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RESEARCH NOTE

Increased production of withanolide A, withanone, and withaferin A in hairy root cultures of *Withania somnifera* (L.) Dunal elicited with methyl jasmonate and salicylic acid

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Abstract *Withania somnifera,* an important medicinal plant that possesses a variety of bioactive secondary metabolites collectively known as withanolides. Hairy roots with an initial inoculum mass of 5 g FW were elicited separately with methyl jasmonate (MeJ) and salicylic acid (SA) at various concentrations for different exposure times after 30 days of culture. Enhanced production of biomass (32.68 g FW and 5.54 g DW; 1.23-fold higher), withanolide A (132.44 mg/g DW; 58-fold higher), withanone (84.35 mg/g DW; 46-fold higher), and withaferin A (70.72 mg/g DW; 42-fold higher) were achieved from 40 day-old harvested hairy roots elicited with 150 μM SA for 4 h exposure time. The present study reports a higher production of withanolide A, withanone and withaferin A from the elicited-hairy roots of *W. somnifera* under optimal inoculum mass, harvest time, elicitor exposure time and its concentration. These results will be useful for biochemical and bioprocess engineering for the viable production of withanolides in hairy root culture.

Keywords Elicitors · Exposure time · Inoculum mass · Time course · Withanolides · *Withania somnifera*

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Withania somnifera (L.) Dunal (*Solanaceae*), commonly known as 'Ashwagandha'/'Indian ginseng' is a highly valued medicinal plant in Indian Ayurvedic and African traditional systems. Pharmacological investigations revealed that the curative properties have been associated with withanolides that are present in the plant leaves and roots (Mirjalili et al. 2009). Field-grown plant materials have generally been used for commercial withanolide production, but the quality and quantity of withanolide constituents are highly affected by genotype and environmental conditions (Sivanandhan et al. 2012b).

As traditional cultivation of Indian ginseng is time-consuming and laborious and the demand of the dried product is increasing, cell and organ cultures have been used for production of secondary metabolites; however, yields of withanolide metabolites have been low. Attempts to increase levels of secondary metabolites in cell and organ cultures should be pursued as these metabolites can be produced in shorter periods of time (Sivanandhan et al. 2012b). Among various plant cell or organ culture systems, hairy root culture is one of the valuable tools for the biosynthesis of secondary metabolites, metabolic engineering studies, and production of root-derived compounds (Rao and Ravishankar 2002).

Previous reports recorded comparatively low yields of withanolides from hairy root cultures (Pawar and Maheshwari 2004; Kumar et al. 2005; Bandyopadhyay et al. 2007; Murthy et al. 2008). Exogenous addition of elicitors of biotic and abiotic origin in culture was considered to be one of the most promising strategies for the increased production of secondary metabolites (Radman et al. 2003). The elicitor molecule in culture interacts with a plant membrane receptor which activates specific genes, resulting in the synthesis of almost all chemical classes of secondary metabolites. Among the various abiotic elicitors, methyl

jasmonate (MeJ) and salicylic acid (SA) have been confirmed as effective elicitors for the induction of secondary metabolites in plant cell/organ cultures (Ketchum et al. 1999; Sivanandhan et al. 2012b). MeJ is a ubiquitous signaling molecule which mediates not only plant responses to environmental stress such as wounding, insect and pathogen attack (Wasternack 2007) but found to enhance a variety of secondary metabolites (Bonfill et al. 2011; Hu et al. 2011; Qu et al. 2011), including hairy root culture (Kim et al. 2009). Similarly, SA is also one of the most widely studied stress-signaling molecules and it influences plant resistance to pathogens and other stress factors (Rao et al. 2000; Kang et al. 2004; Sivanandhan et al. 2012b). Although, the role of MeJ and SA on enhanced production of secondary metabolites has been clearly established in hairy root cultures of several taxa, their effect was species-specific and there is no universal effect of a particular elicitor on different plants or cell culture systems (Radman et al. 2003).

A few reports are available on the elicitor(s) induced enhancement of withanolides in cell suspension culture (Ciddi 2006) and adventitious root culture (Sivanandhan et al. 2012a, b), but none in hairy root culture. Therefore, this investigation has been attempted involving various factors such as root inoculum mass, harvest time, and elicitor's (MeJ and SA) exposure time and their concentrations for an optimized production of hairy roots and withanolides (withanolide A, withaferin A and withanone) in culture. As far as we know, elicitation strategies developed in this investigation provide for the first time, a controlled and sustainable *W. somnifera* hairy root culture system, and higher production of major withanolides from hairy roots.

