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In Vitro Seed Germination and Micropropagation of *Dendrobium densiflorum* Lindl. on Low Cost alternative Substrata

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Abstract

In the present study, successful *in vitro* seed germination and propagation of *Dendrobium densiflorum* were achieved on low cost alternative substrata such as foam, saw dust, straw, sugarcane bagasse, etc. besides agar. Within three weeks of culture, immature embryos of 5 months after pollination (MAP) registered optimum germination (95%) on MS medium containing NAA (á Naphthalene acetic acid) + BA (Benzyl adenine) (3 + 3 µM respectively in combination) and 2% sucrose with sugarcane bagasse as substratum. The shoot buds/PLBs converted into rooted plantlets when they were maintained on optimum regeneration medium containing NAA + BA (6+3µM) in combination and sucrose (3%, w/v). Among the media incorporated withdifferent substrata higher regeneration and multiplication of plantlets were observed on medium containing foam as the alternative substratum followed by medium containing sugarcane bagasse and agar, where as many as 15 shoot buds developed per culture. Thehardened plantlets were transferred to community potting mix. About 80% transplants survived after two months of potting.

Keywords: *Dendrobium densiflorum, agar, alternative substratum, foam, sugarcane bagasse, straw, saw dust.*

Introduction

Orchidaceae represents one of the largest families among the flowering plants exhibiting vast diversity in vegetative and floral characteristics with long shelf life which give high value in floricultural industry. However, germination and subsequent regeneration of orchids in nature is limited due to suppressed endosperm and requirement of fungal stimulus (J. L. Harley, 1959; Temjensangba and Deb, 2006). But, this difficulty has been overcome by the development of tissue culture technique that has opened a new possibility in conservation and commercialization of orchids.

In microbial and plant tissue culture media, agar has been extensively used as a gelling agent due to its stability, high clarity non-toxic nature (McLachlan, 1985; Henderson and Kinnersley, 1988; Babbar and Jain, 2006).In the past, several attempts have been made to look for substratum which can replace agar because of doubts about its inertness and non-toxic nature, fear of over-exploration of its sources, the exorbitant price of tissue culture and bacteriological grade agar (Arnold and Eriicksson, 1984; Zimmerman *et al*., 1995; Babbar and Jain, 1988, 2006; Jain and Babbar, 2002; Deb and Pongener, 2010, 2013).Besides coconut coir, foam and betelnut coir (Deb and Pongener, 2010), alternative substrata such as paddy straw, sugarcane bagasse and saw dust can be used in place of agar in tissue culture medium. They are very cheap, easily available in large volumes, easy to procure, eco-friendly, recyclable and reusable.

Dendrobium densiflorum is an epiphyte that produces attractive yellow flowers with a rich orange lip during March-April (Fig. a). This orchid has immense medicinal as well as horticultural values. Present communiqué describes a successful attempt to propagate *D. densiflorum*on some low cost substrata like foam, saw dust,straw, sugarcane bagasse, against agar.

Materials and Method

Processing of substrata

In the present study, different raw materials like polyurethane foam (foam), paddy straw, saw dust and sugarcane bagasse besides agar were used as substrata in the media. Except agar, the alternative substrata were washed with 'Extron' (a commercial laboratory detergent; 1:100, v/v) followed by rinsing under running tap water and then air dried. They were chopped into small pieces (~0.5 cm size), while the foam was cut into discs (according to the size of the culture vials). These were then autoclaved at 1.05 kg cm^{-2} pressure and 121°C for 1 hr and stored in aseptic conditions.

Explant sources

The green pods of various developmental stages (3 to 7 months after pollination [MAP]) were collected at 1 month interval for the present study. The seed pods were surface cleansed with 'Labolene' (a liquid laboratory detergent, 1:100 ratio, v/v) and washed under running tap water followed by sterilizing in 0.5% (w/v) aqueous solution of $HgCl_2$ for 5 min and subsequently rinsed repeatedly with sterilized distilled water. Finally, the sterilized seed pods were quickly dipped in 70% ethanol and flamed before the immature embryos were scooped out and cultured on different media.

