Research Article

In vitro zygotic embryo germination and propagation of an endangered *Boswellia serrata* Roxb., a source of boswellic acid

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ABSTRACT

This study aims to establish an efficient protocol for development of seedlings of an endangered medicinally important forest tree *Boswellia serrata* Roxb., for mass plantation and consistent supply of *salai guggul*. The green mature fruits served as source of seeds. The excised green zygotic embryos were cultured on Gamborg (B5), McCown and Loyd (WPM) and Schenk and Hildebrandt (SH) media fortified with different concentration of sucrose and on Murashige and Skoog (MS) medium containing 3 % sucrose, polyvinylpyrrolidone (PVP) (0-300 mg l^{-1}), Gibberellic acid (GA_3), Indoleacetic acid (IAA), Naphthaleneacetic acid (NAA), Indole-3-Butyric acid (IBA) or 2,4-dichlorophenoxyacetic acid (2,4 D) and 6-benzylaminopurine (BA) or kinetin (Kin) individually. The highest frequency of embryo germination (96 %) and conversion into seedling was obtained on MS medium containing 3 % sucrose together with 200 mg l^{-1} PVP; other media were either inferior or induced abnormalities in the seedlings including callus formation from the zygotic embryos. Fully developed seedlings could be successfully established in soil with about 94 % survival. The embryos from mature dry seeds did not respond for germination in any of the experiments. In conclusion, selection of zygotic embryo from green mature seeds and their *in vitro* germination is important for propagation of *B. serrata*. **[Physiol. Mol. Biol. Plants 2010; 16(2) : 159-165]** *E-mail : tdnikam@unipune.ernet.in*

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INTRODUCTION

Boswellia serrata Roxb. (Burseraceae) is a perennial deciduous resin producing tree distributed in the tropical parts of Asia, Africa and Middle East (Anonymous, 1962; Gaofeng *et al.,* 2006). It is one of the sources of frankincensences. Its wood is used in paper industry. The gum resin obtained from its bark is called “Indian Olibanum” or “*Salai guggul*”. ‘*Salai guggul’* is classified as non-steroidal anti-inflammatory agent (Singh and Atal, 1986). It has been used in traditional Ayurvedic medicine for the treatment of inflammatory diseases. In recent years the gum resin has been used extensively in pharmaceutical formulations for relieving aches and pain, particularly associated with arthritis (Singh and Atal, 1986; Chikamai, 2002).

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The production of gum resin is declining as a result of poor natural multiplication of the *Boswellia* Woodlands, possibly as a consequence of poor seed germination (10-20 %) and vegetative propagation (Purohit *et al.,* 1995). Limited availability of resources due to absence of leaves on the trees during the entire period of flowering and fruiting, results in decline of natural production of seeds (Sunnichan *et al.,* 2005). The over exploitation of the species, together with low natural regeneration has resulted in a decrease in natural stands, prompting its inclusion in the list of threatened and endangered species (TESS) (Sharma, 1983).

Though, *in vitro* propagation has been reported in *B. serrata* (Purohit *et al.,* 1995), propagation through organogenesis in woody species is of long duration, cost intensive and associated with high mortality of plantlets during hardening and field transfer. Zygotic embryo culture plays an important role in *in vitro* propagation of endangered forest tree species to overcome physical biotic interference (Raghavan, 2003; Rambabu *et al.,* 2006).

This communication describes about the viability of embryo and a protocol for zygotic embryo culture and seedling development in *B. serrata.*

MATERIALS AND METHODS

Plant material and surface sterilisation

The mature green fruits (Fig. 1a) were collected from the trees growing naturally on a hillock (*vetal hill*, in Pune city, in Western India). Uniform and healthy fruits were washed under running tap water, and rinsed twice in sterile distilled water. The surface of the fruits were disinfected by soaking them in 0.1 % $HgCl_2$ for 5 min. followed by washing five times with sterile distilled water. Similar procedure was applied for mature dry fruits.

A cut was made mechanically in the fruit wall and seed coat using a sterile surgical blade. Embryos were excised aseptically (Fig. 1b) and inoculated on to different media in culture tubes, each with one embryo. All these operations were carried out in a laminar air flow cabinet.

