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ORIGINAL PAPER

Multiple shoot induction and jasmonic versus salicylic acid driven elicitation for enhanced andrographolide production in *Andrographis paniculata*

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Abstract Multiple shoot induction at an average of (15.25 ± 2.128 and 16.16 ± 3.851 shoots/explant) was obtained from *Andrographis paniculata* cotyledonary node explants on Murashige and Skoog's (MS) medium supplemented with 2.0 mg/L 6-Benzylaminopurine (BAP) and Zeatin, respectively after 8 weeks of culture. Further proliferation of cultures in MS + BAP medium at a maximum of 150 ± 18.40 multiple shoots was obtained from single cotyledonary node explants after three subculture (84 days) in vitro passage. Elicitation as yield enhancement strategy, a protocol was developed using multiple shoots to treat the cultures with jasmonic acid (JA) and salicylic acid (SA). Various concentrations of JA (1, 5, 10, 25, 50, 75 and 100 µM) and SA (10, 20, 50 and 100 µM) were used for elicitation. Treatment of JA at 1.0 µM concentration resulted in 1.322 % dry weight (DW) with 2.6-fold increase in andrographolide production after fifth week compared to 0.508 % DW in untreated control. However, JA treatment at 25 and 50 µM promoted 3.3, 3.0-fold enhancement in andrographolide production (1.624 and 1.481 % DW), respectively; after eighth week compared to control. Treatment of 10, 20 and 50 µM SA resulted in 3.0, 3.4 and 3.1-fold andrographolide content (1.479, 1.654 and 1.483 % DW), increase after eighth week, respectively; compared to control (0.478 % DW). This is the first report on elicitation of *A. paniculata* multiple shoot cultures using signal molecules (JA and SA). The present findings may be helpful for in vitro manipulation and enhanced production of andrographolides.

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Keywords *Andrographis paniculata* · Andrographolides · Cytokinins · Jasmonic acid · Multiple shoot cultures · Salicylic acid

Abbreviations

BAP	6-Benzylaminopurine
DW	Dry weight
FW	Fresh weight
HPLC	High performance liquid chromatography
JA	Jasmonic acid
Kn	Kinetin
MS	Murashige and Skoog (1962)
SA	Salicylic acid
Zt	Zeatin

Introduction

Andrographis paniculata (Wall. ex Nees) of family Acanthaceae is most popular for its various medicinal applications. It is one of the 28 species of *Andrographis* genus distributed in tropical Asia. This plant is widely used in India, China, Srilanka, Taiwan and other southeast Asian countries for the treatment of respiratory tract infection, common cold, fever, diarrhea, and liver disorders (Negi et al. 2008; Sareer et al. 2014; Wang et al. 2014). This herb is commonly known as Kalmegh which has a bitter taste due to the presence of bioactive diterpene lactones (Kumar et al. 2012). This herb produces many pharmaceutically important molecules such as diterpenoids, flavonoids and polyphenols (Chao and Lin 2010). The primary medicinal constituents of *A. paniculata* are andrographolides and related diterpene lactones which are found abundantly in leaf compared to other plant parts (Parasher et al. 2011).

A. paniculata has diverse pharmacological benefits such as antiviral (Niranjan et al. 2010), anti-malarial (Mishra et al. 2011), anti-helmintic (Singh et al. 2009), anti-oxidant (Lin et al. 2009), anti-bacterial (Burm et al. 2010) and for anti-cancer activity (Subramanian et al. 2012; Parveen et al. 2014). The active ingredients of *A. paniculata* has shown its interference with the viability of HIV-I virus. Further, a phase I trial of andographolide in HIV positive patients and normal volunteers was also undertaken (Calabrese and Berman 2000). Andrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide, have been studied for their anti-allergic, anti-inflammatory and cardiovascular effects (Yoopan et al. 2007; Wang et al. 2014). Currently, there is an increasing demand for plant-based medicines, in primary healthcare worldwide (De et al. 2012).

It is well established that the precursors of diterpene lactone andrographolides i.e. isopentenyl diphosphate and its isomer Dimethylallyl diphosphate are synthesized independently in the plastids through MEP/DXP (2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose-5-phosphate) pathway and in the cytosol through Mevalonic acid pathway (Seetha et al. 2005; Srivastava and Akhila 2010). Productivity of secondary metabolites in vitro can be augmented using different in vitro manipulation strategies and elicitation in particular may play an important role by inducing changes in biosynthetic pathways of the target cell (Leonard et al. 2009; Shilpa et al. 2010). Jasmonates (JAs) are a family of cyclopentanone compounds which are involved in plant stress responses and development (Wasternack and Hause 2013; Wasternack 2014). JA and SA are signal molecules involved in the activation of plant defense responses both in biotic and abiotic stresses and elicit wide range of secondary metabolites in plants (Pieterse et al. 2012; Baenas et al. 2014). It is an established fact that, signaling molecule treated plant cell cultures produce enhanced amount of secondary metabolites. Selection of appropriate elicitor is also important for enhanced secondary metabolite production at commercial scale (Ramakrishna and Ravishankar 2011; Murthy et al. 2014). JA and SA are the most successful chemical elicitors used in plant cell, tissue and organ cultures for production of bioactive compounds (Namdeo 2007; Matsuura et al. 2014; Murthy et al. 2014).

