| 3 | 3 | 0 | 5.7 (0.2)$^{d}$ | 21 | 77 |
| 3 | 6 | 0 | 4.5 (0.3)$^{d}$ | 21 | - |
| **3** | **9** | **0** | **10.4 (0.5)**$^{b}$ | **38** | **86** |
| **6** | **3** | **0** | **9.4 (0.3)**$^{b}$ | **13** | **86** |
| 6 | 6 | 0 | 2.1 (0.2)$^{e}$ | 38 | - |
| 6 | 9 | 0 | 2.3 (0.2)$^{e}$ | 38 | - |
| 9 | 3 | 0 | 3.2 (0.1)$^{e}$ | 13 | 86 |
| 9 | 6 | 0 | 8.7 (0.6)$^{c}$ | 38 | 86 |
| 0 | 0 | 3 | 2.1 (0.2)$^{e}$ | 21 | - |
| 0 | 0 | 6 | 1.2 (0.2)$^{f}$ | 38 | - |
| 0 | 0 | 9 | 1.2 (0.2)$^{f}$ | 38 | - |
| 0 | 0 | 3 | 1.1 (0.1)$^{f}$ | 38 | - |
| 0 | 0 | 6 | 2.3 (0.4)$^{e}$ | 30 | - |
| 0 | 0 | 9 | 1.3 (0.2)$^{f}$ | 38 | - |
| 0 | 0 | 3 | 4.2 (0.1)$^{d}$ | 13 | 165 |
| 0 | 0 | 6 | 1.2 (0.1)$^{f}$ | 13 | - |

* On MS medium containing sucrose (3%) (w/v), # Only the significant treatments are computed here.
** Standard error (Values followed by the same *letters* are not significantly different from each other).
Data represents the mean of three replicates.