Culture medium and conditions

The nutrient media used in this study were MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972), WPM (McCown and Lloyd, 1981) and B5 (Gamborg *et al.,* 1968). Each medium was supplemented with 0, 10, 20, 30 and 40 gm l^{-1} of sucrose. The MS medium was supplemented with BA, Kin, IAA, NAA, 2,4-D and GA_3 individually (0-10.0 µM) and PVP (0 to 300 mg l^{-1}).

Prior to autoclaving at 121 °C for 15 min, pH of the media was adjusted to 5.8 and 0.8 % agar (w/v) was added. The embryos were inoculated with care to ensure that half portion on radicle side was placed into the medium and remaining half portion above the medium. The cultures were maintained in a room at 25 ± 2 °C, 8 h illumination at 30 µmol m^{-2} s^{-1} supplied by cool white fluorescent tubes (Philips India Ltd).

Effect of wet heat treatment, acid scarification and plant growth regulator

The 50 mature dry seeds were wrapped in a clean cotton cloth and then completely submerged in 500 ml hot distilled water set at 60 °C in 1000 ml beaker. The hot water treatment was given separately for 10, 20, 30, 40, 50 and 60 min.

Acid Scarification of Seeds: The seeds were acid scarified in 500 ml beaker by soaking the 50 seeds in concentrated hydrochloric acid (HCl), sulphuric acid (H_2SO_4) and nitric acid (HNO_3) separately for 5, 10, 15, 20, 25 and 30 min. After the specified treatment, the acid was decanted and the seeds were washed under running tap water for 2 h. The control was maintained by submerging the seeds in distilled water for the same duration. Before excision of embryo, wet heat treated and acid scarified seeds were subjected to the treatment of 00, 1, 2, 3, 4 and 5 mM IAA, NAA, 2,4-D, BA, Kin and GA_3 for 12, 24 and 36 h.

Plant acclimatization

The well developed plantlets (3-4 cm height) were removed from the culture tubes and washed with tap water. They were transferred to plastic bag containing sand:soil (1:1) and maintained in the glass house under low light intensity (near 191.14 µmol $m^{-2}s^{-1}$), high humidity 90 ± 5 % and 26 ± 4 °C. After 4 weeks, the seedlings were transferred to natural field conditions with maximum light intensity (765.7 µmol $m^{-2}s^{-1}$), temperature 21 to 28 °C and humidity 65 to 80 %.

Embryo viability: The viability of the embryo was determined using the tetrazolium (TZ) test (Nachlas *et al.,* 1960). The seeds were bisected longitudinally to expose the embryo. Hundred seeds were incubated in 100 ml of 1% (w/v) solution of 2,4,5-triphenyl tetrazolium chloride, prepared in 0.1 M Sorensen's buffer (pH 7.0) for 24 h at 28 °C. Embryos were examined and classified as viable or non-viable according to the staining pattern, viable when the radicle with continuity of staining from the axis to at least 50 % staining of cotyledons. The embryos that turned reddish – pink were scored as viable (Nikam and Barmukh, 2009).

Experiment design and data analysis

The experiments were based on a completely randomised design conducted with 21 replications and one embryo in each culture. Each experiment was repeated at least thrice. For each treatment, the growth of embryo in terms of shoot length, root length and callus formation was observed at weekly intervals for 4 weeks. Combined analysis of variance (ANOVA) based on the mean of three experiments was used to analyze the frequency of embryo showing shoot and root growth without callus formation and those associated with callus formation. Mean comparisons and separations were conducted using Duncan's multiple range test (DMRT) at 5 %, probability level (Duncan, 1955).

