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Influence of biotic and abiotic elicitors on four major isomers of boswellic acid in callus culture of *Boswellia serrata* Roxb

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Abstract

Boswellia serrata Roxb. is an endangered plant, known for its medicinal properties. Enhancing accumulation of the active metabolite, boswellic acid using tissue culture techniques seems a promising approach. This study examined effect of biotic and abiotic elicitors on production of four major components of boswellic acids, 11-keto-β-boswellic acid (KBBA), acetyl-11-keto-β-boswellic acid (AKBBA), β-boswellic acid (BBA) and acetyl-β- boswellic acid (ABBA) in callus culture. Cultures were established from embryo explants on Murashige and Skoog (MS) medium containing 2.5 µM 6-benzyladenine (BA), 15 µM indole-3-acetic acid (IAA) and 200 mg l^{-1} polyvinylpyrrolidone (PVP). *Fusarium oxysporum, Penicillin notatum, Mucor sp. and* yeast extract were among the biotic elicitors and sodium chloride (NaCl), sodium sulphate (Na_2SO_4), ultraviolet light (UV-C) exposure and light intensity were among the abiotic elicitors used. Treatment with elicitors was found to inhibit growth of callus and concurrently enhance boswellic acid biosynthesis. Yeast extract (500 mg l^{-1}) was found to be the most superior biotic elicitor for accumulation of KBBA (27-fold), AKBBA (23-fold), BBA (42-fold) followed by *Fusarium oxysporum* (750 mg l^{-1}) for ABBA (37-fold). Among abiotic elicitors, the optimum content of AKBBA (10-fold) was detected at 10 mg l^{-1} of Na_2SO_4 and KBBA (10-fold) at 15 mg l^{-1} of Na_2SO_4. An exposure time of UV-C (5 min) was effective for production and accumulation of AKBBA (10-fold) and BBA (7-fold) in the callus culture. The present study is the first to report *in vitro* production of boswellic acid from callus cultures of *B. serrata* using various biotic and abiotic elicitors.

Keywords: Boswellic acid; *Boswellia serrata;* Elicitor; Yeast extract; *Fusarium oxysporum.*
Abbreviations: 2,4-D- 2,4-dichlorophenoxyacetic acid; AKBBA- acetyl-11-keto-β-boswellic acid; BA- 6-benzyladenine; BBA- β-boswellic acid; DW- dry weight; FW- fresh weight; HPLC- high performance liquid chromatography; IAA- indole-3-acetic acid; KBBA- 11-keto-β-boswellic acid; KIN- kinetin; MS- Murashige and Skoog; NAA- α-naphthalene acetic acid; PVP- polyvinylpyrrolidone; UV- ultraviolet light.

Introduction

Boswellia serrata Roxb. (Burseraceae), commonly named *Salai guggul* or Indian olibanum is an important medicinal tree which grows in the hilly areas of India (Kapoor, 1990; Shah et al., 2009). Currently, *B. serrata* is categorised among endangered species (Sharma, 1983; Purohit et al., 1995; Ghorpade et al., 2010). '*Guggul*' is one of the oldest fragrant and medicinally important oleo-gum resins (Kapoor, 1990). The oleo-gum resin, obtained by incision made on trunk of the tree is effective for the treatment of inflammatory disorders such as arthritis and in cardiovascular diseases (Ammon, 2006). The pharmaceutical properties of oleo-gum resin is well documented (Gayathri et al., 2007; Krüger et al., 2009) and has been characterized as a rich source of terpenes, triterpenoids and pentacyclic triterpenic acids (boswellic acids) acids (Pardhy and Bhattacharya, 1978a, b). The four major pentacyclic triterpenic acids identified as the active principles of oleo-gum resin are β-boswellic acid (BBA), acetyl-β-boswellic acid (ABBA), 11-keto-β-boswellic acid (KBBA) and acetyl-11-keto-β-boswellic acid (AKBBA) (Pardhy and Bhattacharya, 1978c; Sander et al., 1998). Plant tissue culture techniques are used as an alternative method for the production of these specific metabolites in situations when plant material is rare and difficult to acquire and when chemical synthesis of their metabolites is not possible. These techniques provide well-controlled and sustainable systems for efficient production of desired bioactive compounds (Rao and Ravishankar, 2002). By controlling the composition of the culture medium and the environment, secondary metabolite synthesis may be enhanced *in vitro*. Certain secondary compounds are produced by the plant under the influence of various biotic and abiotic factors that are known as 'elicitors'. Very often biotic elicitors are compounds produced by pathogenic micro-organisms or released from their cell walls by plant enzymes. However, they can also promote the liberation of endogenous elicitors from plant cell walls or stimulate the production and release of plant constitutive endogenous elicitors (Benhamou, 1996). Plant cell cultures offer an alternative to natural biomass for production of useful metabolites. However, the productivity obtained by *in vitro* techniques remains low (Mathur and Ramawat, 2008). Several attempts have been reported in the past for enhanced synthesis of secondary metabolites in cell and organ cultures of different plant species, e.g. indole alkaloid in *Catharanthus roseus* (Zhao et al., 2001a), berberine in *Thalictrum rugosum* (Brodelius et al., 1989), taxol and related taxanes in *Taxus sp.* (Ciddi et al., 1995),

