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SHORT COMMUNICATION

In vitro propagation of Rudraksha (*Elaeocarpus sphaericus* (Gaertn.) K. Schum): a biotechnological approach for conservation

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Abstract The species *Elaeocarpus sphaericus* (Rudraksha) is a religious, medicinally important threatened tree of India. An efficient micropropagation protocol has been developed from nodal explants of this plant species collected from north-east India for large scale production of planting material at favourable sites within the country. Best shoot initiation occurred in MS medium supplemented with 2.2μM BA+2.2μM Kn in combination. Addition of Casein Hydrolysate (CH) (100mg/L) increased the shoot number. Microshoots excised and subcultured in 2.0μM BA further enhanced growth and multiplication. The shoot cultures were maintained in this concentration for 2years with subculturing at 6weeks interval. MS medium containing 5.0μM NAA was most effective for rooting. Successfully acclimatized plants (80%) showed normal growth under suitable habitat conditions.

Keywords Rudraksha · *Elaeocarpus sphaericus* · Nodal explants · Cytokinins · Shoot proliferation · Rooting of microshoots · Acclimatization

Abbreviations

BA	6-Benzyladenine
CH	Casein Hydrolysate
IBA	Indole-3-butyric Acid
Kn	Kinetin
MS	Murashige and Skoog medium
NAA	Naphthalene Acetic Acid
PGRs	Plant Growth Regulators
RH	Relative Humidity

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Introduction

Elaeocarpus sphaericus (Gaertn.) K. Schum (Synonym: *E. ganitrus* Roxb.; family Elaeocarpaceae), popularly known as 'Rudraksha', is a threatened tree species in the tropical wet evergreen forests of North East India (Rao and Haridasan 1983). The tree is well known for its medicinal and religious value in India**.** In Ayurveda, the seed is used for the treatment of hypertension, insomnia, psychoneurosis and mental diseases. The aqueous extract of fruit is hypotensive, sedative, spasmolytic, anticonvulsant, choleretic, bronchodilatory and cardiostimulant and the stem bark is hypoglycaemic (Khare 2004, 2007). Since ancient times, the 'Bodhi beads' (Li et al. 2014) strung together as rosaries or garlands ('malas'), are being used in Eastern religions (Hindus, Sikhs and Tibetan Buddhists) for meditation.

The growing demand and poor germination has affected the plant's natural regeneration in the wild. Various anthropogenic disturbances have resulted in large scale removal of mature Rudraksha trees, plant parts or seeds. *In situ* and *ex situ* conservation methods are recommended in view of its ethnic medicinal importance (Khan et al. 2004) and to derive economic benefits due to increasing demand of Rudraksha beads at various Rudraksha centers in Asia. Plantation in and outside the area of its occurrence would help inachieving this. Propagation through air layering (Singh et al. 1999; Bhojvaid and Negi 2003) and pre-sowing seed treatments (Khan et al. 2003) has been tried with success but while the former has seasonal constraints and callusing during rhizogenesis, the latter may damage or cause absence of seeds.

Since raising of planting stock is a pre-requisite for any plantation programme, biotechnological techniques can be used for mass propagation of this plant species before it falls further in the category of threatened plants. Likewise, the extensive use of several *Elaeocarpus* species for medicinal as

well as timber purpose have critically endangered and threatened a number of species which are in the Red Data List of threatened plants (Saklani et al. 2015). *In vitro* (tissue culture) techniques have become a useful alternative to conventional methods of propagation providing a faster and better means for conservation of such plants. Very limited work has been reported on micropropagation of *Elaeocarpus* species in India and abroad. There has been mention of *in vitro* propagation of selective *Elaeocarpus* species in the past including *E. sphaericus* (Chauhan et al. 2015) and few others such as *E. robustus*, native olive or 'Jalpai' (Roy et al. 1998; Rahman et al. 2003) and *E. tuberculatus* (Arshad and Kumar 2006) whose nuts are used as substitute of Rudraksha (Murti 1993). The present investigation was, thus, undertaken to develop a rapid and efficient *in vitro* propagation method using nodal explants of *E. sphaericus* brought from its natural habitat in North East India for large scale production at regions harbouring subtropical climatic conditions suitable for the growth of this plant species.