RESULTS AND DISCUSSION

Viability and influence of media and sucrose concentration

The germination of embryo excised from green mature seeds (Fig. 1a) started after 7 days of culture. The elongation of root occurred in the second week (Fig. 1c). At the end of two weeks the radicle of 80 % embryos elongated and grew into the medium. The cotyledonary leaves expanded above the medium showing the typical sign of germination (Fig. 1c). 91 % of the excised embryos germinated *in vitro* on 3 % sucrose containing MS medium within four weeks of culture. According to the viability test, seeds from mature green fruits have a viability of about 100 % (data not shown). The embryos from dry mature fruits were dry, shrunken and whitish. Only 4 % of the embryos swelled but failed to show any sign of germination for four weeks, even after subculture for further four weeks on fresh same medium. The remaining embryos neither showed swelling nor any sign of germination during the six weeks of observations (data not shown). In *B. serrata* germination of seeds with mature embryo have been reported very poor (10-20%) (Purohit *et al.,* 1995). On application of TZ test, yellow-red coloured dots appeared on the embryonic portion. The complete embryo did not turn into dark brown-red colour as in case of those obtained form the green mature fruits. These results indicate that the embryos of well matured brown seeds were not viable. This might be possible due to limited availability of resources and absence of leaves on the trees during the entire period of flowering and fruiting (Sunnichan *et al.,* 2005).

Exposure of the embryo excised from dry fruits to different concentrations of sucrose did not show any sign of germination (data not shown). However, data presented in Table 1 reveals that embryo excised from green mature fruits showed maximum germination upto 91 %. Among these germinated embryos, 64 % developed into seedlings and 36 % failed to develop

Table 1. Influence of various concentration of sucrose in MS, SH, WPM and B5 media on *in vitro* germination of zygotic embryo of *B. serrata* after 4 weeks of culture.

Medium	Sucrose % (w/v)	Germination (%)	Plant height (cm)	Primary Root length (cm)	No. of secondary roots/ plant	Survival of seedlings (%)
MS	0	12^{i}	0.5 ± 0.1^{i}	0.3 ± 0.1^{l}	1.0 ± 0.1^{i}	0
	1	54^{f}	1.5 ± 0.1^{g}	0.6 ± 0.0^{j}	1.2 ± 0.0^{efg}	44^{gh}
	2	88^{a}	3.0 ± 0.2^{b}	2.1 ± 0.0^{b}	2.4 ± 0.1^{bc}	52^{de}
	3	91^{a}	3.5 ± 0.1^{a}	2.6 ± 0.1^{a}	3.3 ± 0.1^{a}	64^{ab}
	4	63^{e}	1.0 ± 0.2^{h}	0.6 ± 0.2^{j}	2.2 ± 0.0^{bc}	48^{f}
SH	1	48^{g}	1.1 ± 0.2^{h}	0.7 ± 0.2^{i}	2.3 ± 0.1^{bc}	42^{hi}
	2	75^{cd}	1.2 ± 0.0^{gh}	0.5 ± 0.1^{k}	2.0 ± 0.1^{cd}	53^{d}
	3	89^{a}	2.3 ± 0.2^{de}	2.0 ± 0.0^{c}	3.1 ± 0.0^{a}	67^{a}
	4	59^{e}	2.4 ± 0.1^{cd}	1.7 ± 0.1^{e}	2.1 ± 0.0^{bc}	31^{k}
WPM	1	40^{h}	1.0 ± 0.1^{h}	1.8 ± 0.0^{d}	1.5 ± 0.1^{de}	39^{i}
	2	77^{c}	2.0 ± 0.0^{ef}	2.1 ± 0.1^{b}	2.0 ± 0.1^{cd}	47^{fg}
	3	88^{a}	2.7 ± 0.1^{bc}	1.5 ± 0.1^{f}	3.0 ± 0.0^{a}	62^{bc}
	4	62^{e}	1.3 ± 0.2^{gh}	1.1 ± 0.1^{h}	2.2 ± 0.1^{bc}	49^{ef}
B_5	1	47^{g}	1.1 ± 0.1^{h}	1.1 ± 0.1^{h}	1.0 ± 0.1^{i}	47^{fg}
	2	84^{b}	1.2 ± 0.1^{gh}	1.5 ± 0.0^{f}	1.4 ± 0.1^{ef}	48^{f}
	3	90^{a}	1.9 ± 0.0^{f}	1.7 ± 0.0^{e}	1.3 ± 0.1^{ef}	60^{c}
	4	72^{d}	1.0 ± 0.1^{h}	1.4 ± 0.1^{g}	1.1 ± 0.0^{fgh}	35^{j}

