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A study on the use of low cost substrata against agar for non-symbiotic seed culture of *Cymbidium iridioides* D. Don

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Abstract

Exorbitant price of agar and fear of over-exploitation of its resources necessitated the search for low costs materials as alternative to agar for plant tissue culture. Present study reports the successful attempt to use 'betel-nut coir, coconut coir and polyurethane foam disk' as alternative to agar for non-symbiotic embryo culture of *Cymbidium iridioides*. The processed substrata were incorporated in the culture vials against agar in the medium. Immature embryos of 10 months after pollination was cultured on agar gelled medium and on all other substrata. The embryos registered about 95, 90, 85, 70 and 50% germination on agar gelled medium, betel-nut coir, foam, coconut coir and leaf litter as substrata respectively. Optimum response on all the substrata was registered on MS medium fortified with sucrose (2%, w/v), α-naphthalene acetic acid (NAA) plus benzyl adenine (BA) (3 µM each). The germinated embryos converted into protocorm-like bodies (PLBs) and produced the young plantlets within 3-4 wk. The PLBs differentiated into rooted plantlets on medium containing sucrose (3%) and NAA plus BA (3 and 6 µM respectively in combination) where as many as 13 shoots formed per passage. On agar gelled medium and on foam the germination, plant regeneration, culture proliferation was competitive while, response on other three substrata was comparatively poorer. The rooted plantlets were hardened on ½MS medium containing sucrose (1%) and containing different supporting materials for 4-6 wk before transferring to community potting mix. About ~80% transplants survived after two months of transfer. The outcome of the present study shows that the production cost of the tissue cultures raised plants could be reduced by ~24% by incorporating 'polyurethane foam', betel-nut coir, coconut coir as alternative to agar.

Keywords: Alternative substratum, agar as substratum, betel-nut coir, coconut coir as substratum, cost effective protocol, *Cymbidium iridioides*, plant tissue culture, polyurethane foam as substratum.
Abbreviations: BA: Benzyl adenine, CW: Coconut water, IAA: Indole 3- acetic acid, KN: Kinetin, MAP: Months after pollination, NAA: α-naphthalene acetic acid, PLBs: Protocorm-like bodies, PGRs: Plant growth regulators, WAP: Weeks after pollination.

Introduction

Since agar was introduced as gelling agent more than 100 years ago, it has been extensively used as for microbial and plant tissue culture media (Babbar and Jain, 2006). Agar is useful for the purposes due to its stability, high clarity, nontoxic nature and resistance to its metabolism (McLachlan, 1985; Henderson and Kinnersley, 1988; Babbar and Jain, 2006). In the recent past several attempts have been made to look for suitable substrata that could possibly agar in culture medium because of doubts about its inertness and nontoxic nature, fear of over-exploration of its sources and above all, the high cost of tissue culture grade agar (Babbar and Jain 1998, 2006; Jain and Babbar, 2002, Temjensangba and Deb, 2005; Deb and Temjensangba 2006; Deb and Sungkumlong, 2008; Sungkumlong and Deb, 2008, 2009). In the recent past agarose (Johansson, 1988), alginates (Scheurich et al., 1980), gelrite (Pasqualetto et al., 1988), isubgol (Babbar and Jain, 1998; Jain et al., 1997), xanthan gum (Babbar and Jain, 2006), guar gum (Babbar et al., 2005, Jain et al., 2005), starch (Zimmerman et al., 1995; Nene et al., 1996) etc. have been used with reasonable success as substitutes for agar. However, these are not expected to find universal acceptance for various reasons. Amongst these substances, alginates require specific ions for effective gelling and therefore are not suitable substitute for agar in many circumstances. Agarose is also cost prohibitive for many operators. Starch has poor gelling ability as well as a metabolizable nature that can result in softening of media. While, though isubgol has the potentiality for good gelling agent due to its polysaccharide nature, gel clarity and resistance to enzymatic activity, but due to its high melting point (~70^{o}C) it needs *p*H adjustment and rapid dispensing after autoclaving (Babbar and Jain, 2006).While, polyurethane foam (there after called '*foam*') is very cheap compare to tissue culture grade agar (about 1/4th that of agar), recyclable and has the potential to make plant tissue immobile in liquid medium. Betel-nut coir, coconut coir, chopped forest leaf litters are natural and eco-friendly and have potential application in plant tissue culture. The present investigation was aimed to screen some possible low cost, eco-friendly raw materials for use as alternative substratum against agar in plant tissue culture. In this study the immature embryos of horticultural important orchid *Cymbidium iridioides* was used as plant material. The orchids represent the most evolved and largest family among the flowering plants (Deb and Imchen, 2008, 2010). Most examples exhibit a highly colorful and attractive flower with long shelf life and varied shapes and sizes that commands great value in the international trade in the form of cut flowers and potted plants. However, their regeneration in nature is limited due to suppressed endosperm and requirement of fungal stimulus (Deb and Temjensangba, 2006). This constraint to commercial production has been overcome by the development of tissue culture techniques,