Materials and methods

Potted plants of *E. sphaericus* were collected from Botanical garden at Teju (27°56' N and 96°09' E; 760 ft altitude) situated in Lohit district, Arunachal Pradesh, India and were brought to laboratory at HAPPRC, Srinagar (Garhwal), Uttarakhand. The tree samples of the plants were authenticated from Botanical Survey of India, Dehradun (Accession Number 114647). Surface sterilization procedure of nodal explants included treatments in 0.1 % Tween-20 (*v*/*v*; 15 min), 2 % bavistin, (*w*/*v*; 20 min), 70 % ethanol (30 s) and 0.2 % mercuric chloride (*w*/*v*; 2–3 min) followed by repeated rinses in double distilled water. The antioxidants used were 0.1 % ascorbic acid, 0.05 % citric acid, 0.1 % polyvinylpyrollidone (*w*/*v*) for 15 min. The treated explants were sized and implanted in Murashige and Skoog (1962) medium containing different PGR concentrations for shoot induction and multiplication. The cultures were maintained at 25 ± 1 °C with irradiance (40–50 μmol m^{-2} s^{-1}) under a 16 h photoperiod.

The two cytokinins, BA and Kn (1.0–5.0 μM) alone and in two different combinations (BA+Kn; 1.0 μM each and 2.2 μM each) were tried for shoot induction. The best concentration was further subcultured with CH (100 mg/L). For enhanced shoot multiplication and growth, excised bunches were subcultured in fresh MS medium containing 1.0–15.0 μM of BA or Kn. The root induction was carried out in MS medium containing 2.0–15.0 μM of NAA or IBA. The cultures without PGRs served as controls. Data on different shoot and root parameters were recorded regularly after 5–6 weeks of culture.

The rooted microshoots were dipped in 0.1 % bavistin (*w*/*v*; 5 min), washed and transferred to plastic cups containing sand and soil (1:1; *v*/*v*). Thereafter, they were placed in green house (25 °C and 80 % RH) for acclimatization followed by successful transfer to garden soil and kept outdoors in partially shade nursery condition for growth and survival.

The results were subjected to statistical analysis. Analysis of variance (ANOVA) and significant differences between means were assessed by Duncan's multiple range tests at $p < 0.05$ using SPSS20 software.

Results

The cultures of nodal explants of *E. sphaericus* were 85–90 % infection free with the shoot induction occurring within 2–3 weeks. Best shoot induction in terms of shoot number and length was observed in the cytokinin combination, BA+Kn, 2.2 μM each (3.00 ± 0.41 shoots per explant with shoot length of 1.85 ± 0.10 cm) which was statistically significant ($p < 0.05$) from other cytokinin concentrations taken alone and in combination (Fig. 1a). When compared to lower cytokinin combination concentration of BA+Kn (1.0 μM each) where 50 % shoot induction was observed, the shoot number (2.00 ± 0.00) and shoot length (1.23 ± 0.13 cm) values were lower. Also, these shoot parameters were not significantly different from 3.0 μM BA and 1.0 μM Kn concentrations taken alone. The nodal explants on PGR free medium did not give any results.

After shoot induction, on subculturing the mother explant with regenerated shoots on same concentrations did not result in any significant increase in length and number of shoots per explant. However, in the combination concentration (BA+Kn; 2.2 μM each) showing best results, second subculture with CH (100 mg/L) resulted in profuse multiple shoots production (11.68 ± 0.88) and growth (2.93 ± 0.23 cm) (Fig. 1b). Further subculturing of microshoots excised from this concentration in fresh MS medium supplemented with different BA or Kn concentrations resulted in enhanced multiplication and/or growth.

The results of ANOVA show that differences in both shoot length and shoot number values are statistically significant ($p < 0.05$) with respect to treatments of different concentrations (1.0–5.0 μM) provided under BA and Kn used alone. The response was 100 % with and without PGR. In fact, PGR free medium also showed increase in shoot length. Maximum number of shoots per explant (17.69 ± 1.00 shoots per explant with average shoot length of 3.57 ± 0.19 cm) was obtained on MS medium supplemented with 2.0 μM BA (Fig. 1c). However, Kn under all the concentrations taken gave a slightly better response with respect to shoot length in comparison to BA, but in contrast, lower values were obtained for number of axillary shoots generated per explant where the highest shoot number was observed at 2.0 μM Kn (8.81 ± 0.56 shoots per explant with average shoot length of 3.70 ± 0.17 cm). On the whole, better