Production of andrographolides using in vitro culture systems will be of immense interest because in field cultivated *A. paniculata*, variation exists in growth and diterpene lactone content (Prathunturarug et al. 2007). Medicinal plants have been cultivated with laboratory-generated species based on chemical composition for commercial purpose (Pietrosiuk et al. 2007). Plant regeneration studies in *A. paniculata* are few (Purkayastha et al. 2008; Roy et al. 2009; Bansi and Rout 2013). A few reports on andrographolide production in *A. paniculata* using callus, cell suspension and adventitious root cultures are available (Praveen et al. 2009; Sharma and Jha 2012, 2013a). Recently, multiple shoot culture system has been exploited for elicitation studies and secondary metabolite production (Sivanandhan et al. 2013; Cheruvathur and Thomas 2014). Further, keeping in view the involvement of plastid in MEP/DXP pathway, exploitation of multiple shoot culture system finds its relevance for andrographolide production in vitro. Literature review showed that there was a limited study on elicitation in *A. paniculata* and related yield enhancement strategy. The present communication reports multiple shoot induction and enhanced andrographolide production in jasmonic/salicylic acid elicited multiple shoot cultures of *A. paniculata*.

Materials and methods

Germination of seeds

Seeds of *A. paniculata* were collected from the Regional Centre of CSIR-Central Institute of Medicinal and Aromatic Plants, Hyderabad, India. Seeds were washed using mild detergent (Teepol) solution followed by washing (seeds in mini cheese cloth bag) under running tap water for 15–20 min. Seeds were surface sterilized using 0.1 % (w/v) mercuric chloride solution for 2–3 min and washed 4–5 times with sterile double-distilled water (SDDW). The surface-sterilized seeds were inoculated on to semi-solid MS (Murashige and Skoog 1962) basal medium containing 30 g/L sucrose. Seeds were subsequently, incubated under controlled environmental conditions at 25 ± 1 °C in dark for germination.

Induction and maintenance of multiple shoots

Multiple shoot cultures of *A. paniculata* were induced using cotyledonary node (seedling devoid of root part) explants on full strength MS medium supplemented with 30 g/L sucrose and 1.0, 2.0, 3.0, 4.0 mg/L 6-benzylaminopurine (BAP); 1.0, 2.0, 3.0 and 4.0 mg/L kinetin (Kn) and 1.0, 2.0, 3.0 and 4.0 mg/L zeatin (Zt). The pH of the medium was adjusted to 5.8 ± 0.2 before addition of 0.9 % agar. The media was autoclaved at 121 °C for 15 min. The cultures were maintained in light provided by cool white fluorescent tube lights at 25 ± 1 °C and sub-cultured once at 4-week regular intervals. A minimum of twelve replicates of explants and different cytokinin concentrations was taken for each treatment. A total of thirty-six cotyledonary node explants were used for each treatment involving different cytokinins. The experiments were repeated thrice and data of respective experiments were recorded. Statistical analysis of data using mean, standard deviation and standard error was calculated.

Elicitation by signal compounds

Jasmonic acid (JA) and salicylic acid (SA) elicitors were evaluated for their influence on andrographolide accumulation in multiple shoot cultures of *A. paniculata*. Stock solutions of JA and SA, was prepared by dissolving 250 mg of each signal compound in 0.5 mL of dimethyl sulfoxide (DMSO) and absolute ethanol, respectively. Final stock solution of 25 mg/mL of JA and SA was prepared by adding SDDW and filter sterilized using 0.22 μM bacterial filtration unit (Millipore, Ireland). Filter sterilized JA (1, 5, 10, 25, 50, 75 and 100 μM) and SA (10, 20, 50 and 100 μM) was added aseptically to the melted multiple shoot culture growth agar medium using micropipettes (Thermo Scientifics Ltd. India). The 0.02 g of inoculums (axillary shoots) as explants (size ca. 2.0 cm) was inoculated onto multiple shoot culture media containing different concentrations of filter-sterilized JA and SA. Following elicitor treatment, multiple shoot cultures were incubated in culture room conditions in light. Further, subsequent to elicitor treatment multiple shoot cultures were harvested every week starting from first to eight weeks for the study of growth and andrographolide production.

Growth study for multiple shoot cultures

The fresh weight (FW) of control (untreated) and treated multiple shoot cultures were taken after removing the shoots from the semisolid medium. The fresh shoots were air dried at room temperature for 3 weeks, and dry weight (DW) was determined. The FW and DW data of multiple shoots were taken selecting six shoot bunches as replicates from each flasks extending over 8 weeks.

Ultrasonic extraction and quantitative analysis of andrographolide

Dry powdered material of untreated control and signal compound treated multiple shoot culture samples weighing 50 mg DW, was placed in a vial containing 5 mL of high- performance liquid chromatography (HPLC)-grade methanol and incubated for 1 h. The resulting extract was ultra-sonicated using an ultrasonic cleaning bath (Spectra Lab., Model UCB 30, India). After sonication for 30 min, the extract was initially filtered using Whatman filter paper No. 41. Further, the solution recovered was subjected to final filtration through 0.45 μM membrane (Millex HV, Millipore, Ireland) before its injection into HPLC system. The andrographolide fractions were analyzed using HPLC (Waters, USA). Waters Spherisorb C_{18} column (250 mm × 4.6 mm, 5 μM) was employed. Separation of compounds was carried out by isocratic elution with HPLC-grade methanol as mobile phase at 1 mL min^{-1} flow rate with 0.02 mL injection volume. The column was maintained at 25 °C. The elution was monitored at 230 nm. The software used was Empower pro and detector was photodiode array detector (PDA) with a retention time of 2.6 ± 0.3 min. Commercially available authentic standard andrographolide (98 % purity) was procured from Sigma-Aldrich, USA for analysis. All results were averaged over two consecutive experiments. Andrographolide content was expressed as % DW of control and elicited multiple shoot culture samples.