Table 1. Effects of selected cytokinins and auxins on callus growth (DW and M%) in callus culture of *Boswellia serrata* harvested after 28 days of incubation

Plant growth regulators(PGR, µM)	DW(g culture^{-1})	M (%)
2.5 µM BA	0.120 ± 0.2 o	89
5.0 µM BA	0.129 ± 0.1 m	91
10.0 µM BA	0.216 ± 0.5 d	92
15.0 µM BA	0.225 ± 0.3 c	93
2.5 µM KIN	0.116 ± 0.4 p	81
5.0 µM KIN	0.125 ± 0.5 n	90
10.0 µM KIN	0.156 ± 0.3 l	87
15.0 µM KIN	0.175 ± 0.4 k	85
5.0 µM 2,4-D	0.101 ± 0.2 q	88
10.0 µM 2,4-D	0.110± 0.3 r	92
15.0 µM 2,4-D	0.195 ± 0.1 g	93
5.0 µM IAA	0.179 ± 0.6 j	86
10.0 µM IAA	0.191 ± 0.4 h	87
15.0 µM IAA	0.210 ± 0.2 e	90
5.0 µM NAA	0.190 ± 0.1 h	89
10.0 µM NAA	0.212 ± 0.2 e	92
15.0 µM NAA	0.203 ± 0.5 f	91
15.0 µM IAA + 2.5 µM BA	0.298 ± 0.3 a	91
15.0 µM IAA + 5.0 µM BA	0.232 ± 0.1 b	91
15.0 µM IAA + 2.5 µM KIN	0.195 ± 0.6 g	92
15.0 µM IAA + 5.0 µM KIN	0.175 ± 0.3 k	93
5.0 µM NAA + 5.0 µM BA	0.116 ± 0.1 p	90
5.0 µM NAA + 5.0 µM KIN	0.159 ± 0.2 l	91
5.0 µM BA + 5.0 µM 2,4-D	0.185± 0.4 i	90
5.0 µM KIN + 5.0 µM 2,4-D	0.197 ± 0.2 g	91

Values represent mean ± SE (standard error) calculated on three independent experiments, each based on a minimum of 21 replicates. Values followed by the same letter were not significantly different at $P \leq 0.05$, according to DMRT.

silymarin production in cell suspension cultures (Rahimi et al., 2010) and hairy root cultures (Rahimi et al., 2011; Khalili et al., 2010) of *Silybum marianum*. Till date there is no information available on the influence of elicitors on growth and production of boswellic acid in callus cultures of *B. serrata.* The present study focuses on the effects of selected biotic and abiotic elicitors on growth and accumulation of four major components of boswellic acid viz. KBBA, AKBBA, BBA and ABBA

Results

Callus culture

The embryo exhibited significant response for callus induction after 7 days of incubation on MS medium supplemented with different concentrations of cytokinins alone or in combination with auxins. At the end of second week, callus induction was observed from embryo explants which increased progressively up to 4 weeks. The selected concentrations of plant growth regulators found suitable for optimum callus proliferation from embryo explants are depicted in Table 1. The maximum callus proliferation (DW 0.298 g culture^{-1}) was recorded from embryo explant cultured on MS containing 2.5 µM BA, 15 µM IAA and 200 mg l^{-1} PVP in comparison to other concentrations of cytokinins alone or in combination with auxins (Table 1) and this was used for subsequent experiments.