which have also opened new possibilities in conservation and commercialization of orchids (Pant and Gurung, 2005; Deb and Temjensangba, 2006). Development of these tissue culture techniques for commercial scale production of orchids have included development of low cost protocols including alternative substrata to agar (Agarwal et al., 2006; Deb and Temjensangba, 2006; Temjensangba and Deb, 2005, 2006; Deb and Imchen, 2010) with partial success. Aggarwal et al. (2006) reported the use of coconut coir as support for *in vitro* seed germination of *Cymbidium pendulum* but the success restricted to the germination stage only. Deb and Temjensangba (2006), Deb and Imchen (2010), Temjensangba and Deb (2005, 2006) successfully used forest litter and moss as substrata in the hardening medium for *Arachnis labrosa, Cleisostoma racemiferum* and *Malaxis khasiana*. *Cymbidium iridioides* is an epiphytic/lithophytes that produce attractive flowers during October-November that are 7-8 cm across, brown in color with red streaks and with horticultural value. In Indian domestic market a full bloom spike would cost approximately ~6.0 US$. In this communication we report the potentiality of use of different low cost substrata including 'Foam' 'Coconut coir, 'Betel-nut coir' and 'Leaf litter' as alternatives to agar for non-symbiotic seed germination and plant regeneration of *C. iridioides*.

Results

Culture initiation

The immature embryos/seeds of various developmental ages were cultured on different basal media containing different supplements and different substrata. The green pod age, basal media composition, quality and quantity of PGRs, types of alternative substrata were found to be crucial factors for successful culture initiation. The first sign of germination was recorded as the yellowish nodular swelling of embryos. The swelled embryos formed hairy structure followed by PLBs formation. Immature embryos from green pods up to 8 MAP exhibited delayed germination, while those greater than 14 MAP did not germinate at all. Under the given conditions, optimum germination was achieved from embryos of 10 MAP (data not shown). Amongst the different basal media used for the present study for immature embryo culture, optimum and healthy germination (~95%) was registered on agar gelled MS medium (Table 1). The levels of sucrose and PGRs included in the medium also exhibited striking variation in the non-symbiotic seed germination. On medium devoid of sucrose, there was no germination. When incorporated either NAA or BA singly, did not support healthy seed germination and subsequent culture differentiation. Of the different supplements incorporated, optimum germination was registered on MS medium conjunct with sucrose (2%, w/v) and NAA plus BA (3 µM each in combination) where ~95% of the cultured embryos germinated (Table 1). Incorporation of CW (5%, v/v) exhibited almost 40% faster initiation of germination in comparison to CW control where responding embryos started to swell within 3 wk of culture. However, addition of CW in the germination medium had little effect on germination rate and subsequent development (data not presented). Beside agar, other substrata viz. betel-nut coir, coconut coir, foam disk and leaf litter could be successfully used with differential success for seed germination. Germination rate was slightly higher (~95%) on agar gelled medium compare to foam (85%), betel-nut coir (90%), coconut coir (70%) and leaf litter (50%). Though the germination rate was higher on agar gelled medium, but required longer duration compare to other substrata. Agar gelled medium supported germination only after 58 days of culture while, within 43 to 49 days embryo germination occurred on media with other substrata (Table 1 and 2). Besides time for germination and per cent germination, subsequent differentiation was equivalent to that achieved on agar and in some cases performance on alternative substrata was better than agar. Cultures on betel-nut coir, coconut coir and leaf litter were comparatively poorer compare to agar gelled medium and foam as substratum (Table 2). Cultures maintained on betel-nut coir and coconut coir, germination rate was at lower site (~90 and 70% respectively) as well as PLBs formation was delayed by over ~3 weeks, but cultures on chopped leaf litter failed to proliferate and did not support healthy PLBs formation.