Each value represents mean ± SE, calculated from three separate experiments each with 21 replicates (culture vessels) with each vessel containing one embryo, Mean followed by the same letter within the column are not significantly different using DMRT at 5% level.

into seedling. The type of media (MS, SH, WPM and B5) did not significantly influence the germination of the embryos. But the incorporation of different concentrations of sucrose (0, 1, 2, 3, and 4 %) did influence on embryo germination. 3 % sucrose in the medium proved superior to other concentrations (0, 1, 2, 4 %) for promotion of germination (Table 1, fig. 1d). In an earlier report, Yildirim *et al.* (2007) determined the effects of different carbohydrate source and concentrations of sucrose on *in vitro* cultured embryos of Apricot (*Prunus ameniaca* L. cv Hacihalilaglu) and observed better germination and growth in MS medium containing 3 % sucrose. Rambabu *et al.* (2006) have reported 100 % germination in MS medium containing 30 g l^{-1} sucrose in *Givotia rottleriformis* (var. Tel. Thella Poniki). Kalita and Sarma (2001) also reported highest percentage of seed germination in *Acampe longifolia* on MS medium. The promotion of germination could be attributed to concentration of sucrose since sucrose is known to rapidly transport carbohydrate in plants. It is also known to be a good source of carbon and energy as well as a good osmotic agent (Devlin and Witham, 2000; Taiz and Zeiger, 2006).