Results and discussion

Effect of cytokinins on induction of multiple shoot cultures

Cotyledonary node explants were found suitable for induction of multiple shoots in *A. paniculata*. The effect of various concentrations of BAP (1.0, 2.0, 3.0 and 4.0 mg/L), Kn (1.0, 2.0, 3.0 and 4.0 mg/L) and Zt (1.0, 2.0, 3.0 and 4.0 mg/L) in MS medium on multiple shoot induction showed variable responses after 4 and 8 weeks. Multiple shoot induction in terms of number of shoots/explant in *A. paniculata* gave almost similar results in BAP 2.0 mg/L (3.333 ± 0.465), Kn 2.0 mg/L (3.583 ± 0.711) and Zt 2.0 mg/L (3.916 ± 0.792) after 4 weeks (Fig. 1). The average number of shoots after 4 weeks in 1 mg/L BAP, Kn and Zt were 2.75 ± 3.09, 2.5 ± 0.39 and 2.56 ± 0.228, respectively. Lowest number of shoots in 3 mg/L BAP, Kn and Zt were 2.16 ± 0.29, 2 ± 0.34 and 2 ± 0.30, respectively. A decline in the number of shoots with 4 mg/L BAP, Kn and Zt were 1.58 ± 0.26, 1.41 ± 0.22 and 1.66 ± 0.30, respectively (Fig. 1a).

Further, subculture of multiple shoots for a duration of eight week on 2.0 mg/L BAP, Kn and Zt media generated increased number of shoots to 15.25 ± 2.128, 12.75 ± 3.035 and 16.166 ± 3.851, respectively (Fig. 1b). Minimum number of shoots 12.41 ± 2.44, 9.83 ± 2.65 and 11.33 ± 3.15 after 8 weeks in 1 mg/L BAP, Kn and Zt, respectively. The number of shoots up to 10.58 ± 2.61, 9.66 ± 2.44 and 10.58 ± 2.35 in 3 mg/L BAP, Kn and Zt were obtained, respectively. A decline in number of shoots 9 ± 1.83, 8.5 ± 2.58 and 8.41 ± 2.27 was observed with 4 mg/L BAP, Kn and Zt, respectively. In general, there was a decline in the number of shoots/explant on media with other concentrations of BAP (1.0, 3.0 and 4.0 mg/L), Kn (1.0, 3.0 and 4.0 mg/L) and Zt (1.0, 3.0 and 4.0 mg/L). BAP and Zt (2.0 mg/L) was found suitable for the growth of shoots up to eighth week.

Primary shoots obtained from initially cultured cotyledonary node explants were repeatedly sub-cultured on MS medium supplemented with 2.0 mg/L BAP to generate large number of multiple shoots. BAP 2.0 mg/L was found to be most effective plant growth regulator for multiple

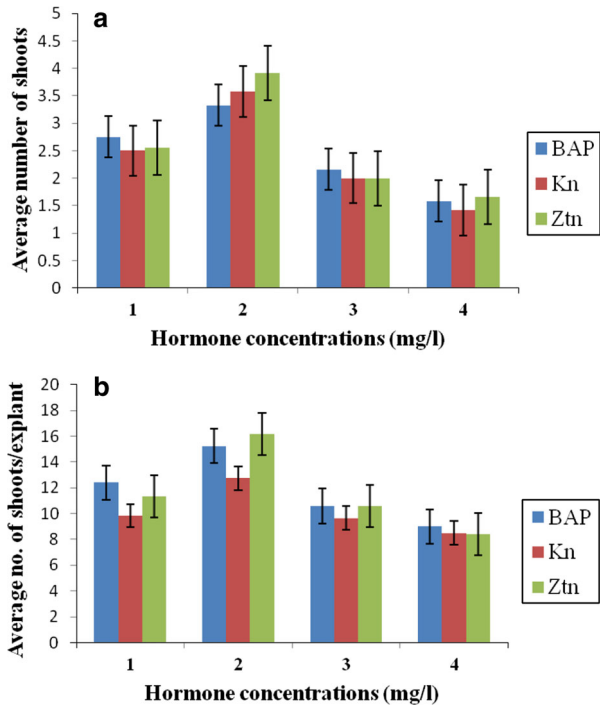

Fig. 1 Effect of different cytokinins and their concentrations on multiple shoot induction from cotyledonary node explants of *A. paniculata* after **a** 4th and **b** 8th week. *Bars* represent the mean ± SE

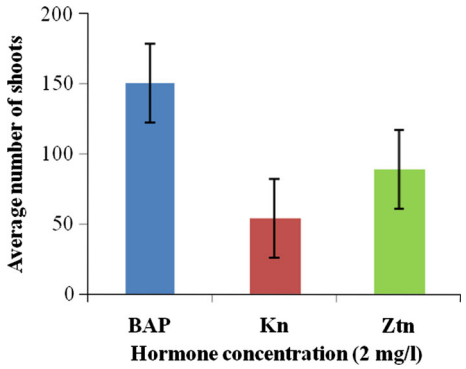

Fig. 2 Effect of cytokinins (2 mg/l) on multiple shoot induction from cotyledonary node explants of *A. paniculata* after 12th week. *Bars* represent the mean ± SE

shoot induction. A total of in an average 150 ± 18.40 shoots can be obtained from single cotyledonary node explant after 12 week (84 days) of culture when 8 week old individual shoots were sub-cultured for further proliferation (Figs. 2, 3a–d). Each single shoot was able to generate minimum of ten shoots in average. However, in a similar earlier report with *A. paniculata* 60–70 shoots was obtained after 90 days of in vitro passage (Purkayastha et al. 2008). In both the aforesaid findings cytokinin BAP was found superior in its response for multiple shoot induction. However, in a different study with *A. paniculata* we had observed, the Zt was found better than both Kn, BAP and thiodiazuron. Although, in this study higher number with an average of 62 shoots was obtained from stem base explants after 4 week of culture. But, maintenance of Zt promoted multiple shoots beyond 4 weeks was not suitable (Roy et al. 2009). However, in the present study this bottleneck was overcome by the induction and maintenance of multiple shoots in BAP containing medium. In fact, the multiple shoot cultures can be maintained in MS + BAP 2.0 mg/L medium beyond 2 years with similar growth pattern.