Initiation of culture

Two different basal media viz., MS (Murashige and Skoog, 1962) and Mitra *et al* (Mitra *et al*, 1976), containing agar and different alternativesubstrata were prepared for culture initiation. The nutrient media were enriched with sucrose (0–4%)(w/v) along with different concentrations of plant growth regulators (PGRs) like NAA (á -naphthaleneacetic acid) and BA (benzyl adenine; 0–9 ìM) either singly or in combination.The pH of the medium was adjusted to 5.6 using 0.1 N NaOH and 0.1 N HCl before autoclaving at 121°Cand 1.05 kg cm^{-2} pressure for 20 min. In each test tube containing different substrata, ~10 ml of liquid medium was poured, and then autoclaved. The immature embryos from the sterilized green pods were then scooped out and cultured on the media. Some of the cultures were maintained in the dark condition for a period of one week and some under full laboratory light condition (40 µmol $m^{-2}s^{-1}$) (12/12 D/L photoperiod) and at 25 ±2°C simultaneously. The germinated seeds formed protocorm like bodies (PLBs) on the germination medium. About 5 ml fresh liquid medium was added every 4–5 weeks in the culture tubes without removing the culture. For eachtreatment, 20 culture tubes were maintained in replicate. Cultures were monitored regularly and all the treatments were repeated at least three times.

Regeneration and mass multiplication

The PLBs/shoot buds developed from the immature embryos were maintained on the optimum initiation condition for 1-2 passages subcultures for further development and differentiation. The advanced stage PLBs/differentiated PLBs were selected for mass multiplication and cultured on MS medium containing various levels concentrations of NAA and BA (0-9 µM either singly or in combination), with different levels concentrations of sucrose (0-4%).The cultures were maintained on the same substrata that was used for germination of immature embryos. The multiple shoot buds/plantlets were taken out from the regeneration medium and cultured on fresh regeneration medium for further multiplication.

Hardening of plantlets and transferring to the potting mix

The well-rooted plantlets (4-5 cm long with 2-

3 roots) were taken out from the regeneration medium and transferred to culture tubes containing charcoal pieces, brick pieces and chopped mosses(1:1:1 ratio) in 1/10th MS medium supplemented with 1% sucrose free from any PGR. The cultures were maintained for 4-6 wk in normal laboratory condition before transferring to community potting mix (CPM) containing sand: brick pieces: coconut husk: charcoal pieces: decayed wood at 1:1:1:1:1 ratio with moss topping. The potted plantlets were watered weekly and were exposed to normal day light for about 1 hour in a day for initial one week and subsequently the exposure period was increased by 2 hours from the second week and finally after a month the plantlets were left in normal full day light condition.

Results and Discussion

The cultured immature embryos started to swell within ten days of initiation which are the first sign of germination. After 3 weeks of culture, swollen seeds formed hair-like structures followed by PLBs formation. The seeds cultured from 5 MAP responded better while the seeds from other pods gave moderate germination. Generally, in most orchids, the developmental stage of the embryos are found to be critical for successful *in vitro* culture and plant regeneration and are found to be species specific (Deb and Temjensangba, 2006; Johnson *et al*., 2007; Pongener and Deb, 2009). The different concentrations of sucrose and PGRs greatly influenced the asymbiotic seed germination. On media devoid of sucrose, no germination was recorded. Of the two media tested in the present study, ~90% germination was achieved on MS medium containing sucrose (2%) and 3 mM NAA and 3 mM BA after 20 days of culture initiation (Table 1) while Mitra *et al* supported ~75% germination. (Data not incorporated). The nutrient regime for orchid culture is species specific and no single culture medium is universally applicable for

Table 1: Effects of different concentrations of PGRs on asymbiotic seed germination of *Dendrobium densiflorum* Lindl.*