Mature seeds of *W. somnifera* were collected from Kolli Hills (Eastern Ghats–1,000 m), Tamil Nadu, India (Kolli Hills variety). The seeds were sterilized by immersion in 2.5 % (v/v) commercial bleach Teepol® (5.25 % Sodium hypochlorite) and in 0.1 % $HgCl_2$ (w/v; Qualigens, Mumbai, India) for 2 min. Then, the seeds were washed 4–5 times with sterilized distilled water and cultured on sterilized-cotton moistened with sterile water. The cultures were maintained at a temperature of 25 ± 2 °C, a 16 h photoperiod, and a light intensity of 50 μmol m^{-2} s^{-1} (white-fluorescent tubes). The leaf (length: 15–30 mm and width: 20–25 mm at the broadest) explants from 45-day-old in vitro seedlings were used to initiate the culture.

An agropine-type strain of *Agrobacterium rhizogenes* namely R1000 was used for hairy root induction. A single colony was cultured in LB medium (30 ml; Himedia, Mumbai, India) in darkness at 28 °C for 12 h on a rotary shaker at 180 rpm. The bacterial cells were pelleted by centrifugation at 8,000 rpm for 10 min followed by washing twice with liquid half strength MS basal medium (Murashige and Skoog 1962). The pelleted-bacterial cells were then dissolved in the same medium. To this bacterial suspension culture (OD_{600} = 1), acetosyringone [AS] (100 μM; Sigma, St. Louis, USA) and MES buffer (20 mM; pH 5.4; SRL, Mumbai, India) were added 1 h before infection. The leaf explants were pricked (10 pricks/explant) with a sterile hypodermic needle on the midrib region of the lamina. The wounded explants were subsequently immersed in the bacterial suspension culture for 15 min and blotted on sterile tissue paper for 10 min. Dried-explants were placed on half strength MS basal medium supplemented with 150 μM acetosyringone, MES buffer (20 mM), 3 % (w/v) sucrose (SRL, Mumbai, India), 0.2 % (w/v) phytagel (Sigma, St. Louis, USA) and co-cultivated in dark at 25 ± 2 °C for 5 days. After 5 days of co-cultivation, the explants were washed first with sterilized-distilled water followed by half strength MS basal liquid medium, with 300 mg/l cefotaxime (Alkime Laboratory, Mumbai, India) and transferred to 30 ml half strength MS basal medium with 3 % (w/v) sucrose, 0.2 % (w/v) phytagel, and 300 mg/l cefotaxime. Hairy roots appeared at the wounded sites of the explants in about 8–12 days of culture. These hairy roots (2–3 cm in length were excised from the explants and transferred to 30 ml half strength MS basal medium supplemented with 3 % (w/v) sucrose, 0.2 % (w/v) phytagel and 300 mg/l cefotaxime. The antibiotic was withdrawn from the medium after the residual bacteria had been completely killed. The hairy root segments were checked for bacterial contamination by culturing them in LB medium. The cultures were incubated under total darkness at 25 ± 2 °C.

Well-developed hairy roots (actively growing one cm root length from root tip) were used to establish hairy root culture with different root inoculum mass (1, 2, 3, 4, 5, and 6 g FW) in 50 ml MS basal half strength liquid medium containing 3 % (w/v) sucrose in 250-ml Erlenmeyer flask on a rotator shaker (80 rpm) at 25 ± 2 °C in darkness. During the culture, the hairy roots were harvested at regular time intervals (10, 20, 30, 40, 50, and 60 days) for the estimation of biomass and withanolides production. The hairy roots were subcultured during their log phase (10–40 days) at 7 days interval.

MeJ and SA (Sigma, St. Louis, USA) were prepared by following the method of Sivanandhan et al. (2012b). Elicitor stock solutions (25 μl) were added in the hairy root culture medium on 30-day-old cultures to yield the final concentrations of 0, 5, 10, 15, and 20 μM for MeJ and 0, 50, 100, 150, and 200 μM for SA. Control replicates received 25 μl of 99.9 % ethanol and 25 μl of Milli-Q water. The hairy roots were allowed to grow in the presence of MeJ and SA separately at different contact period (0, 2, 4, 6, and 8 h) on 30-day-old cultures when the culture exhibited maximum biomass in order to optimize the ample elicitation time for withanolides production. After elicitation treatment for

different contact periods, the hairy roots were again cultured on fresh half strength MS basal liquid medium without elicitors and harvested on 30, 35, 40, 45, and 50 days for the estimation of biomass and withanolides yield.