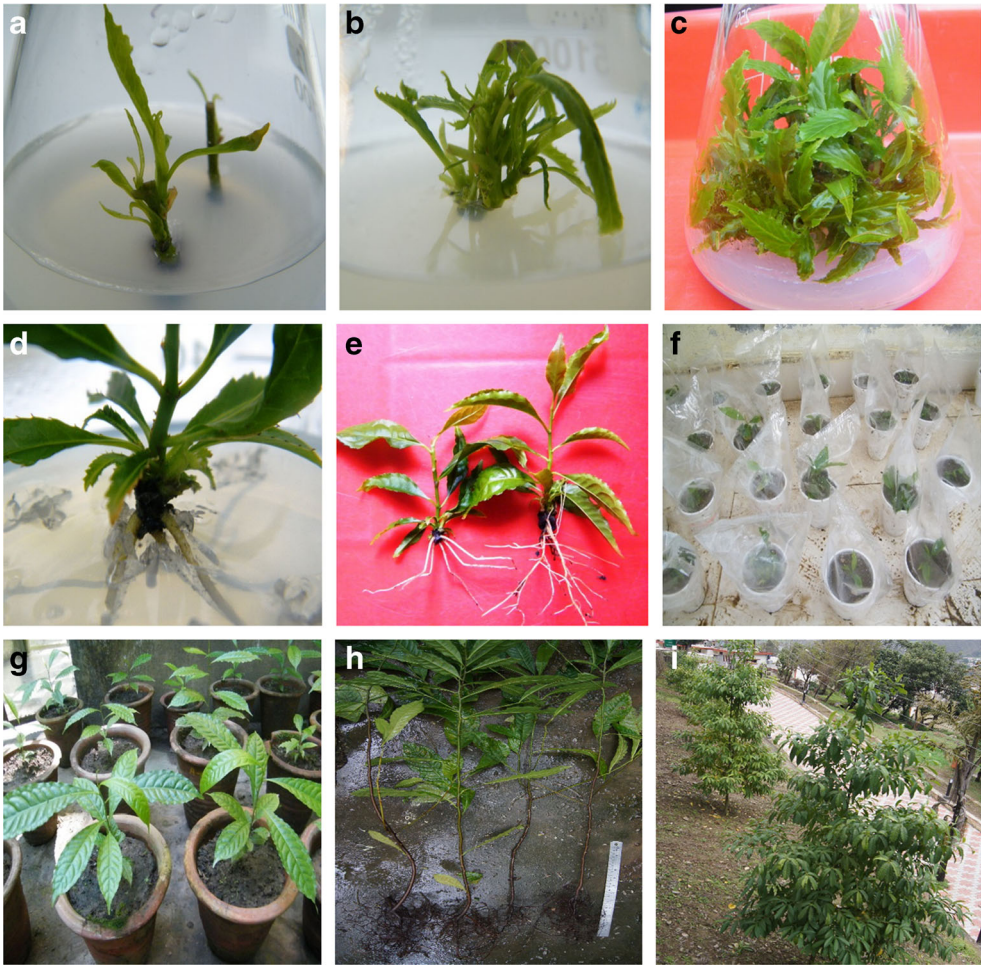

Fig. 1 Different stages of micropropagation of Rudraksha (*Elaeocarpus sphaericus*): Bud breaking in nodal explants (**a**), Multiple shoots formation with casein hydrolysate (**b**), Multiplication and growth in MS medium containing 2 μM BA (**c**), Rooting of microshoots in MS medium (**d**), Rooting of microshoots in MS media containing 5 μM NAA (**e**), Micropropagated plantlets transferred for hardening under green house conditions (**f**), Well acclimatized and grown plants in earthen pots placed under shade net nursery condition (**g**), 2 years old micropropagated plants before transfer to plantation site at HAPPRC premises (**h**) and well grown 3 years old plants in HAPPRC premises at Srinagar-Garhwal (**i**)

results were obtained for BA with respect to number of axillary shoots regenerated, although, at higher concentration (15 μM) of BA or Kn used alone, there was a conspicuous decline in shoot number as well as shoot height. Thus, BA was found to be more effective for axillary shoot proliferation.