PGRs Conc. (µM)		Days taken to germinate	Germination rate (%) (±SE)#	Type of Response
NAA	BA			
0	0	-	-	Nodular swelling only
3	-	28	70	Green PLBs
6	-	22	80	Healthy green PLBs
9	-	32	60	Few PLBs formed
-	3	22	80	Green PLBs formed
-	6	22	90	Small green PLBs formed
-	9	35	60	Few PLBs
3	**3**	**20**	**90**	**Healthy green PLBs which differentiated into well rooted plantlets.**
3	6	22	70	Healthy PLBs formed
3	9	35	60	Delayed germination
6	3	28	50	Few PLBs formed.
6	6	22	90	Healthy green PLBs

* On MS agar gelled medium containing sucrose (2%), and seed pods of 5 MAP.Only the significant treatments are computed.
# Standard error
Note: Data represents the mean of three replicates.

all the orchid species. e.g., *Malaxis khasiana* on MS medium (Deb and Temjensangba, 2006), *Aerides rosea* on Knudson'C', VW and MS media (S.K. Sinha *et al.*, 1998), and *Eulophia alta* on P723 medium (Johnson *et al.*, 2007) were reportedly most suitable over other nutrient media.

Table 2: Asymbiotic seed germination of *Dendrobium densiflorum* (Lindl.)on different alternative substratum*.

Substratum	Days taken to germinate	Germination (%) (±SE)**	Types of response
Agar	20	90	All the embryos formed healthy and green PLBs
Foam	20	90	Nodular swelling and green PLBs formation
Saw dust	35	50	Few PLBs formed
Sugarcane bagasse	22	95	Healthy green PLBs formed.

@ Seeds from green pod of 5 MAP;
* On MS medium containing NAA + BA (3 + 3 µM respectively in combination) and 2% sucrose,
** Standard error
Note: Data represents the mean of three replicates.

Table 3: Effects of different concentrations of PGRs on plantlet regeneration and mass multiplication of *Dendrobium densiflorum* Lindl. *

PGRs Conc. (µM) NAA	BA	No. of shoot buds formed/explant	Days taken for formation of 1st leaf	1st root	Type of response
0	0	1	26	-	Elongated with few leaves.
3	-	5	26	32	Plantlets stunted in growth
6	-	6	26	32	Plantlets healthy
9		3	30	-	Plantlets stunted
-	3	5	16	26	Plantlets stunted
-	6	6	16	32	Plantlets healthy but stunted in growth
-	9	6	30	-	Plantlets etiolated
3	3	8	16	26	Plantlets healthy
3	6	6	26	26	Plantlets quite healthy
3	9	5	30	35	Plantlets etiolated, pale green in colour
6	**3**	**12**	**16**	**26**	**Plantlets healthy with multiple shoot bud and well-developed leaves and roots.**
6	6	9	26	32	Plantlets healthy
9	3	7	26	35	Plantlets stunted

* On MS medium containing sucrose (3%).

Note: Only significant treatments are computed.Data represents the mean of three replicates.

Table 4. Regeneration and mass multiplication of *Dendrobium densiflorum* Lindl. on different alternative substrata *.

Substratum	No. of shoot buds formed/explant	Days taken to form 1st leaf	1st root	Type of response
Agar	12	16	21	Healthy rooted plantlets
Foam	**15**	**16**	**21**	**Well rooted healthy plantlets with multiple shoot buds**
Saw dust	05	22	-	Elongated with small leaves
Straw	06	22	30	Plantlets healthy
Sugarcane bagasse	12	14	21	Healthy plantlets with multiple shoot buds.

*On MS medium containing NAA + BA (6+3µM) in combination, sucrose (3% w/v)
Data represents the mean of three replicates.

Amongst the different media tested, optimum germination was recorded on MS medium containing sucrose (2%), and NAA + BA (3 + 3 µM) in combination where about 90 % of the cultured seeds responded positively. The synergistic effect of NAA and BA in asymbiotic seed germination has been reported in *Dendrobium aphyllum* (Talukdar, 2001), *Cymbidium iridioides* (Pongener and Deb, 2009) where NAA and BA in combination were found superior to all other treatments.

