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Induction of multiple shoots in *Amomum hypoleucum* Thwaites – A threatened wild relative of large cardamom

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Abstract

An efficient and repeatable micropropagation protocol has been established for *Amomum hypoleucum,* a lesser known threatened medicinal plant of the family Zingiberaceae. Eighty percent of the rhizome nodes from greenhouse grown plants, cultured on MS medium supplemented with 1 mg L^{-1} BA and 0.5 mg L^{-1} IAA, showed axillary bud break in 8-10 days. Multiple shoots proliferated from such shoot explants when transplanted to medium with 3 mg L^{-1} BA and 1 mg L^{-1} TDZ. An average of 9.2 shoots could be recovered in two months and about 65-70% of the shoots showed simultaneous rooting. Isolated shoots were also rooted in medium fortified with 0.5 mg L^{-1} NAA. Plantlets, transferred to the field after acclimatization in greenhouse conditions, showed 85% survival.

Key words: *Amomum hypoleucum*, micropropagation, tissue culture, Zingiberaceae

Introduction

Several of the members of the family Zingiberaceae have direct influence on India's agri-produce export trade. Cardamom (*Elettaria cardamomum*), ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), large cardamom (*Amomum subulatum*) etc. are prominent spice members of international trade importance. These domesticated members of the spice family are well focused in research, while their wild relatives have received less attention in propagation, biology, ecology etc. *A. hypoleucum* is a wild relative of large cardamom. Its distribution is restricted to evergreen hilly forests of South India (Kerala and Karnataka) and Sri Lanka (Sabu, 2006).

The genus *Amomum* is represented by 90 species worldwide, out of which 22 are reported from India. *A. hypoleucum* Thwaites which occurs in small scattered populations has been included in the red list of threatened vascular plants (Rao *et al.,* 2003). This wild ginger is a perennial herb with inflorescence having 2-3 small white flowers borne distinctly on long creeping underground rhizomes. Flowering and fruit set are very rarely observed. Recent studies showed that fresh rhizomes and leaves contain 0.03 and 0.04% essential oils, respectively. The rhizome oil constitutes cryptone (15.4%), β-pinene (11.9%), caryophyllene oxide (15.4%) etc, whereas the leaf oil mostly contains (E)-nerolidol (26.5%), α-fenchyl acetate (15%), β- caryophyllene (8.4%) etc. The essential oils have pleasant aroma as well as anti-bacterial activity against *Pseudomonas aeruginosa*, *Salmonella typhi* and *Escherichia coli* (Sabulal *et al.,* 2007).

Apart from the rare fruit setting in *A. hypoleucum*, most of the half-ripe fruits are eaten by rodents in their natural habitat which are the major factors for its rarity in the wild. In lieu of lack of seed set, it is necessary to establish propagation protocols for this rare plant. There are no reports on the micropropagation of *A. hypoleucum* and therefore, the present work was undertaken to develop and establish non-conventional propagation system for increasing the population as well as for possible exploitation of its antimicrobial properties.

Materials and methods

Amomum hypoleucum (Zingiberaceae) plants, collected from Palode Forest Ranges (Thiruvananthapuram, Kerala, India) and maintained in the Tropical Botanic Garden and Research Institute (TBGRI), Palode, Thiruvananthapuram, served as the source of explants. Herbarium specimens were also processed and kept in the TBGRI Herbarium for reference (No. TBGT - 47720). Nodal segments isolated from young rhizomes were washed thoroughly in running tap water for 10 min, followed by treatment in a mixture of 0.2% labolene (Qualigens, Mumbai) and 2% commercial bleach for 60 min and rinsed thrice in distilled water.

The explants were surface sterilised with 15% bleach for 15 min and then in 0.1% $HgCl_2$ for 8 min. They were washed thoroughly in sterile distilled water thrice, trimmed to 1.5-2.0 cm size by keeping the node at the middle. The explants were inoculated onto MS basal medium (Murashige and Skoog, 1962) containing 3% sucrose. Plant growth regulators (PGRs) such as N^6-benzyladenine (BA), thidiazuron (TDZ) and kinetin (KN) at various concentrations (0.5, 1.0 and 2.0 mg L^{-1}) were added either singly or in combination with indole-3-acetic acid (IAA; 0.1, 0.5, 1.0 mg L^{-1}) to the medium for evoking responses. The developed shoots (2-3 cm) were subcultured as decapitated and non-decapitated explants to test their influence. After standardizing the PGR regime for optimum shoot multiplication, effect of mineral salt formulations of MS, B5 (Gamborg *et al.,* 1968), Nitsch (Nitsch, 1969) and WPM (Lloyd and McCown, 1981) were also tested. *In vitro* rooting of micro shoots was studied in MS medium supplemented with auxins – α-Naphthylacetic acid (NAA) and indole-3-butyric acid (IBA).