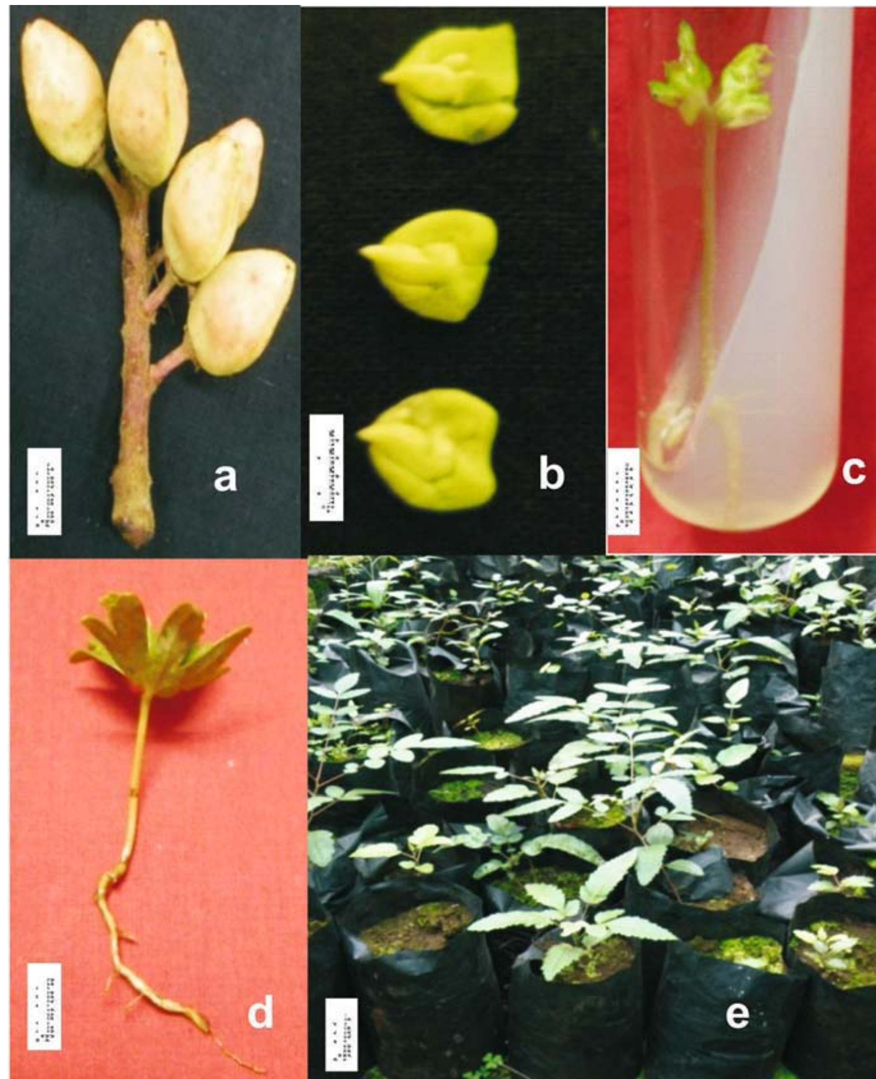

Fig. 1. *In vitro* zygotic embryo germination in *Boswellia serrata.* **(a)** Stem branch with green mature fruits (bar 10 mm). **(b)** Excised green mature embryos (bar 10 mm). **(c)** *In vitro* germinated embryo with well developed root, hypocotyl and expanded green cotyledonary leaves on MS + 3 % Sucrose + 200 mg l^{-1} PVP (bar 10 mm). **(d)** *In vitro* germinated embryos removed from culture tube (bar 10 mm). **(e)** Plants (3 month old) in soil under field conditions. (bar 25 mm)

Influence of PVP

Incorporation of PVP in MS medium did not induce any germination in embryos obtained from dry seeds. But, there was an increase in germination in embryos excised from green mature seeds (96 %) and significant increase in its development into the seedling (82 %) (Table 2). Optimum growth of embryo and less browning in the root and shoots appeared in the presence of 200 mg l^{-1} PVP (Fig. 1c, d). As compared to control, 96 % germinated embryos produced longer shoots (2.3 ± 0.1 cm) and roots (1.6 ± 0.1 cm) after four weeks of culture. However, embryos cultured on MS medium lacking PVP showed stunted growth and browning of margin and appearance of brown spots on newly developed leaf. During six weeks of culture, 18 % seedlings turned brown and succumbed within six weeks of culture. In *Taxus* species, on ½ MS and MS medium, lacking PVP, only 37.5 to 40 % of the embryos produce roots which remain stunted attaining less than 0.5 cm length after 12 weeks in culture. However, on ½ MS medium fortified with PVP, 90 % of the embryos grew with better roots and attained 1 cm length within six weeks of culture. There was similar response of PVP to the cultured embryos and seedling development in *Taxus mairei* on ½ MS medium (Chang and Yang, 1996). This significant effect of PVP could be due to its antioxidant property and its ability to bind with phenolics and toxins (Abdelwahd *et al*., 2008).

Influence of auxins, cytokinins and GA_3

The embryo excised from dry mature untreated seeds and dry mature treated seeds with wet heat, acid scarification, auxins, cytokinins and GA_3, did not show any sign of germination on transferred to nutrient medium On other hand, maximum frequency of germination of excised embryo from green mature seeds was up to 91 % on MS medium lacking PVP and 96 % on MS medium containing 200 mg l^{-1} PVP. However, the conversion of germinated embryos into seedling was 64 % and 82 % respectively. Therefore to improve the conversion of embryos into seedling, the auxins, cytokinins and GA_3 was incorporated in the medium. Embryos from dry mature seeds did not respond for germination on media containing auxins (IAA, IBA, NAA, or 2,4-D) (1.25-2.5 μM). Inclusion of auxins (IAA, IBA, NAA, or 2,4-D) in MS medium (3 % sucrose) resulted in reduction of percentage germination of green mature seeds and promoted white creamy callus formation from entire surface of embryos. According to Rambabu *et al*. (2006) seedling of *Givotia rottleriformis* on medium containing NAA and IAA (0.3-0.5 mg l^{-l}) were also abnormal and associated with callus.

Cytokinins such as BA (5 μM) and Kin (5 μM) caused swelling and induction of callus on radicle side. Cytokinins are regulators of cell division, and show a role in the breaking of bud dormancy, seed germination, callus formation in tissue culture and shoot regeneration process associated to micropropogation (Bhojwani and Razdan, 1996; Taiz and Zeiger, 2006). Root growth is an important factor in the development of seedlings (Chang and Yang, 1996). In the present investigation the inclusion of BA (1.25-2.5 μM) or Kin (1.25-2.5

Table 2. Effect of PVP on germination of zygotic embryos of *B. serrata* after 4 weeks of culture.