Culture differentiation, plantlets regeneration and mass multiplication

The germinating seeds on different substrata converted into PLBs (Fig. 1a-e). Within 7-8 wk of culture on regeneration medium, PLBs started differentiating into rooted plantlets and multiple shoots. Amongst the different PGRs tested in regeneration medium optimum regeneration as well as multiple shoot buds formation was achieved when supplemented with NAA plus BA at 3 µM and 6 µM respectively (Table 3). Under optimum regeneration conditions as many as 13 shoot/buds developed per 3 week subculture cycle (Fig. 1 f-h). Amongst the different substrata incorporated better regeneration and multiple shoot buds formation was registered on media containing agar and foam as substratum where as many as 8 and 13 shoot buds/plantlets formed per subculture cycle respectively. Cultures maintained on agar gelled medium and foam as substratum exhibited identical performance. But other three substrata did not support healthy regeneration and culture proliferation. Release of first set of leaflets in the regenerates was faster on foam, where within 20 days of culture the first leaf sprouted but only after 25 and 30 days of culture similar response was recorded from cultures maintained on agar gelled and coconut coir as substratum (Table 3).

Effect of substratum and cost effectiveness

During the present study coconut coir, betel-nut coir and foam as alternative to agar could be successfully be used for germination, regeneration and culture differentiation of *Cymbidium iridioides*. However, foam supported culture outperformed betel-nut, coconut coir, and leaf litter supported culture in all the three stages. It was found that the initial response on plant regeneration was better on agar gelled medium but, once cultures established on alternative substratum especially on 'foam' and 'coconut coir', culture exhibited healthier growth and more rapid culture proliferation compare to agar gelled medium. This conclusion is based on the following observations/facts: I. The embryos germinated on medium with agar after ~58 days of culture followed by differentiated into healthy green PLBs. On regeneration medium gelled with agar as many as 8-10 shoot buds formed per sub-culture per PLB. II. While, seeds cultured on foam as support otherwise identical condition required 45 days for germination. On regeneration medium about 11-13 shoot buds were formed from a single PLB which differentiated into dark green healthy plantlets about 3-4 cm long with broad leaves and roots (Table 2 and 3).III. Seeds cultured on coconut coir supported medium, seeds germinated after 43 days of culture. Though the PLBs

Table 1. Effects of different levels of PGRs on non-symbiotic seed germination of *C. iridioides.*

PGRs Conc. (µM)		Germination time (days)! (±SE)[#]	% Germination (±SE)[#,!,**]
NAA	BA		
0	0	-	0
3	-	68 (0.6)[c]	80 (1.5)[c]
6	-	80 (0.6)[e]	63 (2.0)[e]
9	-	98 (0.8)	52 (1.0)[f]
12	-	105 (0.9)[c]	50 (1.0)[f]
-	3	67 (0.7)[c]	50 (2.0)[f]
-	6	79 (0.8)[e]	50 (0.5)[f]
-	9	117 (0.6)[h]	32 (1.0)[h]
-	12	105 (0.8)[b]	30 (1.0)[i]
3	3	58 (0.6)[b]	95 (1.50)[a]
		45[!!] (0.8)[a]	!!85 (0.75)[b]
3	6	64 (0.6)[c]	70 (0.5)[d]
3	9	70 (0.4)[d]	60 (2.5)[e]
6	3	103 (0.4)[g]	50 (2.0)[f]
9	3	93 (0.6)[f]	45 (1.0)[g]
12	3	103 (0.4)[g]	35 (1.5)[h]

On MS medium containing sucrose (2%), and seed pods of 10 MAP; ** Only the significant treatments are computed.
Standard error (In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ± standard error). ! On agar gelled medium, !! On medium with 'Foam' as substratum; Note: Data represents the mean of five.