Influence of signal compounds (JA, SA) on growth and andrographolide production

In the present work, the effect of elicitation on growth and total andrographolide production in *A. paniculata* multiple shoot cultures were evaluated from first to 8 weeks. JA showed positive effect on growth and biomass production. However, on the other hand SA significantly reduced the biomass production in most of the treatments irrespective of all the concentrations used when compared to control shoot cultures. The effects of both the elicitors at varying concentrations on total andrographolide accumulation in multiple shoot cultures of *A. paniculata* showed changeable responses.

A comprehensive study on the effect of signal compounds such as JA and SA on fresh and dry weight of *A. paniculata* multiple shoot cultures revealed variable responses (Figs. 4a, b, 5a, b). FW of JA treated multiple shoot cultures were increased after first (all the concentrations), third (1 and 5 μM), fourth (all the concentrations except 100 μM), sixth (all the concentrations except 10, 50 and 100 μM), seventh (1 and 5 μM) and eighth (1 and 5 μM) week when compared to untreated control cultures. A decline in growth of shoots were observed from second to eighth week with 100 μM JA treatment compared to untreated control. However, in an earlier report all the concentrations (50-200 μM) of MeJA decreased the growth of *Bacopa monnieri* shoots (Sharma et al. 2013b). The growth study starting from first to eighth week revealed that highest biomass production of shoots (0.546 ± 0.097 g FW) was obtained with 5 μM treated multiple shoots after fourth week of culture compared to other concentrations of JA and untreated control (0.221 ± 0.040 g FW) shoot cultures (Fig. 4a). Best growth of control shoots (0.359 ± 0.139 g FW) was observed after fifth week. Seventh week JA (1, 5 μM) treated shoot cultures showed more FW when compared to control cultures. However, the treatment with these two concentrations (1, 5 μM), a meager increase in growth was observed after eighth week compared to control. There was an increase in growth of shoots at 25 and 75 μM JA dosage after sixth week compared to1 and 5 μM JA. Increased treatment of JA (50, 100 μM) decreased the growth drastically even lower than untreated control shoot cultures after sixth week. Similar

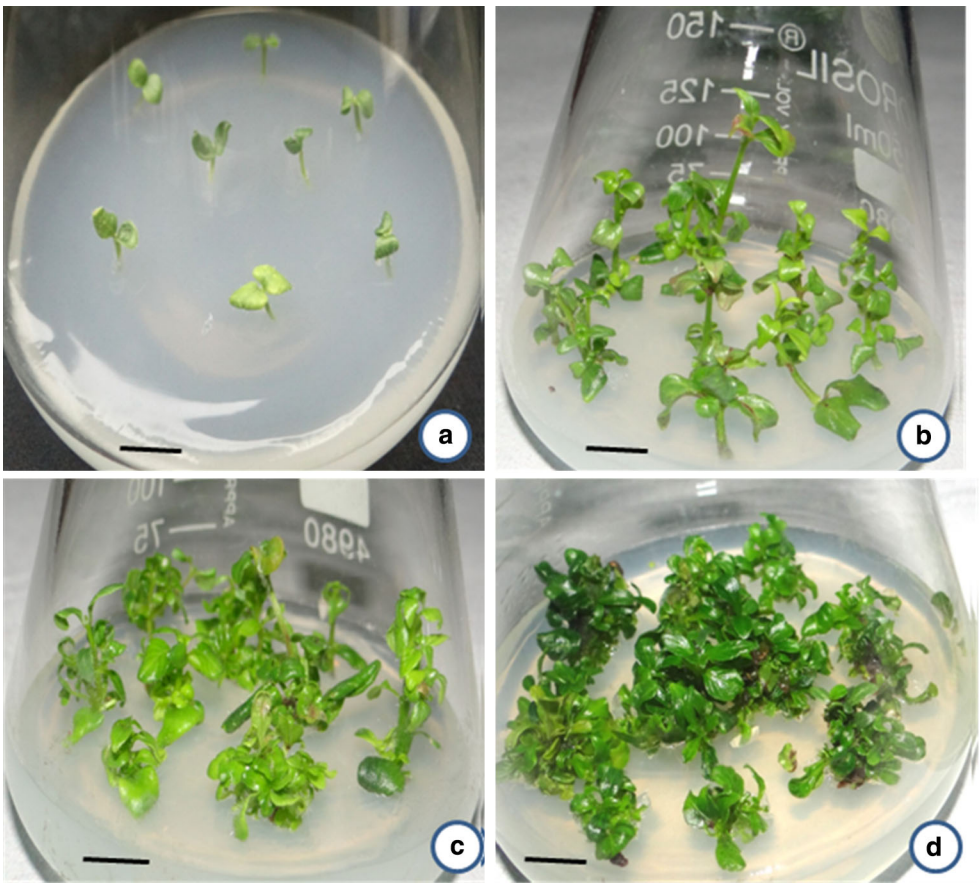

Fig. 3 High frequency multiple shoot from cotyledonary node explants of *A. paniculata.* **a** Cotyledonary node explants; **b** 4 week old multiple shoot cultures derived from cotyledonary node explants; **c** 8 week old multiple shoot cultures; **d** 12 week old multiple shoot cultures (*Bar* = 1.0 cm)

type of response was also reported in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum* (Coste et al. 2011). In the present study, there was no significant difference in biomass between 1 µM JA treated and control shoots after fifth week but at 25 µM concentrations there was inhibitory effect on growth of shoots when compared to control shoot cultures (Fig. 4a). Elicitors are signal compounds, which induce or enhance the biosynthesis of metabolites by activating pathways in response to exogenous stresses (Kuzmaa et al. 2009). Jasmonic acid and its derivatives are stress signaling molecules involved in plant defense and regulate various metabolic pathways along with other plant hormones (Avanci et al. 2010; Ballare 2011; Pirbalouti et al. 2014). These molecules are responsible for enhanced production of different secondary metabolites and act in plant chemical defense mechanism (Frankfater et al. 2009; Murthy et al. 2014). In the past, a number of elicitors were used for increased production of plant secondary metabolites. JA and its derivatives were proven very effective in enhancing secondary metabolite production in different plant cell cultures (De Geyter et al. 2012).