Estimation of biomass accumulation, extraction of withanolides and HPLC analysis of withanolides were performed by following the method of Sivanandhan et al. (2012a, b). Withanolide A standard was obtained from Chromadex Inc. (Laguna Hills, CA, USA). Withaferin A, and withanone standards were procured from Natural Remedies (Bengaluru, Karnataka, India). A completely randomized design was used for all treatments. All the experiments were repeated thrice with three replicates for each treatment. Data were statistically analyzed using analysis of variance (ANOVA), and were presented as the mean ± standard error (SE). The mean separations were carried out using Duncan's multiple range test and significance was determined at 5 % level (SPSS 11.5).

The transformed hairy roots cultured in LB medium did not show any bacterial growth proving the absence of live *A. rhizogenes,* in line with observations by Hayta et al. (2011) in the hairy root culture of *Gentiana cruciata*. In this study, a high transformation rate (90 %) was obtained in leaf explants infected with R1000 strain, with the production of 28.2 hairy roots (2–3 cm root length) per explant after 12 days of culture. The hairy roots produced were thin, soft and highly branched (Fig. 1). This extensive branching, due to the presence of many meristems, accounted for higher growth rates of hairy roots in culture as this phenomenon was common for members of *Solanaceae* (Flores and Filner 1985). The newly formed hairy roots were initially white and subsequently turned brown excepting the growing root tips and they exhibited the typical features of the hairy root syndrome, i.e., plagiotropic growth, hormone independence and extensive lateral branching. Plagiotropism of hairy roots was characteristic as the result of *A. rhizogenes*-mediated transformation (Gelvin 1990). In the present study, the hairy roots got emerged from the deep wounded sites of the midrib of leaf explants as observed by Tiwari et al. (2007) in *Gentiana macrophylla*. Nilsson and Olsson (1997) hypothesized that cells that contain high level of auxin and sucrose are ideal targets for hairy root induction. Tiwari et al. (2007) observed that the phloem cells, positioned deep in plant organs, could be the target of *A. rhizogenes*.

Of different inoculum mass and time course tested, 5 g FW hairy root inoculum mass yielded maximum accumulation of biomass (26.36 g FW, and 4.47 g DW) and withanolides [withanolide A (2.25 mg/g DW), withanone (1.81 mg/g DW) and withaferin A (1.65 mg/g DW)] after 40 days of culture (Figs. 2, 3). Our earlier reports confirmed that the production of withanolides and changes in the product yield could be obtained by optimizing the

Fig. 1 High frequency of hairy root induction from leaf explants of *W. somnifera* infected with R1000 strain after 12 days (*bar* 1 cm)

inoculum mass and harvest time in *W. somnifera* adventitious root culture (Sivanandhan et al. 2012a, b). Similar to our results, 5 g FW of inoculum mass favored the accumulation of biomass and ginsenoside production in ginseng hairy root culture (Kim et al. 2009). Enhanced secondary metabolite production and changes in product profile could be obtained by optimizing the inoculum size in culture (Kanokwaree and Doran 1997). The optimization of inoculum mass is a known fundamental factor in determining the success of tissue cultures for secondary metabolite production (Lee and Shuler 2000). Tiwari et al. (2007) correlated higher alkaloid production in hairy roots with increased root biomass. Pavlov et al. (2003) cultured hairy roots of *Beta vulgaris* up to 14-days and obtained highest betalain yield. Similarly, 45-day-old hairy roots of *Echinacea purpurea* yielded higher levels of cichoric acid and caftaric acid (Liu et al. 2002). It is clear from the present study that 5 g FW of initial inoculum mass and a time course of 40 days of culture are required for optimal biomass accumulation and withanolides production in the hairy root culture of Kolli Hills variety of *W. somnifera*.

MeJ and SA were added separately to 30-day-old hairy root cultures (10 days prior to the harvest period) for specific exposure times. The elicitor-treated hairy roots were then analyzed at regular intervals from 30-day-old cultures up to 50th day for withanolide production, since the biomass reached a maximum on 40-day-old cultures. The culture turned brown after 40 days in MeJ-treated hairy root cultures with subsequent death of roots after 50 days (Table 1). Our results are in agreement with Kuźma et al. (2009) and Sivanandhan et al. (2012b) who observed similar browning in *Salvia sclarea* and