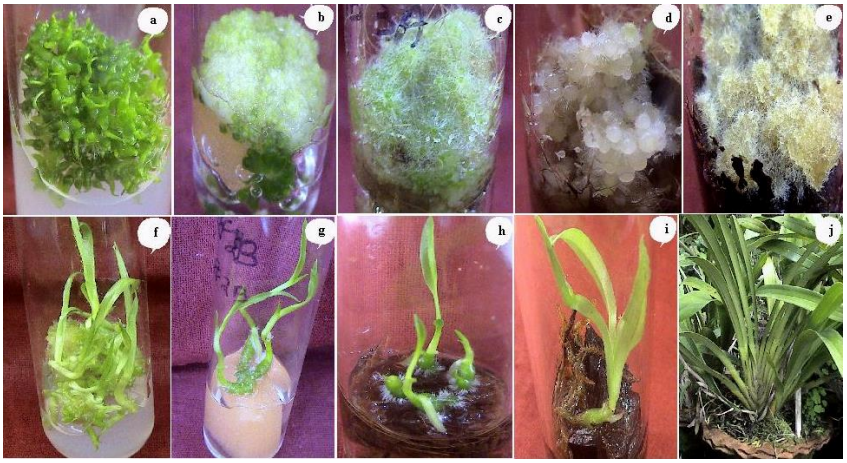

Fig 1. Different stages of non-symbiotic seeds germination, plantlet regeneration, hardening and transplanting of regerenrates in community potting mix of *Cymbidium iridioides*. (a-e): non-symbiotic seed germination on different substratum formed PLBs (a): on agar gelled medium, (b): on medium containing foam disk as substratum, (c): coconut coir as substratum, (d): betal-nut coir as substratum, (e): forest leaf litter as substratum), (f-h): regeneration of plantlets and multiple shoot/buds on different substratum (f: regeneration on agar gelled medium, g: regeneration of foan disk and h. on coconut coir as substratum), (i): A rooted plantlet in hardening condition and (j): An established plant in the pot.

were smaller in size and delayed differentiation compared to agar gelled and foam supported media, but resulted into dark green and healthy plantlets (Fig. 1i).

Hardening and transplantation to community potting mix

The well rooted plantlets (~6-7 cm long with roots) were taken out from the regeneration medium and transferred into culture vials containing ½MS liquid medium conjunct with sucrose (1%), and no PGRs. In the culture vials different types of supporting materials like charcoal pieces, brick pieces and chopped mosses (at 1:1 ratio) was incorporated and maintained for 4-6 wk in normal laboratory condition as described in materials and methods (Fig. 1j). The plants were exposed in normal day light for about 1 hr in a day for initial 1 wk and subsequently to normal light condition. During this process the plantlets turned deep green. About 80% of the transplants survived after two months of transplantation.

Discussion

Culture initiation

For orchid propagation immature embryos serve as deal material allowing large scale production of plantlets in a relatively shorter period of time (Deb and Temjensangba, 2006; Sungkumlong and Deb, 2008). The successful non-symbiotic seed germination of orchids are greatly influenced by several factors like seed pod age, quality of nutrient medium, media supplements, PGRs etc. (Sharma and Tandon, 1990; Temjensangba and Deb, 2005; Deb and Sungkumlong, 2008, 2009). In the present study with *C. iridioides*, a key factor for successful non-symbiotic seed germination was the developmental age of immature embryos. Different species of orchids exhibit a particular threshold, a factor genetically structured in the organism. The influence of physiological age varies with the genus, species