Apart from agar, other materials like foam, paddy straw, saw dust and sugarcane bagasse could be successfully used with differential success for seed germination. In some cases, germination rate and subsequent differentiation were at par with agar and in some cases the alternative substratum out performed agar (Table 2). Amongst the different substrata tested, better germination and subsequent differentiation were observed on medium containing sugarcane bagasse as substratum followed by medium containing foam. Medium containing saw dust as substratum supported poor germination.

Germinating seeds on different substratagot converted into PLBs (Figure *b–e*). PLBs formed on germination medium differentiated into young rooted plantlets with multiple shoots/buds. Amongst the different concentrations ofPGRs tested for plant regeneration and mass multiplication, optimum regeneration as well as multiple shoot buds formation were achieved when the medium was supplemented with both NAA and BA(6 + 3 ìM) (Table 3). In optimum regeneration conditions as many as 12 shoot/buds developed per subculture (Figure *f–j*) with the first sign of a leaf and root after 16 and 26 days of culture respectively.

Amongst the different substrata incorporated in the regeneration media, better regeneration and multiple shoot buds formation were observed on media containing foam disc as substratum (15 shoot buds) followed by medium with sugarcane bagasse and agar as substrata (Table 4). Well rooted healthy plantlets were transferred for hardening and then to the community potting mix (Fig. k-l). About 80% of the transplants survived after two months of potting.

During the last two decades, a number of substances, viz. agarose (Johansson, L. B., 1989), alginates (Scheurich, P. *et al.*, 1980), gelrite (Pasqualetto, P. L. *et al.*, 1988), isubgol (Babbar and Jain, 1998), starch (Nene, Y. L., *et al.*, 1996) etc. have been used with reasonable success as substitutes of agar. These substrata are not expected to find universal acceptance for various reasons. Alginates gel only in the presence of specific ions and therefore are not suitable substitute of agar while, agarose is cost prohibitive.

Starch has inferior gelling ability, poor clarity and metabolizablenature, which leads to softening of the media. Isubgol, due to its polysaccharidic nature, good gelling ability, resistance to enzymatic activity, and gel clarity has the potential to become a universal gelling agent for plant tissue culture media. But due to its high melting point (~70°C) it needs pH adjustment and fast dispensing (Babbar and Jain, 2006), and use of these gelling agents do not substantially reduce the production costs. Deb and Temjensangba (2006) successfully used forest litter and moss as substratum in the hardening medium while Deb and Pongener (2010) succeeded with coconut coir, betel nut coir and foam as substrata. In the present study, foam, paddy straw, saw dust and sugarcane bagasse could be successfully used as alternative to agar for germination and mass multiplication of *D. densiflorum*. Agar congealed cultures demand subculturing every 3–4 weeks on fresh medium which many at times result in unwanted microbial contamination. This problem can be ruled out in alternative substratum as fresh medium can be poured in the same culture tubes at regular intervals and only the proliferated propagules are transferred to fresh culture tubes. Moreover, most of the substances used in the present study arenatural and renewable sources, their increased demand scan be met without any fear of exploitation of itsresources and also does not pose any threat to the environment. The use of these low costs substrata could be commercially feasible and would reduce the production costs considerably.

