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RESEARCH ARTICLE

# Micropropagation and non-steroidal anti-inflammatory and anti-arthritic agent boswellic acid production in callus cultures of *Boswellia serrata* Roxb.

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**Abstract** Micropropagation through cotyledonary and leaf node and boswellic acid production in stem callus of a woody medicinal endangered tree species *Boswellia serrata* Roxb. is reported. The response for shoots, roots and callus formation were varied in cotyledonary and leafy nodal explants from in vitro germinated seeds, if inoculated on Murshige and Skoog's (MS) medium fortified with cytokinins and auxins alone or together. A maximum of 8.0±0.1 shoots/cotyledonary node explant and 6.9±0.1 shoots/leafy node explants were produced in 91 and 88 % cultures respectively on medium with 2.5 μM 6-benzyladenine (BA) and 200 $mgl^{-1}$ polyvinylpyrrolidone (PVP). Shoots treated with 2.5 μM IBA showed the highest average root number (4.5) and the highest percentage of rooting (89 %). Well rooted plantlets were acclimatized and 76.5 % of the plantlets showed survival upon transfer to field conditions. Randomly amplified polymorphic DNA (RAPD) analysis of the micropropagated plants compared with mother plant revealed true-to-type nature. The four major boswellic acid components in calluses raised from root, stem, cotyledon and leaf explants were analyzed using HPLC. The total content of four boswellic acid components was higher in stem callus obtained on MS with 15.0 μM IAA, 5.0 μM BA and 200 $mgl^{-1}$ PVP. The protocol reported can be used for conservation and exploitation of in vitro production of medicinally important non-steroidal anti-inflammatory metabolites of *B. serrata*.

**Keywords** Anti-arthritic · Anti-inflammatory · *Boswellia serrata* · Boswellic acid · Burseraceae · Callus · Indian olibanum · Micropropagation · Salai guggul · Steroids

**Abbreviations**

| | |
|---|---|
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| ABBA | Acetyl-β-boswellic acid |
| AKBBA | Acetyl-11-keto-β-boswellic acid |
| BA | 6-Benzyladenine |
| BBA | β-boswellic acid |
| IAA | Indole-3-acetic acid |
| KBBA | 11-keto-β-boswellic acid, |
| Kin | Kinetin |
| MS | Murashige and Skoog (1962) medium |
| NAA | α-Naphthaleneacetic acid |
| PVP | Polyvinylpyrrolidone |
| RAPD | Randomly amplified polymorphic DNA |
| TDZ | Thidiazuron |
| HPLC | High performance liquid chromatography |

## Introduction

*Boswellia serrata* Roxb. (Burseraceae) is an endangered, dry deciduous, moderate branched tree species. It is a native to India and has been naturalised in the tropical parts of Asia, Africa and Middle East (Gaofeng et al. 2006). It has been reported as a unique non-coniferous source of terpentine employed for the manufacture of paints and varnishes. The plant is also used as a source of pulp in paper industry and timber for making furniture (Anonymous 1988). Since it does not require frequent irrigation and due to its adaptation

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to arid environment, this species is recommended for plantation on arid, sandy, rocky underutilized lands. The gum-resin exudate from tree trunk is called '*Salai guggul*' or 'Indian Olibanum'. It is a non-steroidal anti-inflammatory agent extensively used in ayurvedic formulations for relieving ache and pain, particularly associated with arthritis (Gaofeng et al. 2006). In the gum-resin, boswellic acid is regarded as an active principle which comprises pentacyclic triterpenes. Recently reported medicinally active major triterpenes of boswellic acid are β-boswellic acid (BBA), 11-keto-β-boswellic acid (KBBA), acetyl-β-boswellic acid (ABBA) and acetyl-11-keto-β-boswellic acid (AKBBA) (Safayhi et al. 2009).

The rate of natural propagation of *B. serrata* is too slow where older trees get propagated through root suckers. However, the root sucker technique has not been exploited commercially. Poor fruit setting (2.6 to 10 %) under open pollination, inadequate production of viable seeds and scanty seed germination (10–20 %) restricts the distribution and therefore limits the availability of natural source (Purohit et al. 1995; Sunnichan et al. 2005). As a consequence, *B. serrata* has been grouped in an endangered and threatened category of plant species (Purohit et al. 1995; Ghorpade et al. 2010). This has resulted in declining the natural stand and production of medicinally important gum-resin as well as active metabolites of boswellic acid. Therefore, there is need to apply the ex situ and in situ conservation strategies as well as to develop alternative methods for production of active metabolites of boswellic acid.

Tissue culture technique is one of the efficient tools to achieve the goals of conservation of rare, endangered and threatened plants (Ahire et al. 2011). The technique has also been interestingly applied for genetic improvement of some medicinally important tree species (Goodger and Woodrow 2009). In vitro culture of medicinal plants have the ability to synthesize the chemicals *de novo* under controlled conditions and can be utilized to produce the medicinally important compounds similar to that of mother plants. Few reports are available on in vitro propagation of *B. serrata* by using cotyledonary nodal segments (Purohit et al. 1995; Suthar et al. 2011) and in vitro zygotic embryo germination (Ghorpade et al. 2010). However, in the said protocols, the regenerated shoots were either less in number or required several subcultures to achieve higher number of regenerants. The regenerants after several subcultures might have lead to the formation of somaclonal variants which was not tested in the said protocols (Purohit et al. 1995; Suthar et al. 2011). Availability of zygotic embryos is limited because of natural barriers in seed formation (Ghorpade et al. 2010). Therefore, in the present investigation, the attempts were made to develop a micropropagation protocol for rapid multiplication and boswellic acid production in the callus of *Boswellia serrata*.

## Materials and methods

### Plant material and explant preparation

The explants (young leaf and stem, mature leaf and stem, stem bark and green seeds) excised from the naturally occurring trees and seedlings (root, leafy node and leaf explants) at Vettal Hill, Pune, India, were brought to the laboratory. They were initially washed in the running tap water, followed by 4 min wash with 0.01 % (v/v) Tween-20 solution and then with sterilized distilled water. The explants were surface sterilized with 0.1 % (w/v) $HgCl_2$ for 5 min followed by 10 times washing with sterilized distilled water. The embryos were excised aseptically and inoculated for germination on Murashige and Skoog (MS, 1962) basal medium. The root, cotyledonary node (CN), leafy node (LN) and leaves obtained from in vitro raised 5 week old seedlings were also used as explants and the response was compared for organogenesis and callus formation with the explants of naturally grown plants and seedlings.