Influence of biotic elicitors on KBBA, AKBBA, BBA and ABBA accumulation

The concentration of the four major components of boswellic acids extracted from callus cultures treated with different biotic and abiotic elicitors was calculated from the HPLC chromatogram (data not shown). Each of the four components viz., KBBA, AKBBA, BBA and ABBA showed their separation at retention times 7.154 11.477, 13.404 and 21.412 min, respectively. The individual concentration of these four boswellic acid components and their total content is shown in Figure 1, 2 (a), 3 and Table 2. Yeas extract (500 mg l^{-1}) significantly improved the content of the boswellic acid components. At this optimum concentration the content of KBBA, AKBBA and BBA was 27-, 23- and 42-folds higher as compared to control (Fig. 1a, b, c). However, the content of ABBA was 37-folds higher than control when *F. oxysporum* (750 mg l^{-1}) was used (Fig. 1d) *P. notatum* and *Mucor sp.* were comparatively less effective for production of all four isomers of boswellic acid. When the total boswellic acid content was calculated, it was observed that the yeast extract (500 mg l^{-1}) proved to be a superior elicitor followed by *F. oxysporum* (750 mg l^{-1}). *Mucor sp.* (500 mg l^{-1}) followed by *P. notatum* (750 mg l^{-1}) were relatively inferior (Fig. 2a).The dry weight of the callus was maximum at the optimum concentration of yeast extract (500 mg l^{-1}) as compared to other concentrations as well as other biotic elicitors. However, the dry weight of the control was higher when compared to that of yeast extract (Fig. 2b). There was no such correlation between the dry weight of callus and content of boswellic acid(s) in case of *F. oxysporum, P. notatum* and *Mucor sp.*

Influence of abiotic elicitors on KBBA, AKBBA, BBA and ABBA accumulation

Among the four abiotic elicitors used (Fig 3a, b, c, d), significant increase in the content of KBBA (10-folds) was obtained in medium supplemented with Na_2SO_4 (15 mg l^{-1}). Na_2SO_4 (10 mg l^{-1}) followed by UV-C exposure (5 min) improved the content of AKBBA by 10-folds. The content of BBA was highest (7-folds) with UV-C exposure (5 min). Supplementation of 5 mg l^{-1} of Na_2SO_4 increased the content

Table 2. Effect of abiotic elicitor on growth of callus and total boswellic acid content in callus culture of *Boswellia serrata* harvested after 28 days of incubation.

Abiotic elicitor		DW(g culture^{-1})	M (%)	Total boswellic acid content (µg g^{-1} DW)
Control	0	0.317 ± 0.01 b	93	20.01 ± 0.6 n
NaCl (mg l^{-1})	2.5	0.280 ± 0.03 e	95	131.74 ± 0 c
	5	0.264 ± 0.08 g	93	90.68 ± 0.8 f
	10	0.252 ± 0.04 i	91	32.83 ± 0.5 k
	15	0.236 ± 0.03 k	94	29.39 ± 0.4 l
Na_2SO_4 (mg l^{-1})	2.5	0.287 ± 0.03 d	95	74.2 ± 0.7 h
	5	0.272 ± 0.02 f	91	111.61 ± 0.7 e
	10	0.259 ± 0.04 h	90	130.37 ± 0.9 cd
	15	0.245 ± 0.01 j	94	78.92 ± 0.8 g
UV-C (min)	5	0.284 ± 0.02 d	92	159.18 ± 0.9 a
	10	0.269 ± 0.03 f	91	145.83 ± 0.8 b
	15	0.246 ± 0.05 j	89	127.09 ± 0.9 d
	20	0.218 ± 0.01 l	82	79.96 ± 0.8 g
Light intensity (Watt)	25	0.279 ± 0.03 e	89	25.87 ± 0.6 m
	40	0.309 ± 0.02 c	91	38.38 ± 0.6 j
	60	0.327 ± 0.04 a	91	45.75 ± 0.9 i
	100	0.285 ± 0.01 d	85	13.15 ± 0.3 o

Values represent mean ± SE (standard error) calculated on three independent experiments, each based on a minimum of 21 replicates. Values followed by the same letter were not significantly different at *P≤0.05*, according to DMRT.

of ABBA to 2.5 folds. When concentration of total boswellic acid was calculated, UV-C exposure (5 -10 min) followed by Na_2SO_4 (10 mg l^{-1}) was found beneficial (Table 2). NaCl and Light intensity were not as effective in enhancing accumulation of any of the four isomers of boswellic acid as compared to Na_2SO_4 and UV-C light. Although the growth was maximum when at a light intensity of 60 watt, this did not effect in the content of total boswellic acid or its isomers (Table 2).