References

1. Arnold, S. V. and Ericksson, T (1984) Effect of agar concentration ongrowth and anatomy ofadventitious shoots of *Picea abies* (L.) Karst. . *Plant Cell, Tissue and Organ Culture* **3**:257–264.
2. Babbar, S. B. and Jain, R.(1998) 'Isubgol' as an alternative gelling agent for plant tissue culture media. *Plant Cell Report* 17:318–322.
3. Babbar, S. B. and Jain, R. (2006) .Xanthan gum: an economical partial substitute for agar in microbial culture media *Current Microbiology* 52: 287–292.
4. Deb C.R. and Pongener A.(2013) .A study on the use of low cost substrata against agar for non-symbiotic seed culture of *Cymbidium iridioides* D.Don. *Australian Journal of Crop Sciences* 7(5): 642-649.
5. Deb C.R. and Pongener A.(2010) Search of alternative substratum for agar in plant tissue culture. *Current Science* 98 (1):99-102.
6. Deb C.R. and Pongener A. (2011) Asymbiotic seed germination and in vitro seedling development of *Cymbidium aloifolium* (L.) Sw.: a multipurpose orchid. *Journal of Plant Biochemistry and Biotechnology* 20(1):90–95.
7. Deb, C. R. and Temjensangba. (2006).*In vitro* propagation of threatened terrestrial orchid, *Malaxix khasiana* Soland ex. Swartz throughimmature seed culture. *Indian Journal of Experimental Biology* 44: 762–766.
8. Henderson, W. E. and Kinnersley, A. M. (1988). Corn starch as an alternativegelling agent for plant tissue culture., *Plant Cell, Tissue and Organ Culture* 15:17–22.
9. Jain, N. and Babbar, S. B. (2002) Gum katira – a cheap gelling agent for plant tissue culture media. *Plant Cell, Tissue and Organ Culture* 71:223–229.
10. Johansson, L. B (1988).Increased induction of embryogenesis and regeneration in anther cultures of *Solanum tuberosum* L. *Potato Research* 31, 145–149.
11. Johnson T.R, Stewart S.L, Daniela D, Kane M.E, Richardson L.(2007). Asymbiotic and

symbiotic seed germination of *Eulophia alta*(Orchidaceae)—preliminary evidence for the symbiotic culture advantage. *Plant Cell, Tissue and Organ Culture* 90:313–323.

12. McLachlan (1985). Macroalgae (seaweeds): industrial resources and their utilization. *Plant Soil* 89:137–157.
13. Mitra G.C, Prasad R.N, Roy Chowdhury A.R. (1976) Inorganic salts and differentiation of protocorm in seed callus of an orchid and correlation changes in its free amino acid content. *Indian Journal of Experimental Biology* 14:350–351.
14. Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* 15:473–497.
15. Nene, Y. L., Shiela, V. K. and Moss, J. P. (1996) Tapioca – a potential substitute for agar in plant tissue culture media. *Current Science* 70:493–494.
16. Pasqualetto, P. L., Zimmerman, R. H. and Fordham, I. M. (1988).The influence of cation and gelling agent concentrations on vitrification of apple cultivars *in vitro*. *Plant Cell, Tissue and Organ Culture* 14:31–40.
17. Pongener A and Deb C.R. (2009) Asymbiotic culture of immature embryos, mass multiplication of *Cymbidium iridioides* D.Don and the role of different factors. *International Journal of Pharma and BioSciences* 1(1):1-14.
18. Sinha S. K., Singh L. S. and Hegde S. N.. (1998). *In vitro* multiplication of *Aerides rosea* Loddiges ex. Paxt. through asymbiotic seed germination. *Arunachal For. News* 16:38-44.
19. Scheurich, P., Schnabl, H., Zimmermann, U. and Klein (1980) Immobilisationand mechanical support of individual protoplasts. J. *Biochimica Biophysica Acta* 598:645–651.
20. Talukdar A.(2001) Multiple shoot induction in *Dendrobium aphyllum* Roxb. *Journal of Orchid Society of India* 15:35–38.
21. Zimmerman, R. H., Bhardwaj, S. V. and Fordham, I. M. (1995) Use of starch-gelled medium for plant tissue culture of some fruit crops. *Plant Cell, Tissue and Organ Culture* 43:207–213.