within the genus. There is a short window period of seed development for every orchid species, which support optimum *in vitro* germination. Earliest stage at which embryos could be cultured successfully varies within the orchid genotype (Sauleda, 1976; Deb and Temjensangba, 2006). In the present study, the green pod age of 10 MAP supported ~95% germination. The green pod age <8 MAP either failed to germinate or delayed germination. Earlier in *Dactylorhiza hatagirea*, seeds of 16 WAP (Vij et al., 1995), in *Malaxis khasiana*, seeds of 8 WAP (Deb and Temjensangba, 2006), in *Arachnis labrosa*, seeds of 16-18 WAP (Temjensangba and Deb, 2005), in *Coelogyne suaveolens*, immature embryos of 13 MAP (Sungkumlong and Deb, 2008) registered optimum germination. Besides this, the requirements of nutrient regime is known to be species specific and no single culture medium being universally applicable for all the orchid species (Pongener and Deb, 2011). Amongst the different media tested, optimum germination was achieved on MS basal medium followed by Mitra et al. and Knudson 'C' media. Orchids of different species exhibit a preferential requirement to specific nutrient media for seed germination but as such no standard medium could be prescribed for all the orchid taxa (Rao et al., 1998; Vij et al., 1995; Temjensangba and Deb, 2005; Stewart and Kane, 2006; Johnson et al., 2007; Li and Xu, 2009).The nutrient media were conjunct with different PGRs and optimum germination was achieved on MS medium fortified with sucrose (2%), NAA plus BA at 3 µM each in combination. The synergistic effect of NAA and BA on non-symbiotic seed germination similar to that recorded in the present study has been reported in *Dendrobium aphyllum* (Talukdar, 2001), *Aerides odorata* (Pant and Gurung, 2005), *Cleisostoma racemiferum* (Temjensangba and Deb, 2006), *Coelogyne suaveolens* (Sungkumlong and Deb, 2008) where NAA and BA in combination was found to be superior over other treatments. Besides agar as gelling agent, some low costs substrata were screened as potential alternative to agar for seed germination. Of the different substrata including agar used in the present study, germination was faster on foam supported medium. On this substratum seed germination was registered after 43 days of culture, but on medium with agar as gelling agent germination was achieved after 58 days of culture. Amongst the different substrata screened, performance on agar gelled medium and foam supported medium was found to be comparative and competitive, but the response on betel-nut coir, coconut coir and leaf litter were poorer and there was significant delay in germination and subsequent culture differentiation. During the last two decades, number of substances viz. agarose (Johansson, 1988), gelrite (Pasqualetto et al., 1988), guar gum (Babbar et al., 2005), xanthan gum (Babbar and Jain, 2006) etc. have been used as substitutes of agar. However they are not acceptable universally for various reasons (Babbar and Jain, 2006). In literatures there is not much information available on the use of low cost natural substrate and foam as alternative to agar except Aggarwal et al. (2006), who used coconut coir as substratum for seed germination of *Cymbidium pendulum* and success was restricted only to the initial swelling of seeds.

Culture differentiation, plantlets regeneration and mass multiplication

Quality and quantity of PGRs had a pronounced effect and elicit different responses in the seedling development. Inclusion of PGRs in the regeneration medium was obligatory for successful plant regeneration and mass multiplication. In the absence of PGRs, cultures remained recalcitrant and degenerated subsequently. Both the auxins (NAA and IAA) when used singly, impaired regeneration compared to cytokinins (BA and KN). Of the different levels of PGRs tested for plant regeneration and culture proliferation, optimum response was achieved on medium supplemented with NAA and BA (3 and 6 µM respectively in combination) where as many as 13 shoot buds developed per subculture. When compared the performance on different substrata, it was found that cultures maintained on agar gelled medium and foam supported medium exhibited identical performance but performance on other three substrata was not competitive. Inhibitory effect of auxins especially IAA on seedling development has been reported on *Dactylorhiza purpurella* (Hadley, 1970). While, IAA (0.1 mgl^{-1}) was found to promote seed germination, seedling development of *Cymbidium punctulata* (Sharma and Tandon, 1986). But Vij and Aggarwal (2003) reported that NAA alone favored the development of multiple shoots in *Vanda coerulea*. Bhadra and Hossain (2004) reported highest number of shoots formation from nodal segments of *Micropera pallida* on medium supplemented with NAA and BA (2 mgl^{-1}) in combination.

Effect of substrata and cost effectiveness

The goal of the present study was to investigate low cost alternatives to agar for use in orchid tissue culture to reduce the production costs. According to our study it was found that in agar gelled medium, agar constitutes about 25% of the media cost excluding PGRs. In plant tissue culture one of the costliest ingredients is gelling agent and in most of the cases agar is used as gelling agent and this makes the tissue culture as costly technique. For example average cost of one liter of tissue culture medium with agar (0.8%, w/v) is ~8.0 US$ where the cost of tissue culture agar is ~2.0 US$. For making the plant tissue culture technique more user friendly and integrate the same in floriculture/horticulture and conservation programmes, we should look for alternatives to agar. One way to reduce the production cost is use of low cost substrata such as betel-nut coir, coconut coir, foam, leaf litter' as used in the present study. These materials have certain advantages over agar or other gelling agents. Agar and other comparatively cheaper gelling agents like phytagel need to be incorporated every time when medium is prepared, as they dissolve in the medium and are discarded in every sub-culture. Conversely, materials like foam, coconut coir, betel-nut coir and forest litters could potentially be recycled for several cycled. 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 low cost substrata to replace agar. In most of the cases success was restricted to either initiation of culture or hardening stage. Aggarwal et al. (2006) used coconut coir for seed germination of *Cymbidium pendulum*, but success restricted to swelling of embryos only. Deb and Temjensangba (2006), Deb and Imchen (2010) could successfully use forest litter and moss as substrata in the hardening medium. In the present study we could successfully use coconut coir, betel nut coir and polyurethane foam disk as alternative to agar for germination, regeneration. Superior germination, regeneration of plantlets, multiple shoot bud formation was achieved on foam disk compared to other substrata. According to our estimation in the present study, the overall production cost could be sustainably reduced to ~24% compare to agar gelled culture. This distinct cost advantage is mostly due to:

Table 2. Asymbiotic seed germination[@] of *C. iridioides* on different alternative substratum[*].

