



# Innate endophytic fungus, *Aspergillus terreus* as biotic elicitor of withanolide A in root cell suspension cultures of *Withania somnifera*

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## Abstract

In the present study, root cell suspension cultures of *W. somnifera* were elicited with mycelial extract (1% w/v) and culture filtrate (5% v/v) of their native endophytic fungus *Aspergillus terreus* 2aWF in shake flask. Culture filtrate of *A. terreus* 2aWF significantly elicits withanolide A at 6H ( $12.20 \pm 0.52$  µg/g FCB). However, with *A. terreus* 2aWF mycelial extract, withanolide A content was higher at 24H ( $10.29$  µg/g FCB). Withanolide A content was maximum with salicylic acid (0.1 mM) treatment at 24H ( $8.3 \pm 0.20$  µg/g FCB). Further, expression analysis of withanolide pathway genes, hydrogen peroxide production, and lipid peroxidation was carried out after 48H of elicitation with 2aWF mycelial extract and culture filtrate. The expression levels of withanolides biosynthetic pathway genes, viz. *HMGR*, *DXR*, *FPPS*, *SQS*, *SQE*, *CAS*, *SMT1*, *STE1* and *CYP710A1* were quantified by real time PCR at 48H of elicitation. In all the treatments, the expression levels of key genes were significantly upregulated as compared to untreated suspension cells. Hydrogen peroxide was noticeably enhanced in SA, mycelia extract and culture filtrate, at 20% ( $115 \pm 4.40$  nM/g FCB), 42% ( $137.5 \pm 3.62$  nM/g FCB), and 27% ( $122.8 \pm 1.25$  nM/g FCB) respectively; however, lipid peroxidation was  $0.288 \pm 0.014$ ,  $0.305 \pm 0.041$  and  $0.253 \pm 0.007$  (µM/gm FCB) respectively, higher than the control ( $0.201 \pm 0.007$  µM/gm FCB).

**Keywords** *Withania somnifera* · Withanolide A · Endophyte · Elicitor · Suspension culture

## Introduction

*Withania somnifera* bioactives are a part of traditional medicine in various health supplements that can enhance immunity and longevity. Root and leaf bioactive compounds

from *W. somnifera* have been reported to be adaptogenic, cardio-protective, anti-oxidative, immunomodulatory, and possessing anti-cancer and anti-Alzheimer properties [1–3]. Pharmaceutically important bioactive compounds within leaf or root tissue of *W. somnifera* contain a group of 28 carbon steroidal lactone triterpenoids, collectively called ‘Withanolides’ that includes withaferin A, withanolide A, withanolide D, withanoside IV or VI, withanolide sulfoxide, etc. [4]. Withaferin A also disrupts cancerous cell by generating ROS, leakage of mitochondrial membrane potential [5], inhibiting telomerase [6], and changing the signaling pathways [7].

With increasing demand for withanolides, various attempts were made to increase the production of withanolides under *in-vivo* or *in-vitro* conditions. Under these circumstances, the application of biotechnological approaches to enhance withanolide production was made possible through cell/organ culture, which could provide new landscape to fulfill market demands. Ever since last decade, numerous avenues have successfully been explored to improve withanolides production through cell and organ

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culture [8]. Production of withanolide A, withaferin A, withanone, and others minor withanolides was successfully reported from shoot cell suspension, hairy root, and root suspension culture [9–12]. Increase in biomass and withanolide A accumulation in suspension culture was attempted by application of plant growth regulators, and nitrogen and carbon sources ratio in suspension media [13, 14]. Maximum biomass accumulation (147.81 g/L fresh weight) was recorded in medium containing 0.5× concentration of  $\text{NH}_4\text{NO}_3$ , however, maximum production of withanolide A content (4.36 mg/g DW) was recorded in the 2× concentration of  $\text{KNO}_3$  [13]. Ciddi et al. [9] inoculated salacin (750  $\mu\text{M}$ ) into cell suspension culture in production medium, leading to significantly enhanced production levels of withaferin A compared to unelicited controls. Sivanandhan and his group applied a variety of abiotic and biotic elicitors to hairy root culture and suspension culture to achieve maximum production of withanolides with enhanced biomass [15–17]. Amendment of aluminium chloride to hairy root culture results in substantial enhancement of withaferin A, withanolide A, withanolide B, withanoside V, and withanoside IV, as compared to control [16, 17]. However, treatment with biotic elicitor chitosan (100 mg/L) to root suspension culture leads to substantial enhancement of the total withanolides content both in shake-flask culture and bioreactor [17]. Squalene feeding to root suspension culture also plays a role in syntheses of key precursors withanolide A, withanolide B, withaferin A, withanone, 12 deoxywithanstramonolide, withanosides IV and withanosides V [18].

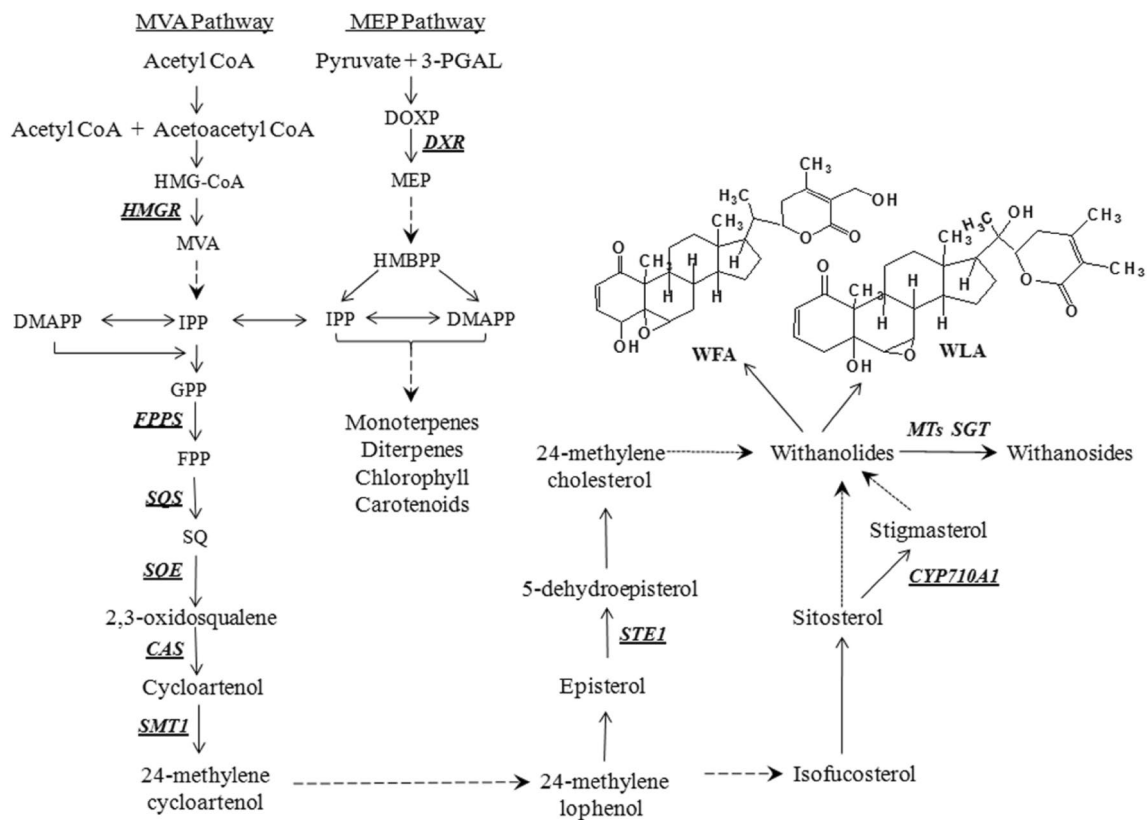
Among biotic elicitors, the application of fungal elicitors resulted in substantial augmentation in the production of a number of phytochemicals in plant tissue cultures [19, 20]. Cell wall-released heat-soluble elicitor prepared from *Rhizoctonia solani* was found to increase specific indirubin content in suspension culture of *Polygonum tinctorium* by 168% of the unelicited cell [19]. *Catharanthus* suspension cultures elicited with fungal extract (5% v/v) of *T. viride* for 48 H showed a threefold increase in ajmalicine content, whereas *A. niger* and *F. moniliforme* induced twofold increase in ajmalicine content in control sample [20]. Ahlawat and his group worked on the application of fungal cell homogenate and culture filtrate to bring about enhancement in withanolides content in suspension culture of *W. somnifera* [21, 22]. Culture filtrates and cell homogenate of *A. alternata*, *V. dahliae*, *F. solani* and *P. indica* at different concentration (1.0, 3.0, 5.0 and 7.0% v/v) have been applied to the suspension culture of *W. somnifera* in shake flask but *P. indica* cell homogenates (3.0% v/v) were resulted in maximum production of withanolide A ( $7.12 \pm 0.07$  mg/g DW), withaferin A ( $0.33 \pm 0.04$  mg/g DW) and withanone ( $5.97 \pm 0.06$  mg/g DW) [22]. Amendment of 10% v/v *T. harzianum* culture filtrate enhanced maximum biomass accumulation and total alkaloid content both in *Vinca minor* hairy roots culture as well as in cell suspension cultures [23]. However,

