

## Search of alternative substratum for agar in plant tissue culture

Chitta Ranjan Deb\* and Aolemla Pongener

Department of Botany, Nagaland University, Headquarters: Lumami (University Branch), Mokokchung 798 601, India

**In plant tissue culture, agar has been the most commonly used gelling agent. But the exorbitant price and stress on its sources have necessitated the search for low cost alternatives. In the present study, a successful attempt has been made to standardize low cost plant tissue culture substratum. Different substratum like 'polyurethane foam disc', chopped coconut coir, betel nut coir and leaf litter were processed and used as substratum in place of agar and a comparative study was done. Successful asymbiotic immature seed germination and plant regeneration of *Cymbidium aloifolium* was achieved on different substratum like foam disc and coconut coir. Amongst the different substratum used, optimum germination, regeneration and multiple shoot buds formation was registered on polyurethane foam containing medium followed by agar medium and coconut coir containing media. The results of the present study offer a new possibilities of using low cost raw materials which will reduce production costs considerably and will help in popularizing plant tissue culture techniques.**

**Keywords:** Agar, alternative substratum, coconut coir, polyurethane foam.

AGAR has been extensively used as gelling agent in microbial as well as plant tissue culture media over the last 100 years<sup>1</sup>. It is useful due to its stability, high clarity, nontoxic nature and resistance to its metabolism<sup>1-3</sup>. In the recent past, several attempts have been made to look for some suitable substratum which can replace agar in the plant tissue culture media as well as microbial culture medium because of doubts regarding its inertness and nontoxic nature, fear of exhausting its sources and above all, the exorbitant price of tissue culture and bacteriological grade agar<sup>1,4-15</sup>. Earlier xanthan gum<sup>1</sup>, isubgol<sup>9,16</sup> and guar gum<sup>17,18</sup> were used as alternative gelling agents in plant and microbial culture media, but most of them are comparatively costlier.

Polyurethane foam (foam) is very cheap, eco-friendly and has the potential to make the culture immobile in liquid medium. Coconut coir, betel nut coir, chopped forest leaf litters are natural and have potential similar to foam. The present study aims at screening some low cost raw materials which could be used in plant tissue culture media as agar substitutes. In this communication, we report the successful use of different low cost substratum like

polyurethane foam disc, coconut coir, betel nut coir, leaf litter for asymbiotic seed germination and plant regeneration of *Cymbidium aloifolium*, a horticulturally important orchid.

In the present study, different substratum like agar, betel nut coir, coconut coir, polyurethane foam, and leaf litter were selected. Except agar all other materials were soaked in 'Extron' (a commercial laboratory detergent; 1:100) (v/v) for about 2 h followed by washing under running tap water till clean and then air dried. The natural substratum was 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<sup>-2</sup> pressure and 121°C for 1 h and stored in aseptic conditions.

Immature green pods of about 12 months after pollination were harvested and used for initiation of culture. The seed pods were first surface sterilized with aqueous solution of 0.3% mercuric chloride (w/v) for 3 min and rinsed 3-4 times with double distilled sterile water. The green pods were slightly flamed after dipping in 90% ethanol (v/v) prior to scooping out embryos from the pods. The immature embryos were inoculated on MS medium<sup>19</sup>. The nutrient medium was enriched with sucrose (0-4%) (w/v) along with different levels of plant growth regulators (PGRs) like NAA ( $\alpha$ -naphthaleneacetic acid) and BA (benzyl adenine; 0-12  $\mu$ M) 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°C and 1.05 kg cm<sup>-2</sup> pressure for 20 min. In each test tube ~10 ml of liquid medium was poured, different substratum were introduced and then autoclaved.

Immature embryos from the sterilized green pods were scooped out and cultured on media containing different substratum and cultures were maintained at 40  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> light intensity, 12/12 D/L photoperiod and at 25  $\pm$  2°C. The germinated seeds formed protocorm like bodies (PLBs) on the same germination medium. About 5 ml fresh liquid medium was replaced every 2-3 weeks in the same culture vial without removing the culture. 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.