Substratum	Days taken to germinate	Germination (%) (±SE)[**]	Types of response
Agar	58 (0.6)[c]	95 (1.50)[a]	All the embryos formed healthy and green PLBs
Polyurethane Foam	45 (0.8)[a]	85 (0.75)[c]	Nodular swelling and green PLBs formation
Betel-nut coir	49 (1.0)[b]	90 (0.75)[b]	Green PLBs formed but delayed differentiation
Coconut coir	43 (0.8)[a]	70 (1.00)[d]	Green PLBs formed and delayed differentiation
Leaf litter	45 (1.0)[a]	50 (1.50)[e]	Nodular swelling but few green PLBs formed

@ Seeds from green pod of 10 MAP; * On MS medium containing NAA + BA (3 + 3 µM respectively in combination) and 2% sucrose; ** Standard error (In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ± standard error). Note: Data represent the mean of five replicates.

Table 3. Effects of different levels of PGRs for plantlet regeneration and mass multiplication of *C. iridioides* on different substrata*

PGRs Conc. (µM)				No. of shoot buds formed/explant**(±SE)[#] (on medium containing different substratum)			Days taken for 1st leaf formation (±SE)[#]		
NAA	IAA	BA	Kn	Agar	Foam	Coconut coir	Agar	Foam	Coconut coir
0	0	0	0	0	0	0	0	0	0
-	-	3	-	2 (0.5)[e]	3 (0.33)[f]	0	30 (2.0)[b]	0	0
-	-	6	-	10 (0.33)[a]	11(0.33)[b]	6 (0.66)[a]	25(3.0)[a]	20 (3.0)[a]	30(3.5)[a]
-	-	9	-	3(0.5)[d]	5 (0.33)[d]	4 (0.66)[c]	55 (3.5)[g]	80 (3.0)[i]	70(3.0)[f]
-	-	-	3	4 (0.33)[c]	3 (0.33)[f]	2 (0.33)[e]	40 (3.5)[d]	35 (3.0)[d]	40 (3.0)[b]
-	-	-	6	3 (0.33)[d]	8 (0.33)[c]	4 (0.33)[c]	35 (3.5)[c]	20 (3.5)[a]	30 (3.5)[a]
-	-	-	9	2 (0.5)[e]	4 (0.66)[e]	2 (0.33)[e]	65 (3.0)[h]	118 (2.0)	75 (3.0)[g]
3	-	3	-	0	4 (0.33)[e]	3 (0.66)[d]	50 (3.5)[f]	60 (3.0)[h]	115 (3.0)[i]
3	-	6	-	8 (0.5)[b]	13 (0.66)[a]	5 (0.33)[b]	25(3.5)[a]	30 (3.0)[c]	115 (3.5)[i]
3	-	9	-	2 (0.33)[e]	5 (0.66)[d]	4 (0.66)[c]	80 (4.0)[i]	120 (2.0)[k]	120 (3.0)
-	3	-	3	2 (0.5)[e]	2 (0.33)[g]	2 (0.33)[e]	78 (3.5)[i]	100 (3.0)[j]	90 (3.0)[h]
-	3	-	6	2 (0.33)[e]	2 (0.33)[g]	2 (0.33)[e]	120 (4.0)[j]	140 (3.0)	120 (3.5)[j]
-	3	-	9	4 (0.5)[c]	5 (0.33)[d]	3 (0.33)[d]	45 (2.0)[e]	25 (3.5)[b]	42 (3.0)[b]
6	-	3	-	4 (0.33)[c]	5 (0.33)[d]	3 (0.33)[d]	40 (2.5)[d]	40 (3.0)[e]	45 (3.0)[c]
3	-	-	3	2 (0.5)[e]	3 (0.33)[f]	2 (0.33)[e]	50 (3.0)[f]	45 (3.5)[f]	65 (3.5)[e]
3	-	-	6	4 (0.33)[c]	5 (0.5)[d]	3 (0.33)[d]	55 (4.0)[g]	55 (3.0)[g]	65 (3.0)[e]
3	-	-	9	3 (0.33)[d]	4 (0.33)[e]	2 (0.33)[e]	50 (3.0)[f]	55 (3.5)[g]	60 (3.5)[d]

*On MS medium containing sucrose (3%); **Only significant treatments are computed' # Standard error (In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ± standard error); Note: Data represents the mean of five replicates.