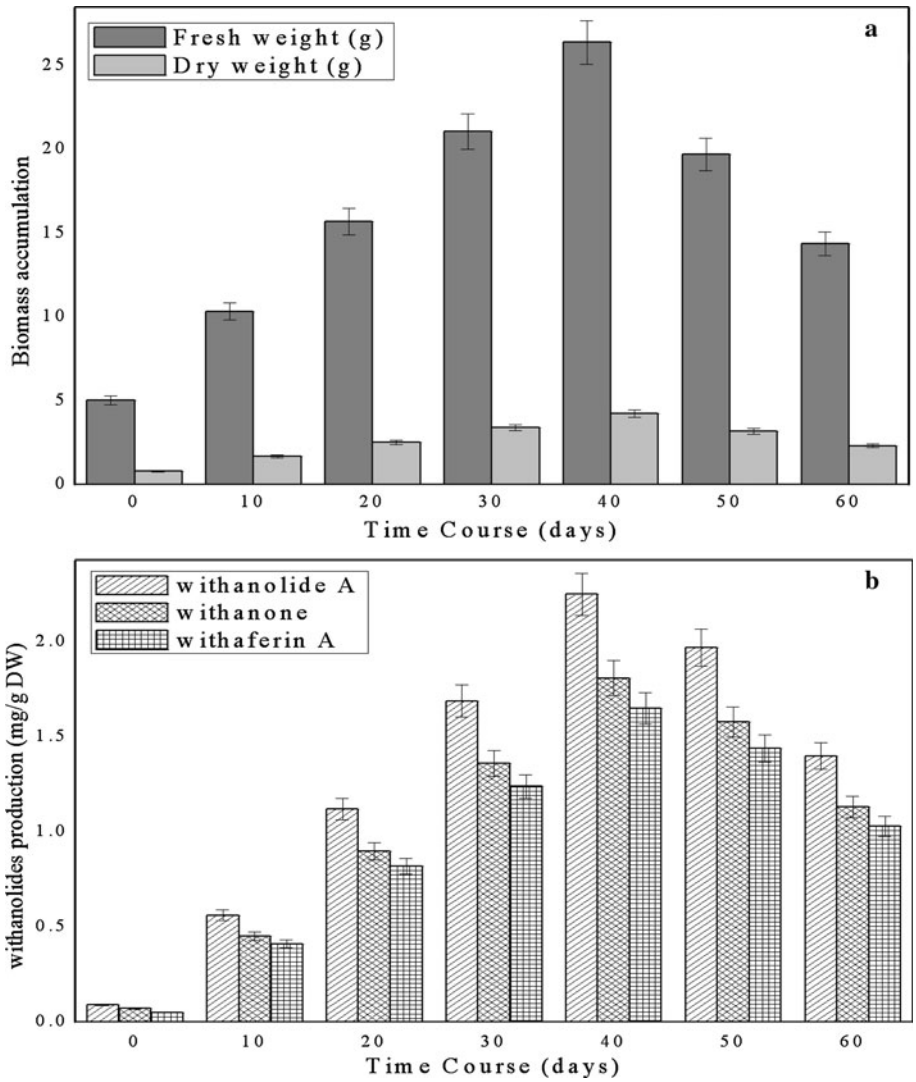

Fig. 2 Effect of inoculum mass and time course studies on biomass accumulation (**a**) and withanolides production (**b**) in *W. somnifera* hairy root culture. Values represent the mean ± standard error. The cultures were established with 5 g FW of initial inoculum mass (hairy roots derived from R1000 infected leaf explants) in 50 ml MS basal liquid medium

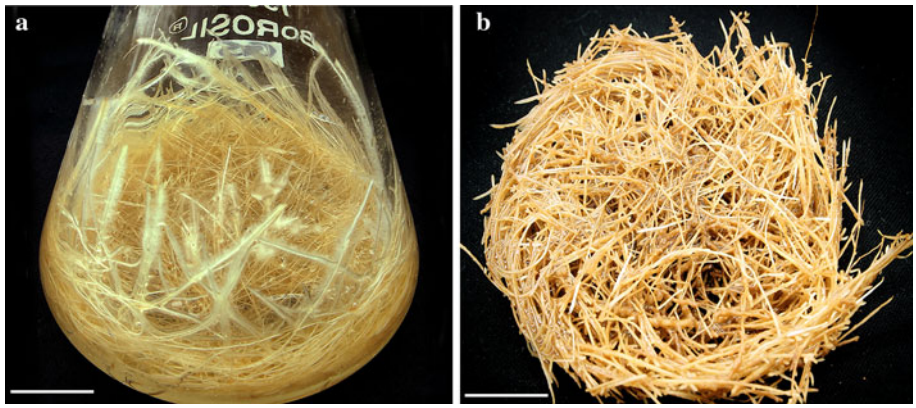

Fig. 3 Growth of hairy roots in *W. somnifera.* **a** Hairy root proliferation derived from R1000 infected leaf explants after 40 days of culture in 250 ml flask; **b** open view of hairy roots harvested after 40 days of culture in 250-ml Erlenmeyer flask. The cultures were established with 5 g FW as an initial inoculums mass in MS basal liquid medium supplemented with 3 % sucrose (*bar* 1 cm)