It is well known that SA is an endogenous signaling molecule involved in plant defense and growth responses (Hayat et al. 2010; Boatwright and Pajerowska-Mukhtar 2013). In general, the findings in the present study indicated that SA was not a good promoter for growth rather generated less biomass compared to untreated control. SA concentrations, such as 10, 20 and 50 µM showed optimum growth, but; still lesser than control and further reduction in growth at 100 µM salicylic acid (Fig. 4b). Thus, increased concentration of SA suppressed the growth and production of andrographolides in shoot cultures of *A. paniculata*. Similar observations on reduced biomass production in *Digitalis purpurea* shoots with increasing concentrations of SA has also been reported (Patil et al. 2013). SA (10, 20, 50 and 100 µM) was very effective after fifth, sixth, seventh and eighth week. SA treatment resulted in adverse influence on overall growth of multiple shoot cultures. SA significantly reduced the biomass production irrespective of all concentrations used when compared to control shoots except with 10 µM SA after second week. The second week 10 µM SA treated shoots gave higher

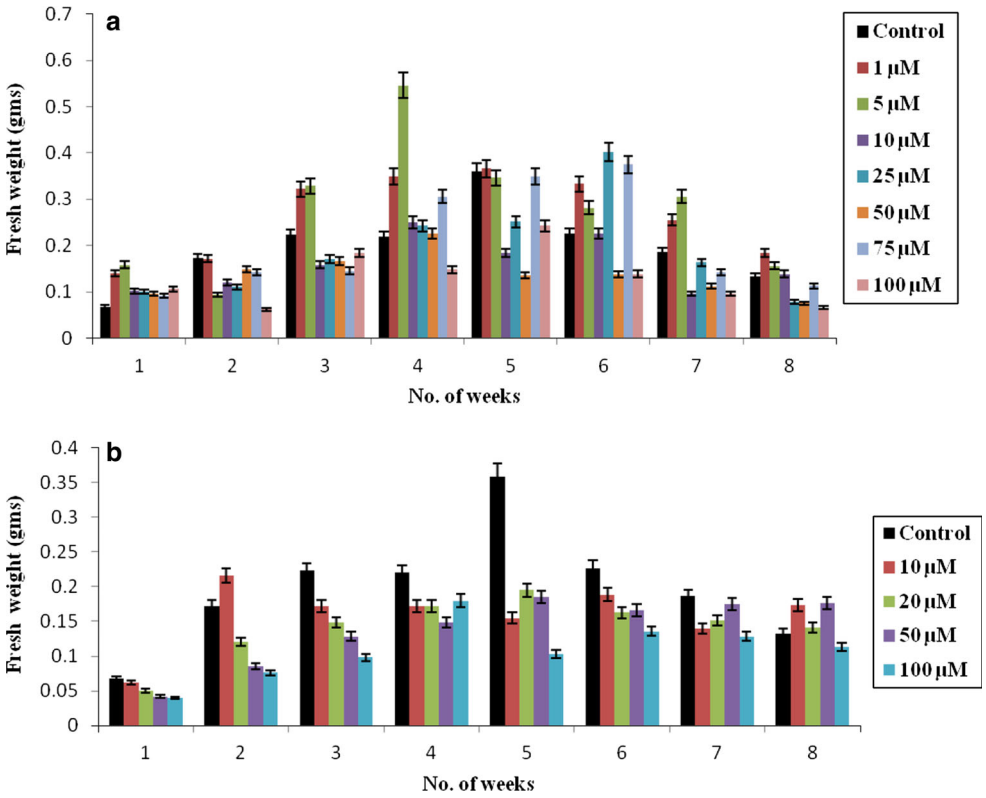

Fig. 4 Influence of different concentrations of elicitors **a** JA, **b** SA on multiple shoot culture growth (fresh weight) in *A. paniculata*. *Bars* represent the mean ± SE

