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In vitro propagation of Lesser Galangal (*Alpinia calcarata* Rosc.) - a commercially important medicinal plant through rhizome bud culture

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An efficient protocol has been established for clonal propagation of *Alpinia calcarata*, a commercially important medicinal plant on Murashige and Skooge medium using rhizome bud explants. Of the different concentrations of 6-benzylaminopurine (BAP) and BAP in combination with different levels of kinetin, the best response of axillary shoot proliferation was achieved in a combination of 1.5 mg/l of kinetin in combination with 0.5 mg/l of BAP producing 13.6 shoots per explant in 6-8 weeks of culture followed by 2 mg/l kinetin and 0.5 mg/l BAP with an average of 6.2 shoot buds from each of the explants. Rooting of the shoots also occurred in the same medium in 3 weeks of subculture. Shoots transferred to half strength MS medium with 0.5 mg/l IBA was optimum for healthy rooting. The healthy *in vitro* rooted plants were hardened on plastic cups in sterile sand and were transferred to pots containing potting mixture under green house conditions for 3-4 weeks for acclimatization. The survival rate was 87-90% and the plants established well in the field and developed rhizomes after 4-6 weeks of growth under shade house. This protocol proves its utility for rapid propagation of *A. calcarata*, which can be exploited for pharmaceutical and commercial purpose.

Keywords: *Alpinia calcarata*, rhizome bud, shoot multiplication, regeneration

The commercially important aromatic medicinal plant, *Alpinia calcarata* Rosc. (Zingiberaceae), native to India and China (Mangaly and Sabu, 1992) commonly known as Cheriyarasna, Chittaratha and Lesser galangal, is extensively used in the indigenous systems of medicine in Asian countries. In India, the dried rhizome forms a major ingredient of several Ayurvedic drug formulations as *Rasna* in the preparation of *Ashwagandharistam, Rasnadichurnam*, *Rasnadikashayam* and *Rasnadithailam* (Sabu, 2006). Drug prepared by using rhizomes are used in the treatment of rheumatism, bronchial catarrh and asthma. It is used against infection of the skin and also possesses antibacterial activity. It is also used to stimulate digestion, purify blood, prevent bad breath, improve the voice and also to treat inflammation and arthritis (Husain *et al.* 1992). Rhizomes of *A. calcarata* yield essential oil that contains 1,8-ciniole and α-fenchyl acetate which are useful as commercial herbicide and pesticide in addition to pharmaceutical use. The plant is propagated vegetatively through rhizomes due to its rare flowering and lack of seed set which seems insufficient to meet the present day demand especially in the northern districts of Kerala where approximately 1.70 tons of dried rhizome is required annually (Sasidharan and Muraleedhara, 2000). Considering the high demand and greater economic and medicinal values of *A. calcarata*, it is

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necessary to develop a suitable protocol for *in vitro* production of disease free stocks through tissue culture techniques. Protocol for *in vitro* propagation of *A. calcarata* and evaluation of their chemical fidelity through comparison of volatile compounds has been reported (Sudha *et al*., 2012).

Materials and Methods

Healthy and actively growing young axillary shoot buds derived from the rhizomes of the field harvested 12 month old stock plants from the accession IC373610 (Fig.1) maintained in the herbal garden of NBPGR Regional Station, Thrissur, Kerala served as the source of explants for the present experiments. The explants were cleaned thoroughly in running tap water after removing the roots and scale leaves. The trimmed out rhizome bud explants were initially wiped with 70% alcohol and were taken in a conical flask and surface sterilized using 2% extran detergent (v/v) for 10 minutes with vigorous shaking and then rinsed repeatedly under tap water and then rinsed thrice in distilled water. This was followed by treating the explants with 70% alcohol for one minute and then treated with 0.1% $HgCl_2$ for 10 minutes followed by rinsing with sterile water 3-5 times under the laminar air flow chamber.