only 3.3% v/v *T. harzianum* culture filtrate accelerated biomass growth and sanguinarine production ( $0.090 \pm 0.008\%$  DW) in *Papaver somniferum* cell suspension culture [24]. Many research groups have not explored the biotic elicitors of inherent endophytic fungi in cell suspension cultures, which could probably bring up an immense potential for mass production of secondary metabolites.

The complete biosynthetic pathways of withanolides and their derivatives are still elusive [25]. Since withanolides originated from plant sterols, any modulation in sterol biosynthetic pathway genes could directly affect withanolides content in plants [26]. Thus, sterols of endophytic fungus of native host plant could also elicit withanolides production.

In plants, the formation of 3-isopentenyl pyrophosphate (IPP) follows the common chloroplastic MEP and cytoplasmic MVA pathway; from IPP to withanolide precursor 24-methylenecholesterol, various steps proceed through GPP, FPP, SQ, 2,3-oxidosqualene, cycloartenol, cycloartenol, 24-methylene cycloartenol, 24-methylene lupinol, episterol, and finally 5-dehydroepisterol (Fig. 1). Till date, no report is available for the production of transgenic *W. somnifera* at commercial level, which could be cultivated for substantially enhancing the withanolides content within plants. Due to limited knowledge of the complete pathway, some transgenic plants were developed which expressed an intermediate pathway gene; For example, the overexpression of *WsCAS* or *GrDXS* gene in transgenic *W. somnifera* have been shown to exhibit higher withanolides content in leaves as compared to control [27, 28]. Overexpression of squalene synthase (*SQS*) in *W. somnifera* leads to enhanced withanolide biosynthesis in roots stem and leaves [29, 30]. However, the transient suppression of squalene synthase gene in *W. somnifera* results into substantial decrease in phytosterol and withanolide content in leaves, and plants become susceptible for pathogens [31].

The present study was designed to evaluate the elicitation potentiality of native endophytic fungus, *Aspergillus terreus* 2aWF, in root cell suspension cultures of *W. somnifera*. Here we report, for the first time, the effect of culture filtrate and mycelial extract of native endophytic fungus, *A. terreus* 2aWF in root cell suspension cultures of *W. somnifera*, in eliciting withanolide A and modulation of key withanolide biosynthetic pathways genes in comparison to salicylic acid (SA). Additionally, lipid peroxidation and hydrogen peroxide content was estimated in the presence of SA, culture filtrate, and mycelial extract of *A. terreus*.



**Fig. 1** Schematic presentation of important steps of withanolides biosynthetic pathway in *W. somnifera*. Solid arrows indicate single-step reactions, dashed arrows indicate several steps, dotted arrows represent unidentified steps and the double arrow show isomers conversion or the exchange between cytosolic and plastid compartments. Bold with underlined genes are quantified in this study. CAS (cycloartenol synthase), CYP710A1 (C-22 sterol desaturase), DMAPP (dimethylallyl diphosphate), DOXP (1-deoxy-D-xylulose 5-phosphate), DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase), FPP (farnesyl diphosphate), FPPS (FPP synthase), GPP (geranylge-

ranyl diphosphate), GPPS (geranyl diphosphate synthase), HMBPP (4-hydroxy-3-methylbut-2-enyl diphosphate reductase), HMG-CoA (3-hydroxy-3-methylglutaryl-CoA), HMGR (HMG-CoA reductase), IPP (isopentenyl diphosphate), MEP (2-C-methyl-D-erythritol 4-phosphate), MTs (methyl transferase), MVA (Mevalonate), 3-PGAL (glyceraldehyde 3-phosphate), SGT (sterol glycosyl transferase), SMT1 (sterol methyltransferase 1), SQE (squalene monooxygenase/epoxidase), SQS (squalene synthase), SQ (squalene), STE1 (C-5 sterol desaturase). WFA withaferin A, WLA withanolide A

## Materials and methods

### Aseptic plantlet development and establishment of callus culture

*W. somnifera* Dunal var. Poshita seeds were washed 5 times with autoclaved distilled water, then soaked in 0.01% mercuric chloride for 30 s and washed 5 times with autoclaved distilled water. Thereafter, seeds were treated with 70% ethanol for 90 s, and washed 5 times with autoclaved distilled water. Surface sterilized seeds were inoculated in tissue culture bottle containing half strength MS media [32] and maintained at  $25 \pm 2$  °C at 16H light and 8H dark cycle for 5 weeks. Five weeks grown roots were used as explants for tissue culture. Aseptically treated roots were chopped into small pieces (0.5 cm), transferred onto callus induction media (CIM) plate (MS 4.4 g/L, sucrose 3%, 2,4-dichlorophenoxyacetic

acid (2,4-D) 2 mg/L, kinetin 0.2 mg/L, agar 0.8%, pH 5.8) [33], and kept at  $25 \pm 2$  °C in dark for 4 weeks. The pH of the medium was maintained using 0.1 N NaOH/HCl prior to the addition of agar. The medium and accessory materials were autoclaved at 121 °C at 15 lbs for 20 min.

### Cell suspension culture establishment

After 4 weeks, creamish color callus was formed around the roots; the new growth was subcultured onto fresh callus induction media. After 4 weeks of subculture, around 500 mg of fragile creamish color callus was inoculated in 250 mL Erlenmeyer flask containing 50 mL of cell suspension medium supplemented with the same hormone concentration without agar. The cultures were maintained on gyratory shaker at 100 rpm under total darkness at  $25 \pm 2$  °C. Cell suspension cultures were maintained in 250 mL Erlenmeyer

flask through sub-culturing every 4 weeks at 1:4 dilutions in 50 mL of cell suspension medium supplemented with the same hormone concentrations without agar.