W. somnifera root cultures, respectively, upon MeJ elicitation. It has been suggested that root browning could be due to either phenolic compounds in response to elicitor-induced stress (Zhao et al. 2001), or due to direct toxic effect of MeJ and loss of viability of the cultures (Kuźma et al. 2009). There was a significant difference in biomass production between control and MeJ- elicited hairy root cultures. The inhibition of root growth was observed at higher concentrations (10–15 μM) of MeJ when compared to non-elicited hairy roots in line with observation of Veerashree et al. (2012) in *Gymnema sylvestre*. On the other hand, SA did not inhibit the root biomass when

Table 1 Effect of elicitation with MeJ and SA and exposure time on biomass accumulation in hairy root culture of *W. somnifera*

Elicitors (μM)	Exposure time (h)									
	0		2		4		6		8	
	FW	DW	FW	DW	FW	DW	FW	DW	FW	DW
MeJ										
0	26.36 ± 0.13^{a}	4.47 ± 0.11^{a}	26.36 ± 0.14^{c}	4.47 ± 0.12^{b}	26.36 ± 0.10^{d}	4.47 ± 0.18^{b}	26.36 ± 0.15^{b}	4.47 ± 0.13^{a}	26.36 ± 0.14^{b}	4.47 ± 0.19^{a}
5	26.36 ± 0.17^{a}	4.47 ± 0.16^{a}	24.41 ± 0.17^{e}	4.13 ± 0.14^{b}	18.62 ± 0.13^{e}	3.15 ± 0.15^{c}	13.73 ± 0.18^{e}	2.32 ± 0.17^{c}	7.26 ± 0.18^{f}	1.23 ± 0.14^{c}
10	26.36 ± 0.11^{a}	4.47 ± 0.14^{a}	20.32 ± 0.13^{g}	3.44 ± 0.13^{d}	14.43 ± 0.15^{f}	2.44 ± 0.13^{d}	9.68 ± 0.11^{f}	1.64 ± 0.11^{d}	4.43 ± 0.12^{g}	0.73 ± 0.17^{d}
15	26.36 ± 0.10^{a}	4.47 ± 0.10^{a}	14.52 ± 0.11^{h}	2.46 ± 0.17^{e}	8.28 ± 0.12^{g}	1.40 ± 0.10^{e}	3.31 ± 0.14^{g}	0.56 ± 0.15^{e}	1.72 ± 0.15^{h}	0.29 ± 0.16^{d}
20	26.36 ± 0.15^{a}	4.47 ± 0.17^{a}	8.82 ± 0.10^{i}	1.49 ± 0.19^{f}	3.22 ± 0.11^{h}	0.54 ± 0.11^{f}	1.12 ± 0.19^{h}	0.18 ± 0.13^{e}	0.43 ± 0.13^{i}	0.07 ± 0.13^{d}
SA										
0	26.36 ± 0.11^{a}	4.47 ± 0.15^{a}	26.36 ± 0.13^{c}	4.47 ± 0.16^{b}	26.36 ± 0.19^{d}	4.47 ± 0.16^{b}	26.36 ± 0.12^{b}	4.47 ± 0.15^{a}	26.36 ± 0.17^{b}	4.47 ± 0.14^{a}
50	26.36 ± 0.14^{a}	4.47 ± 0.13^{a}	22.35 ± 0.15^{f}	3.79 ± 0.14^{c}	26.74 ± 0.17^{d}	4.53 ± 0.13^{b}	20.66 ± 0.14^{d}	3.50 ± 0.13^{b}	18.55 ± 0.12^{e}	3.14 ± 0.16^{b}
100	26.36 ± 0.15^{a}	4.47 ± 0.11^{a}	25.83 ± 0.17^{d}	4.38 ± 0.10^{b}	29.48 ± 0.11^{c}	4.99 ± 0.15^{b}	24.43 ± 0.19^{c}	4.14 ± 0.12^{a}	22.34 ± 0.15^{d}	3.78 ± 0.18^{b}
150	26.36 ± 0.17^{a}	4.47 ± 0.19^{a}	29.56 ± 0.11^{a}	5.01 ± 0.09^{a}	32.68 ± 0.13^{a}	5.54 ± 0.17^{a}	28.12 ± 0.17^{a}	4.76 ± 0.14^{a}	27.61 ± 0.18^{a}	4.68 ± 0.13^{a}
200	26.36 ± 0.19^{a}	4.47 ± 0.10^{a}	27.77 ± 0.20^{b}	4.40 ± 0.18^{b}	30.72 ± 0.10^{b}	5.20 ± 0.10^{a}	26.68 ± 0.10^{b}	4.52 ± 0.10^{a}	24.12 ± 0.14^{c}	4.09 ± 0.11^{a}