The surface sterilized explants were trimmed to appropriate sizes (0.6-1.0 cm) by the removal of exposed scale leaves and cut base of the buds and were inoculated on to MS (Murashige & Skooge, 1962) basal solid medium, supplemented with myo-inositol (100 mg/l) and 3% (w/v) sucrose and different concentrations of 6-benzylaminopurine (BAP) (0.5-3.0 mg/l) and combinations of BAP (0.5 mg/l) and kinetin (0.5-3 mg/l) for shoot multiplication. Individual regenerated shoots were excised and used for rooting. Root induction was carried out on subculture to the same medium and on to full and half-strength solid medium supplemented with 0.5 mg/l IBA. The pH of the media was adjusted to 5.8 prior to gelling with 0.7% agar and dispensed into the culture tubes and sterilized by autoclaving at 121^0C and 108 Pa for 18 min. The well prepared explants were inoculated into the sterile media. The cultures were incubated in the culture room at 24 ± 2^0C under 16/8 h (light/dark) photoperiod with light provided by white fluorescent tubes at 2000 lux. The Rooted individual shoots were removed from the culture medium, rinsed in water to remove the media and transferred to plastic cups with sterile soil for hardening and were then transferred to pots containing potting mixture to green house conditions for 3-4 weeks for acclimatization and subsequently to the field.

Results and Discussion

Experiments were conducted with a view to find out the optimum concentration of BAP and also combination of BAP (0.5 mg/l) with different concentrations of kinetin for maximum shoot multiplication from the culture of rhizome bud explants (Fig.2). The multiple shoots were cultured from rhizome bud explants of *Alpinia calcarata* on MS solid medium supplemented with different concentrations of BAP and 0.5 mg/l BAP in combination with different levels of kinetin after 4-8 weeks of incubation (Table-1). Among the different treatments, the best response was obtained in a combination of 1.5 mg/l of kinetin with 0.5 mg/l of BAP (Table-1). In this combination almost 90% of the inoculated explants showed shoot bud regeneration within 4-6 weeks of inoculation and the average number of shoots per explant was 13.6 (Fig.3). The second highest response was observed in a combination of 2 mg/l Kinetin and 0.5 mg/l BAP where an average of 8.2 shoot buds were regenerated from each of the explants (Fig.4). These results are in agreement with the report of shoot induction and multiplication in *A. zerumbet* in a combination of BAP (1.5 mg/l) and kinetin (0.5 mg/l) (Rikkimuthu *et al*., 2011). Among the different concentrations of BAP, maximum shoot induction was recorded in 0.5 mg/l BAP where 6.4 shoots/explant

were produced (Fig.5) followed by 1mg/l with the production of 5.2 shoots/explant (Fig.6) compared to the control MS solid basal medium without any growth hormones producing only 1.87 shoots/explants (Fig.7). As the concentration of BAP was increased to 3 mg/l, the number of shoots induced showed a reduction to 2.8 per explant (Fig.8), suggesting that lower concentration of BAP as best for shoot proliferation in the species. A gradual reduction in the BAP concentration facilitating rapid induction of normal plants during subsequent subculture was reported by Sudha *et al.*, 2012 in *A. calcarata*. An efficient regeneration protocol from rhizome bud explants in MS medium with BAP and kinetin was reported in *A. purpurata* (Kochuthressia *et al*., 2010) and *Kaempferia galanga* (Kochuthressia *et al*., 2012). Multiplication can be continued by transferring each of the separated shoot explants to the same media or to a medium with BAP at 0.5 mg/l (Fig.9) in jam bottles. Individual shoots on subculture to the same shoot initiation medium after 3-4 weeks initiated roots spontaneously (Fig.10). Similar result supporting spontaneous rooting of shoots in the same concentration of shoot induction media with cytokinins BAP and kinetin was reported in *A. purpurata* (Kochuthressia *et al*., 2010) and simultaneous production of shoot and roots in MS medium with kinetin 3 mg/l from rhizome bud explants was reported in *A. galanga* (Borthakur *et al*., 1999). Rhizome bud as potent explants for root induction from shoots in shoot inducing medium itself was reported in *Curcuma longa* and *Zingiber officinale* (Balachandran *et al*., 1990), *Alpinia calcarata* (Agretious *et al*., 1996) and *A. galanga* (Borthakur *et al*., 1999). Healthy and higher rooting (95%) was obtained in the shoots sub-cultured to half strength MS solid medium with 0.5 mg/l IBA (Table-1; Fig.11). Optimum shoot production in half-strength MS medium with 0.2 mg/l IBA was reported in *A. calcarata* (Sudha *et al*., 2012), in 0.5 mg/l IBA in *A. officinarum* (Selvakumar *et al*., 2007) and *A. zerumbet* (Rikkimuthu *et al*., 2011) and in 1 mg/l IBA in *Kaempferia galanga* (Kalpana and Anbazhagan, 2009).

