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Micropropagation and genetic fidelity analysis in *Amomum subulatum* Roxb.: A commercially important Himalayan plant

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ABSTRACT

An efficient protocol for in vitro regeneration of a commercially important plant, *Amomum subulatum* Roxb., was developed using small pieces of rhizome explants taken from mature plants. Murashige and Skoog (MS) medium supplemented with 4.0 μM 6-benzylaminopurine (BAP) and 1.0 μM α-naphthalene acetic acid (NAA) resulted in maximum shoot numbers (32.6) with highest shoot length of 14.00 cm and on an average 61.4 roots and with 16.90 cm root length per explant. This could be repeated when individually separated shoots were transferred again on the same medium. Thus, on an average, about 30 plantlets could be obtained per culture cycle of three weeks. Ninety percent survival was recorded at the end of the 5 weeks of acclimatization, and cent percent survival was observed after 150 days of transfer of acclimatized plantlets into earthen pots, containing a mixture of soil and farmyard manure (3:1, v/v) when the pots were kept in the open nursery with partial shade. Random amplified polymorphic DNA (RAPD) marker analysis of ten randomly selected tissue culture raised plantlets confirmed their genetic fidelity with the mother plant. High multiplication rate associated with observed genetic stability clearly indicates the efficacy of the present in vitro clonal propagation protocol of this important medicinal plant of high commercial value.

1. Introduction

Large cardamom (*Amomum subulatum* Roxb., family: Zingiberaceae, Hindi: 'Bari Elaichi') is a tall perennial rhizomatous herb and an important cash crop cultivated between 600 and 2000 m asl. It is mainly used as a spice and India is the largest producer of large cardamom with production of 4465 metric tons in 2013–14 and exported 1110 metric tons during the same year (Anon, 2015). In India, it is cultivated in the states of Sikkim, West Bengal, Arunachal Pradesh, Nagaland, Mizoram, Manipur and Uttarakhand (Bisht et al., 2010). It is also cultivated in neighboring countries like Nepal and Bhutan. The seed has a pleasant aromatic odour due to which it is extensively used for flavoring food preparations, and has also been used in Ayurvedic medicines (Sharma et al., 2000). Seeds are also considered as an antidote to snake and scorpion venom; it is also used as preventive as well as curative agent for throat troubles, congestion of lungs, inflammation of eye lids, digestive disorders and in the treatment of pulmonary tuberculosis (Verma et al., 2010; Bisht et al., 2011).

The volatile oil present in the seeds of large cardamom is a major constituent responsible for the typical odour. The seeds contain 3% essential oil (Gupta et al., 1984), which is dominated by 1, 8-cineole (Bal Krishnan et al., 1984; Gurudutt et al., 1996; Bhandari et al., 2013); seeds also contain of a number glycosides, namely petunidin 3, 5-diglucoside, leucocyanidin 3-o-β-D-glucopyranoside subulin, aurone glycoside, cardamomin-α-chalcone and alpinetin-α flavanone (Shankaracharya et al., 1990). In nature the plant propagates through seeds and rhizomes (vegetatively); however, low (29%) seed germination (Bisht et al., 2010) and slower rate of vegetative multiplication fail to cope up with ever increasing demand of plant propagules for expanding cultivation. One of the important problems associated with this crop is the occurrence of viral and fungal diseases (Sharma et al., 2009) which affect productivity and also result in high plant mortality. In order to overcome these problems, use of in vitro method of propagation is considered to offer an alternative approach for effective and rapid means of multiplication of elite genotypes. Although in vitro regeneration protocol of *A. subulatum* has been reported earlier (Sajina et al., 1997), data are

Abbreviations: IBA, Indole-3-butyric acid; BAP, 6-benzylaminopurine; NAA, α-naphthalene acetic acid; MS, Murashige and Skoog medium; PGRs, plant growth regulators; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA.

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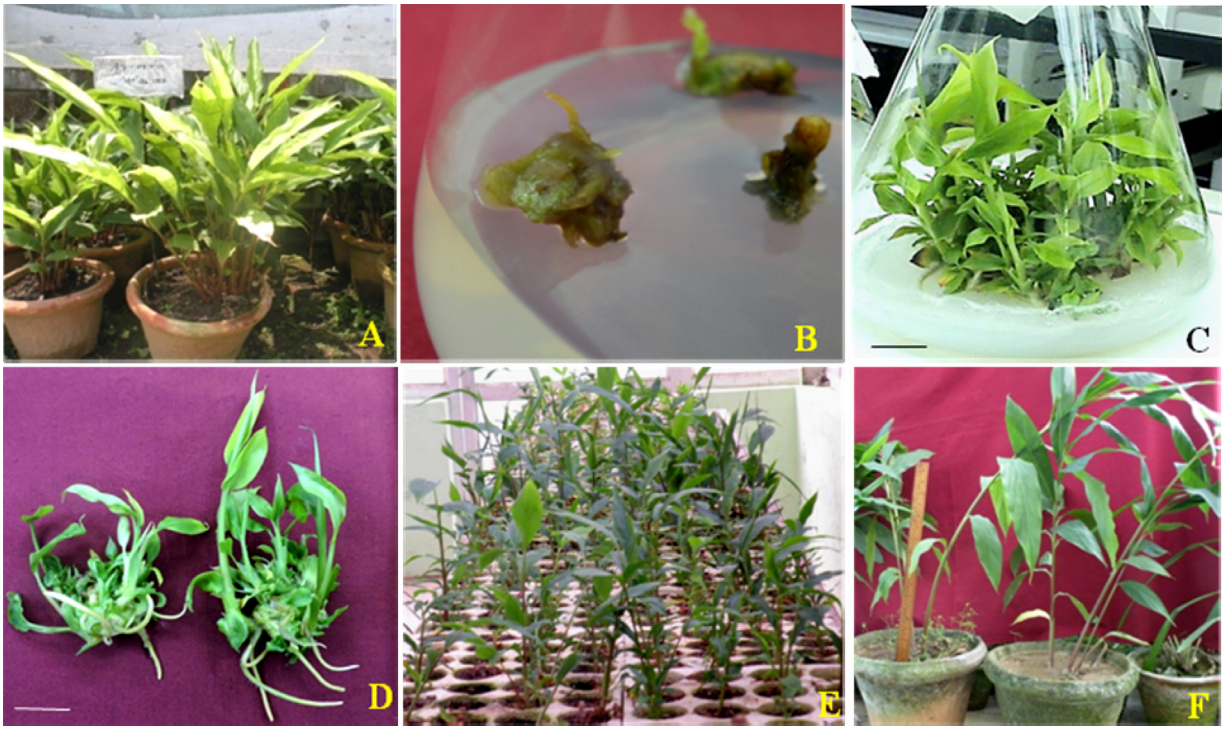