MeJ and SA were added to 30-day-old cultures. The samples were taken at day 10 after post elicitation (after 40 days of culture). Data represents the mean ± standard error of three replicates; each experiment was repeated thrice. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5 % level

compared to MeJ elicitation. The biomass of hairy roots remained unaffected up to 150 μM SA during 50 days of culture and beyond 150 μM, the root growth declined marginally. SA at 150 μM for 4 h exposure time increased hairy root growth significantly on the 40th day of culture. Root growth was increased up to 1.23-fold higher in SA treatment (at 150 μM) when compared to MeJ (at 15 μM) (Table 1). On the other hand, elicitation with SA inhibited the biomass of adventitious root culture of *Scopolia parviflora* (Kang et al. 2004). Krzyzanowska et al. (2012) stated that elicitor concentration and the time of incubation with elicitor are crucial for the elicitation process. Elicitor specificity, its concentration and time of its exposure, as well as the culture conditions and growth stage of the cultured cells, influence the elicitation process (Vasconsuelo and Boland 2007).

Retention times of the standard compounds run by HPLC were as follows: withanolide A 12.5 min, withaferin A 14.3 min, and withanone 4.8 min. Production of withanolide A (2.25 mg/g DW), withaferin A (1.65 mg/g DW), and withanone (1.81 mg/g DW) was estimated in the non-elicited (control) hairy roots harvested after 40 days of culture (Fig. 2b). Both MeJ and SA elicitation exerted different effects on withanolide production in hairy root cultures. Production of withanolide A was increased with the addition of MeJ up to 15 μM and SA up to 150 μM. Withanolide A production was 114.38 mg/g DW (50-fold higher than control) at 15 μM MeJ elicitation for 4 h exposure time. The production of withanolide A was highest (132.44 mg/g DW) at 150 μM SA after 4 h exposure time and it was 58 times higher when compared to control (Figs. 4, 5). The maximum production of withanolide A with MeJ (15 μM) elicitation coincided with a decrease in biomass. Inhibition of root growth and promotion of secondary metabolite synthesis by MeJ treatment were observed in the hairy root culture and adventitious root culture of *Salvia sclarea* and *W. somnifera,* respectively (Kuźma et al. 2009; Sivanandhan et al. 2012b). Pauwels et al. (2008) reported that MeJ mediates the reprogramming of cellular metabolism and cell cycle progression via the regulation of jasmonic acid biosynthesis. Zhao et al. (2005) proposed a hypothesis that cellular process and regulatory principles are involved in the activation of plant secondary metabolite biosynthesis. Accordingly, an extracellular or intracellular signal is perceived by a receptor on the surface of the plasma membrane or endomembrane; the elicitor signal perception initiates a signal transduction network that leads to activation or de novo biosynthesis of transcription factors, which regulate the expression of biosynthetic genes involved in plant secondary metabolism as observed by Kim et al. (2009) in ginseng hairy root cultures. The resulting enzymes catalyze the biosynthesis of target secondary metabolites.

Withaferin A content also increased with the addition of MeJ up to 15 μM and SA up to 150 μM. Maximum withaferin A production was 57.46 mg/g DW (34-fold higher than control) with the elicitation of MeJ (15 μM) and its production was 42 times higher (70.72 mg/g DW) with SA elicitation at 150 μM when compared to control (Figs. 4, 5). Production of secondary metabolites in plant tissue, cell cultures and adventitious root cultures could be enhanced through elicitation with SA as in *Glycyrrhiza glabra* and *W. somnifera*, respectively (Shabani et al. 2009; Sivanandhan et al. 2012b). Ciddi (2006) reported 50-fold enhancement of withaferin A production (25 mg/l) using salacin as an elicitor in cell suspension culture of *W. somnifera*. In contrast to earlier reports, the hairy root culture reported in the present study recorded a higher withaferin A production.