### Preparation of elicitor from native leaf endophytic fungi

*Aspergillus terreus* strain 2aWF as native endophytic fungus was previously isolated from the leaf of *W. somnifera* (L) Dunal. Complete procedure for elicitor's preparation from native endophytic fungi is depicted in Fig. 3. Agar plugs, 6 mm each, were punched out from actively growing edges of the PDA plates with fungus growth across the plate, cultured with a sterilized cork-borer, and inoculated in 500 mL Erlenmeyer flask containing 100 mL modified CzapekDox (Sucrose 15 g/L,  $\text{NaNO}_3$  1 g/L,  $\text{KH}_2\text{PO}_4$  1 g/L,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g/L, KCl 0.5 g/L,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.1 g/L) and kept at 28 °C for 10 days in static conditions. Thereafter, mycelia were separated from the culture filtrate with the help of Whatman filter paper No. 1 and washed twice with autoclaved distilled water to remove traces of modified Czapek Dox culture filtrate. Filtered culture filtrate was considered as an elicitor, as previously reported in [23]. Filtered culture filtrate (100% v/v) was sterilized with syringe filter membrane (0.22  $\mu\text{m}$ , Merck Millipore) and directly used as elicitor called 'culture filtrate'. Washed mycelia were semidried with blotting paper followed by grinding in liquid nitrogen with the help of mortar pestle and finally dissolved in double distilled water (10% w/v). Water dissolved mycelial extract was sterilized by autoclaving, followed by filtration of the supernatant with syringe filter membrane (0.22  $\mu\text{m}$ , Merck Millipore), and used as elicitor called 'mycelia extract'.

### Elicitor treatment in cell suspension culture and biomass estimation

Cell suspension culture was maintained in the same hormone composition of medium without agar, subcultured every 4 weeks in 1:4 dilutions, and kept on gyratory shaker at 100 rpm under total darkness at  $25 \pm 2$  °C. Exponential growing cell suspension cultures (28th day of subculture) were accounted for culture filtrate and mycelia elicitor treatment. 2.5 mL of culture filtrate (100%) or 5 mL of water extract mycelia elicitor (10% w/v) were added individually to 50 mL of growing cell suspension to reach final concentrations 5% (v/v) and 1% (w/v) respectively. Here, we used reported salicylic acid concentration (SA 0.1 mM) as positive elicitor, which was induced the withanolides content in leaves of *W. somnifera* [34]. After treatment, samples were collected at different time points, such as 6H, 12H, 24H and 48H, for biomass and withanolide estimation. For RNA extraction, samples were stored at  $-80$  °C. At each time point, suspension cells culture were collected from

suspension media and filtered with Whatman paper for fresh biomass measurement. Each treatment was preceded with three biological replicates.

### Withanolides isolation from fresh cell biomass and HPLC analysis

Fresh cell biomass (2.5 g) was dissolved in 5 mL ethyl acetate for withanolide extraction and kept overnight on shaker at 100 rpm at room temperature. Ethyl acetate extract was collected and the procedure repeated thrice for complete extraction. Pooled ethyl acetate extract was dried at room temperature and dissolved in HPLC grade methanol. Dissolved samples were subjected to HPLC analyses (Shimadzu 10AVP; Shimadzu, Kyoto, Japan) Zodiac C-18 (250 mm  $\times$  4.6 mm, 5  $\mu$ , S.N.220814A516). Two solvents were used for the analysis that consisted of 0.1M of ammonium acetate ( $\text{CH}_3\text{COONH}_4$ ) dissolved in 18 M $\Omega$  MQ water (solvent A) and acetonitrile (solvent B). Gradient program was set for 40 min run, having solvent A at 95%, comprising 55% A at 18 min and then to 20% A at 25 min. Constantly, 20% A was maintained from 25 to 28 min, and elevated to 55% of A at 38 min and finally run stabilized to 95% A at 40 min. The flow rate was set at 0.5 mL/min, and the chromatograms were monitored at a wavelength of 227 nm with UV detector. Peaks of withanolide A were identified by comparing with their retention time similar to standards.

### RNA extraction and cDNA synthesis

Total RNA was isolated from 100 mg of treated and untreated cell suspension cultures using the RNA isolation kit (Sigma-Aldrich) according to the manufacturer's instructions. Genomic DNA contamination in extracted RNA was removed by treatment with RNase-free DNase I enzyme (Ambion). Quantity and quality of RNA were checked at OD 260/280 by using the spectrophotometer (Eppendorf biospectrometer). 2  $\mu\text{g}$  of RNA was preceded for first-strand cDNA synthesized using the ReadyScript® cDNA Synthesis Kit (Sigma-Aldrich) according to the manufacturer's instructions.

### Real-time-quantitative PCR (RT-qPCR) analysis

Primers for the real-time quantitative PCR (RT-qPCR) were followed as previously designed by Singh and his group [31] (Suppl. Table 1). RT-qPCR was performed using SYBR® Green JumpStart™TaqReadyMix™ (Sigma-Aldrich) detection on technical replicates of triplicate biological samples from each of the three plants. PCR conditions followed were: initial denaturation for 10 min at 95 °C, followed by 40 cycles as of denaturation at 95 °C for 15 s, and annealing or extension at 60 °C for 1 min. Fluorescent signal was

captured and analyzed by StepOnePlus™ Real-Time PCR System (Applied Biosystems). For specificity, evaluation amplicons were subjected to melting curve analysis using the dissociation method (Applied Biosystems). Threshold cycle ( $C_t$ ) value of each gene was recorded and normalized against the  $C_t$  value of 18S gene (as the constitutive reference transcript). For relative quantification,  $2^{-\Delta\Delta C_t}$  method was followed, which was carried out with average  $C_t$  values of three technical repeats of each transcript in treated and untreated cell suspension culture [35].

### Hydrogen peroxide determination

Hydrogen peroxide production was estimated at 48H of elicitor treatment according to Junglee and co-worker [36]. 200 mg of cell suspension culture was grounded in 1 mL of 0.1% (w/v) TCA in chilled mortar-pestle and then centrifuged at 12,000 rpm for 15 min at 4 °C. 400  $\mu$ L of supernatant was mixed with 400  $\mu$ L of 10 mM potassium phosphate buffer (pH 7.0) and 800  $\mu$ L of 1M potassium iodide (KI) in a 2 mL eppendorf tube. All mixtures were gently mixed and measured for absorbance at 390 nm;  $H_2O_2$  production was determined with standard Hydrogen peroxide. Standard curve was prepared for absorbance against concentration of  $H_2O_2$  in solution.

### Lipid peroxidation

Extent of lipid peroxidation was determined according to [37] by estimating the malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction. 200 mg of cell suspension culture were homogenized in 1 mL of TBA (0.25% w/v) solution in 10% TCA with the help of chilled mortar and pestle. Homogenized mixture was kept at 95 °C for 30 min and then quickly cooled on ice to stop reaction. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C and absorbance of the supernatant measured at 532 nm for MDA-TBA complex. Non-specific absorbance was corrected by subtracting the absorbance at 600 nm, and the amount of MDA-TBA concentration determined by its molar extinction coefficient (155  $mM^{-1} cm^{-1}$ ).

### Trypan blue staining of suspension cells

The viability of suspension cells was determined by the trypan blue staining method [38]. 0.4% trypan blue solution was prepared (40 mg of trypan blue dissolved in 10 mL lactic acid, 10 mL phenol pH 7.5–8.0, 10 mL glycerol, 10 mL of distilled water) and used for staining of suspension cells. 100  $\mu$ l of suspension culture treated with equal volume of trypan blue solution was mixed uniformly and kept at room temperature for 5 min. After incubation, 20  $\mu$ l of dye treated

suspension cells were placed on microscopic glass slide and visualized under bright field microscope.

### Statistical analysis

Three biological replicates were considered for each experiment and presented as mean  $\pm$  SD. For statistical analysis one-way ANOVA, with Dunnett as a post-test, was calculated using Graph Pad prism, version 5.01 (GraphPad Software, San Diego, CA). The statistical significance of differences between control and treated samples was at different P-values, and are reported in figure legends.