Discussion

The results of present investigation reveals significant callus induction and proliferation from embryo explants of *Boswellia serrata* on medium supplemented with lower concentration of cytokinin (2.5 µM BA) together with higher levels of auxins (15 µM IAA). In addition, the auxins and cytokinins when used alone also showed callus inducing ability from embryo explant, although at a relatively slower rate. Our results are in agreement with those of callus induction and proliferation for *Acacia mangium* (Xie and Hong, 2001) and woody tree, *Taxus wallichiana* (Das et al., 2008) on the medium containing auxins (2,4-D) and cytokinins (Kin). callus induction frequency from different explants of deciduous tree, *Catalpa bungei* on the medium supplemented with NAA in combination with BA has also been reported by Lin et al. (2010). Thus, the results of the present investigation reveal the important role of auxins in callus induction. Furthermore, the lower concentrations of cytokinins facilitate the effect of auxins in callus induction. One of the primary actions of the bio industry is to improve production of commercially valuable compounds through elicitor application. It is effected by several parameters such as specificity, concentration and exposure time of the elicitor, as well as culture conditions (nutrient composition of the medium, growth regulators and light). The growth stages of the cell culture also play an important role (Wiktorowska et al., 2010). In the present investigation, yeast extract and fungi viz. *F. oxysporum,* significantly improved the production of KBBA, AKBBA, BBA and of ABBA respectively in the callus biomass. Concurrent to the observations in our study enhancement of thiophene production in *Tagetes sp.* by *F. conglutinans* has been reported (Mukundan and Hjarttso, 1990). In contrast to the results of the current study negative effects of *F. oxysporum* for anthocyanin production in callus culture of *Daucus carota* L (Rajendran et al., 1994) and for α-topcopherol and pigment production in cell cultures of *Carthamus tinctorius* L (Chavan et al., 2010) have been reported. Limited accumulation of sanguinarine by *F. oxysporum homogenates* along with methyl jasmonate in *Argemone mexicana* cell cultures has also been reported (Trujillo et al., 2010). The results of the current study indicate a promising effect of yeast extract among the biotic elicitors in accumulation of boswellic acid. The stimulating effect of yeast extract on saponin production and accumulation was also reported by Lu et al. (2001) in cell cultures of *Panax ginseng.* Yeast extract is composed of variety of compounds, apart from amino acids vitamins and minerals: it is also possible that elicitation effects might be due to other components that still remain unidentified (Ertola and Hours, 1998). The elicitation induced by yeast extract could perhaps be attributed to the content of some cations like Zn, Ca and Co in the extract (Suzuki et al., 1985), which act as abiotic elicitors. Elicitors are known to induce changes in plasma-membranes permeability and have specific binding sites that act as receptors. Such interaction of elicitors with binding sites trigger secondary metabolite production. Translation of this external signal into internal information within the cell may involve gene expression. (Sudha and Ravishankar, 2003). UV light is a known inhibitor of plant growth, that affects plant morphology and further affects plants secondary metabolism during lengthy exposures causing a drought stress. Artificially increased levels of UV radiation have been reported to have significantly increased the accumulation of flavonoids, a class of secondary metabolite in *Aquilegia spp*. (Larson et al., 1990). UV light has also been reported to induce accumulation of several monoterpene indole alkaloids by expression of *tdc* gene in *C. roseus* (Ouwerkerk et al., 1999). In the present investigation, callus growth decreased with increasing time of exposure to UV light. When callus was exposed to UV light the effect of drought stress on growth was higher than on unexposed calli thus effectively resulting in a low fresh weight. This reduction in fresh weight post UV exposure may have been caused by the reduced amount of endogenous growth regulators, especially the cytokines, as a result of break down or lack of synthesis due