### Culture media and conditions

Explants were cultured on MS basal medium supplemented with and without auxins (IAA, NAA, and 2, 4-D) and/or cytokinins (BA, Kin and TDZ) at various concentrations. In the pilot experiments, the medium was supplemented with different concentrations of PVP (Polyvinylpyrolidone) or ascorbic acid to avoid browning of the explants. The medium was supplemented with 3 % sucrose and solidified with 0.8 % (w/v) agar (Hi Media, Mumbai, India). The pH of medium was adjusted to 5.8 before addition of agar and then medium was autoclaved for 15 min at 121 °C. The cultures were incubated under controlled conditions viz., 25±2 °C temperature, 60±10 % relative humidity and 8/16 h photoperiod (30 $\mu mol\, m^{-2} s^{-1}$) provided from fluorescent tubes (Philips, Kolkata, India).

### Multiple shoot induction

The effect of age and position of explants on shoot regeneration was examined by using CN and LN explants from one to five week old in vitro seedlings raised from either zygotic embryos or shoots from cotyledonary and leafy node. Each explant was placed on optimized shoot regeneration medium (MS+2.5 μM BA+200 mg/l PVP). The number of explants producing shoots (≥0.5 cm) was recorded at the end of fourth week of culture. As the availability of CN explants was less due to limited seed germination, the shoot regeneration protocol was standardized by using LN explants from in vitro raised shoots. The three consecutive LN explants from basal position of four-week old in vitro

raised shoots gave best response and therefore were used for shoot multiplication over a period of 2 years.

### In vitro rooting and acclimatization

The in vitro raised shoots (3–5 cm in height with two or three leaflets) derived from CN and LN explants were transferred to rooting medium containing NAA, IBA or IAA (0–10 μM). The well rooted plantlets were removed from the culture vessels and washed gently with water to remove the agar. Plantlets were then transferred to small plastic pots (7.5 cm in diameter) containing autoclaved vermi-compost and kept in a plant growth chamber at 26 °C temperature, 85 % relative humidity and illuminated with light at 50 μmol $m^{-2}s^{-1}$ from white fluorescent tubes. The relative humidity was reduced gradually, and the plantlets were then transferred to large polythene bags (18 cm in diameter) containing soil-sand mixture (1:1) and were maintained under diffused light (16/8 h photoperiod). Potted plantlets were covered with a transparent polythene membrane to ensure high humidity, initially irrigated with 1/4 strength MS medium on every alternate day for 3 weeks, followed by tap water at the same interval. Finally, plantlets were transferred to pots containing normal garden soil and maintained in a greenhouse.

### Maintenance and growth of callus

Approximately 100 mg fresh calluses were sub-cultured on the same fresh medium at the interval of three weeks and maintained over a period of 24 months. Fresh weight (FW) of calluses was recorded after harvesting at the end of the fourth week. The calluses were dried in an oven at 60 °C till a constant dry weight (DW) was obtained. The dried calluses were further analysed for boswellic acid content.

### Data analysis

A completely randomized design (CRD) was used in all the experiments. Each experiment was repeated at least thrice. The data were subjected to ANOVA followed by Duncan's Multiple Range Test (DMRT) at $P \leq 5$ %.

### RAPD analysis

Nineteen randomly selected field-transferred one year old plants and a mother plant (control) was subjected to RAPD analysis. Young leaves (500 mg) were ground in liquid nitrogen, and the DNA was isolated by following CTAB method as described by Warude et al. (2003). DNA yield and quality was checked by electrophoresing on 0.8 % agarose gel stained with ethidium bromide (EtBr).

Among 20 random decamer RAPD primers (set A; procured from Operon Biotechnologies GmbH, Germany) 17 primes showed amplification and were used for fidelity analysis (Table 3). Amplification was performed using 25 μL PCR mixture consisting of 2.5 μL 1X PCR buffer, 2.0 μL dNTP (10 mM: 2.5 mM each of the dNTPs), 0.3 μL *Taq* polymerase, 1.5 μL DNA (50 ng/μL), 2.5 μL primer (5 pM), 2.0 μL $MgCl_2$ (50 mM) (GeNei™, Banglore, India) and 14.2 μL MiliQ water. Amplifications were carried out in a Thermal Cycler (Eppendorf Cycler Gradient) with an initial denaturation at 94 °C for 5 min, followed by 40 cycles, each consisting denaturation (94 °C) for 1 min, annealing (36–38 °C) for 1 min, extension (72 °C) for 2 min, with a final extension for 5 min at 72 °C followed by hold at 4.0 °C. The PCR products were mixed with 1 μL gel loading dye (6X), and separated on 2 % agarose gel (SeaKem LE Agarose, Lonza, USA) prepared in 1X TAE buffer, on a Midi subsystem electrophoresis unit (GeNei™, Banglore, India) at 70 V for 2 h. A 100 bp DNA ladder was used as a molecular standard. Gels were visualized under UV-visible transilluminator and then documented.

### Scoring and data analysis

Only the primers which displayed reproducible, scorable bands were considered for analysis. Such bands were scored as present (1) or absent (0). The band was considered to be 'polymorphic' if present in some of the clones and absent in others. Data were used for computing Jaccard's similarity coefficients by using NTSYS-Pc (Numerical Taxonomy System, Applied Biostatistics Inc., New York, USA, software version 2.02e) developed by Rohlf (1998).

## Estimation of boswellic acid

### Preparation of standard and sample

The standard boswellic acid components BBA, ABBA, KBBA and AKBBA were obtained from Alexis Biochemicals (Axxora platform, UK). One milligram powder was dissolved in 1 ml of methanol and used as stock, which was diluted to obtain the final concentration of 25 μg/ml. One gram powdered biomass of gum-resin of naturally grown plant, and calluses were suspended in 5 ml methanol and sonicated for 20 min. The supernatants obtained after centrifugation at 3,000 rpm for 10 min were filtered through 0.22 μM nylon membrane filter.

### HPLC equipment and conditions

The HPLC system by Dionex (Germany) equipped with a hypersil BDS C18 column (250 mm×4.6 I.D., Thermo

Electron Corporation) was used with the following analytical conditions: an isocratic mobile phase of acetonitrile–water (90:10 v/v) adjusted to pH 4 by glacial acetic acid with a flow-rate of 1.0 ml/min at 25 °C. The system had a P-680 solvent delivery pump, connected to a Rheodyne valve and a 170 UV detector operated at a wavelength of 210 nm and 254 nm. The sample injection volume was 20 μL. The absorbance for BBA and ABBA were recorded at 210 nm, while those for KBBA and AKBBA were recorded at 254 nm (Shah et al. 2008). The components of boswellic acid in the samples were identified on the basis of characteristic absorption spectra, retention time, and spiking the sample with the standard and were quantified by using an external standard.