## Results and discussion

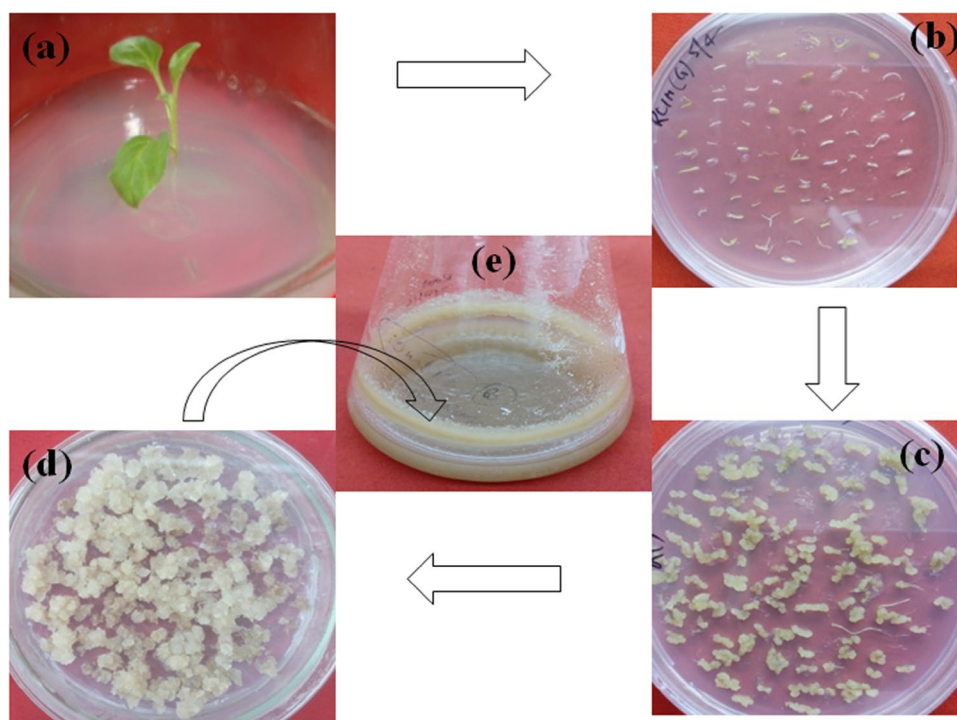
### Callus development and kinetics of biomass accumulation in cell suspension culture

All steps, starting from plant explants till the suspension culture development, are shown in Fig. 2. Plants were successfully grown in tissue culture bottles and the chopped root explants (0.5–1 cm) aseptically transferred onto root callus induction media. After 15 days of transfer, callus formation started to appear around the root surface and cut root ends. After 4 weeks, the calli were transferred on fresh callus induction media plate for more proliferation and appeared fragile creamish in color. Maximum percentage of callus induction was induced in combination with 2,4-Dichlorophenoxyacetic acid (2 mg/L) and Kinetin (0.2 mg/L) from root explants [33]. This creamish color callus was maintained on the same composition of semi-solid media for the further experiments.

Approximately 500 mg of fragile, creamish color callus was inoculated in suspension culture media and kept on rotatory shaker at 100 rpm at  $25 \pm 2$  °C for 30 days. From the first batch of suspension culture, 10 mL suspension cell was inoculated into Erlenmeyer flask (250 mL) containing freshly prepared 40 mL suspension media. After inoculation from 10th days at every 5 day of interval for 30 days, 10 mL of suspension culture was taken for fresh cell biomass estimation. Cell biomass increased with time, it was 0.9, 1.18, 1.77, 2.23 and 2.33 g/10 mL suspension culture at 10, 15, 20, 25 and 30 days respectively, which could be an equivalent of ‘exponential growth phase’ (Fig. 4a). Fresh cell biomass did not change significantly in the period from 25 to 30 days of subculture; here we considered 30th day for measurement of maximum cell growth (23–25% w/v FCB). Sivanadhan and his co-worker found exponential growth of root suspension culture for the period of 7–28 days, maximum growth reaching on the 28th day (14.72 g FCB/30 mL) [18].

Viability of suspension cells, prior to elicitor treatment was determined by staining the cells with trypan blue and

**Fig. 2** Different steps for callus and cell suspension culture development from root of *W. somnifera* (a) 5 weeks old plantlets grown under controlled conditioned on  $\frac{1}{2}$  MS media in tissue culture bottle at  $25 \pm 2$  °C (b) aseptically transfer of chopped root explant on callus induction media for 4 weeks (c) after 4 weeks callus was formed round root and cut end (d) well grown creamish color fragile callus after 30 days of subculture on callus induction media (e) suspension culture in 250 mL Erlenmeyer flask after 30 days inoculation of fragile callus, grown under dark condition at 100 rpm and  $25 \pm 2$  °C



observed under bright field microscope (Fig. 4b). Generally trypan blue staining differentiates live and dead cells in suspension culture [38]. In a viable cell trypan blue is not absorbed; however, it traverses the membrane in a dead cell. It enters the dead cell through plasma membrane and stains complete cytoplasm in blue color. Because of negatively charged trypan blue, enters damaged cell membrane and gives intense blue color to cytoplasm, while viable cells maintains the membrane integrity hence do not take dye and remained unstained [39]. Arrow in Figure No 4(b), indicates actively growing, banana shaped cells of *W. somnifera* root suspension cells. Similar to this observation, previous reports on cell suspension culture of strawberry and tobacco suspension cells have shown round and elongated cells [40, 41].

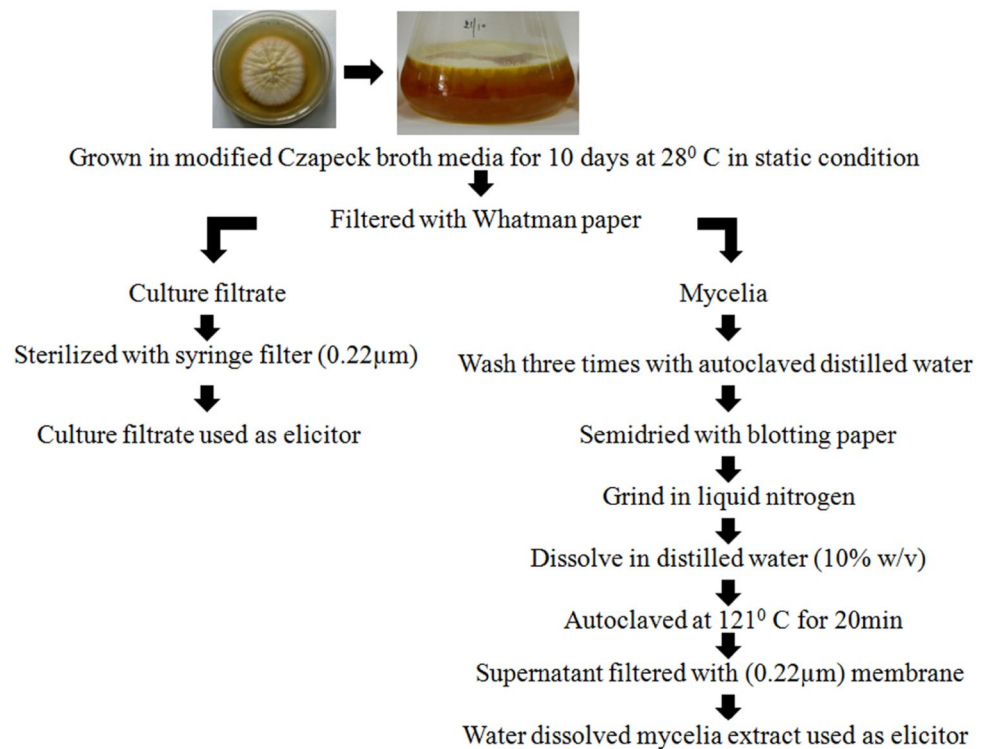
### Impact of elicitors on biomass accumulation