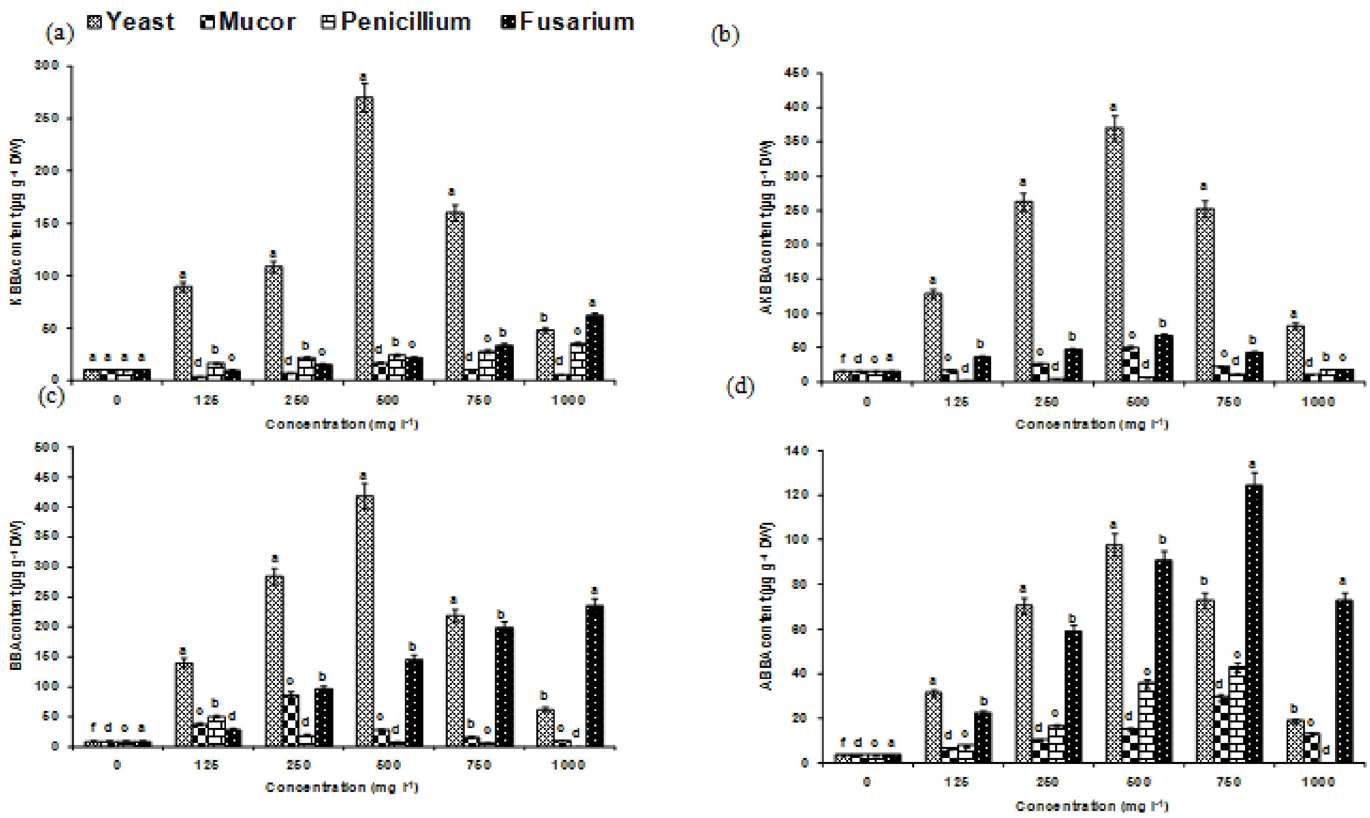

Fig 1. Influence of biotic elicitors (yeast, *Mucor sp.*, *F. oxysporum* and *P. notatum*) on accumulation of four major components of boswellic acid in callus culture of *B. serrata*.. (a) 11-keto-β-boswellic acid (KBBA), (b) acetyl-11-keto-β-boswellic acid (AKBBA), (c) β-boswellic acid (BBA) and (d) acetyl-β-boswellic acid (ABBA). The *error bar* indicates the standard error (SE). The *bars with different letters* revealed significantly different values at $P \leq 0.05$, according to DMRT.

to irradiation or genetic or epigenetic (physiological) changes (Ensanpour and Razaviza, 2005). In the current study, an optimum exposure time of 5 min to UV light was effective not only for accumulation of AKBBA and BBA but also for total boswellic acid content in the callus culture. Similar results were recorded by Antognoni et al. (2007) in flavonoid production by UV-B radiation in *Passiflora quadrangularis* callus culture. Several studies have been reported on possibilities of UV light activating certain genes and their corresponding enzymes which may be responsible for accumulation of the secondary metabolites (Broetto and Crocomo, 1995; Ouwerkerk et al., 1999). UV radiation and high light intensity have been reported to induce biosynthetic enzymes that produce phenolic compounds (Zhang and Björn, 2009). Plants can perceive abiotic stress and elicit appropriate responses to metal ions. Sodium, calcium, magnesium, zinc, copper, etc., at appropriate concentrations could act as signalling molecules and trigger many common reactions in the stressed tissue of plants or enzymes present in pathways leading to the synthesis of functional and structural metabolites (Rontein et al., 2002). In present study, it was observed that abiotic elicitors like NaCl and Na_2SO_4, reduced the growth of the callus culture. This suggests that salt stress exerts an unfavourable effect on the growth. Addition of the salt in the medium increased the accumulation of sodium ions (Na+) and / or chloride ions (Cl) in the plant tissue and/or organs and produced osmotic stress that inhibited growth, as in other species (Jaleel et al., 2007). A positive effect of salt stress on secondary metabolite production has been reported earlier in some tropane alkaloid producing plants and in other medicinal plants. Ajungla et al. (2009) reported increasing concentration of NaCl increase in the accumulation of both hyoscyamine and scopolamine in root culture of *Datura metel* L. Increased accumulation of ajmalicin alkaloid in *Catharanthus roseus* cell suspension culture was noted after treatment with increasing concentration of NaCl (Carpin et al., 1997). However, in the current study NaCl was not as beneficial as Na_2SO_4 in accumulation of boswellic acid. This is in contrast to observations of Ajungla et al. (2009) where Na_2SO_4 was less stimulatory as compared to NaCl for accumulation of alkaloids. Light is a predominant factor in the control of various life processes such as growth, development and stress responses in plants. Many biotic stress responses in plants are specifically adjusted by light conditions (Kumar et al., 2009).The results of the positive effect of light on growth of callus (Zhao et al., 2001b) and increase in vindoline and other indole alkaloids (Zhao et al., 2001a) has been reported in *C. roseus* callus culture. A significant effect on increasing calli fresh weight by light in *Brassica napus* L. (Afshari et al., 2011) has been reported. In the current study, illumination did not favour increased accumulation of boswellic acid.