Withanone content exhibited 38-fold higher production (69.89 mg/g DW) in the MeJ-elicited (15 μM) hairy roots, and it was 46-fold higher (84.35 mg/g DW) in SA (150 μM) elicitation when compared to their respective control (Figs. 4, 5). Therefore, the hairy roots of *W. somnifera* constitute an ideal system for the synthesis of withanolides in germ-free conditions and in the absence of expensive phytohormones in the culture system as advocated by Kumar et al. (2005). In the present study, we have optimized hairy root culture factors and clearly established a positive correlation between elicitor treatment and withanolide A, withaferin A and withanone production in the hairy root cultures of *W. somnifera* for the first time. In addition, hairy root culture established in the present study provides continuous production of withanolides in in vitro

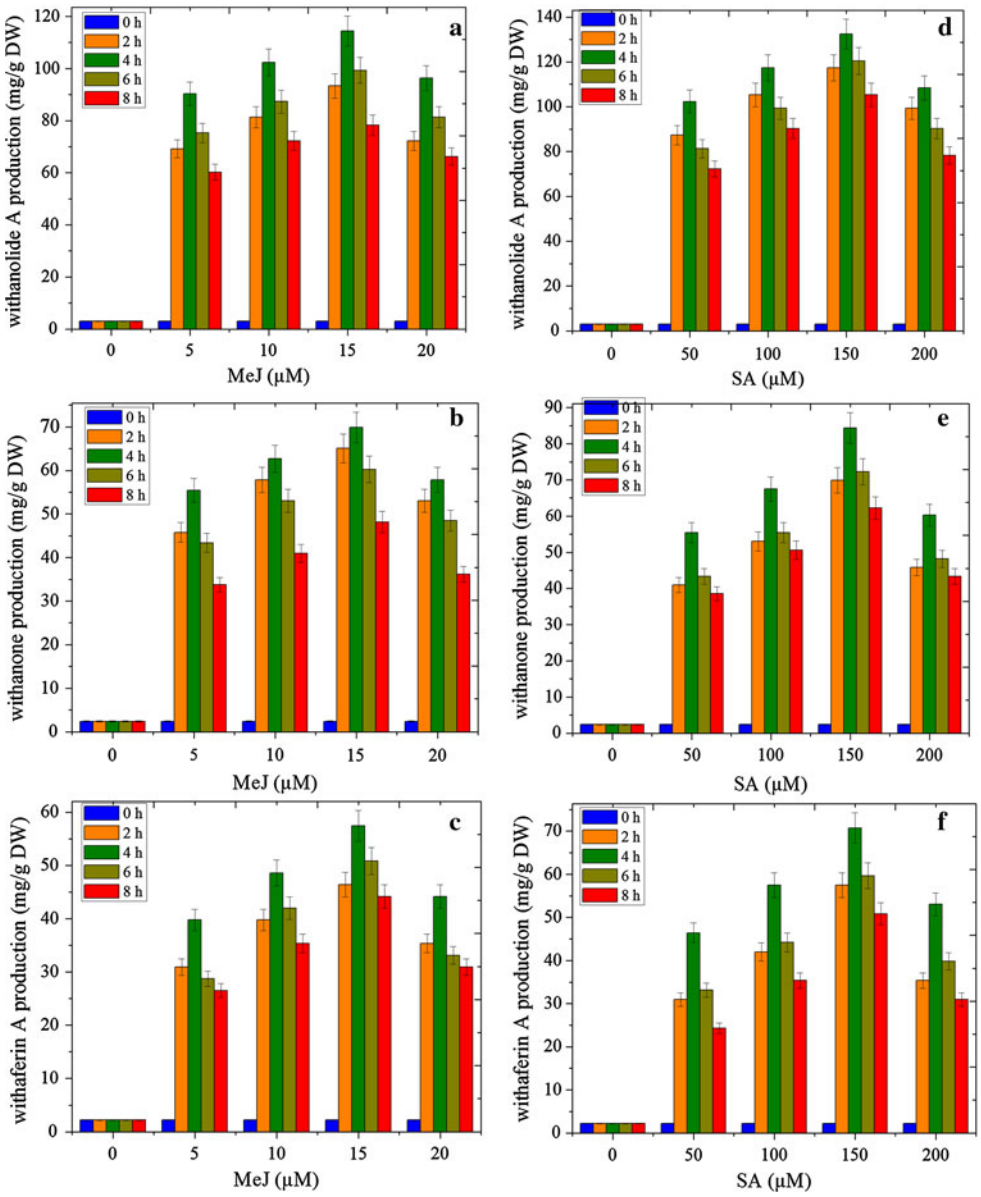

Fig. 4 Enhanced production of withanolides from hairy roots treated with various concentrations of MeJ and SA at different exposure time. Withanolide A (**a** and **d**), withanone (**b** and **e**) and withaferin A (**c** and **f**). Values represent the mean ± standard error of three replicates. The data were recorded after 40 days of culture. The cultures were established with 5 g FW of initial inoculum mass in 50 ml MS basal liquid medium