The PLBs developed from the cultured embryos on different substratum were maintained on optimum germination media for further development and differentiation. The advanced stage PLBs/fully differentiated plantlets with first set of leaflets were selected for mass multiplication and cultured on MS medium containing various levels of PGRs like IAA (indole-3-acetic acid), NAA, BA and Kn (kinetin) singly or in combination supplemented with 3% sucrose. The cultures were maintained on different substratum as in germination of immature embryos. The resultant multiple shoot buds/plantlets were removed from the regeneration medium and cultured on fresh regeneration medium for further multiplication.

\*For correspondence. (e-mail: debchitta@rediffmail.com)

**Table 1.** Asymbiotic seed germination\* on *C. aloifolium* on different substratum\*\*

Substratum	Days taken to germinate	Germination (%) ( $\pm$ SE)***	Types of response
Agar	58	80 (1.50)	All the embryos formed healthy and green PLBs
Betel nut coir	49	65 (0.75)	Green PLBs formed but delayed differentiation
Coconut coir	43	75 (1.00)	Green PLBs formed and delayed differentiation
Foam	45	90 (0.75)	Nodular swelling and green PLBs formation
Leaf litter	45	50 (1.50)	Nodular swelling but few green PLBs formed

\*Seeds from green pod of 12 months after pollination; \*\*On MS medium containing NAA + BA (3 + 6  $\mu$ M respectively in combination) and 2% sucrose; \*\*\*Standard error.

Note: Data represents the mean of five replicates.

The well rooted plantlets (about 6–7 cm long with 2–3 roots) were taken out from the regeneration medium and transferred to culture vials containing  $\frac{1}{2}$ MS medium supplemented with 1% sucrose and devoid of PGRs. In the culture vials, charcoal pieces, brick pieces and chopped moss (at 1 : 1 ratio) were used as substratum. The cultures were maintained for 4–6 week in normal laboratory conditions before transferring to community potting mix (CPM) containing sand and brick pieces, coconut husk, charcoal pieces and decayed wood at 1 : 1 ratio with a moss topping and covered with a transparent polybag with holes for aeration. The potted plants were watered weekly. The potted plants were exposed to normal day light for about 1 h in a day in the first week and subsequently the exposure period was increased by 2 h from the second week onwards and finally after one month the plantlets were left in normal full day light conditions.

In the present study, the cultured immature embryos started swelling within two weeks of initiation as the first sign of germination. After 6–7 weeks of culture, swollen seeds formed hair-like structures followed by PLBs formation. The levels of sucrose and PGRs exhibited a great variation in influencing the asymbiotic seed germination. On media devoid of sucrose, no germination was registered. Amongst the different adjuncts incorporated, optimum germination was registered on MS medium containing sucrose (2%), and NAA + BA in combination. NAA or BA singly did not support healthy seed germination and subsequent culture differentiation (data not presented). About 90% of the cultured seeds responded positively to medium containing NAA + BA (3 + 6  $\mu$ M respectively in combination). The synergistic effect of NAA and BA in asymbiotic seed germination has been reported in *Dendrobium aphyllum*<sup>20</sup>, *Aerides odorata*<sup>21</sup>, *C. racemiferum*<sup>22</sup>, *C. suaveolens*<sup>14</sup>, where NAA and BA in combination were found superior to all other treatments. However, combined treatment of NAA and KN favoured optimum germination in *Vanda coerulea*<sup>23</sup>.