## Results and discussion

### Shoot regeneration

The axenic seedling explants (root, stem, cotyledon and leaf) cultured on MS medium supplemented with various concentrations (0 to 20.0 μM) of cytokinins (BA, Kin, TDZ) and auxins (IAA, NAA, 2,4-D), alone and in combination, showed variable response for organogenesis and callus formation. The response also varied with the age, type, and orientation of explants on regeneration medium. The cultures showed browning of tissue and adjoining medium which affected the proliferation of explant and induced callus. Therefore in the preliminary experiments, PVP or ascorbic acid at varying concentrations (0–300 mg/l) was incorporated in the nutrient media (Data not presented). Among these, inclusion of 200 mg/l of PVP effectively prevented browning of explants and adjoining nutrient medium. Consequently, in all the subsequent experiments, the nutrient media were enriched with 200 mg/l PVP.

Root, stem, cotyledon and leaf explants tended to produce roots with little or no callus growth on medium containing 2.5 μM to 10.0 μM IAA and 2.5 to 5.0 μM NAA; while nodal explants responded for shoot regeneration on medium with 0.5 to 10.0 μM BA, Kin and TDZ, (described in later section). However, inclusion of 2.5 to 10.0 μM 2,4-D either alone or in combination induced only callus formation.

Cotyledonary node (CN) and leafy node (LN) explants did not respond morphogenically to a growth regulator-free medium. Incorporation of 2.5 μM BA and 200 $mgl^{-1}$ PVP was highly effective for induction of shoot, increasing mean number of shoots per explant and shoot elongation as well. A maximum of 8.0±0.1 shoots/cotyledonary node and 6.9± 0.1 shoots/leafy node explant were produced in 91 % and 88 % cultures respectively (Table 1; Fig. 1a). Inclusion of BA at a lower (0.5 and 1.25 μM) or higher (5, 7.5 and 10 μM) concentrations significantly reduced the shoot regeneration response. However, extensive callus induction was observed at higher concentrations of BA from both explant types (Table 1). Incorporation of auxins (IAA, NAA, 2,4-D) together with the optimal concentration of BA (2.5 μM) affected not only the frequency of explants responding for shoot regeneration but also the mean number of shoots per explants. However, it supported prolific callus growth at the cut end of explants (data not presented). On the contrary, in vitro shoot formation was observed from cotyledonary nodes on the MS medium containing 0.5 mg/l BA together with 0.05 mg/l NAA (Purohit et al. 1995). The differences in the results on the requirement of growth regulators for in vitro shoot formation in the present investigation and that of Purohit et al. (1995) might be attributed to the differences in the source of seeds, and age of seeds used for generating explants.

In the present investigation, TDZ and Kin supplementation also was found effective for shoot induction from cotyledonary and leaf node explants. However, the percent shoot regeneration and number of shoots per explant was comparatively less than those observed on medium containing BA (Table 1). On the contrary, the shoot multiplication within 50 days of culture initiation in *Boswellia ovalifoliata* has been reported from cotyledonary nodal explants cultured on MS medium containing TDZ (Chandrasekhar et al. 2005), thereby indicating that the growth regulator requirement for shoot regeneration changes with the species of *Boswellia*.

Katalin et al. (2010) suggested that, although TDZ and BA have been shown efficient for shoot multiplication, their efficiency depends on genotype, and other factors such as explant type, size and age of the explant. Previous reports suggest that, among cytokinins, BA alone was effective for induction and elongation of multiple shoots in several plant species (Pandey and Agrawal 2009). Although, its mechanism of action and reasons for differential responses are uncertain; the superiority of BA predicted might be due to its easy permeability and increased affinity for active cell uptake (Dal Cin et al. 2007; Pandey and Agrawal 2009). Therefore, these results suggest the use of BA for efficient shoot multiplication from the cotyledonary and leafy node explants of *B. serrata* for its conservation.

The age and position of the explant influenced its performance in the culture. One and two weeks old CN explants showed better response for multiple shoot formation as compared to 3, 4 and 5 weeks old explants. At the same time, the LN explants from the three weeks old seedlings also showed considerable axillary multiple shoots formation. The stem tip and LN explants taken from below the stem tip did not respond for shoot formation. Besides media, the age and explant type have influenced the multiple shoot induction in several tree species like *Agel marmelos* (Ajithkumar and Seeni 1998),

**Table 1** Effect of cytokinins on multiple shoot induction from CN and LN explants of *B. serrata*

| Cytokinins (μM) | Cotyledonary node (CN) | | | Leaf node (LN) | | |
|---|---|---|---|---|---|---|
| | Percent of explants producing shoots (%) | Number of shoots/explant | Shoot length (cm) | Percent of explants producing shoots (%) | Number of shoots/explant | Shoot length (cm) |
| BA | | | | | | |
| 0.00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 0.50 | 60 f | 3.7±0.4 f | 1.3±0.7 kl | 50 jk | 2.3±0.2 hi | 2.2±0.1 de |
| 1.25 | 85 b | 5.2±0.3 c | 2.2±0.1 efh | 80 c | 3.3±0.3 f | 2.6±0.2 bc |
| 2.50 | 91 a | 8.0±0.1 a* | 3.6±0.4 a | 88 a | 6.9±0.1 a | 3.0±0.5 a |
| 5.00 | 83 b | 5.3±0.1 bc* | 3.0±0.4 b | 85 ab | 4.7±0.1 c* | 2.9±0.2 cde |
| 7.50 | 78 c | 4.3±0.2 de** | 2.1±0.2 fhi | 70 ef | 4.0±0.3 de** | 2.0±0.1 e |
| 10.0 | 54 g | 3.0±0.1 ij ** | 1.8±0.2 ij | 55 hi | 3.7±0.4 ae** | 1.4±0.1 fg |
| Kin | | | | | | |
| 0.50 | 55 g | 3.5±0.1 fg | 2.2±0.5 l | 52 ij | 2.1±0.8 hi | 2.2±0.2 g |
| 1.25 | 73 d | 4.6±0.2 d | 2.5±0.2 de | 75 d | 2.8±0.3 g | 2.4±0.1 de |
| 2.50 | 90 b | 5.4±0.7 bc* | 3.0±0.1 b | 85 ab | 5.2±0.1 b | 2.8±0.4 b |
| 5.00 | 72 d | 3.0±0.3 ij* | 2.6±0.2 cdf | 82 bc | 3.1±0.3 fg* | 2.8±0.4 b |
| 7.50 | 63 f | 2.7±0.4 jk** | 2.2±0.4 efh | 70 k | 2.8±0.4 g** | 2.3±0.2 cde |
| 10.0 | 40 i | 2.2±0.1 l** | 1.9±0.2 hij | 65 f | 2.2±0.8 hi** | 1.6±0.1 f |
| TDZ | | | | | | |
| 0.50 | 49 h | 3.3±0.5 ghi | 2.6±0.5 jk | 48 k | 2.0±0.2 i | 2.2±0.8 g |
| 1.25 | 71 d | 4.2±0.3 e | 2.8±0.2 def | 73 de | 2.4±0.1 h | 2.5±0.2 bcd |
| 2.50 | 82 b | 5.6±0.1 b* | 2.9±0.1 bc | 80 c | 4.2±0.3 d | 2.6±0.7 bc |
| 5.00 | 78 c | 3.4±0.4 gh* | 2.3±0.2 def | 68 fg | 3.9±0.6 de* | 2.4±0.4 cd |
| 7.50 | 67 e | 3.1±0.7 hi ** | 2.0±0.4 hi | 56 h | 2.9±0.2 g** | 2.2±0.3 de |
| 10.0 | 53 g | 2.6±0.2 k** | 1.3±0.2 kl | 48 k | 2.1±0.5 hi** | 1.6±0.1 f |