Mycelial extract and culture filtrate elicitor were prepared from *W. somnifera* native endophytic fungus *A. terreus* grown in modified Czapek Dox media in static conditions for 10 days at 30 °C (Fig. 3). Earlier reports highlighted dose dependent application of (3.3, 6, and 10% v/v) culture filtrate of endophytic fungi to induce the biomass and sanguinarine production from *P. somniferum* suspension culture [24]. In this study, 5% (v/v) culture filtrate of native endophyte *A. terreus* and only 1% (w/v) of their mycelia extract were used. Elicitors were applied during exponential growth stage in 28 day old suspension culture, and fresh

cell biomass was approximately 23.8–25% w/v FCB in all culture shake flask. Fresh cell biomass after treatment with culture filtrate at 6H, 12H, 24H and 48H of culture filtrate was determined to be  $2.5 \pm 0.059$ ,  $2.55 \pm 0.012$ ,  $2.62 \pm 0.080$  and  $2.65 \pm 0.082$  gm FCB/10 mL suspension culture, respectively. However, in mycelia extract treatment, cell biomass was found to be  $2.47 \pm 0.079$ ,  $2.5 \pm 0.07$ ,  $2.55 \pm 0.12$ , and  $2.61 \pm 0.062$  g/10 mL suspension culture at 6H, 12H, 24H and 48H of treatment, respectively. In case of abiotic elicitor SA also did not show any significant change in cell biomass even at 6H ( $2.38 \pm 0.047$  g/10 mL) and 48H ( $2.38 \pm 0.082$  g/10 mL), and it remained the same (Fig. 4). Hence, it was imperative that the application of mycelia extract or culture filtrate did not show any significant change on cell growth.

Secondary metabolite accumulation in suspension cell culture started after the 7th day of subculture, and synthesized profusely as cell growth entered the exponential stage [42]. The application of endophytic fungus *P. indica* cell homogenate (3% v/v) for 7 days to *W. somnifera* suspension culture on 15th day significantly enhanced biomass ( $15.2 \pm 0.56$  g/L) and withanolide accumulations ( $4.9 \pm 0.23$  mg/L) [21]. Addition of sea weed extract of *Sargassum wightii* and *Gracilaria edulis* in shoot suspension culture of *W. somnifera* resulted in significantly enhanced cell biomass and withanolide production [43]. Exposure of suspension culture after 5 weeks for 24 h to 40% weed extract of *G. edulis* and 50% of *S. wightii* significantly evoked biomass production up to 62.4 g FW and 56.80 g

**Fig. 3** Diagrammatic representation of different steps for biotic elicitor preparation from endophyte *A. terreus*

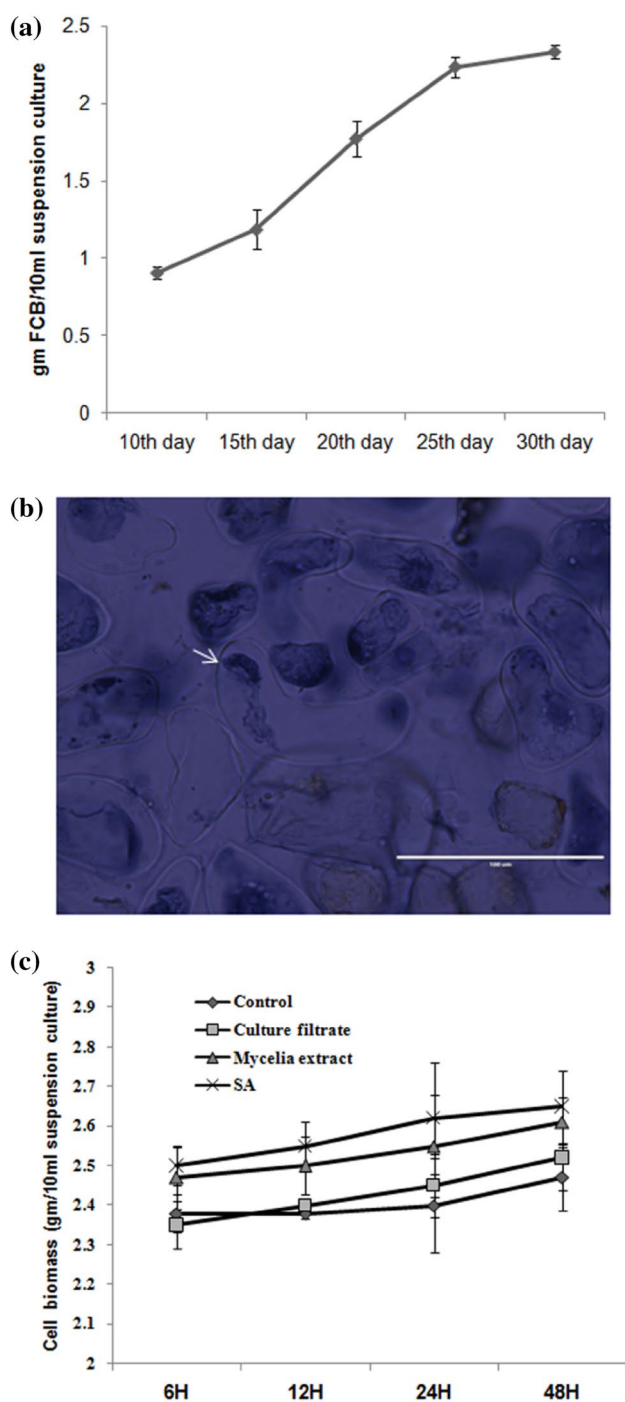


FW respectively [43]. Verma and her group applied fungal elicitor from endophytic fungi, *T. harzianum* culture filtrate, at the dose of 3.3% v/v, and induced maximum biomass accumulation ( $GI = 293.50 \pm 14.82$ ) as well as highest sanguinarine production ( $0.090 \pm 0.008\%$  dry wt.) [24]. However, 10% culture filtrate of *C. globosum* and *A. niveoglaucus* showed maximum growth index of  $202.00 \text{ m} \pm 25.40$  and  $187.50 \pm 2.80$ , respectively, but showed lesser sanguinarine accumulation in 5.0 L bioreactor [24]. In the present study, none of the elicitors showed significant changes in suspension cell biomass accumulation between the exposure times 6–48 H; the possible reason behind this might be that the endophytic fungi neither secreted any growth promoting exogenous elicitors in culture filtrate nor was any component present in mycelia extract to induce the cell suspension growth within treatment point.

### Effect of elicitors on withanolides production in suspension cell culture

*Withania somnifera* roots are known to be a prominent source for the production of withanolide A, so we examined the effect of abiotic elicitor SA (0.1 mM), and biotic elicitor culture filtrate and mycelia extract on withanolide A production in suspension culture at various times point (6H, 12H, 24H and 48H) in shake flask. SA and Me-JA are well known for hormone production in plant under abiotic or biotic stress conditions, although its external application enhances secondary metabolite accumulation

and resistance against the environmental stress [44–46]. All the three elicitors significantly induce withanolide A content at each time point compared to control (Fig. 5). In SA treatment, withanolide A content was found to be enhanced by 1.34 fold ( $4.31 \pm 0.45 \mu\text{g/g FCB}$ ), 2.10 fold ( $7.56 \pm 0.1 \mu\text{g/g FCB}$ ), 2.94 fold ( $8.31 \pm 0.20 \mu\text{g/g FCB}$ ), and 1.79 fold ( $5.39 \pm 0.13 \mu\text{g/g FCB}$ ), irrespective of 6H, 12H, 24H and 48H of treatment respectively, which was significantly higher than the control ( $3.06 \pm 0.21 \mu\text{g/g FCB}$ ) (Fig. 5a–d). A maximum value of  $8.31 \pm 0.20 \mu\text{g/g FCB}$  withanolide A was observed in SA treatment at 12H, which thereafter decreased with time up to 48H. Treatment of 40 day old *W. somnifera* hairy root culture with SA for 4 h significantly enhanced the withanolide A ( $132.44 \text{ mg/g DW}$ ; 58-fold higher), withanone ( $84.35 \text{ mg/g DW}$ ; 46-fold higher), and withaferin A ( $70.72 \text{ mg/g DW}$ ; 42-fold higher) content compared to control [16]. In culture filtrate treatment, enhanced production of withanolide A content by 3.81 fold ( $12.20 \pm 0.52 \mu\text{g/g FCB}$ ), 2.22 fold ( $8.02 \pm 0.23 \mu\text{g/g FCB}$ ), 2.28 fold ( $6.43 \pm 0.30 \mu\text{g/g FCB}$ ) and 1.66 fold ( $4.99 \pm 0.12 \mu\text{g/g FCB}$ ) corresponding to time 6H, 12H, 24H and 48H of treatment respectively, was observed, compared to control ( $3.06 \pm 0.21 \mu\text{g/g FCB}$ ) (Fig. 5a–d). Culture filtrate drastically induced withanolide A accumulation at early stages of treatment (6H), followed by decline in the content with increasing time onward. Elicitor derived from the mycelial component of *A. terreus* was employed parallel to other elicitors under same conditions. At various time points likewise, 6H, 12H, 24H, and 24H, increase in withanolide