PVP (mg l^{-1})^Y	Germination (%)	Plant height (cm)	Root length (cm)	No. of roots/plant	Survival of Seedling %
0	91^{bc}	0.4 ± 0.1^{d}	0.5 ± 0.1^{g}	1.1 ± 0.2^{f}	64^{d}
50	93^{ab}	0.5 ± 0.0^{d}	1.0 ± 0.1^{f}	2.0 ± 0.0^{d}	69^{c}
100	95^{ab}	1.1 ± 0.1^{c}	1.2 ± 0.0^{d}	3.2 ± 0.1^{a}	72^{bc}
150	95^{ab}	2.2 ± 0.0^{a}	1.4 ± 0.1^{b}	2.1 ± 0.0^{cd}	74^{b}
200	96^{a}	2.3 ± 0.1^{a}	1.6 ± 0.1^{a}	2.8 ± 0.1^{b}	82^{a}
250	88^{c}	2.0 ± 0.0^{a}	1.3 ± 0.0^{c}	2.2 ± 0.1^{c}	62^{d}
300	48^{d}	1.7 ± 0.1^{b}	1.1 ± 0.0^{e}	1.4 ± 0.0^{e}	46^{e}

^{Y}Cultures were grown on MS medium containing 30 g l^{-1} sucrose and different concentration of PVP.
Each value represents mean ± SE, calculated from three separate experiments each with 21 replicates (culture vessels) with each vessel containing one embryo, Mean followed by the same letter within the column are not significantly different using DMRT at 5 % level.

µM) in the embryo culture medium was not found suitable as it resulted in inhibition of radicle growth and caused swelling and callusing on radicle side of the cultured embryos.

Although role of GA_3 as a growth regulator for breaking the seed dormancy and regulate germination in several ways has been reported (Taiz and Zeiger, 2006; Nikam and Barmukh, 2009). However, the treatment of GA_3 was not effective in induction of germination in embryos from dry mature seeds (data not shown). Gibberellins occur at relatively high concentrations in developing seeds (Manrique *et al.,* 2005). In the present study, the embryos used for germination were excised from green seeds before shedding it from parental plant. They may have had sufficient quantity of endogenous gibberellins to promote the germination. Increase in the concentration of exogenous gibberellin (1.25-10 µM) did not help much to influence the difference in germination (data not shown).

Hardening and acclimatization

Different culture media, type of growth regulator, and their concentration and PVP concentration in the nutrient medium influence the survival of seedlings on transferred to soil and field condition (Table 1 and 2). All well developed seedlings were transferred directly to soil and showed yellowing of leaves, followed by defoliation within 5-7 days. However, 94 % of the seedlings derived from *in vitro* germinated embryos on MS medium containing 3 % sucrose together with 200 mg l^{-1} PVP and lacking of growth regulator survived when transferred to the sand soil mixture (1:1). They were further maintained for four weeks under glasshouse conditions (diffuse natural light near 191.14 µmol m^{-2} s^{-1}, temperature 26 ± 4 °C and humidity 90 ± 5 % watered at 3 days interval). After 4 weeks of glass house acclimatization, 500 seedlings were transferred to field conditions (light intensity maximum 765.7 µmol $m^{-2}s^{-1}$ temperature in the range of 21 to 28 °C and humidity 65 to 90 %). Among these 94 % seedlings survived and were morphologically identical (Fig. 1e) to those that grew in the seed source plantation area. Survival of seedling produced on media lacking PVP and growth regulator remained in range of 31 to 67 % whereas the seedlings produced on growth regulator (Auxin, Cytokinin and GA_3) containing media were susceptible to environmental stress. This might be possible due to the high endogenous levels of plant growth regulators and exogenous supply of growth regulator that may cause deformation in the developing seedling especially in the radicle.

In conclusion, the results on *in vitro* germination of excised embryos from green mature seeds on different media containing sucrose and MS media containing 3 % sucrose, PVP and different growth regulators, suggest that the MS medium fortified with 3 % sucrose and 200 mg l^{-1} PVP was the best for embryo germination and seedling development. The established protocol can be applied for preparation of large number of seedlings in a short period of time. Finally, this will help in conservation, mass plantation and utilization of *B. serrata* Roxb., for production of frankinsences and medicinally important anti-inflammatory active metabolite boswellic acid.

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