Data are means±SE of 21 replicates from three independent experiments. Values followed by the same letter in a line are not significantly different ($P \leq 0.05$) as described by one-way ANOVA. * indicates callus formation and ** Extensive callus proliferation

*Pterocarpus marsupium* (Husain et al. 2008), and *Pinus taeda* (De Oliveira et al. 2012). However, explant position along the stem did not influence multiple shoot formation in *Humulus lupulus* L. (Gurriarán et al. 1999), and *Acer grandidentatum* (Bowen-O'Connor et al. 2007).

The multiple shoot formation was rare in leaf, stem, and stem apex explants excised from naturally grown seedlings and mature trees. Similar observations were recorded in *Thea sinenensis* (Jha and Sen 1992), *Tylophora asthmatica*, and *Uraria picta* (Mukundan et al. 2002), where the nodal segments taken from propagated shoots responded better as compared to explants taken from field grown plants and suggested that in vitro raised explants have greater potential for morphogenesis than tissues excised directly from field grown plants. In accordance to this, in the present investigation, the difference in shoot regeneration and callus proliferation in explants from field grown seedlings and mature plants and in vitro raised shoots can be attributed to the effects of environment on the physiology of explants.

The shoot multiplication was always intermingled with callus in CN explants, whether their placement on shoot regeneration medium was horizontal, oblique or even vertical. In the previous report, shoot regeneration in cotyledonary node was also intermingled with callus formation (Purohit et al. 1995). On the other hand, poor seed germination and each germinated seed giving a single cotyledonary node limit the availability of CN explants. However in the present investigation, shoot regeneration was direct in vertically placed LN explants on the MS medium with optimal concentration of BA (2.5 μM). A possible explanation for callus intervention during shoot multiplication in CN explants and shoot multiplication without callus intervention in LN explants could be variation in absorption, transportation, degradation, maintenance of endogenous levels of PGR and their interaction with exogenously applied cytokinins. The response of shoot regeneration depends not only on the type of explant chosen, but also the way explants are placed in vitro on the culture media for tomato (Bhatia et al. 2005). This study demonstrated that the vertical placement of LN explants induce better shoot regeneration and produce true-to-type shoots (described in later section). According to Suthar et al. (2011), subculturing of shoot cluster at every

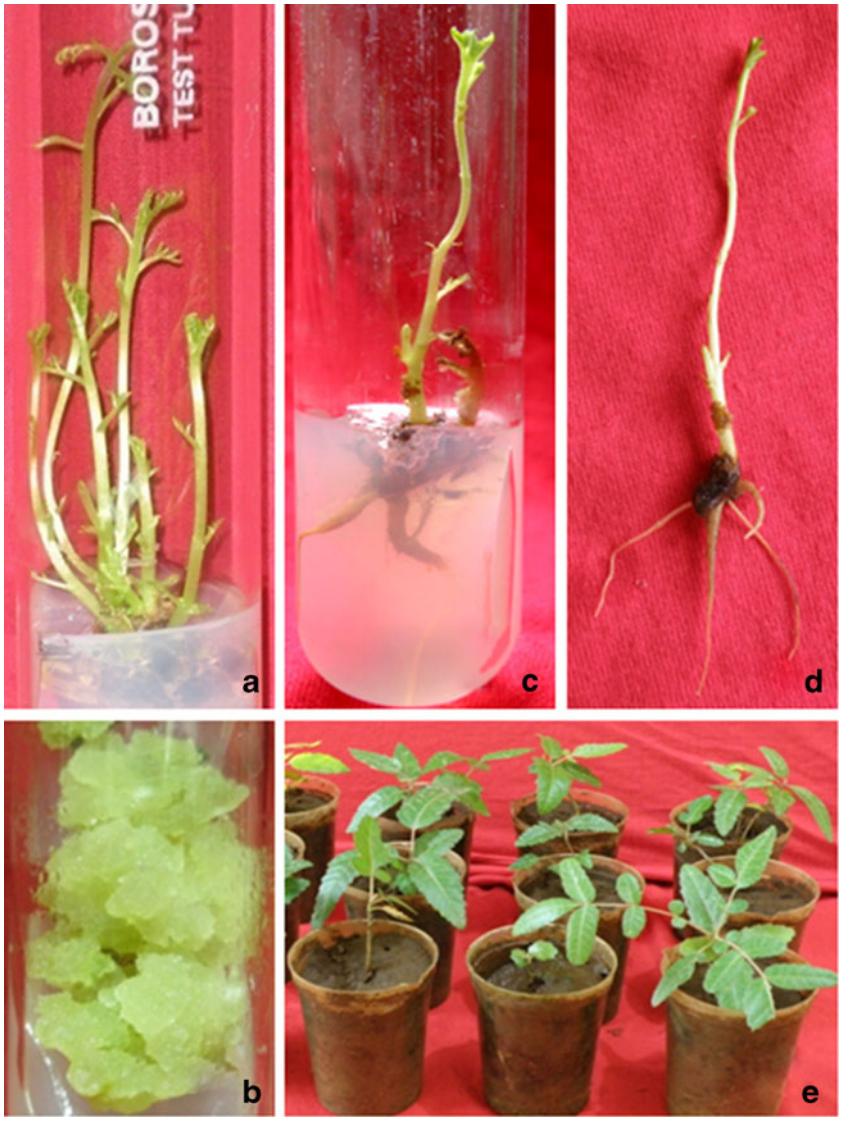


**Fig. 1** In vitro propagation of *B. serrata.* **a**. multiple shoot induction from leafy node explant on MS with 2.5 μM BA after 4 week **b**. Callus derived from stem explant on MS with 15.0 μM IAA, 5.0 μM BA and 200 $mgl^{-1}$ PVP after 4 week **c, d**. Rooting of in vitro grown shoot on MS with 1.25 μM IBA **e**. Micropropagated plant established in plastic pots under ex vitro condition after 4 week of transfer

2 weeks interval, without harvesting the shoots and their maintenance for 42 days under the same conditions resulted in maximum shoot formation in liquid MS medium fortified with 0.5 mg/l BA+0.05 mg/l NAA (21.0±3.03 shoots per culture). However, this is a three step process to achieve maximum shoots multiplication requiring longer duration, more labour and higher cost. The present protocol demonstrates a one step method that requires placement of leafy nodal explants from in vitro developed four weeks old shoots on the regeneration medium (MS+2.5 μM BA+200 mg/l PVP). In the experiments described here, each 4 weeks old shoot produced at least three regenerative leafy nodal explants. On an average, 7 shoots were produced from each leafy nodal explant, and 21 nodal explants were available from each culture. The subculturing of 21 nodal explants produced about 147 shoots. Thus, by following the protocol presented, it is possible to produce large number of leafy nodal explants, shoots and plantlets of *B. serrata*.