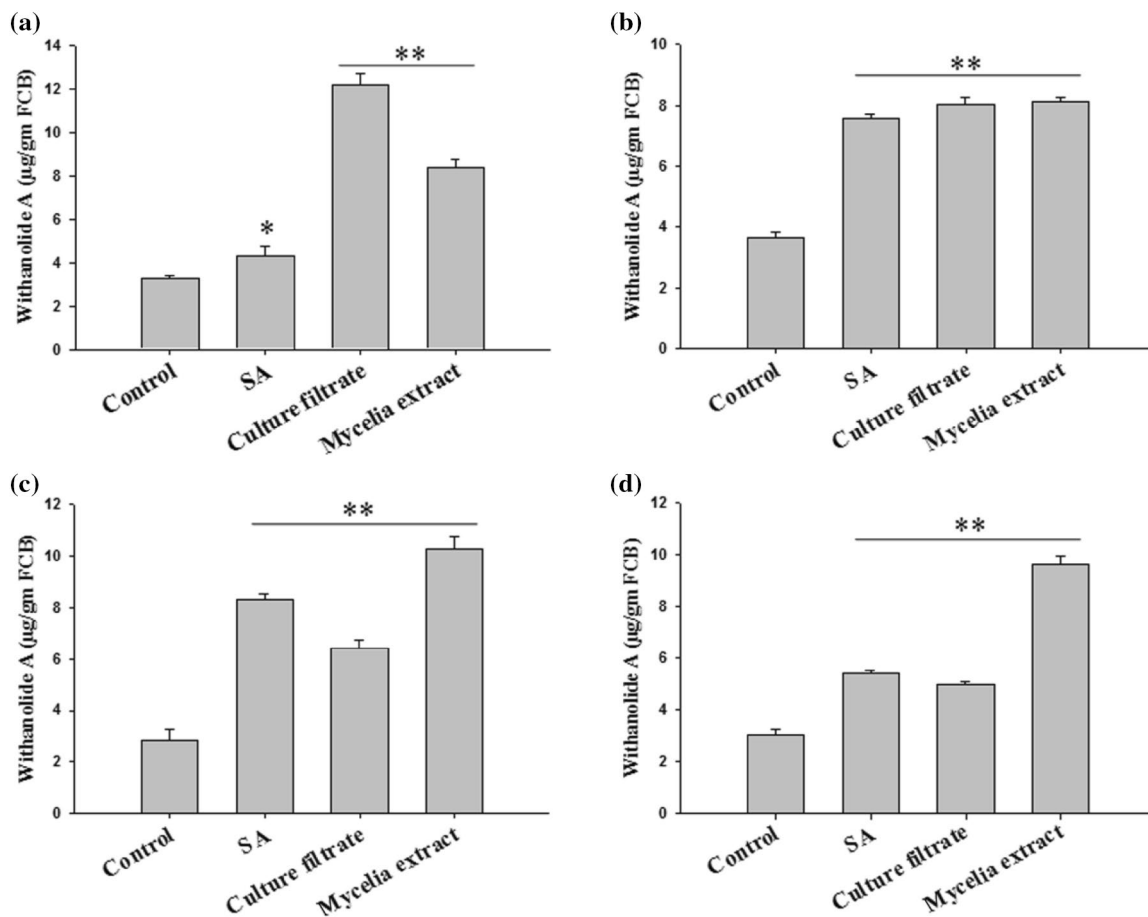
**Fig. 4** Growth kinetic of suspension culture, microscopy of suspension cell culture and effect of different elicitor on cell biomass **a** growth kinetic of suspension culture after subculture at 10th, 15th, 20th, 25th and 30th days, **b** bright field microscopic view of actively growing suspension cell before subjecting for treatments. Arrow indicates actively growing root suspension cells (transparent and banana shaped) of *W. somnifera*. **c** Effect of different elicitors on fresh cell biomass at different time point 6H, 12H, 24H and 48H post treatment. Cell stained with trypan blue. Scale bar represented at 100 μm

A content by 2.62 fold ( $8.38 \pm 0.42 \mu\text{g/g FCB}$ ), 2.25 fold ( $8.13 \pm 0.13 \mu\text{g/g FCB}$ ), 3.65 fold ( $10.29 \pm 0.45 \mu\text{g/g FCB}$ ) and 3.21 fold ( $9.63 \pm 0.33 \mu\text{g/g FCB}$ ) respectively, was achieved, compared to control ( $3.06 \pm 0.21 \mu\text{g/g FCB}$ ) (Fig. 5a–d). Mycelial extract treatment showed higher withanolide A content at 24H ( $10.29 \pm 0.45 \mu\text{g/g FCB}$ ), which then reduced marginally at 48H ( $9.63 \pm 0.33 \mu\text{g/g FCB}$ ), but was higher compared to 6H or 12H of the same treatments ( $8.38 \pm 0.42 \mu\text{g/g FCB}$ ). Interestingly, the results obtained after treatment of mycelial extract and culture filtrate with regard to withanolide A showed greater elicitation potential as compared to abiotic elicitor, however, in control sample, content remained approximately constant.

In an earlier study, different elicitors such as cadmium chloride, aluminium chloride, and chitosan, and precursors such as cholesterol, mevalonic acid and squalene were applied to induce the withanolide accumulation in root suspension culture at different time points (0–72 H) [18]. Within 4H of exposure with chitosan (100 mg/L), there was a substantial enhancement in major and minor withanolides such as withanolide A (14.76 mg/g DW), withanolides B (9.34 mg/g DW), withaferin A (6.68 mg/g DW), withanone (12.27 mg/g DW), 12 deoxywithanstramonolide (4.62 mg/g DW), withanosides IV (2.48 mg/g DW), and V (3.62 mg/g DW) [18]. Abiotic elicitor aluminium chloride (10 mg/L) and Cadmium chloride (15 mg/L) exposed for 4H elicitation resulted in enhanced withanolides production without causing any significant changes in biomass accumulation. Although when 48H was employed with their precursor such as mevalonic acid (200 μM), cholesterol (200 μM) and squalene (6 mM), a reduction in the biomass accumulation was seen, but withanolide accumulation was significantly higher than control.