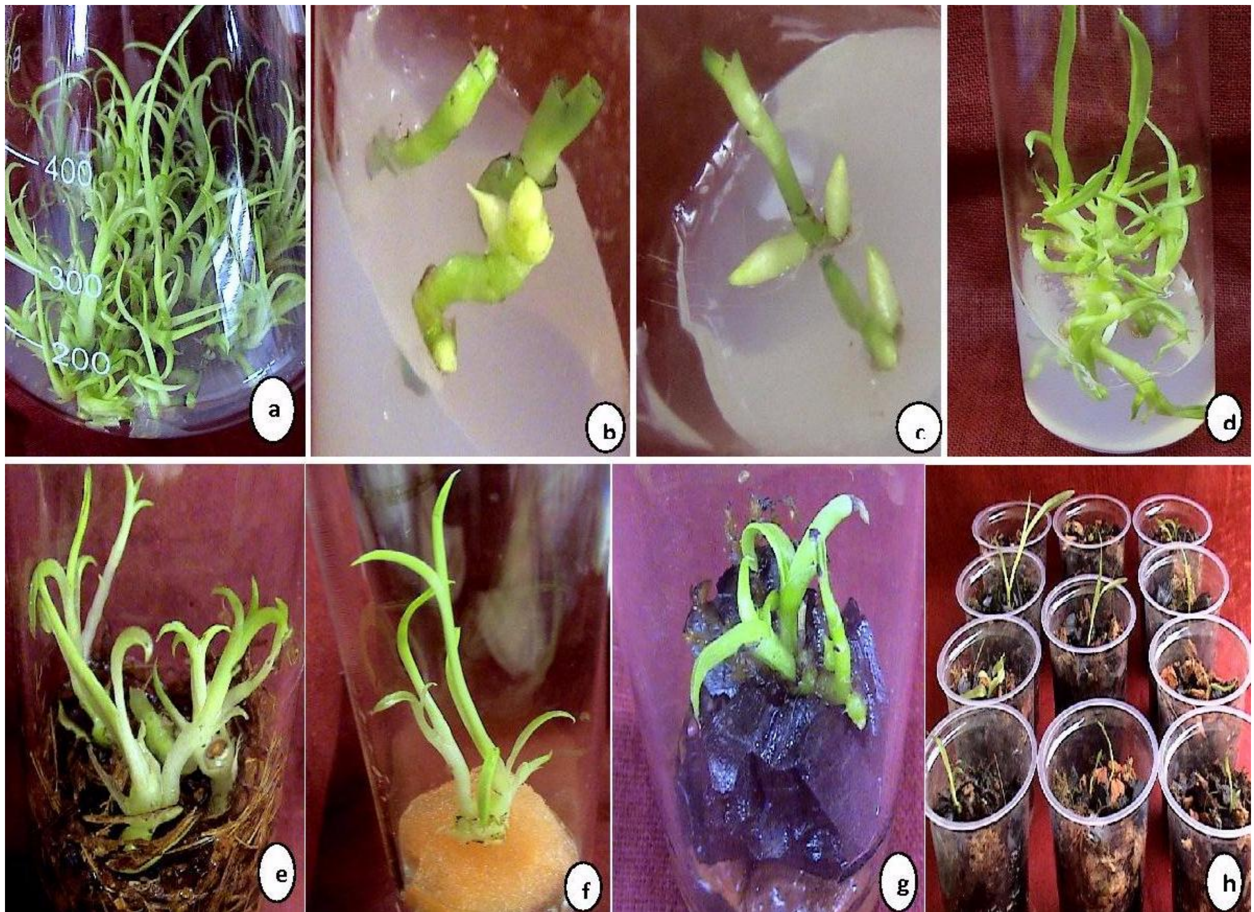


**Figure 1:** Different stages of *in vitro* morphogenetic response of nodal explants of *Cymbidium aloifolium*. a. Donor plants from where nodal explants are collected; b. Fresh nodal explants on initiation medium; c. Nodal explants initiated morphogenetic response showing sprouting of shoot buds; d. Multiple shoots on agar gelled regeneration medium; e. Regeneration of plantlets on coconut coir as substratum; f. Regeneration of plants on foam; g. Plants under hardening condition and h. Transplants in community potting mix.

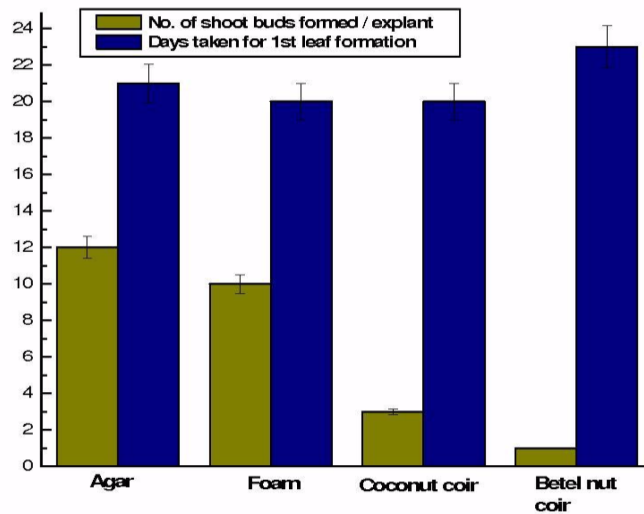


**Figure 2:** Effects of different substrata on regeneration and mass multiplication of *C. aloifolium* plantlets (*with MS medium containing 3% sucrose, 3 BA μM).*

*vitro* raised plants [34-35]. In the present study about 6-7 cm long well rooted plantlets (with 3-4 roots) were hardened for considerable period prior to transferring in the potting mix as mentioned in materials and methods (Fig. 1 g). To transplant the hardened plantlets, the CPM was prepared by mixing different substrates like sand: brick pieces: coconut husk: charcoal pieces: decayed wood (at 1:1 ratio) with a layer of moss (Fig. 1h). The hardened plants were transferred to CPM along with the contents in the culture vials under hardening condition and plants were covered with holed transparent poly bags. The potted plants were maintained in a shaded place and fed with $1/10^{th}$ strength MS salt solution weekly for 2-3 wk. About 80% of the transplants survived to form fully developed plants after two months of transfer.

## 4. Conclusion

The outcome of this study opens up the route for clonal mass multiplication of this economically important orchid from alternative explants source whose population is under check in its natural habitat. Besides this, during the present study we could successfully use some low cost substratums against agar for culture initiation and regeneration of plantlets which will help in reducing the production cost and commercial exploitation. Further studies are in progress to screen some other low cost substratums which could be used as alternative to agar in plant tissue culture.

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