(i) The agar gelled cultures demands subcultures at every 3-4 wk on fresh medium which many a times invites unwanted microbial contamination. But with the cultures on alternative substratum this problem could be ruled out as the fresh medium can be poured in the same culture vials at regular interval and only the proliferated propagules are transferred to fresh culture vials. Besides this it cuts the manpower costs. (ii) As most of the substances used in the present study is either the waste of households or are very cheap. (iii) One liter of tissue culture medium with tissue culture grade agar is ~8.0 US$ where the cost of the agar is ~2.0 US$. But the cost of the foam for one liter medium is ~0.5 US$ and we could successfully recycle the foam disk up to 10 cycles. Therefore, the cost of 10 liters of medium with agar as gelling agent would be ~80 US$ but with foam as substratum it could be ~60.5 US$ only. Hence the medium cost with foam is 76% in comparison to agar gelled medium thereby reduces the production cost by ~24%. Apart from costs effectiveness, use of these substrata as in the present study does not exert pressure on nature and environment as most of the substrata are natural and renewable sources except 'foam' which is synthetic. Their increased demands can be met without any fear of exploitation of its resources and also does not pose much threat to environment on being disposed after use. It is further observed that the cultures maintained on alternative substrata establish better in the community potting mix compare to the cultures maintained on agar gelled medium as in most of the cases the roots of the regenerates adheres to the substratum as does by velamenous roots of orchids in their natural habitats.

Hardening and transplantation to community potting mix

The hardening of *in vitro* raised plantlets is essential for better survival and successful establishment. Losses of micro propagated plants after transferring to nature are attributed to low humidity, high levels of light and non-sterile condition of the *in vivo* environment (Lavanya et al., 2009; Deb and Imchen, 2010). Conventionally the tissue-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 substrata with manipulation in salt solution were employed for hardening of different angiospermic *in vitro* raised plants by various workers viz. soilrite for *Carica papaya* (Agnihotri et al., 2004), soaked cotton for sugar cane (Gill et al., 2004) etc. In the present study, the regenerates were maintained on ½ strength MS salt solution with 1% sucrose and some low cost substrata like charcoal pieces, brick pieces and moss and maintained at normal laboratory condition, followed by transferring to CPM. The plantlets in the hardening condition were found to develop newer roots. These newly developed roots attached to the support medium with the passages of time. The roots attached themselves mostly to the charcoal pieces and mosses, which strongly suggest the suitability of the materials for the purpose.

Materials and methods

Plant material

Cymbidium iridioides plants were collected from the wild and maintained in the Botanical Garden, Nagaland University. Immature green pods of various developmental ages (6 to 18 MAP) were harvested from the garden and used for initiation of culture.

Preparation of substrata

Different types of substrata including 'agar, betel-nut coir, coconut coir, foam, and leaf litter' were selected for comparison with plant tissue grade agar (Hi-media, India). '*Foam*' was collected from the local market at Nagaland, India that is generally used for preparation of mattresses. Other substrata like 'betel-nut coir, coconut-coir were extracted from the dried fruits and chopped into small pieces according to our requirements while, 'leaf litter' was collected from the forest floor before they are decayed. Except for agar all other materials were soaked with 'Extron' (a commercial laboratory detergent, Merck, India) at 1:100 (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 substrata except foam were chopped into small pieces (~0.5 cm size), while the foam was cut into disks according to the culture vials size. In the present study borosilicate test tubes (25 mm diameter x 150 mm length; Borosil, India) was used as culture vessels. All substrata except agar were then autoclaved at1.05 Kg cm^{-2} pressure and 121^{o}C for one hr before putting them in the culture vials.