Excised microshoots (2.0–4.0 cm long) when implanted in MS medium containing different concentrations of auxins, either NAA or IBA, showed significant results ($p < 0.05$), with respect to number of roots per shoot and the root length. The microshoots in auxin free MS medium failed to develop roots. Highest number of roots per shoot (4.60 ± 0.39 roots per shoot) was observed in 5.0 μM NAA and statistical analysis showed that this was significantly different from others (Fig. 1d, e). However, in case of IBA, while no rooting occurred at 2.0 μM concentration and although rooting was observed at other concentrations, highest at 10 μM IBA (2.15 ± 0.20 roots per shoot with root length of 2.43 ± 0.15 cm), the overall results were poor. Further, it was also observed that NAA showed good response with respect to root length at all concentrations with maximum root length (3.49 ± 0.23 cm) occurring at 5.0 μM concentration. The comparative analysis revealed that NAA was more effective auxin than IBA for rooting in terms of percent root response as well. For instance, the different concentrations of NAA used alone resulted in 62–82 % rooting with maximum root induction (82 %) at 5.0 μM NAA. In comparison, IBA at different concentrations resulted in 14–22 % rooting only. Higher concentrations were inhibitory for both.

The microshoots with well-developed roots were taken out after 6 weeks and transferred to plastic cups with great care avoiding damage to the roots. These cups were covered with polythene bags to maintain high humidity (Fig. 1f). The well rooted plantlets which were shifted from laboratory to green house (25 °C, 80 % RH) and then nursery showed healthy growth with 80 % survival (Fig. 1g). Growth conditions of the tissue culture raised plants were monitored for 2 years (Fig. 1h) and are growing well in the HAPPRC premises, Srinagar-Garhwal, Uttarakhand (Fig. 1i).

Discussion

The results indicated that a very simple and efficient clonal propagation can be successfully achieved for *E. sphaericus*

through tissue culture technique. The importance of cytokinins for shoot proliferation is well established (Van Staden et al. 2008) and is an essential step to successful micropropagation. In the present study, both BA and Kn were ineffective when used alone for shoot proliferation and growth in the initial stages but showed good results when used in combination and better results were obtained along with growth additive, casein hydrolysate. Similar investigations have been reported in the same species (Chauhan et al. 2015) and other species such as *E. robustus* by Roy et al. (1998) wherein equal concentrations of BA and Kn along with casein hydrolysate were used for optimal shoot multiplication. Further, for routine sub culturing of multiple shoots of *E. sphaericus*, BA and Adenine sulphate were used. However, in *E. tuberculatus* (Arshad and Kumar 2006), axillary bud proliferation was achieved in MS medium supplemented with BA alone but *in vitro* maintenance was carried out by sub culturing on the same medium containing equal concentrations of BA and Kn. Contrary to this, in the present investigation, the multiple shoots were further subcultured in BA or Kn which resulted in enhanced proliferation and much better growth than the earlier report on the same species and were maintained in MS medium with 2.0 μM BA for 2 years with subculturing at 6 weeks interval but without any growth additives.

The present findings also indicate that a permissive balance of cytokinins may be required by a plant which can be species specific as is evident from various *in vitro* shoot multiplication protocols of *Elaeocarpus* species. The synergistic effect of growth additives with PGRs on shoot multiplication and elongation of other plant species (Sridhar and Aswath, 2014) and enhanced multiplication on subculturing of proliferated shoot culture at regular intervals (Biswas et al. 2009) have also been reported. A successful micropropagation protocol for any plant species rests on the ability of the clonal plantlets to acclimatize under *ex vitro* conditions which further is related to the rooting status of the microshoots. Both the auxins exhibited rooting response in the present investigation but much better rooting results were observed at all concentrations of NAA than reported earlier by Chauhan et al. (2015) in IBA supporting the fact that rooting also depends on factors like shoot length, light and age of cultures (Singh 2015). The well rooted plants acclimatized and grew into healthy plants. Therefore, this method is a useful alternative to the conventional methods of propagation and will help in conservation of this threatened tree species under suitable habitat conditions in India.

Conclusion

Although the state of Uttarakhand in Northern India does not fall in the natural habitat of *E. sphaericus*, the species is being cultivated in different regions having subtropical climatic conditions suitable for growing Rudraksha. Rudraksha beads have immense value in Asia and are being used for spiritual and medicinal purposes. As such, the beads are sold at various Rudraksha centres located in Northern India and are of great interest to Indian and foreign tourists who traverse the Himalayas for adventure and for spiritual purpose. In view of its threatened status in northeast India and the related importance, the present protocol has been devised from the plants which have been brought from their natural habitat and not from the Rudraksha trees already growing in Uttarakhand for the past so many years but belonging to the same species. Therefore, in the present perspective, there is an urgent need to carry out genetic diversity studies and phylogenetic relationships of this highly religious, medicinally important plant species from these regions and also establish a link between cultivated plants grown in Uttarakhand region and those from the native habitat of this species.

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