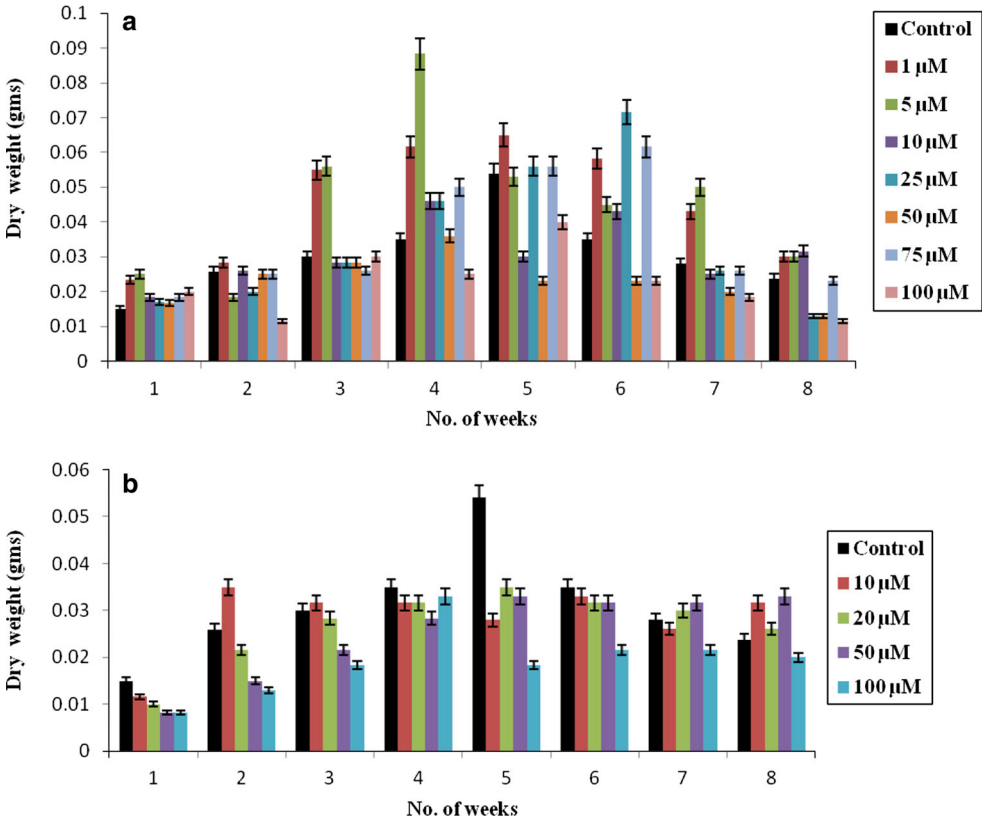

Fig. 5 Influence of different concentrations of elicitors **a** JA, **b** SA on multiple shoot culture growth (dry weight) in *A. paniculata*. *Bars* represent the mean ± SE

biomass (0.216 ± 0.055 g FW) compared to untreated control. Nevertheless, compared to control a marginal increase in growth was observed after eighth week when multiple shoots were treated with both 10 and 50 µM SA.

Compared to other concentrations of JA and control shoot cultures, DW was high after third and fourth week when 1 and 5 µM JA treatment was given. However, maximum DW (0.088 ± 0.011 g DW) of shoots was obtained with 5 µM JA compared to control (0.035 ± 0.004 g DW) after fourth week of culture. Higher DW was obtained after sixth week with 1, 25 and 75 µM JA action. On the other hand, shoot DW was more after seventh week following 1 and 5 µM JA application. Very low growth of shoot cultures was observed with 1, 5 and 10 µM JA on eighth week when compared to control shoots cultures (Fig. 5a). SA treated shoot cultures at different concentrations and culture duration revealed differential growth responses. Maximum shoot biomass (0.035 ± 0.007 g DW) was more with 10 µM SA after second week and 10 and 50 µM after eighth week when compared to control shoot cultures (Fig. 5b). Previous reports on the effect of SA on biomass production gave similar credence to the results observed in the present study. High concentration of SA (100 µM) resulted in inhibition of biomass from first to eight weeks and similar observations were also observed with multiple shoot cultures of *Ruta graveolens* (Diwan and Malpathak 2010) and *Withania somnifera* (Sivanandhan et al. 2013).

The influence of signal molecules JA and SA on andrographolide production in *A. paniculata* multiple shoot cultures revealed irregular responses. HPLC analysis of standard andrographolide, untreated control and randomly selected elicited multiple shoot cultures was carried out (Fig. 6a–c). Addition of 75 µM JA resulted in increased andrographolide production (0.813 % DW) when compared with untreated control cultures (0.548 % DW) after first week. Cultures treated with 50 µM JA and the analysis after second week resulted in comparable amount of andrographolide production (0.793 % DW) compared to 0.582 % DW in control cultures. Earlier, a treatment of 50 µM MeJA (derivative of JA) promoted enhanced production of bacoside A in multiple shoot cultures of *B. monnieri* (Sharma et al. 2013b). Further, a marginal decline in andrographolide content (0.776 % DW) was observed with 5 µM JA treatment after third week with control (0.590 % DW). Addition of JA at concentrations 1 and 25 µM and analysis of shoot cultures at the end of fifth week was effective and andrographolide content significantly enhanced up to 1.322 % (2.6-fold) and 1.253 % (2.4-fold), respectively; compared to control cultures (0.508 % DW). An increase in andrographolide content (1.012 % DW) was observed with 1 µM JA dosage after seventh week compared to control (0.891 % DW). However, a significantly sharp increase in andrographolide content of 1.624 (3.3-fold) and 1.481 % DW (3.0-fold) was obtained after eighth week with 25 and 50 µM JA concentrations, respectively (Fig. 7a). In an earlier finding it was found that a, longer exposure of *Salvia officinalis* shoots to high concentrations of MeJA treatment resulted in lower diterpenoid content, although the content were significantly higher compared to controls (Izabela and Halina 2009). In an earlier report threefold increase in bacoside content was obtained with an elicitor treatment of 1.0 mg/L JA (Sharma et al. 2015). A general trend of increase in andrographolide content was observed after eighth week with all treatments ranging from 1, 5, 10, 75 and 100 µM JA when compared to control. All the concentrations of elicitor (MeJA) facilitated higher bacoside A content, however; 50 µM MeJA treatment resulted in highest (twofold) production (Largia et al. 2015). JA at 1, 25, and 50 µM resulted in increased production of andrographolide. Higher concentrations of JA (75 and 100 µM) resulted in reduced andrographolide production. Comparable results on higher concentrations of JA (500 µM) was observed with decreased hypericin content in *H. hirsutum* and *H. maculatum* (Coste et al. 2011).