## Rooting of shoots

The success of micropropagation relies on efficient rooting of regenerated shoots and survival of plantlets under field conditions. IBA is a common auxin used for inducing rooting in several woody plant species (Han et al. 2009; Pop et al. 2011). In the present investigation, the best rooting response (89 %) was observed on MS medium supplemented with IBA (2.5 μM) (Table 2; Fig. 1c, d). At this concentration, 4.5±0.2 roots were produced from regenerated shoots within two weeks of culture. The developed roots were thin, whitish and attained an average length of 6.3±0.2 cm. Callus intervening root formation was observed in shoots subcultured on medium containing NAA, whereas incorporation of IAA and 2,4-D in the medium induced only callus from the basal portion of the shoots. This differential requirement for auxin type may be influenced by the level of endogenous auxins in cultured shoots.

**Table 2** Effect of auxins on rooting of in vitro regenerated shoots of *B. serrata*

| Auxins (μM) | Rooted shoots (%) | Mean no. of root/shoot | Mean root length (cm) | Survival % |
|---|---|---|---|---|
| NAA | | | | |
| 0.00 | 0 | 0 | 0 | 0 |
| 0.50 | 34 | 2.4±0.1 e | 2.3±0.2 g | 32.1 |
| 1.25 | 47 | 3.0±0.1 d | 3.2±0.1 e | 42.1 |
| 2.50 | 30 | 2.2±0.2 e | 2.8±0.2 f | 38.9 |
| 5.00 | 26 | 1.4±0.3 f | 2.1±0.3 g | 27.8 |
| IBA | | | | |
| 0.50 | 59 | 3.1±0.2 d | 4.3±0.3 d | 54.8 |
| 1.25 | 75 | 3.7±0.3 c | 5.7±0.1 b | 71.3 |
| 2.50 | 89 | 4.5±0.2 a | 6.3±0.2 a | 76.5 |
| 5.00 | 67 | 4.1±0.1 b | 5.1±0.2 c | 65.4 |

The values represent the mean±SE experiments repeated at least three times, each based on minimum 21 replicates. Values followed by same letters were not significantly different at $P \leq 0.05$ according to DMRT

Similar results have been observed for root initiation in the in vitro shoots of *B. serrata* (Purohit et al. 1995), and *B. ovalifoliolata* (Chandrasekhar et al. 2005) cultured on the medium containing IBA and NAA, alone or in combination and the subsequent acclimatization of plants to pot and field conditions.

## Hardening

The six weeks old rooted plantlets were taken out of the medium, washed with water to remove the agar and transferred to pots containing fine soil: sand mixture (80:20). Each plantlet was covered with a glass bottle such that a slight gap for ventilation was left at the bottom. These plantlets were maintained in a glass-house for two weeks under low light intensity (near 191.14 $\mu mol m^{-2} s^{-1}$), 90±5 % humidity and 26±4 °C temperature. The bottles were frequently removed during the day time and replaced again, thus adjusting the humidity levels (60–70 %). The plantlets were then transferred and grown in a shade net house (light intensity near 250.16 $\mu mol m^{-2} s^{-1}$, 27±4 °C temperature, and 60 % humidity) for about four to five weeks. Finally, the plantlets were transferred to the field conditions (light intensity 765.7 $\mu mol\ m^{-2} s^{-1}$, 21 to 28 °C temperature and 65–80 % humidity). Few rooted plantlets were infected by fungi, which resulted in rottening and wilting within one to two weeks after transferring to soil. A total of 408 plants were taken out for hardening and a survival percentage of 76.5 % was achieved. The propagated plantlets grew well and did not show morphological abnormalities (Fig. 1e).

## Genetic fidelity analysis using RAPD

Several minor genes and environmental factors affect regeneration through tissue culture, particularly in callus and cell cultures (Ebrahimie et al. 2006). In general, the interventions of callus during shoot regeneration increase the incidence of chromosomal abnormalities and somaclonal variation (Bairu et al. 2006). Maintaining shoot cultures for a longer time in vitro during micropropagation is known to result in undesirable clonal variability in many important crops as well as medicinal plants (Ryynanen and Aronen 2005; Lattoo et al. 2006). Therefore, true-to-type clonal fidelity is one of the most important pre-requisites in the in vitro propagation of forest tree species (Goto et al. 1998). The variations in the plants regenerated in vitro can be determined at the morphological, cytological or molecular levels with several techniques. Molecular markers suitable for generating DNA profiles have been proved to be an effective tool in assessing the genetic stability of regenerated plants (Rani and Raina 2000). In the present investigation, RAPD markers were employed to study the genetic stability of the micropropagated plantlets of *Boswellia serrata* Roxb., and the results are presented in Table 3 and Fig. 2. About 126 clear reproducible bands were obtained by total 17 random decamer RAPD primers analysed for micropropagated plantlets and mother plant (control) of *B. serrata*. The number of bands for each primer varied from five in primer OPA 10 to ten in primer OPA 5, OPA 12, OPA 15 and OPA 18 (Table 3). The primers produced on an average 7.4 bands and ranged in size from 0.2 to 2.0 kb (Fig. 2). The true-to-type nature of the micropropagated plants was confirmed as the amplification profile produced homogenous and monomorphic banding pattern from the primers used. Similar studies have been conducted on the genetic fidelity of in vitro regenerated plantlets produced from different explants of *Betula pendula* (Ryynanen and Aronen 2005), *Agel marmelos* (Pati et al. 2008), *Phoenix dactylifera* (Abd-

**Table 3** Details of randomly amplified polymorphic DNA (RAPD) primers used for genetic fidelity analysis of *B. serrata* regenerants

| Primer name | Primer Sequences | Primer name | Primer Sequences |
|---|---|---|---|
| OPA-1 | 5′-CAGGCCCTTC-3′ | OPA-10 | 5′-GTGATCGCAG-3′ |
| OPA-2 | 5′-TGCCGAGCTG-3′ | OPA-12 | 5′-TCGGCGATAG-3′ |
| OPA-3 | 5′-AGTCAGCCAC-3′ | OPA-13 | 5′-CAGCACCCAC-3′ |
| OPA-4 | 5′-AATCGGGCTG-3′ | OPA-14 | 5′-TCTGTGCTGG-3′ |
| OPA-5 | 5′-AGGGGTCTTG-3′ | OPA-15 | 5′-TTCCGAACCC-3′ |
| OPA-6 | 5′-GGTCCCTGAC-3′ | OPA-18 | 5′-AGGTGACCGT-3′ |
| OPA-7 | 5′-GAAACGGGTG-3′ | OPA-19 | 5′-CAAACGTCGG-3′ |
| OPA-8 | 5′-GTGACGTAGG-3′ | OPA-20 | 5′-GTTGCGATCC-3′ |
| OPA-9 | 5′-GGGTAACGCC-3′ | | |