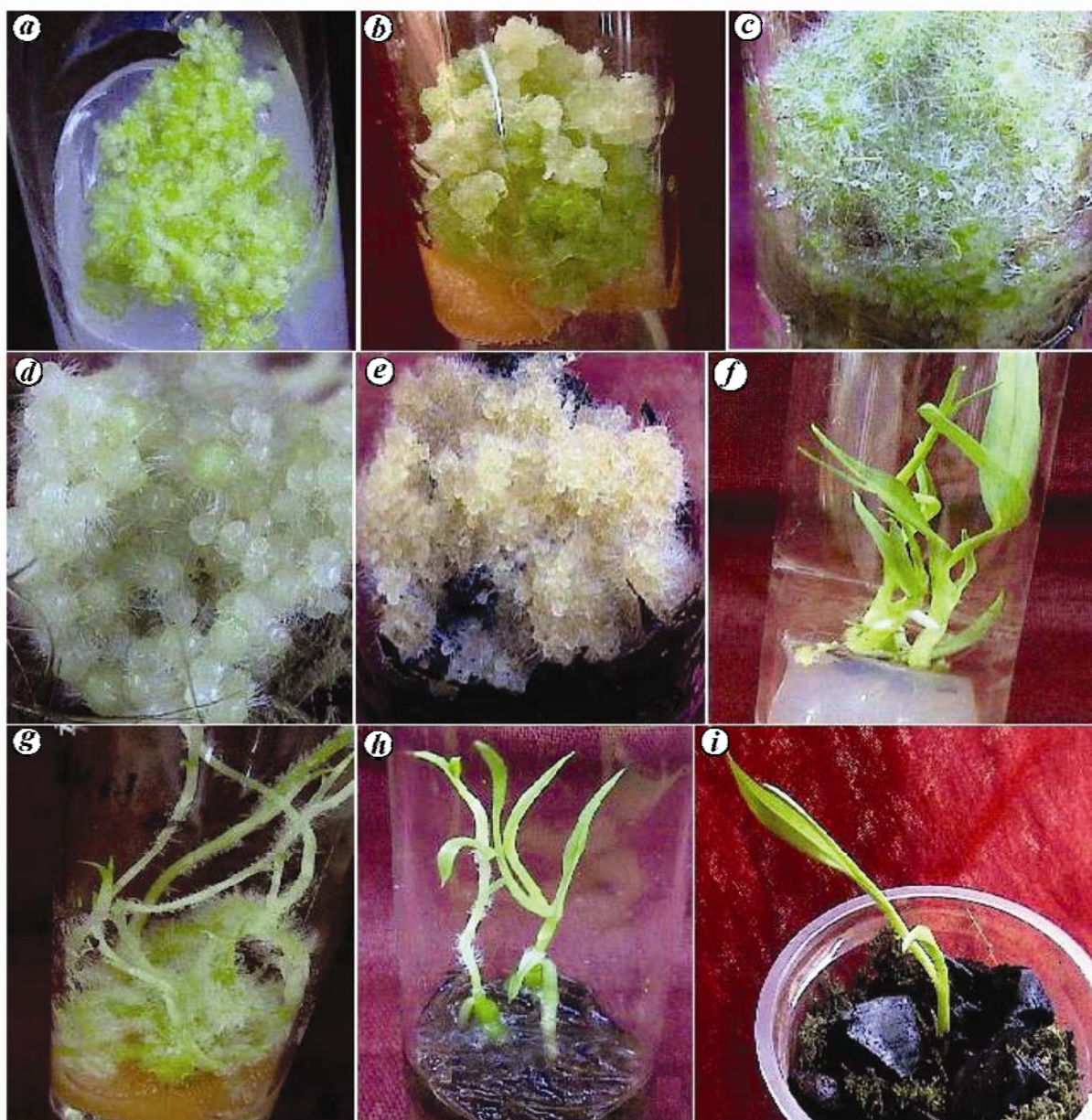
Apart from agar, other materials like betel nut coir, coconut coir, foam disc and forest leaf litter could be successfully used with differential success for seed germination. In some cases, germination rate and subsequent differentiation was at par with agar and in some cases the

alternative substratum outperformed agar (Table 1). Though the germination rate was slightly higher on agar medium, germination was faster on media containing alternative substratum. Amongst the different substratum tested, better germination and subsequent differentiation was registered on medium containing foam as substratum. Medium containing betel nut coir and coconut coir supported moderate germination but delayed differentiation, and cultures on chopped leaf litter failed to differentiate properly.