Kumar and co-workers (2012) reported that inoculation of 3% (v/v) *P. indica* culture filtrate (filter-sterilized) in *Linum album* hairy roots culture for 48H led to 1.4 fold increase in biomass and 3.5–4.5 fold increase in secondary metabolite [47]. Successively, the same group revealed elicitation potential of *P. indica*, and compared known elicitors like methyl jasmonate (Me-JA), chitin and chitosan in the suspension cultures of *Lantana camara* [48]. So, the application of Me-JA at 62.5 μM and 2.5% (v/v) culture filtrate of *P. indica* enhanced ursolic acid (3.5-fold), oleanolic acid (5.6-fold), and betulinic acid (7.8-fold) production to similar levels [48]. Similarly, inoculation of *P. indica* cell homogenate to *W. somnifera* hairy root culture led to biomass and secondary metabolite induction [49]. The treatment of *Tessaria absinthioides* suspension culture with elicitor preparations of *Verticillium* sp., *Monodictis cataneae*, *Acremonium* sp., and *Aspergillus niger* substantially enhanced the sesquiterpene tessaric acid (TA) production by 281%, 197%, 149%, and 139% respectively compared to the untreated control [50].





**Fig. 5** Effect of elicitor treatments on withanolide A accumulation in cell suspension culture at different time point **a** withanolide A content per gm of cell biomass at 6H of post elicitors treatment, **b** withanolide A content per gm of cell biomass at 12H of post elicitors treatment, **c** withanolide A content per gm of cell biomass at 24H of post

elicitors treatment, **d** withanolide A content per gm of cell biomass at 48H of post elicitors treatment. Error bars represent standard deviation of three biological replicates. Asterisks indicate a significant difference from the control (Student's *t* test; \* $P < 0.05$ , \*\* $P < 0.01$ )

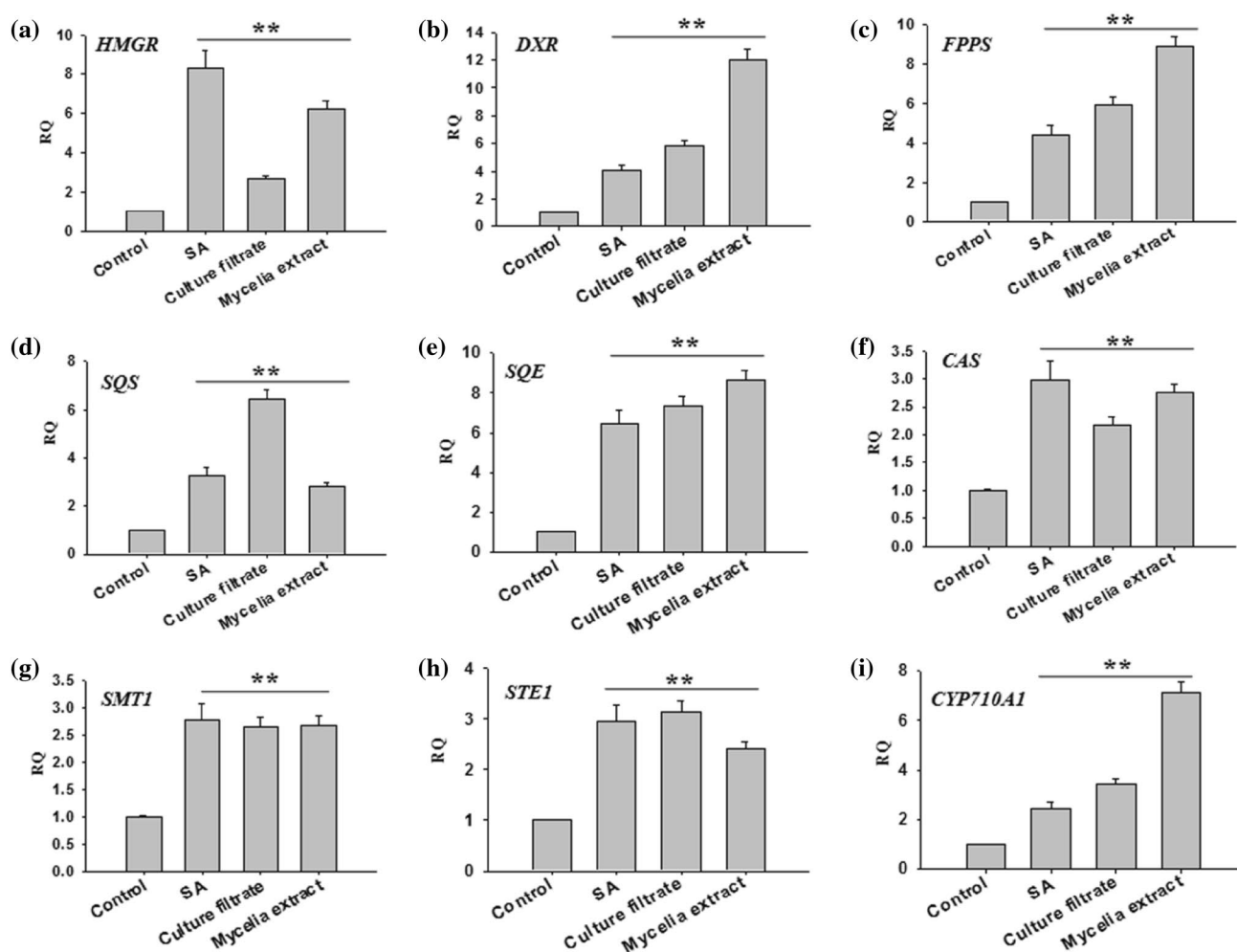
Novelty of our work is in application of native, endophytic fungi (*Aspergillus terreus*) of *W. somnifera* as an elicitor for synthesis of withanolide A in root suspension cells. Previous researcher have explored the application of biotic elicitors from random sources such as *A. alternata*, *F. solani*, *V. dahlia* and most of the cases these eliciting organisms were not endophytes [22]. Few cases of non-native and non-specific endophytes such as *P. indica* has also been used as biotic elicitors for induction of secondary metabolites in hairy root and shoot suspension culture [21, 22]. In this studies, instead of non-native fungal elicitors, we deployed crop specific functional endophyte of *W. somnifera* [51], for secondary metabolite production in root suspension cultures of *W. somnifera*. We hypothesized, to use potential crop specific endophyte *Aspergillus terreus*, which is indeed native to *W. somnifera* plant, as a biotic elicitor during suspension cell culture for synthesis of withanolide A. Thus, this could be our novel approach so far and application of functional endophyte of medicinal

plant which assists in *in planta* synthesis of bioactive compound and also acts as a potential biotic elicitor during suspension culture system is an added advantage. Hence it will have an edge over conventional approaches, in terms of its industrial acceptability – since the biotic elicitors used, is from inherent endophyte of plant system and a non-pathogen of the host plant. To substantiate, we report for the first time, culture filtrate and mycelia extract of innate, functional endophytic fungus (*Aspergillus terreus* 2aWF) of *W. somnifera* has enhanced maximum withanolide A induction from less treatment duration in root suspension cells. *A. terreus* 2aWF culture filtrate, at 6H induction time resulted in maximum withanolide A (3.81 times) while with mycelia extract at early 6H yielded 2.6 times higher further maintained upto 48H (3.2–3.6 times) over the untreated culture cells, which is better than non-native *P. indica* biotic elicitor in shoot suspension culture (1.8 times after 7 days exposure to elicitor) [22].