The cultures were incubated at 25±2^{0}C under 16 h light period provided by cool white fluorescent tubes (1000 lux). All well rooted *in vitro* plantlets were deflasked and washed thoroughly in tap water. Plantlets free of agar were dipped in 3% commercial fungicide (Indofil M-45) for 5 min prior to planting in clay pots containing river sand and coarse charcoal (3:1). All the plantlets

were maintained in semi shade (50% shade) and high humid (80-90% RH) net houses and sprinkled with water 3-4 times a day. Well established plants were potted and kept in the same net house conditions till field transfer.

All the experiments were set up in completely randomized block design. Each treatment was replicated 8 times. The data were analysed by single factor analysis of variance and the means were compared using Duncan's Multiple Range Test (DMRT) at P=0.05.

Results and discussion

Culture initiation: Nodal explants from underground rhizome of *A. hypoleucum* were initially established with 70 percent survival. The initial response of the primary explants varied according to the PGRs present in the medium. A combination of BA (1 mg L^{-1}) and IAA (0.5 mg L^{-1}) favoured the best culture initiation and showed 80 percent bud break within 10 days (Fig. 1A), while only 30-60 % bud break was observed in combinations of BA (0.5 - 2 mg L^{-1}) + IAA (0.1-1 mg L^{-1}) and BA (0.5- 2 .0 mg L^{-1}) used alone. KN was least effective of all the cytokinins tested. The superiority of BA over KN for multiple shoot formation was also demonstrated in *Jetropha integerrima* (Sujatha and Dhingra, 1993), *Sapium sebiferum* (Siril and Dhar, 1997), and *Bombax ceiba* (Chand and Singh, 1999). Though single buds were developed in PGR supplemented media, the explants failed to initiate bud break in hormone-free medium. The newly sprouted axillary buds developed into shoots with distinct nodes and internodes. The developed shoots were subcultured on to the same medium for multiplication and the elongated shoots (2-3 cm) were used for all further experiments. Decapitated and intact shoots explants in the medium showed varied responses. The intact shoot explants though elongated, scarcely produced axillary branches, while the decapitated explants showed frequent sprouting of axillary buds and therefore were selected as potential explants. In *Musa,* decapitation of apical dome was found necessary for the development of new shoots (Ma and Shii, 1972; Doreswamy *et al.,* 1983), while longitudinally split shoot halves showed better multiplication than whole explant in *Curcuma haritha* (Bejoy *et al.,* 2006), *Allium wallichi* (Wawrosch *et al.,* 2001) and *Opuntia polyacantha* (Mauseth and Halperin, 1975), whereas rhizome bud explants longitudinally divided into four equal parts showed better response in *C. longa* (Parthanturarug *et al.,* 2003). The present study showed that the removal of apical meristem facilitated sprouting of axillary buds overcoming the apical dominance reminiscent of the *in vivo* systems.

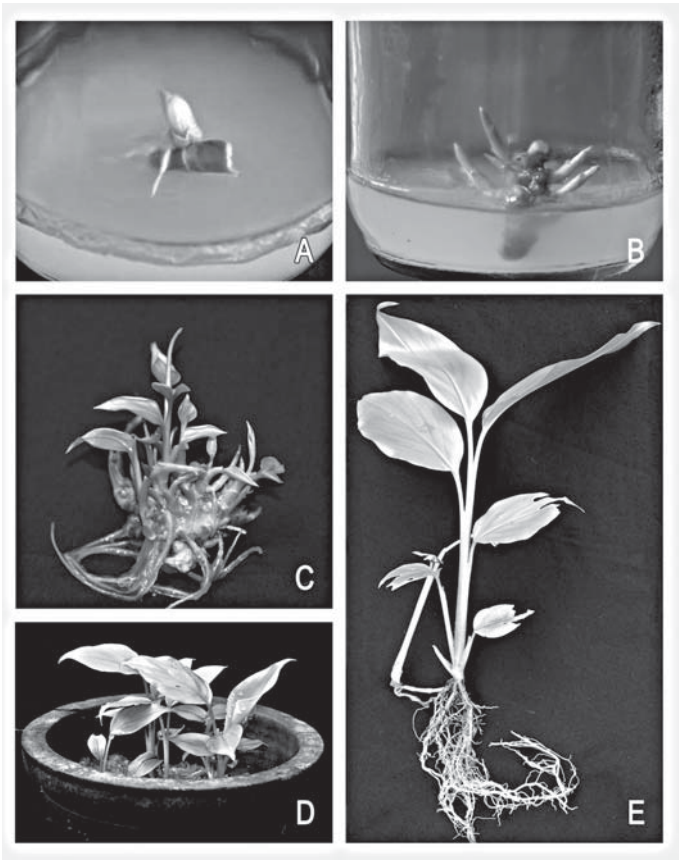

Fig. 1. *In vitro* propagation of *A. hypoleucum* from shoots of rhizome origin: 1A. Proliferating axillary bud from rhizome; 1B. Shoot development from decapitated shoot; 1C. Induction of multiple shoots with roots in BA (3mg L^{-1}) + TDZ (1mg L^{-1}) supplemented medium; 1D. Initial establishment of *in vitro* plantlets after one month; 1E. Well established plantlet showing newly formed rhizome developing into shoot.