The rooted plants thus obtained were washed well in running tap water to make it free from agar gel and were separated (Fig.12) and transferred to plastic cups with sterile sand for hardening (Fig.13). After 3 weeks, these plants were transferred to pots with potting mixture in greenhouse (Fig.14) and were then transferred to the field and the survival rate was recorded.

Table 1: Effect of different concentrations of BAP alone and BAP (0.5 mg/l) in combination with kinetin (0.5-3 mg/l) in MS basal solid medium on shoot multiplication from rhizome bud explants and rooting of the induced shoot buds in *Alpinia calcarata*

Concentration of BAP (mg/l)	Concentration of Kinetin (mg/l)	Number of shoots/ explant	Percentage of regeneration	Rooting Percentage
MS Basal	--	1.87	80	58
0.5	--	6.4	87	65
1.0	--	5.2	76	78
2.0	--	4.2	82	79
3.0	--	2.8	79	79
0.5	0.5	3.58	85	85
0.5	1.0	5.32	92	80
0.5	1.5	13.6	90	82
0.5	2.0	8.2	85	75
0.5	3.0	3.4	78	80
Shoots on transfer to MS basal medium with 0.5 mg/l IBA				85
Shoots on transfer to ½ MS basal medium with 0.5 mg/l IBA				95

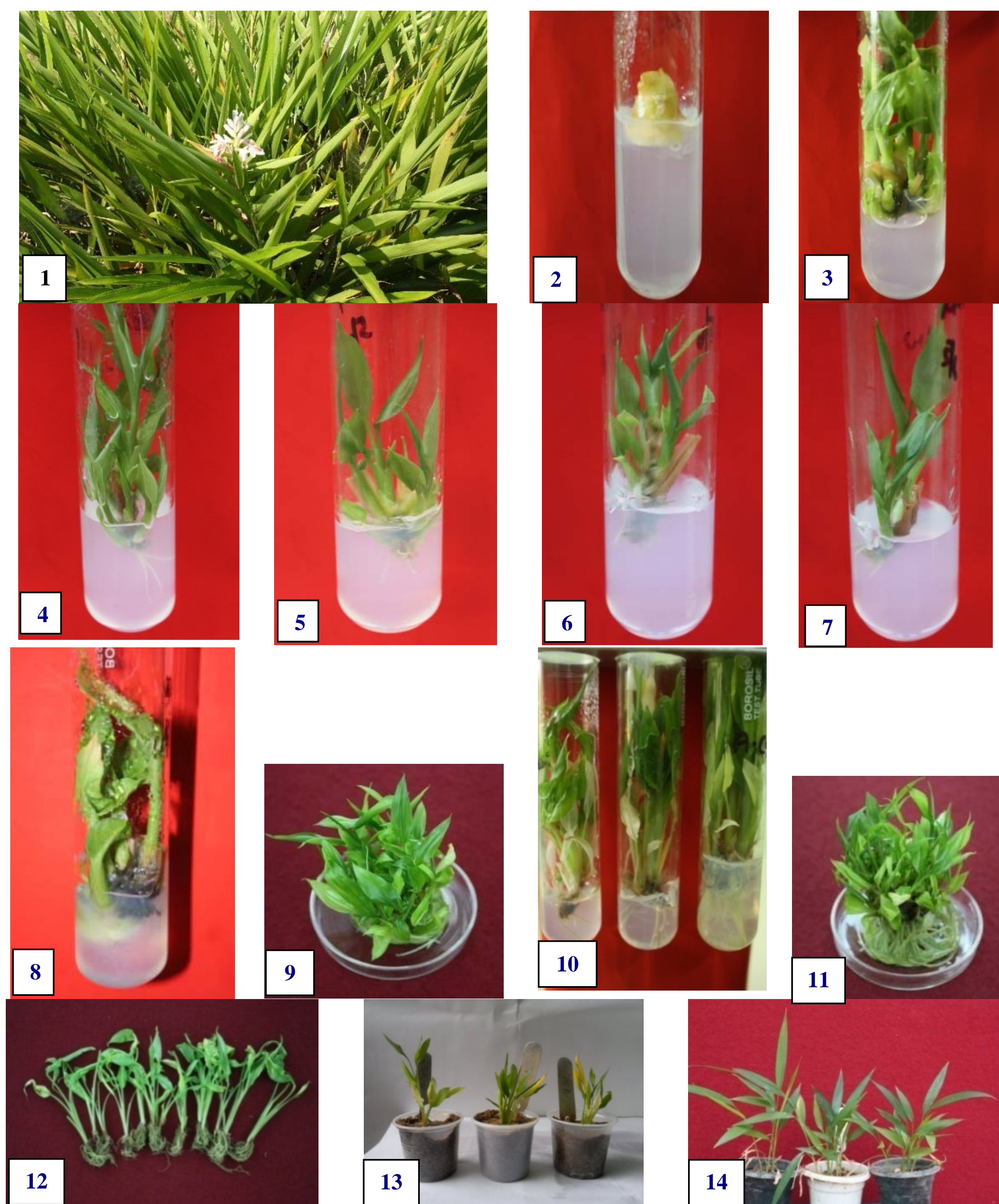

Figure 1. IC373610, the accession of *Alpinia calcarata* maintained in the herbal garden of NBPGR Regional Station, Thrissur, Kerala, mother plant for the rhizome bud explants. 2. Surface sterilized rhizome bud inoculated on to the medium for shoot initiation. 3. Multiple shoot induction (13.6) from rhizome bud explant in MS medium with 0.5 mg/l BAP and 1.5 mg/l kinetin. 