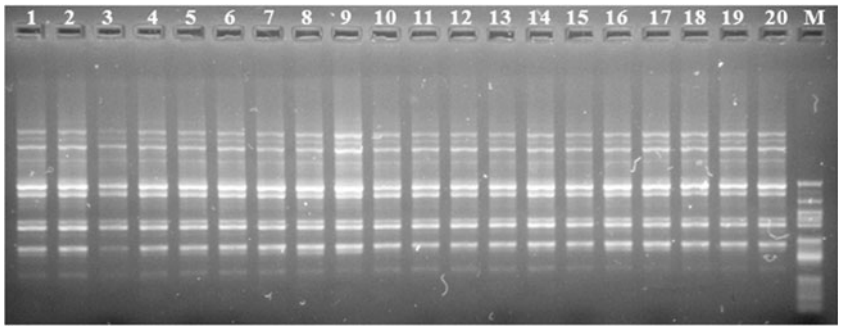


**Fig. 2** RAPD banding profile obtained with primer OPA-5 for propagated and mother plant of *B. serrata.* Lane 1: mother plant and lane 2–20 in vitro propagated plants. M: 100 bp DNA ladder

Alla 2010), and the plantlets were shown to be true-to-type in nature. In conclusion, the in vitro propagation protocol for *B. serrata* reported in the present investigation can be utilized for large scale production of plantlets and for conservation of this important medicinal tree species.

## Callus induction and maintenance

Callus formation was observed in root, stem, cotyledon and leaf explants on MS medium fortified with 10 to 15 μM BA, KIN, IAA, NAA, and 2.5 to 5.0 μM 2,4-D used individually. The explants started to form callus at their cut end after 2–3 weeks of culture initiation. The type of auxin and cytokinin, and their concentrations in the medium affected the growth of callus (Table 4). On incorporation of cytokinins and auxins separately, extensive callus proliferation was observed with 15 μM BA or IAA, followed by Kin, NAA and 2,4-D. These results indicate that the cytokinins or auxins when used alone can induce the callus in seedling explants of *B. serrata*. However, callus proliferation in explants was enhanced by addition of 2.5 to 5.0 μM BA or Kin along with 10.0 to 15.0 μM IAA, NAA or 2.5 to 5.0 μM 2,4-D, than when used singly. No apparent difference was observed in the frequency of callus formation. These results revealed that, the lower concentrations of cytokinins enhanced the effect of auxin in callus induction. The induced calluses on media containing auxin and cytokinin except 2, 4-D were friable and faint green in colour, whereas on 2,4-D containing media, the calluses were loose and whitish or yellowish in colour. As compared to root and stem, less callus formation was observed in cotyledon and leaf explants (Table 5). Such differences could be due to differences in endogenous growth regulator levels in the explant sources or different tissue sensitivities to these plant growth regulators (Rani and Nair 2006). Calluses were isolated from the explants and subcultured at the three weeks interval onto fresh medium of the same composition.

From a total of more than 256 combinations of different auxins and cytokinins at various concentrations (2.5 to 20.0 μM) that have been tested, the significant callus production from seedling explants (root, stem, cotyledon and leaf) was obtained on the medium with an auxin:cytokinins ratio>1. The incorporation of 15.0 μM IAA together with 5.0 μM BA in the medium was effective for callus induction as well as proliferation in all explants (Table 5) though good callus growth was also obtained with other combinations of growth regulators. In general, the growth of callus was slightly more on medium containing IAA than that with NAA and 2, 4 - D.

Auxins and cytokinins alone or in combination have been reported as most widely used growth regulators for callus induction from different types of explants (Gang et al. 2003). The present investigation proves that the combination of auxins and cytokinins enhance the callus production but, nevertheless, the individual cytokinin or auxin treatments are also effective for callus production (Tables 4 and 5). These results are in agreement with the callus induction and proliferation reported for *Acacia mangium* (Xie and Hong 2001) and *Taxus wallichiana* (Das et al. 2008), on the medium containing auxins (2,4-D) and cytokinins (Kin). Recently, Lin et al. (2010) reported higher frequency of callus induction from different explants of a deciduous tree, *Catalpa bungei* on the medium supplemented with NAA in combination with BA. Irrespective of the source of explant, type and concentration of auxin and cytokinin, the calluses started appearing brownish in the fourth week of culture and subsequently become completely dark brown to black. However, the healthy 18–20 days old calluses of the different explants when transferred to fresh medium of same composition showed proliferation, but not organogenesis. The calluses could be maintained over a period of 24 months by repeatedly subculturing to fresh parental medium at 3 weeks interval. Growth of calluses (in terms of dry weight) derived from different explants on media containing various growth regulators remained constant over 24 months. Thus, calluses of *B. serrata* can be maintained for a prolonged period.

## Boswellic acid production in callus

The source of callus and growth regulator(s) in the medium influenced the accumulation of boswellic acid components. Callus grown on media containing auxins (IAA, NAA, 2,4-D) or cytokinin (BA, Kin) alone showed the accumulation of components of boswellic acid (CBA). The levels of CBA were