Sterilization of plant material and initiation of culture

The green pods were first surface sterilized with 0.3% mercuric chloride (w/v) for 3 min and rinsed 3-4 times with double distilled sterile water. The pods were slightly flamed by dipping in 90% ethanol (v/v) prior to scoping out the embryos from the pods. The immature embryos were inoculated on different basal media viz. Murashige and Skoog medium (MS) (Murashige and Skoog, 1962), Mitra et al., medium (Mitra et al., 1976) and Knudson 'C' medium (Knudson, 1946). The nutrient media were enriched with various levels of sucrose (0-4%, w/v), CW (0-20%, v/v) in addition to different levels of PGRs including NAA (0-12 µM) and BA (0-12 µM) either singly or in combination. Coconut water was obtained from young coconuts purchased from the local market (Nagaland, India). The *p*H of the media was adjusted to 5.6 using 0.1 N NaOH and 0.1 N HCl before autoclaving at 121^{o}C and 1.05 kg cm^{-2} pressure for 20 min. In each test tube ~10 ml of liquid medium was poured. A part of the medium was gelled with 'agar (0.8%) (w/v)', while remaining media were prepared as liquid. About 10-12 ml of liquid medium was dispensed in each test tube and different preprocessed (as described above) substrata like betel-nut coir, coconut coir, foam, leaf litter were used as supporting materials before media autoclaving. All the additives except CW are added before autoclaving while CW was filter sterilized and added to the medium after medium is autoclaved.

Initiation of culture

Immature embryos extracted from sterilized green pods were cultured on media containing different substrata cultured in a 12/12 hr photoperiod with light provided at 40 µmol $m^{-2}s^{-1}$ at a temperature of 25±2^{o}C. About 5 ml fresh liquid medium was replaced every 2-3 wk in the same culture vial without removing the culture material. For each treatment 20 culture vials were cultured in each replicate. Cultures were monitored regularly and all the treatments were tested at least five times.

Culture differentiation, plantlets regeneration and mass multiplication

The PLBs developed from the cultured immature embryos were maintained on the optimum germination media for further development and differentiation. The advanced stage PLBs (PLBs with the first set of leaflets) were selected for mass multiplication and cultured on MS medium containing various levels IAA, NAA, BA and Kn singly or in combination supplemented with 3% (w/v) sucrose. The cultures were maintained on different substrata as used for germination of immature embryos. The resulting multiple shoot buds/plantlets were separated from the regeneration medium and cultured on fresh regeneration medium for further multiplication.

Hardening and transplanting to community potting mix

Well rooted plantlets (about 6-7 cm long with 2-3 roots) were removed from the regeneration medium and transferred to test tubes containing ½MS inorganic salt solution basal salt solutions supplemented with sucrose (1%) and no PGRs. In the culture vials, different types of supporting materials were incorporated including charcoal pieces, brick pieces and chopped mosses (at 1:1 ratio). Charcoal was collected from the open market and made to small pieces of ~5 mm size, while normal bricks used in construction work were made to small pieces of ~5-7 mm size (brick pieces). Moss was collected from the hilly terrain near Nagaland University Campus and used for the experimental purpose. All these materials were washed thoroughly under running tap water and autoclaved at 1.05 Kg cm^{-2} pressure and 121^{o}C for one hr before placing into the culture vials. The cultures were maintained for 4-6 wk in 12/12 hr photoperiod with light provided at 40 µmol $m^{-2}s^{-1}$ at a temperature of 25±2^{o}C before transferring to community potting mix (CPM) consisting of sand: brick pieces: coconut husk: charcoal pieces: decayed wood at 1:1:1:1 ratio with a moss topping. For potting purpose, disposable plastic glass (90 mm diameter x 120 mm length) was used and covered with holed transparent poly bag. The potted plants were maintained in a shaded place and watered weekly. The potted plants were exposed to normal day light for about 1 hr in a day for initial one wk and subsequently increased the exposure period by 2 hr from the second week and finally after one month the plantlets were left in the normal full day light condition.

Experimental design and data analysis

Completely randomized experimental design was performed. In all the experiments, each treatment had five replicates and there were 20 culture vials per treatment. All the cultures were sub cultured at 4-5 wk interval. Performance was evaluated based on *per cent* germination response, number of shoot/plantlets/PLBs formed per explants. Data was analyzed at a threshold of 5% (Newman-Keuls, ± standard error).

Conclusion

The present investigation offers a newer possibility of using the low costs raw materials like foam, coconut coir, betel-nut

coil, leaf litter as alternative to agar which will reduce the production cost and will help in popularizing the plant tissue culture technique. Works are in progress with considerable success to use these substratums in non-orchid species.

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