4. Induction of 8.2 shoots from rhizome bud explant in MS medium with 0.5 mg/l BAP and 2 mg/l kinetin. 5. Multiple shoot induction (6.4) from rhizome bud explant in MS medium with 0.5 mg/l BAP. 6. Induction of 5.2 shoots from rhizome bud explant in MS medium with 1 mg/l BAP. 7. Initiation of 1.87 shoots from rhizome bud explants in MS basal medium. 8. Reduction in the number of shoots initiated to 3 in MS medium with 3 mg/l BAP. 9. Multiplication of shoots on transfer to fresh media in jam bottles. 10. Spontaneous rooting of shoots in shoot induction medium itself. 11. Vigorous rooting of shoots on transfer to MS solid basal medium supplemented with 0.5 mg/l IBA. 12. Well rooted shoots separated out. 13. Shoots transferred to plastic cups with sterile sand for hardening. 14. Hardened plants in pots in the greenhouse.

Almost 90% of the regenerated plants survived and showed vigorous growth of rhizome and roots without showing any morphological variations. Successful establishment of regenerated plantlets with 95% survival rate was reported in *A. calcarata* (Sudha *et al*., 2012), 93% in *A. officinarum* (Selvakumar *et al*., 2007) and 80% survival within 10-12 weeks in *A. galanga* (Borthakur *et al*., 1999). The present protocol on *in vitro* propagation of *A. calcarata* proved viable to be exploited for mass multiplication of the species for pharmaceutical and commercial purpose.

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