**Table 4** Influence of plant growth regulators on growth and accumulation of boswellic acid in callus of *B. serrata*

| Callus source and PGR (μM) | Callus DW (gm) | Components of boswellic acid in callus | | | | Total boswellic acid components in callus (μgg$^{-1}$ DW) |
|---|---|---|---|---|---|---|
| | | KBBA (μgg$^{-1}$ DW) | AKBBA (μgg$^{-1}$ DW) | BBA (μgg$^{-1}$ DW) | ABBA (μgg$^{-1}$ DW) | |
| Stem | | | | | | |
| BA (15) | 0.24±0.1 a | 9.2±0.3 a | 128.4±0.5 a | 66.7±0.3 a | 504.4±0.4 a | 708.7±1.5 a |
| Kin (15) | 0.21±0.2 a | 8.4±0.3 b | 056.3±0.2 b | 29.9±0.3 b | 268.4±0.4 b | 294.3±1.0 b |
| IAA (15) | 0.23±0.1 a | 7.1±0.2 c | ND | 09.9±0.2 c | 231.7±0.4 c | 243.7±0.7 c |
| NAA (10) | 0.22±0.1 a | 4.1±0.1 d | 018.7±0.1 c | 07.2±0.2 e | 199.8±0.2 d | 230.9±0.6 d |
| 2,4-D (5) | 0.23±0.4 a | 2.1±0.1 e | 011.6±0.2 d | 08.3±0.2 d | 116.6±0.3 e | 211.2±1.1 d |
| Root | | | | | | |
| BA (15) | 0.22±0.1 a | 8.8±0.3 a | 099.9±0.5 a | 58.4±0.6 a | 279.9±0.6 a | 447.0±2.0 a |
| Kin (15) | 0.19±0.2 ab | 7.9±0.2 b | 052.7±0.4 b | 28.1±0.3 b | 202.3±0.2 b | 223.2±1.0 b |
| IAA (15) | 0.21±0.1 a | 7.2±0.3 c | ND | 08.8±0.2 c | 198.5±0.5 c | 214.2±0.5 c |
| NAA (10) | 0.18±0.1 b | 3.9±0.1 d | 016.8±0.2 c | 07.9±0.3 d | 174.5±0.2 d | 203.1±0.8 d |
| 2,4-D (5) | 0.21±0.3 a | 3.1±0.1 e | 010.4±0.1 d | 07.1±0.1 e | 110.8±0.3 e | 199.5±1.2 e |
| Cotyledon | | | | | | |
| BA (15) | 0.21±0.1 a | 7.7±0.4 a | 092.7±0.6 a | 53.1±0.7 a | 258.4±0.4 a | 411.9±2.1 a |
| Kin (15) | 0.18±0.1 b | 7.2±0.2 ab | 049.8±0.3 b | 22.9±0.2 b | 098.9±0.5 b | 178.8±1.2 b |
| IAA (15) | 0.20±0.2 a | 6.9±0.3 b | ND | 08.1±0.3 c | 089.4±0.3 c | 101.3±0.8 c |
| NAA (10) | 0.19±0.2 ab | 3.6±0.2 c | 014.9±0.1 c | 07.6±0.2 d | 077.7±0.3 d | 099.8±0.6 d |
| 2,4-D (5) | 0.18±0.1 b | 2.8±0.1 d | 009.8±0.1 d | 06.9±0.1 e | 068.5±0.4 e | 095.1±1.0 e |
| Leaf | | | | | | |
| BA (15) | 0.20±0.2 a | 7.5±0.4 a | 096.5±0.3 a | 55.3±0.4 a | 178.1±0.3 a | 337.4±1.4 a |
| Kin (15) | 0.18±0.1 b | 7.1±0. 3 a | 051.7±0.1 b | 26.7±0.2 b | 094.4±0.2 b | 163.0±1.0 b |
| IAA (15) | 0.20±0.1 a | 6.7±0.2 b | ND | 09.2±0.2 c | 077.9±0.5 c | 105.2±0.7 c |
| NAA (10) | 0.19±0.1 ab | 4.1±0.1 c | 015.9±0.2 c | 08.1±0.3 d | 067.7±0.2 d | 094.1±1.0 d |
| 2,4-D (5) | 0.19±0.1 ab | 2.7±0.2 d | 011.9±0.2 d | 07.4±0.3 e | 053.6±0.4 e | 082.8±0.9 e |

*KBBA* 11-keto-β-boswellic acid; *AKBBA* Acetyl-11-keto-β-boswellic acid; *BBA* β-boswellic acid; *ABBA* Acetyl-β-boswellic acid. Data for callus dry weight (DW) are means±SE of 21 replicates from three independent experiments. Boswellic acid content was measured from three independent replicates and repeated thrice. Values followed by the same letter in columns are not significantly different ($P \leq 0.05$) according to DMRT. ND=No detection of the compound

observed to be higher in the stem derived callus raised on BA fortified medium, followed by Kin, IAA, NAA and 2,4-D enriched media (Table 4). Among the CBA in root, stem, cotyledon and leaf callus, the trend of accumulation of ABBA was higher followed by AKBBA, BBA, KBBA. The callus derived from stem explant showed significantly higher levels of CBA as compared to root, cotyledon and leaf derived callus on BA enriched medium (Table 4). Cytokinins have been demonstrated to stimulate accumulation of alkaloids in cultures of periwinkle (Garnier et al. 1996), *Nothapodytes foetida* (Thengane et al. 2003), and *Stephania tetrandra* (Kuo et al. 2011). Contrasting to these, cytokinins were inhibitory for naphthoquinone formation in *Lithospermum erythrorhizon* (Fujita et al. 1981). Alkaloid accumulation was not affected by cytokinins in callus cultures of *Leonurus heterophylus* (Yang et al. 2008), and *Solanum torvum* (Moreira et al. 2010).

Incorporation of auxins together with cytokinins was promotive for growth of callus as well as accumulation of CBA in the calluses derived from stem as well as root, cotyledon and leaf explant (Table 5). The medium with an auxin: cytokinin ratio>1 enhanced the growth of callus cultures and accumulation of CBA. However, for 2,4-D: cytokinins ratio of≤1was beneficial. The maximum contents of components of boswellic acid, KBBA, AKBBA, BBA, ABBA and total boswellic acid in stem callus on MS medium containing 15 μM IAA and 5.0 μM BA were 14.3, 312.0, 98.5, 814.5 and 1,239.5 μgg$^{-1}$ DW respectively. This trend of CBA accumulation remained the same irrespective of source of callus, type and concentrations of growth regulator. Among the explants, callus derived from stem explant contained higher levels of CBA followed by the callus derived from root, cotyledon and leaf explant. A considerable accumulation of CBA also appeared in calluses raised on other media (Table 5). The content of CBA was higher in stem derived callus as compared to that reported earlier (Ghorpade et al. 2011) in embryo derived callus of *B.*

**Table 5** Influence of auxins and cytokinins together on growth and accumulation of boswellic acid in callus of *B. serrata*