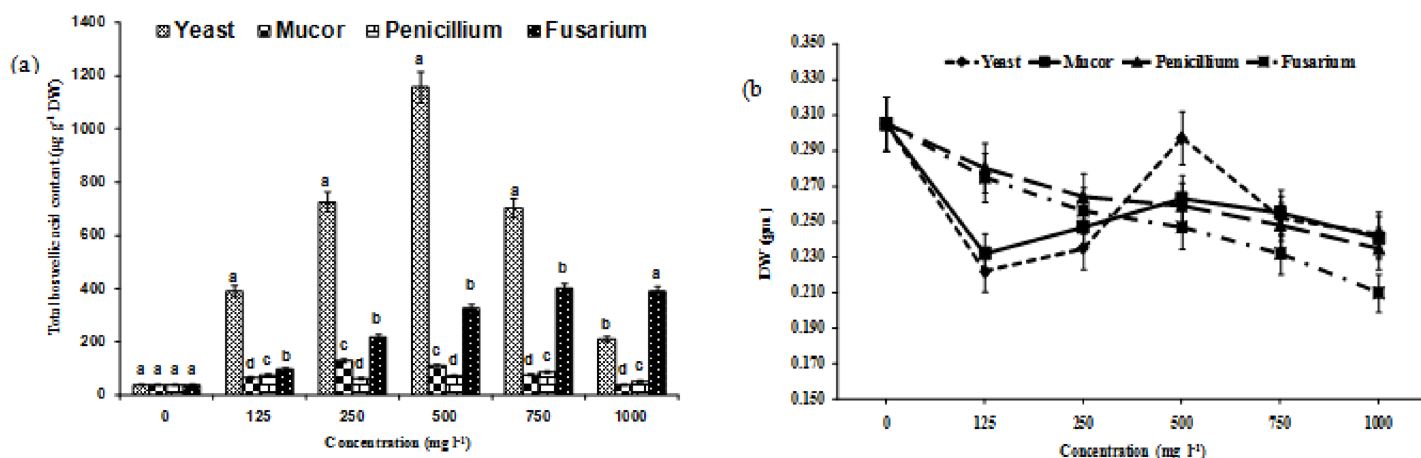

Fig 2. Influence of biotic elicitors on growth and total boswellic acid content in callus culture of *B. serrata* in presence of different fungi. Total boswellic acid content (b) Effect of elicitor on growth in terms of dry weight in callus culture. The *error bar* indicates the standard error (SE). The *bars with different letters* revealed significantly different values at *P≤0.05*, according to DMRT.

Materials and methods

Source of plant material and callus culture establishment

Mature green fruits of naturally growing *B. serrata* were used as source of plant material for establishment of callus cultures. The seeds were surface sterilized with 0.1% (w/v) mercuric chloride for 5 min and rinsed thoroughly with sterilized water. The clean and sterilized seeds were cut longitudinally and embryos were isolated and placed on solid MS (Murashige and Skoog, 1962) medium supplemented with 3% (w/v) sucrose, 200 mg l^{-1} (w/v) PVP and various concentrations of cytokinins [0, 2.5, 5, 10, 15 or 20 µM 6-benzyladenine (BA) and kinetin (KIN)] alone and in combination with auxins [0, 2.5, 5, 10, 15 or 20 µM indole acetic acid (IAA), α-naphthalene acetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D)].The pH of the medium was adjusted to 5.8 and solidified with agar-agar prior to autoclaving at 121 °C for 15 min. The cultures were incubated at 25 ± 2 °C daily for 16 h in light [40 µmol $m^{-2}s^{-1}$ photon flux density] and 8 h in darkness. After 4 weeks, fresh weight (FW, g) of the calli per culture was determined. Dry weight (DW, g) was recorded after oven-drying the calluses at 60 °C for 48 h. The moisture content (M%) of the calluses were determined using the equation [(FW-DW)/FW]*100. The concentrations of cytokinins alone or in combination with auxins which found most suitable for callus induction and proliferation were selected and used for the maximum production and maintenance of calli. Sub culturing was performed on the fresh parental medium at intervals of 28 days.