Fig. 1. In vitro regeneration of *A. subulatum* and establishment of plants in the soil (A–F).
A: Mother plants of *A. subulatum*, the source of explants,
B: Rhizome explants cultured on MS medium,
C: Profuse shoot multiplication in a 3-week old culture; 1 bar = 1 cm,
D: A close view of plantlets along with roots from a 3 week old culture; 1 bar = 1 cm,
E: Acclimatization of tissue culture raised plantlets after transfer of such plantlets in to thermocole trays kept for 3 weeks in a green house,
F: Established tissue culture raised plants after transfer of acclimatized plants in clay pots.

Table 1
Effect of different PGRs on shoot multiplication, elongation and rooting in *A. subulatum*.

Plant growth regulators (Concentration, μM)		Number of shoots	Shoot length (cm)	Number of roots	Root length (cm)
BAP	NAA				
0.0	0.0	3.20 ± 0.59^{c}	4.80 ± 0.67^{d}	7.0 ± 0.72^{d}	4.00 ± 0.72^{e}
2.0	0.5	4.00 ± 0.45^{c}	7.80 ± 0.67^{bc}	14.8 ± 1.49^{cd}	6.90 ± 0.69^{d}
2.0	1.0	3.20 ± 0.59^{c}	6.00 ± 1.16^{c}	14.6 ± 3.4^{cd}	9.40 ± 1.23^{bc}
3.0	0.5	6.40 ± 0.52^{c}	8.00 ± 2.99^{bc}	15.0 ± 3.23^{cd}	7.30 ± 0.59^{cd}
3.0	1.0	11.8 ± 0.87^{b}	8.08 ± 0.61^{b}	35.0 ± 3.59^{b}	6.80 ± 0.86^{d}
4.0	0.5	12.8 ± 0.59^{b}	4.00 ± 0.45^{d}	11.6 ± 1.09^{cd}	2.30 ± 0.26^{e}
4.0	1.0	32.6 ± 2.60^{a}	14.00 ± 1.11^{a}	61.4 ± 3.36^{a}	16.90 ± 1.05^{a}
5.0	1.0	4.20 ± 0.87^{c}	8.40 ± 0.94^{bc}	18.4 ± 2.42^{c}	11.70 ± 0.89^{b}

Note:- Values are mean ± standard error; Mean values with same letters in a column are not significantly different ($P < 0.05$; DMRT). The data have been recorded on per explant basis and recorded after 3 weeks of inoculation.

missing on the clonal fidelity and survival of tissue culture raised plants in the field. The present study reports the development of an efficient in vitro propagation protocol, using rhizome pieces with bud as explants, and assessment of genetic stability of regenerated plants and their subsequent cent percent survival 5 month after transfer to pots.

2. Materials and methods

2.1. Plant material and tissue culture

Plants of *Amomum subulatum* cv. Sawney were collected (courtesy Dr. K.K. Singh, Senior Scientist at Sikkim unit of GBPIHED located at Tadong near Gangtok) from Kabi area (altitude 1500 m asl; 27°15′05″ N; 88°39′24.27″ E) in Sikkim during July 2007, and these were brought to the Institute at Kosi – Katarmal, Almora (altitude 1150 m asl; 29°38′22.54″ N; 79°37′24.87″ E). These were grown in earthen pots (20 cm height and 18 cm diameter) containing potting mixture (soil and farmyard manure; 3:1, v/v) under open nursery conditions with partial sun light using green shade nets (Fig. 1A). After several months, the rhizomes were removed from an identified pot, washed under running tap water for 15–20 min to remove adhering soil and other debris. The mother plant was labeled and kept in the green house (80% relative humidity, 25 ± 1 °C, 18/6 h day/night, light- 50% of ambient) for further growth and subsequent use. While initiating the experiment the rhizomes (from mature plant) were washed and cleaned (as mentioned above) and then immersed in water containing liquid detergent (0.2% Tween-20, v/v; Hi Media, India) for 20 min, and washed with sterile distilled water (x4). The explants were then treated with Bavistin solution (2%, w/v; a systemic fungicide; BASF, Mumbai, India) on a shaker for 30 min, and then rinsed again with sterile distilled water (x4) in a Laminar air flow cabinet (Thermadyne, India), followed by treatment with freshly prepared mercuric

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