| Callus source and PGR (μM) | Callus DW (gm) | Components of boswellic acid in callus | | | | Total boswellic acid components in callus (μg g$^{-1}$ DW) |
|---|---|---|---|---|---|---|
| | | KBBA (μg g$^{-1}$ DW) | AKBBA (μg g$^{-1}$ DW) | BBA (μg g$^{-1}$ DW) | ABBA (μg g$^{-1}$ DW) | |
| Stem | | | | | | |
| IAA (15)+BA (5) | 0.44±0.2 a | 14.3±0.4 a | 312.2±0.7 a | 98.5±0.3 a | 814.5±0.5 a | 1239.5±1.9 a |
| IAA (15)+Kin (5) | 0.35±0.2 a | 12.7±0.2 b | 268.3±0.4 b | 63.3±0.3 b | 407.2±0.4 b | 0720.5±1.5 b |
| NAA (10)+BA (5) | 0.37±0.1 a | 09.5±0.2 c | 244.7±0.5 c | 55.9±0.4 c | 371.9±0.6 c | 0643.1±1.7 c |
| NAA (10)+Kin (5) | 0.34±0.1 a | 08.7±0.1 d | 198.4±0.6 d | 49.7±0.2 d | 347.4±0.6 d | 0583.3±1.4 d |
| 2,4-D (5)+BA (5) | 0.33±0.1 a | 05.4±0.2 e | 188.8±0.6 e | 31.7±0.3 e | 217.4±0.5 e | 0522.8±1.4 e |
| 2,4-D (5)+Kin (5) | 0.32±0.2 a | 04.8±0.1 f | 177.5±0.5 f | 27.4±0.2 f | 209.1±0.5 f | 0430.1±1.4 f |
| Root | | | | | | |
| IAA (15)+BA (5) | 0.42±0.2 a | 10.1±0.3 a | 218.3±0.5 a | 77.8±0.4 a | 379.8±0.7 a | 0686.0±1.9 a |
| IAA (15)+Kin (5) | 0.32±0.3 a | 09.8±0.3 a | 209.4±0.5 b | 54.2±0.1 b | 355.6±0.7 b | 0615.0±1.8 b |
| NAA (10)+BA (5) | 0.35±0.1 a | 09.1±0.2 b | 195.9±0.6 c | 40.2±0.3 c | 345.3±0.5 c | 0604.5±1.4 c |
| NAA (10)+Kin (5) | 0.32±0.1 a | 08.2±0.4 c | 185.8±0.3 d | 37.7±0.3 d | 234.8±0.4 d | 0466.5±1.4 d |
| 2,4-D (5)+BA (5) | 0.34±0.1 a | 05.8±0.1 d | 144.7±0.5 e | 28.5±0.4 e | 212.5±0.6 e | 0391.5±1.6 e |
| 2,4-D (5)+Kin (5) | 0.32±0.1 a | 05.1±0.1 e | 121.5±0.4 f | 23.7±0.2 f | 199.3±0.2 f | 0349.6±0.9 f |
| Cotyledon | | | | | | |
| IAA (15)+BA (5) | 0.41±0.2 a | 12.9±0.2 a | 206.7±0.5 a | 72.5±0.3 a | 311.3±0.7 a | 0603.4±1.7 a |
| IAA (15)+Kin (5) | 0.32±0.1 a | 09.8±0.3 b | 188.9±0.3 b | 42.8±0.5 b | 209.9±0.8 b | 0447.0±1.7 b |
| NAA (10)+BA (5) | 0.34±0.1 a | 08.7±0.2 c | 169.2±0.3 c | 38.4±0.3 c | 195.7±0.3 c | 0416.4±1.3 c |
| NAA (10)+Kin (5) | 0.32±0.2 a | 08.1±0.1 d | 155.4±0.2 d | 35.7±0.4 d | 167.5±0.5 e | 0366.7±1.2 d |
| 2,4-D (5)+BA (5) | 0.33±0.2 a | 04.9±0.3 e | 139.8±0.5 e | 30.5±0.3 e | 188.2±0.5 d | 0363.4±1.6 e |
| 2,4-D (5)+Kin (5) | 0.32±0.1 a | 04.8±0.2 e | 115.4±0.4 f | 24.4±0.2 f | 167.9±0.3 f | 0312.5±1.1 f |
| Leaf | | | | | | |
| IAA (15)+BA (5) | 0.41±0.1 a | 13.4±0.3 a | 199.3±0.5 a | 77.1±0.2 a | 309.4±0.4 a | 0599.2±1.4 a |
| IAA (15)+Kin (5) | 0.31±0.2 a | 10.7±0.2 b | 178.8±0.4 b | 40.5±0.2 b | 199.3±0.5 b | 0425.6±1.5 b |
| NAA (10)+BA (5) | 0.34±0.1 a | 09.2±0.2 c | 163.6±0.5 c | 37.1±0.2 c | 189.7±0.3 c | 0403.0±1.2 c |
| NAA (10)+Kin (5) | 0.32±0.2 a | 09.4±0.1 c | 151.3±0.4 d | 36.8±0.4 d | 165.8±0.3 e | 0363.6±1.0 d |
| 2,4-D (5)+BA (5) | 0.34±0.1 a | 06.2±0.3 d | 138.4±0.3 e | 25.7±0.1 e | 175.6±0.3 d | 0345.9±1.0 e |
| 2,4-D (5)+Kin(5) | 0.31±0.2 a | 05.9±0.2 d | 110.7±0.3 f | 23.1±0.3 f | 132.4±0.3 f | 0272.1±1.1 f |

*KBBA* 11-keto-β-boswellic acid; *AKBBA* Acetyl-11-keto-β-boswellic acid; *BBA* β-boswellic acid; *ABBA* Acetyl-β-boswellic acid. Data for callus dry weight (DW) are means±SE of 21 replicates from three independent experiments. Boswellic acid content was measured from three independent replicates and repeated thrice. Values followed by the same letter in columns are not significantly different ($P \leq 0.05$) according to DMRT. ND=No detection of the compound

*serrata* produced on MS medium containing IAA together with BA and lacking elicitor (about 0.02 mg of boswellic acid per g DW of callus) and the similar medium containing yeast extract as elicitor (about 1.01 mg of boswellic acid per g DW of callus). In general, the plant growth regulators enhanced the growth, metabolism and differentiation in the cultures, which ultimately lead to change in cytoplasmic conditions for product formation (Misra et al. 2005). The results of the present investigation are in agreement with the assertion that the source of callus, the type, concentrations and combinations of auxin and cytokinin affect accumulation of product (Constable and Vasil 1987).

Although as compared to callus from other explants, the boswellic acid production was more in callus derived from stem explant (1.2 mg g$^{-1}$ DW) but it was comparatively less than the natural gum (40–50 mg g$^{-1}$ DW). In nature, the exudation of gum which is used for commercial purpose appears only after maturation of *B. serrata* tree and only in summer season. The plant is deciduous, mainly found in rocky soil and being a slow growing tree, takes several years for establishment and maturity. On the other hand, the natural stand available is limited and the species has been grouped in endangered and threatened category (Purohit et al. 1995; Ghorpade et al. 2010). Therefore, though the contents of boswellic acid in callus and natural gum are not comparable, it is possible to grow the callus on a large scale round the year and to use it as an alternative source of boswellic acid.

## Conclusion

Ayurvedic and pharmaceutical companies largely depend on the gum-resin exudates of field grown plants which occur only in dry season. The overexploitation of natural population for gum resin exudates and pulp and also due to difficulty in seed germination and lack of systematic cultivation of this plant species, it is justifiable to develop an efficient in vitro propagation method and protocol for callus culture and its use as a source of boswellic acid. Results of this study show that the calluses derived from stem explants accumulate the considerable levels of boswellic acid components. The calluses can be maintained over period of 24 months by regular subculturing. The in vitro propagation and callus culture protocol can be utilized round the year for production of plantlets for germplasm conservation, commercial cultivation and by employing approaches like elicitation and precursor feeding for enhancing the boswellic acid content.

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