Biotic elicitor preparation and administration

Commercially available yeast extract (Hi Media) was used. The fungal strains of *Penicillium notatum* (NCIM 745), *Fusarium oxysporum* (NICM 1008) and *Mucor sp*. (NICM 881) were procured from National Collection of Industrial Micro-organism (NCIM), National Chemical Laboratory (NCL, Pune, India). These fungal strains were cultured on liquid potato-dextrose medium containing potato (200 g l^{-1}), dextrose (20 g l^{-1}) and pH 5.5 with 10% tartaric acid (Rajendran et al., 1994). The cultures were incubated for 28 days under the same controlled condition as described above and subsequently autoclaved at 121 °C for 15 min The resulting fungal mycelial mats were harvested for drying in a hot oven at 60 °C for 48 h, ground to fine powder and added at various concentrations (0.0, 125, 250, 500, 750 and 1000 mg l^{-1}) in the nutrient medium before adjusting pH and sterilization. A set of nutrient media which was devoid of fungal homogenate was maintained as control.

Abiotic elicitors

Salts such as sodium chloride (NaCl) and sodium sulphate (Na_2SO_4) at varying concentrations (0.0, 2.5, 5.0, 10.0 and 15.0 mg l^{-1}) and short wave UV-C light (Sankyo Denki G15T8 UV-C lamp, 254 nm wave length) were used as abiotic elicitors. The callus was exposed to UV-C irradiation at a distance of 45 cm from UV-C source for different time duration (5, 10, 15 and 20 min) in a sterile cabinet. Callus cultures were also exposed to white light source (25, 40, 60 and 100 watt; Philips, India).The cultures (~500 mg wet biomass) were innoculated onto MS medium containing (2.5 µM BA + 15 µM IAA + 200 mg l^{-1} PVP) at various concentrations of the above mentioned biotic or abiotic elicitors. The cultures were incubated for 4 weeks under the controlled conditions as described above. Using fresh weight and dry weight of the callus the percentage of the moisture content of the callus was calculated. The cultures were then used for the quantitation of boswellic acids components.

Boswellic acid extraction and quantification

The dried powder (1 g) of callus treated with biotic and abiotic elicitors was suspended in 5 ml high performance liquid chromatography (HPLC) grade methanol and sonicated for 20 min. The homogenate was centrifuged at 5000 rpm for 10 min. The supernatant was filtered through 0.22 µm nylon membrane filter (Milli-Q) and subjected for HPLC analysis.