Shoot multiplication: The growth response of shoots in terms of shoot elongation was better in BA alone supplemented media.

Table 1. Effect of plant growth regulators on morphogenetic response of shoot explants of *A. hypoleucum**

BA (mg L^{-1})	TDZ (mg L^{-1})	IAA (mg L^{-1})	Number of of shoots**	Shoot length (mm)**	Number of roots**
0.5			1.8 ±0.4a	50.8±7.3n	20.0±2.5q
1.0			2.8±0.6ab	41.1±5.5lm	18.0±3.5p
2.0			4.3±0.6bcd	44.4±4.6m	23.8±3.4r
3.0			3.7±0.4bcd	31.5±2.7ikj	14.4±1.4no
	0.01		2.7±0.4ab	40.2±6.2l	13.0±3.9mn
	0.05		3.1±0.6ab	32.7±4.2jk	4.6±1.0ghi
	0.10		3.8±0.8bcd	31.3±4.2ijk	5.3±1.3hi
	0.50		3.7±0.8bcd	22.7±4.4fg	4.0±0.6fgh
	1.0		5.0±0.7bcd	15.8±2.3abc	0.8±0.4a
	2.0		5.8±0.9cd	13.9±1.5a	3.2±0.8efg
	3.0		6.3±0.8d	17.6±1.7cde	1.6±0.6abc
	4.0		4.6±0.9bcd	15.3±1.9ab	2.0±1.3bcd
2.0	0.5		4.0±0.8bcd	21.2±2.0def	4.2±1.1ghi
2.0	1.0		5.2±0.7bcd	19.8±1.7def	3.6±0.6fgh
2.0	2.0		6.3±0.8d	17.2±1.3bcd	3.0±0.7efg
3.0	0.5		4.3±1.0bcd	18.7±2.8cde	2.0±0.9efg
3.0	1.0		9.2±0.8e	18.0±1.6cde	2.8±1.0efg
3.0	2.0		4.1±0.5bcd	18.3±2.6cde	1.0±0.5ab
1.0		0.1	3.2±0.8abc	16.9±4.6abc	8.8±2.1j
1.0		0.5	3.8±1.2bcd	32.0±3.8jk	4.3±0.8ghi
1.0		1.0	5.2±0.9bcd	34.8±4.4k	15.2±2.4o
2.0		0.1	3.0±0.7ab	49.0±3.9n	12.0±2.3lm
2.0		0.5	3.5±0.6abc	30.2±3.0ij	9.0±3.4jk
2.0		1.0	4.8±0.4bcd	21.7±2.5ef	10.6±2.1kl
	1.0	0.1	3.8±0.8bcd	28.1±4.1hi	2.1±0.4cde
	1.0	0.5	5.1±0.5bcd	25.6±2.8gh	2.5±1.0efg
	1.0	1.0	6.3±0.8d	18.6±1.3cde	2.3±0.7def
	2.0	0.1	4.3±0.6bcd	23.0±1.9fg	4.0±0.6fgh
	2.0	0.5	5.2±0.7bcd	15.0±1.4ab	6.0±0.9i
	2.0	1.0	4.6±0.7bcd	21.3±5.0ef	1.0±0.4ab

*Basal medium: MS + 3% sucrose + 0.7% agar & pH 5.7; all data after 60 days of incubation; **Mean ± Standard error followed by different letters within a column denotes significant difference at P =0.05 by Duncan's multiple range test.

Maximum elongation of *in vitro* shoots was achieved in 0.5 mg L^{-1} BA (50.8 mm). The shoots produced in medium supplemented with TDZ above 3 mg L^{-1} were short, stout and occasionally produced malformed leaves. Though BAP initiated multiple shoots, TDZ was found to be more effective. Thidiazuron, a substituted phenylurea (1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea) has been identified for its cytokinin-like activity in various explants cultured *in vitro* (Mok *et al.,* 1982; Huetteman and Preece, 1993). As per available literature, TDZ has been very rarely tested in members of Zingiberaceae, except in *C. longa* (Parthanturarug *et al.,* 2003). However, cytokinins were individually effective, their combined use improved the rate of multiplication. Addition of BA along with TDZ was the most potential hormonal regime for evoking almost 50% more multiplication (Table 1). The explants developed an average 9.2 shoots when treated with 3 mg L^{-1} BA and 1 mg L^{-1} TDZ in 9 weeks. This is in line with the reports of Vincent *et al.* (1992) and Anish *et al.* (2008), where the synergistic action of two cytokinins resulted in maximum shoot production. In contrast, cytokinin supplemented with an auxin was found optimal in other species, *C. amada* (Prakash *et al.,* 2004) and *C. longa* (Salvi *et al.,* 2002). Application of an auxin (IAA) along with BA did not improve the rate of multiplication in *A. hypoleucum*.