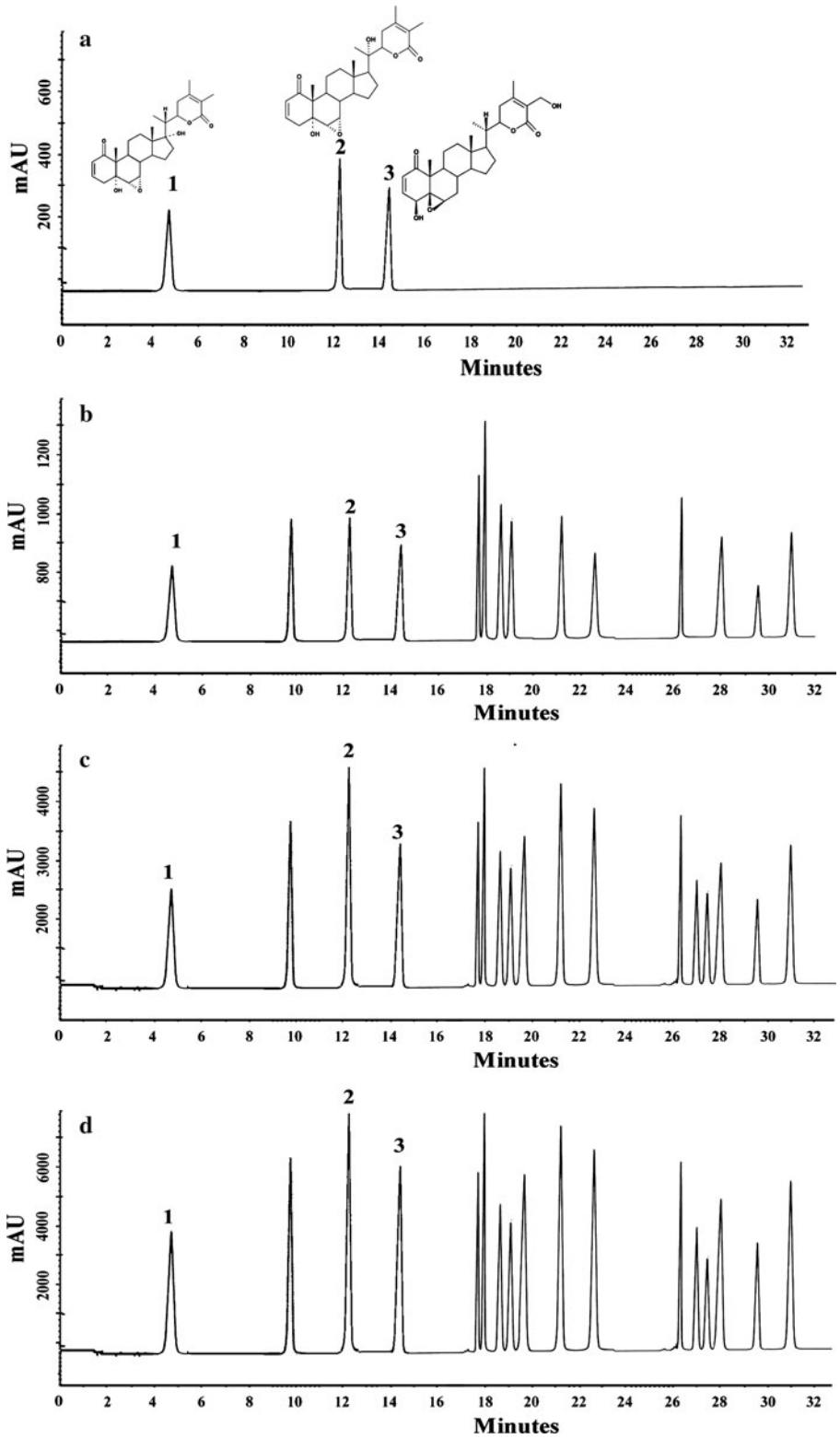

Fig. 5 HPLC analysis of methanol extracts from hairy root cultures of *W. somnifera*. **a** Standards of withanone (1), withanolide A (2) and withaferin A (3); **b** Control; **c** Hairy root cultures treated with 15 μM MeJ at 4 h exposure time; **d** Hairy root cultures treated with 150 μM SA at 4 h exposure time

all through the year by passing the vagaries of natural conditions. Further work is required to ascertain the exact mechanism of elicitation on the biochemical pathways that would trigger the higher production of withanolides in the hairy root culture of *W. somnifera,* since these compounds invite great commercial interest.

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