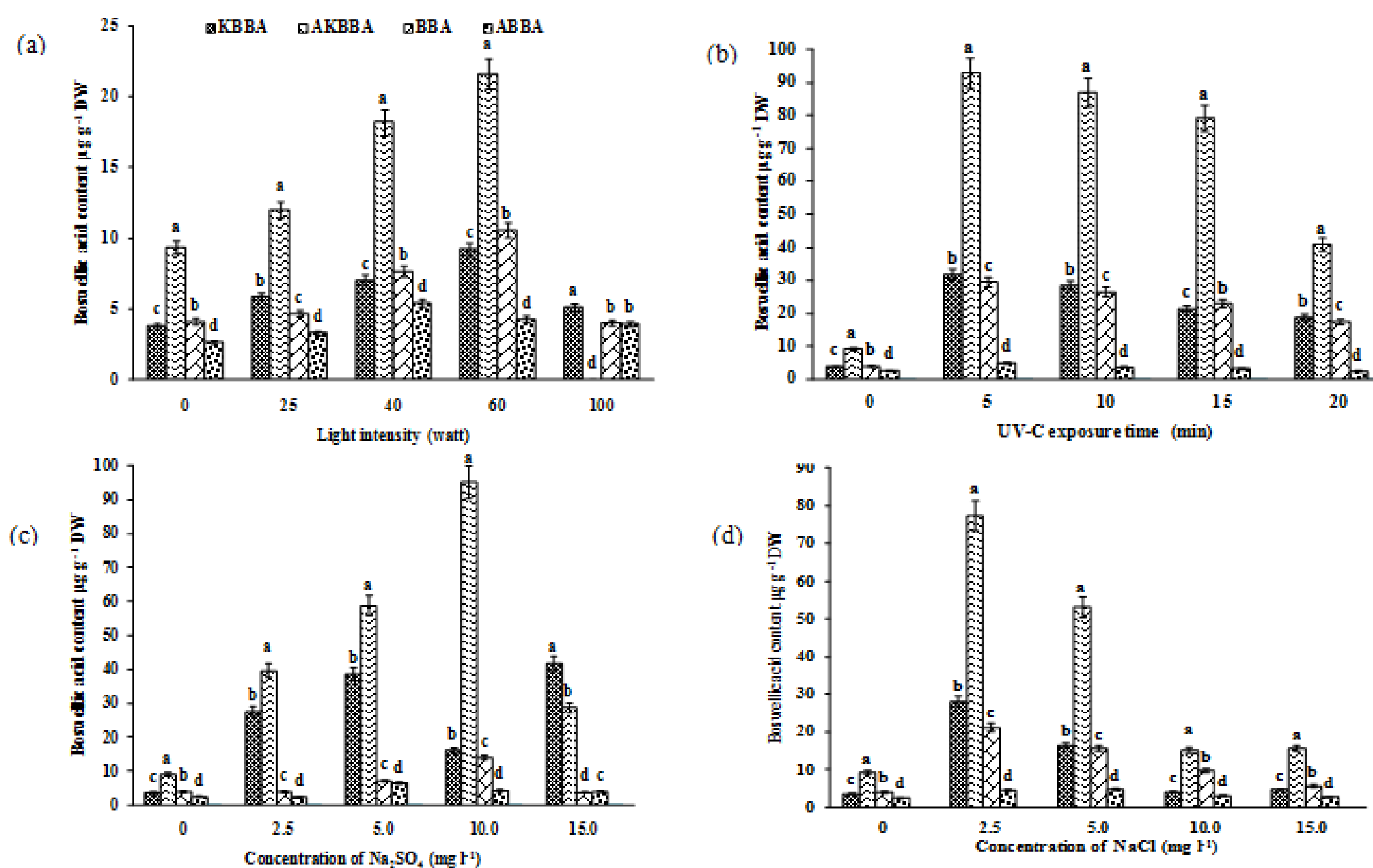

Fig 3. Influence of abiotic elicitors on accumulation of 11-keto-β-boswellic acid (KBBA), acetyl-11-keto-β-boswellic acid (AKBBA), β- boswellic acid (BBA) and acetyl-β-boswellic acid (ABBA) in callus culture of *B. serrata* in presence of (a) Light intensity (b) UV-C light (c) Na_2SO_4 and (d) NaCl. The *error bar* indicate the standard error (SE). The *bars with different letters* revealed significantly different values at *P≤0.05*, according to DMRT.

Preparation of boswellic acid standards

The working standard mixture of boswellic acid isomers in the current study [composition 1.89% 11-keto- β- boswellic acid (KBBA), 34.88% acetyl-11-keto-β-boswellic acid (AKBBA), 8.21% β-boswellic acid (BBA) and 5.39% acetyl-β-boswellic acid (ABBA)] was procured from Alexis Biochemicals (Axxora platform, UK). The standard was dissolved (10 mg) in 2.0 ml HPLC grade methanol and used as stock solution. Serial dilutions of the stock solution were made to obtain a working concentration ranging from 9.45-47.25 µg ml^{-1} of KBBA, 174.4-872 µg ml^{-1} of AKBBA, 41.1-205.3 µg ml^{-1} of BBA and 27.0-134.8 µg ml^{-1} of ABBA. Each working solution of standard was filtered through 0.22 µm nylon membrane filter (Milli-Q) and subjected for HPLC analysis

HPLC analysis

The isocratic HPLC system (DIONEX 170 AU, Germany) comprised a P-680 solvent delivery pump, a BDS Hypersil C18 (250 × 4.6 mm, 5 µm) column (Thermo scientific, Part No. 28105-254630), and a UV detector (170 U). The mobile phase involved acetonitrile:water (90:10 v/v, pH 4 adjusted with glacial acetic acid) with a flow rate of 1.0 ml min^{-1} at 25 °C. Samples (20 µl) were injected and the absorbance was recorded at 210 nm (for BBA and ABBA) and at 254 nm (for KBBA and AKBBA) (Shah et al., 2008). The content of boswellic acid components was quantified by comparing the retention time and peak area of BBA, ABBA, KBBA and AKBBA in the sample with that of standards as well as by spiking the samples in combination with standards. The boswellic acid components was quantified using calibration curve derived from the working standard and were expressed in terms of µg g^{-1} DW.

Statistical analysis

The experiments were carried out in a randomized block design with three independent biological replicates and repeated three times. Values in tables and figures are expressed as mean ± SE (standard error). The statistical significance of the data was analysed using univariate analysis of variance ($P \leq 0.05$) (one-way ANOVA; SPSS for Windows, version 10.0).

Conclusion

This study represents the first successful callus culture based approach for the production of four major components of

boswellic acid from the Indian species of *B. serrata* Roxb. The problem of low content in plant callus cultures is addressed through addition of biotic and abiotic elicitation, which resulted in significant improvement in four derivatives of the boswellic acid production. The present study indicates the potential of these biotechnology-based methodologies for mass production of four isomers of boswellic acids. The changes in boswellic acid production can be influenced by manipulation of type of elicitor, elicitation time and also their concentration and type of culture *in vitro.* This study sheds light on increasing the productivity of secondary metabolites in large scale process enterprises. Therefore, the protocol developed for *in vitro* production of boswellic acid could be used as promising and alternative source which will help to reduce the risk of harvesting and destruction of the plant from natural resources and improve the conservation status of *B. serrata.*

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