Addition of 10, 20 and 100 µM SA resulted in increased andrographolide content of 1.259 % DW, 1.323 % DW and 1.243 % DW, respectively; after fifth week of culture when compared to 0.804 % DW content following 50 µM treatment. Treatment of 20 and 50 µM SA was effective after sixth week old cultures in increasing the andrographolide content of 1.551 % and 1.494 %, respectively; than control (0.877 % DW). Further, a minor increase in andrographolide content of 1.169 % and 1.132 % DW was observed in 10 and 100 µM treated cultures, respectively after six week of culture. Previously, 50 µM SA resulted in enhanced digitoxin and bacoside production in *D. purpurea* and *B. monnieri* shoots; respectively (Patil et al. 2013; Sharma et al. 2015). The presence of higher levels of salicylic acid (100 µM) decreased the andrographolide production when compared to other concentrations. This observation is similar to previous reports on secondary metabolites production in multiple shoot cultures of *H. hirsutum* and *H. maculatum* (Coste et al. 2011). Addition of 10, 20, 50 and 100 µM SA resulted in enhanced andrographolide content of 1.481, 1.442, 1.467 and 1.352 % DW, respectively; compared to control (0.891 % DW) after seventh week. Treatment of shoots with 10, 20, 50 and 100 µM SA also resulted in improved andrographolide production of 1.479 % (3.0-fold), 1.654 % (3.4-fold), 1.483 % (3.1-fold) and 1.275 % DW(2.6-fold), respectively; contrast to control (0.478 % DW) after eighth week (Fig. 7b). Further, elicitation with 50 µM SA produced about threefold higher content of bacoside A; respectively, when compared to untreated control shoots (Largia et al. 2015). In the present study similar response was observed

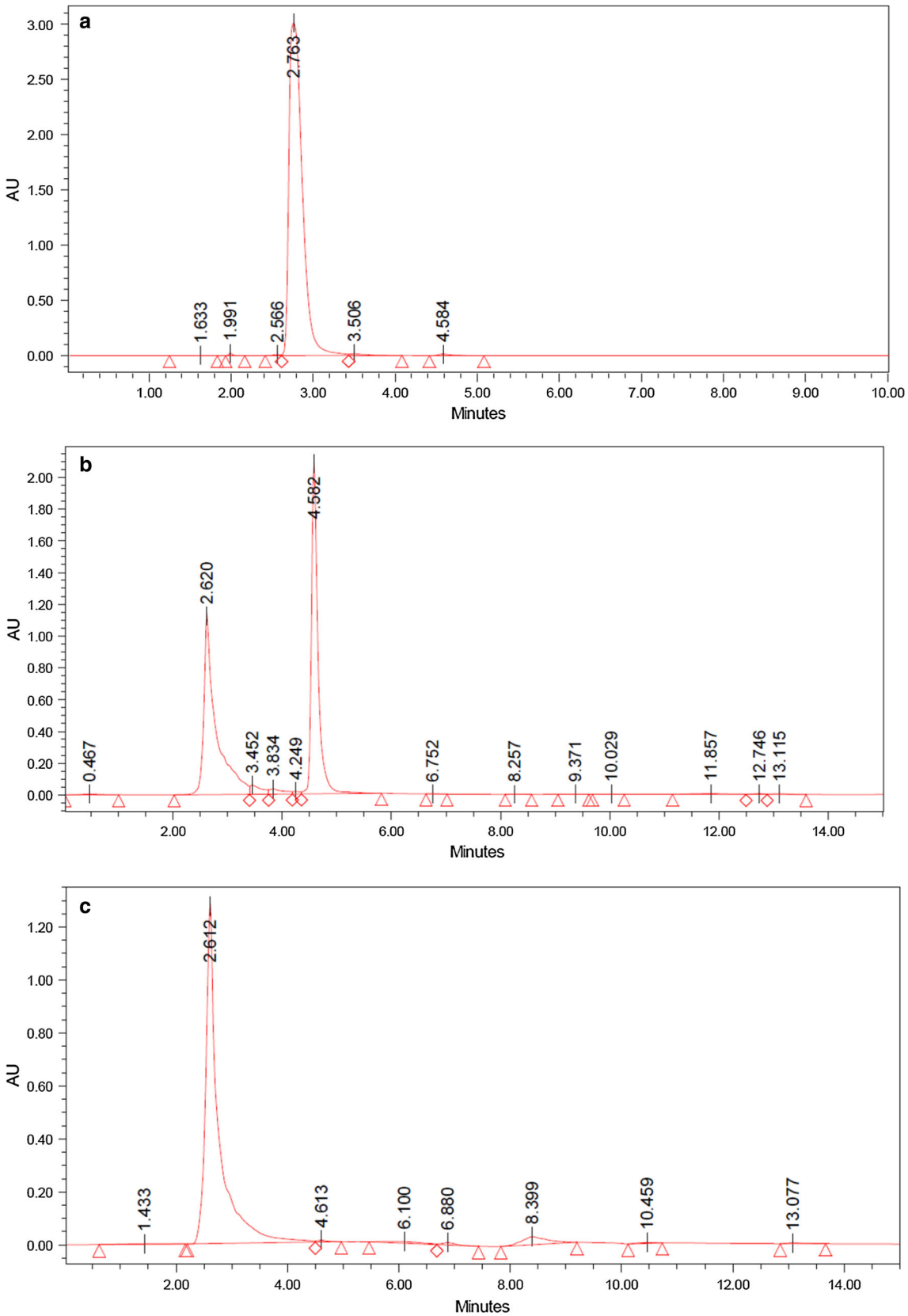

Fig. 6 HPLC chromatograms of **a** andrographolide standard; **b** control untreated cultures; **c** randomly selected elicited cultures