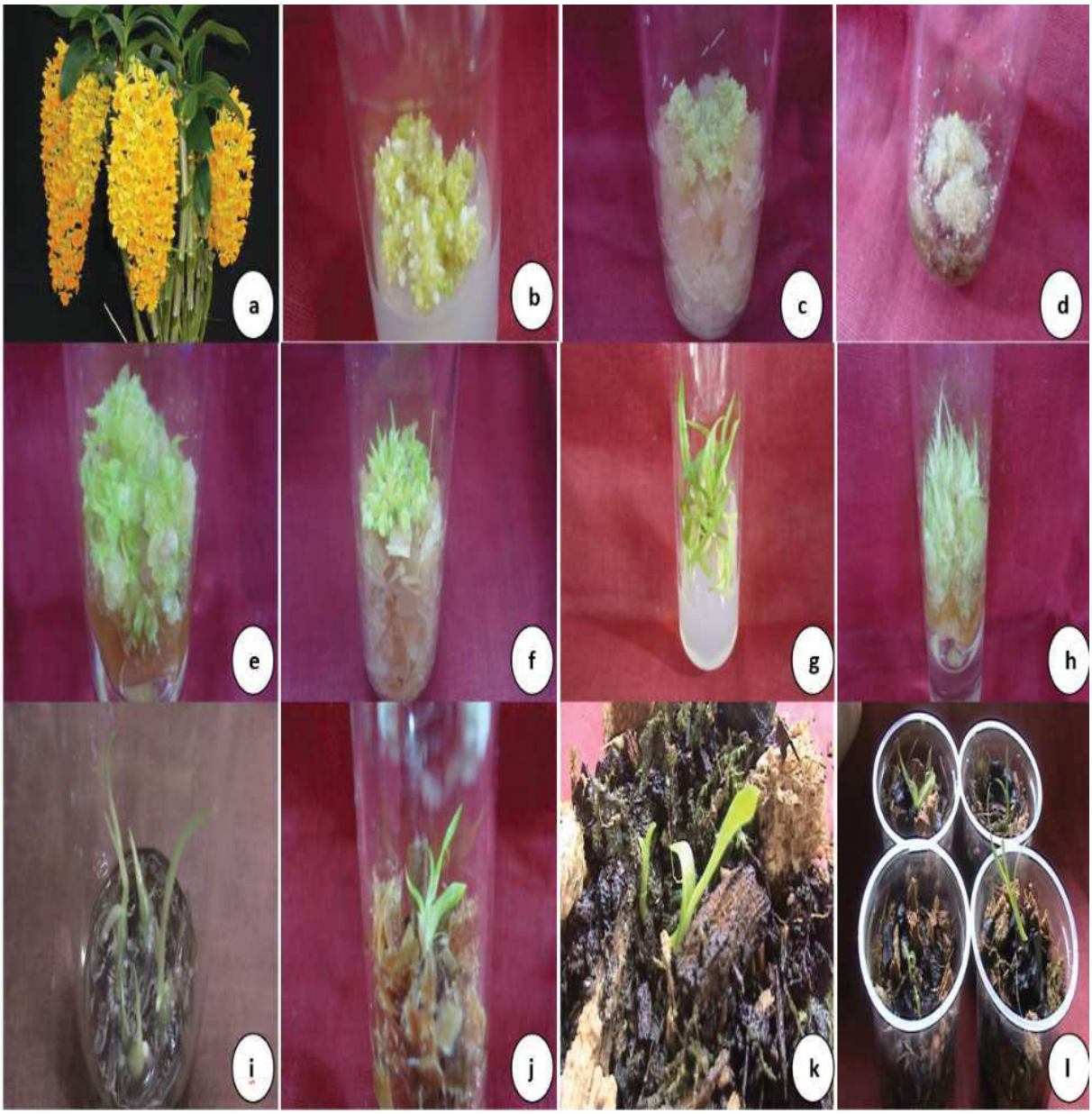

Figure: Different stages of asymbiotic immature embryo culture and plantlet formation of *Dendrobium densiflorum*. **a**. An inflorescence; **b-e.** Germinated embryos formed PLBs on different substratum (**b.** on agar gelled medium; **c**. on medium containing sugarcane bagasse as substratum; **d**. saw dust as substratum; e. foam disc as substratum); ***f–j.*** Regeneration of plantlets and multiple shoot/ buds on different substratum (***f***, regeneration on sugarcane bagasse; **g**. on agar gelled medium; ***h.*** on foam disc; **i.** on straw; and **j**. on saw dust as substratum); **k**. A plant in hardening condition; **l**. Transplants in community potting mix.