Germinating seeds on different substratum are converted into PLBs (Figure 1a–e). PLBs formed on germination medium started differentiating into young rooted plantlets and multiple shoot/buds. Amongst the different levels of PGRs tested for plant regeneration and mass multiplication, optimum regeneration as well as multiple shoot buds formation was achieved when supplemented with BA (3  $\mu$ M; data not presented). In optimum regeneration conditions as many as 12 shoot/buds developed per subculture (Figure 1f–h). Treatment using only one of the auxins could not support differentiation while the cultures supplemented with only BA started releasing the first leaflet within 3–4 weeks of culture. Amongst the different substratum incorporated in the regeneration media, better regeneration and multiple shoot buds formation was registered on media containing foam disc as substratum. The time taken for culture differentiation was similar to agar media (Table 2). Finally, 85% of the plantlets survived after hardening and two months of potting.

It was observed that the initial response for germination, regeneration was better on agar gelled medium as cultures establish on this medium faster compared to different substratum. But once the cultures establish themselves especially on ‘foam disc’ and ‘coconut coir’, they exhibit healthier growth and exhibit rapid culture proliferation compared to agar medium.

During the last two decades, a number of substances, viz. agarose<sup>24</sup>, alginates<sup>25</sup>, gelrite<sup>26</sup>, isubgol<sup>9</sup>, starch<sup>8,27</sup>, etc. have been used with reasonable success as substitutes of agar. These 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.



**Figure 1.** Different stages of asymbiotic seed germination, plantlet regeneration, hardening and transplanting of regenerates in community potting mix of *Cymbidium aloifolium*. *a–e*, Germinated embryos formed PLBs on different substratum (*a*, on agar gelled medium; *b*, on medium containing foam disc as substratum; *c*, coconut coir as substratum; *d*, betel 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 on foam disc and *h*, on coconut coir as substratum). *i*, A hardened plant transferred to community potting mix.

**Table 2.** Regeneration of plantlets and mass multiplication of *C. aloifolium* on different substratum\*

Substratum	No. of shoot buds formed/explant	Days taken for formation of		Type of response
		1st leaf	1st root	
Agar	4	21	21	Plantlets healthy
Betel nut coir	1	69	–	Slightly elongated shoot with small leaves
Coconut coir	3	20	35	Plantlets growth stunted
Foam	10	20	36	Healthy plantlets

\*On MS medium containing BA (3  $\mu$ M), sucrose (3% w/v).  
Data represents the mean of five replicates.

Starch has inferior gelling ability, poor clarity and metabolizable nature, 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<sup>1</sup>. But use of these gelling agents does not substantially reduce the production costs.

In most of the cases, success has been restricted to part of the culture phase only. Deb and Temjensangba<sup>12</sup> & Temjensangba and Deb<sup>11,22</sup> could successfully use forest litter and moss as substratum in the hardening medium. In the present study, we could successfully use coconut coir, betel nut coir and polyurethane foam disc as alternative to agar for germination, regeneration, etc. Agar gel cultures demand subculturing every 3–4 weeks on fresh medium which many a time results in unwanted microbial contamination. This problem can be ruled out in alternative substratum as fresh medium can be poured in the same culture vials at regular intervals and only the proliferated propagules are transferred to fresh culture vials. Moreover, most of the substances used in the present study are natural and renewable sources, their increased demands can be met without any fear of exploitation of its resources and also does not pose any threat to the environment. It is further observed that the cultures maintained on alternative substratum establish better in the community potting mix compared to the cultures maintained on agar medium.

Using low costs raw materials will reduce production costs considerably and will help in popularizing plant tissue culture techniques. Research on using alternative substratum for non-orchid species is in progress in our laboratory.

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