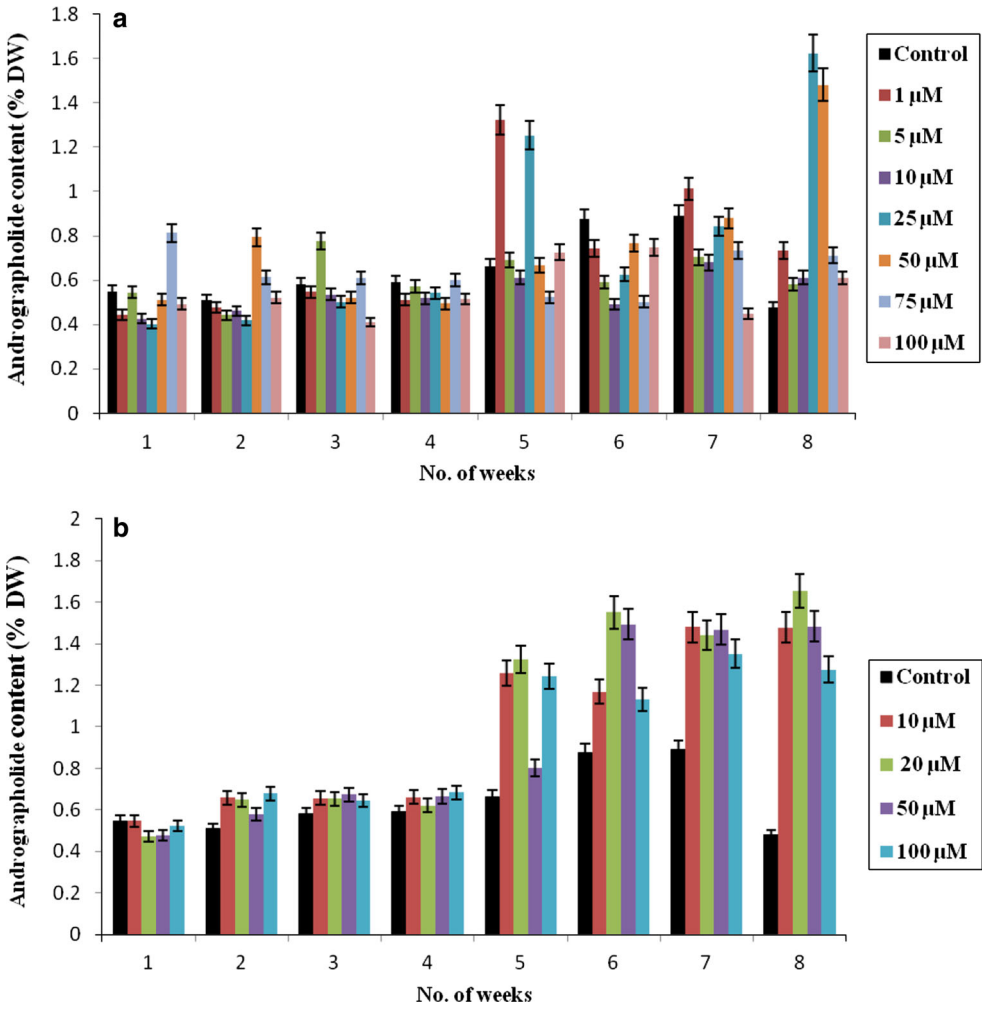

Fig. 7 Influence of different concentrations of elicitors **a** JA, **b** SA on enhanced andrographolide production in *A. paniculata* multiple shoot cultures. *Bars* represent the mean ± SE

following 50 µM SA elicitation. After eighth week low concentration of SA (20 µM) enhanced the production of andrographolide and it was the highest compared to other concentrations of SA. However, higher concentration of 100 µM SA decreased the andrographolide content. SA at higher concentrations (200 and 250 µM) decreased withanolides production, although their accumulation was significantly higher when compared to untreated control (Sivanandhan et al. 2013). Decrease in bacoside content with increased concentration of SA (100 and 200 µM) was observed in *B. monnieri* shoot cultures (Sharma et al. 2015).

There are several reports on the influence of many signal compounds including JA, SA and other chemical elicitors on in vitro culture systems (callus, cell suspension, adventitious roots, hairy roots and multiple shoot cultures etc.) for enhanced secondary metabolites production (Karuppusamy 2009; Ramakrishna and Ravishankar 2011). A number of other reports include valtrate production in *Valeriana amurensis* adventitious root cultures (Cui et al. 2012), glucosinolates production in *Sinapis alba* hairy root cultures (Kastell et al. 2013) and enhancement of vindoline and vinblastine production in suspension-cultured cells of *Catharanthus roseus* by artemisinic acid elicitation (Liu et al. 2014). Besides the use of signal molecules as elicitor heavy metals can also influence improved production of total andrographolides in cell suspension culture of *A. paniculata* (Gandi et al. 2012).

Conclusion

These results suggest that, andrographolide accumulation in multiple shoots of *A. paniculata* was influenced by both the elicitors (JA, SA) bringing about enhancement and variation. Out of the two elicitors, JA could result in increase of biomass at certain concentrations but SA showed inhibitory effect irrespective of different concentrations used. However, both the elicitors were found to be very effective in eliciting increased andrographolide production.

Our results showed that the application of signal molecules allowed optimal production of andrographolide in multiple shoot cultures of *A. paniculata.* Synthesis of secondary metabolites requires the plant tissue to perceive and react to various environmental signals in an interactive manner. The exogenous presence of elicitors (signal molecules), helps to alter the cellular dynamics and regulate the cellular environ (Kohli et al. 2013; Murthy et al. 2014). Outcome of the present study may be exploited for further enhancement in andrographolide production through biotechnological interventions in the diterpene lactone biosynthetic pathway using molecular approaches.

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Conflict of interest None.

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