Effect of nutrient formulations: Mineral nutrition has also a controlling influence over morphogenic response of explants cultured *in vitro* (Preece 1995; Ramage and Richard, 2002). Hence, effect of four basal media (MS, B5, Nitsch and WPM) with constant PGR regime (3 mg L^{-1} BA and 1 mg L^{-1} TDZ) was investigated (Fig. 2). There were significant differences in the culture responses depending on the nutrient media used. The rate of multiplication was 9.2, 5.2, 4.6 and 2.8 in MS, Nitsch, WPM and B5 formulations, respectively revealing the superiority of MS medium in shoot multiplication for *A. hypoleucum*. Improved regeneration using MS has been reported in many members of Zingeberaceae such as *A. microstephanum* (Thoyajaksha and Rai, 2006), *C. haritha* (Bejoy *et al.,* 2006), *Kaempferia rotunda* (Anand *et al.,* 1997) and *Z. officinale* (Balachandran *et al.,* 1990). Shoot elongation (25.4 mm) was better in B5 compared to 17.8 mm in MS medium although the shoots developed in B5 medium showed pale yellow and narrow leaves when compared to other media. In contrast, B5 was found to be the best nutrient formulation for shoot multiplication in *C. amada* (Barthakur and Bordoloi, 1992). Rhizogenesis also varied in accordance with the nutrient composition. In the present study, Nitsch medium induced maximum number of roots (17.0) when compared to WPM (7.8), MS (2.8) and B5 (2.5).

Rooting and *ex vitro* establishment: In *A. hypoleucum,* about 70 percent of the shoots rooted in multiplication media. This is in agreement with reports in other Zingiberaceae members such as *C. haritha* (Bejoy *et al.,* 2006), *Paracautleya bhatii* (Rai and Thoyajaksha, 2001), *B. pulcherrima* (Anish *et al.,* 2008) etc, where rooting was achieved during shoot multiplication. Such concurrent root formation along with shoot development, reported in many Zingiberaceae members, may be due to an inherent root inducing capability of rhizomatous tissues. Simultaneous root development from microshoots also made the multiplication system easier and cost effective as reported in *Anthurium andraeanum* (Bejoy *et al.,* 2008). Since 30 percent shoots did not show simultaneous root development, a rooting medium was also adopted. Shoots without roots were rooted with the help of rooting hormones such as NAA and IBA (Table 2). The time required for root initiation was lesser (12-14 days) in NAA supplemented medium, when compared to that with IBA. Maximum number of roots per shoot (8.3) was also observed in 0.5 mg L^{-1} NAA. The necessity of a separate rooting phase in Zingiberaceae has been very rarely reported (Thoyajaksha and Rai, 2006).

Table 2. Effect of auxins on *in vitro* rooting response in *A. hypoleucum**

NAA (mg L^{-1})	IBA (mg L^{-1})	Days to root initiation	Percent response	Number of roots/shoot**
0.1	-	12-14	98	6.2 ± 1.2^{ab}
0.5	-	12-14	99	8.3 ± 1.1^{b}
-	0.1	15-18	95	5.4 ± 0.6^{a}
-	0.5	14-16	97	7.8 ± 0.6^{ab}

* Basal medium: MS+3% sucrose+0.7% agar & pH 5.7; all data after 4 weeks of incubation; **Mean ± Standard error followed by different letters within a column denotes significant difference at ∞ =0.05 by Duncan's multiple range test.

After 4-5 weeks of incubation in the rooting media, plantlets with good root system were deflasked and transplanted to the *ex vitro* condition. The young plants showed 85% survival and started producing new leaves in 6-8 weeks in community pots (Fig. 1D). They were then transferred to 8 inch clay pots containing river sand and soil mixture. The plantlets grew to 30-45 cm size in 6-7 months and developed fresh rhizome with vigorous shoot system (Fig. 1E). The regenerated plants were morphologically similar to their mother plants *in vivo*.

This is the first report on *in vitro* multiplication of *A. hypoleucum*. The multiplication programme established for the species has considerable practical importance to establish new population for the restoration of this rare species with minimum samples, suitable for its conservation.

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