## Effect of elicitors on withanolide biosynthetic pathway genes

Elicitors that enhance withanolide A content also modulate withanolide biosynthetic pathways genes. Therefore, we examined the gene expression profile in elicitor treated and untreated cell suspension cultures after 48H, and quantified few selected withanolide biosynthetic pathway genes, viz. *HMGR*, *DXR*, *FPPS*, *SQS*, *SQE*, *CAS*, *SMT1*, *STE1* and *CYP710A1*, which play an important role in withanolide biosynthesis [31, 52]. However, the complete pathway of withanolides biosynthesis still remains elusive and is challenging research problem [53]. *HMGR* and *DXR* are upstream genes for MVA and MEP pathway for the formation of downstream common precursor isopentenyl

diphosphate (IPP). In the treatment of SA, culture filtrate and mycelia extract, *HMGR* expression levels increased significantly by 8.3, 2.6 and 6.5 fold respectively, compared to control (Fig. 6a). The higher expression levels of *HMGR*, *DXS*, *DXR*, *FPPS*, *SQS*, *SQE* and *CAS* genes were observed following application of 3% cell homogenate, 3% culture filtrate, and fungal disc of *P. indica* in *W. somnifera* suspension culture or callus treatment [21]. However, *DXR* expression level was 4, 5.8 and 12 fold higher in SA, culture filtrate, and mycelia extract treatment respectively (Fig. 6b). From IPP onward, *FPPS* gene was observed to have enhanced 4.3, 5.9 and 8.8 fold by SA, culture filtrate and mycelia extract treatment respectively (Fig. 6c). Squalene formed by gene *SQS*, which were increased by 3.2, 6.4 and 2.7 fold in corresponding to SA,



**Fig. 6** Effect of different elicitor on withanolide biosynthesis genes studied at 48H of treatment. At each time point withanolide A content was higher in elicitor treated than control, so only at 48H treated suspension cell taken for withanolides biosynthesis pathway genes expression level quantification. Real-time qPCR analysis showing relative expression levels of upstream and downstream withanolides pathway genes **a** *HMGR*, **b** *DXR*, **c** *FPPS*, **d** *SQS*, **e** *SQE*, **f** *CAS*, **g**

*SMT1*, **h** *STE1*, **i** *CYP710A1*. Expression levels of these genes were normalized to 18S rRNA and are represented in comparison with control. Data are mean  $\pm$  SD (n=3 biological replicates) and y axis represents relative quantity (RQ). RQ was calculated using the equation;  $RQ = 2^{-\Delta\Delta C_t}$ . Error bars represent standard deviation. Asterisks indicate a significant difference from the control (Student's *t* test;  $**P < 0.01$ )

culture filtrate and mycelia extract treatment (Fig. 6d). Upon treatment with seaweed extract of *G. edulis* (50%) and *S. wightii* (60%) to *W. somnifera*, hairy root culture for 48H exposure significantly induced the expression levels of *HMGR*, *FPPS*, *SQE* and *SQS* gene [54]. 2, 3-oxidosqualene is formed through squalene precursor that is a multi-branch point for other metabolites such as  $\beta$ -amyrin, lupeol, lanosterol and cycloartenol, which proceeded in presence of *SQE* gene. The treatment of SA, culture filtrate, and mycelia extract profoundly induced the *SQE* expression by 6.4, 7.3 and 8.6 fold higher level relative to the untreated cells (Fig. 6e). In the biosynthesis of cycloartenol, *CAS* gene was observed to be upregulated by 2.9, 2.1 and 2.7 fold in SA, culture filtrate and mycelia extract treatment respectively (Fig. 6f). It was recently shown that applied biotic elicitor, apart from *P. indica*, such as *V. dahliae*, *A. alternata* and *F. solani* fungal culture filtrate or cell homogenate, also induced the expression levels of *HMGR*, *DXS*, *DXR*, *FPPS*, *SQS*, *SQE* and *CAS* gene in shake flask [22]. In all the three treatments *SMT1* expression level was enhanced by approximately 2.7 fold, which was similar to the values in SA, culture filtrate and mycelia extract treatment as compared with the control suspension cell (Fig. 6g). Further, the dehydrating gene *STE1* was quantified and was found to increase by 2.9, 3.1 and 2.4 fold in SA, culture filtrate and mycelia extract treatment respectively (Fig. 6h). Last gene in this study, *CYP710A1* in stigmasterol biosynthesis was seen to increase by 2.4, 3.4 and 7.1 higher SA, culture filtrate and mycelia extract treatment respectively, over control suspension cell (Fig. 6i). The employment of 15  $\mu$ M Me-JA for 4H to *W. somnifera* hairy root increased expression levels of the *SQS* (6.58 fold), *SQE* (5.38 fold), *CAS* (5.7 fold), *SMT-1* (6.9 fold), *ODM* (8.24 fold) and *SDS* (9.78 fold) [49]. However, with treatment with *P. indica* cell homogenate (3%) to hairy root for 48H, the expression levels of some important genes of withanolides pathway like *SQS* (2.87 fold), *SQE* (3.25 fold), *CAS* (3.08 fold), *ODM*

(4.42 fold), *SMT1* (4.81 fold) and *CYP710A1* (5.024 fold) were significantly higher as compared to the control [49].

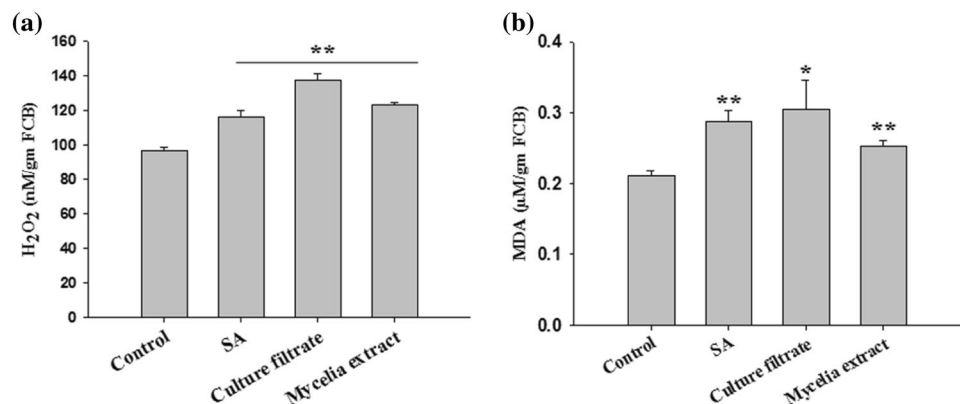
The external application of universal abiotic elicitor SA (2 mM) to leaf of *W. somnifera* for 6H exposure was observed to differentially elicit important MEP pathway genes such as *WsDXS* and *WsDXR* than control [55]. Otherwise if *W. somnifera* seedling roots dipped for 4H in SA (2 mM) that also enhanced transcript expression level of *WsCAS* by 2.49 fold [28]. In another experiment, lower concentration of SA (0.1 mM) exposed for 24H and 48H also influenced to induce the accumulation of P450s A-type monooxygenases *WsCYP98A* and *WsCYP76A* transcript upto 2.5 and threefold, respectively [34].

The amendment of known abiotic elicitor SA and crude biotic elicitor extracted from endophytic fungi *A. terreus* differentially regulated the withanolide biosynthetic pathway genes for withanolides accumulation. Here, we have noticed that native endophyte *A. terreus* in the form of culture filtrate or their mycelia extract could act as elicitor molecules to induce secondary metabolites in suspension culture.

### Effect of elicitor on hydrogen peroxide accumulation

Salicylic acid is a well-known hormone that induces hydrogen peroxide formation and secondary metabolites within plants, and induces systemic resistance against biotic stress [56, 57]. In this study, we examined the effect of SA as well as culture filtrate and mycelia extract on hydrogen peroxide production by fresh cell biomass at 48H in all elicitor treated suspension culture. In SA treated suspension culture, hydrogen peroxide production ( $115 \pm 4.40$  nM/g FCB) was enhanced by 20% as compared to control ( $96.5 \pm 2.17$  nM/g FCB). However, in culture filtrate and mycelia, extract treated suspension culture were produced by 42% ( $137.5 \pm 3.62$  nM/g FCB) and 27% ( $122.8 \pm 1.25$  nM/g FCB) higher, respectively, than untreated suspension culture  $96.5 \pm 1.25$  nM/g FCB (Fig. 7a). In the present study, we found that not only SA elicited  $H_2O_2$  production, culture

**Fig. 7** Effect of elicitor treatments on hydrogen peroxide and MDA production in treated suspension culture at 48H of treatment. **a** hydrogen peroxide production per gm of cell biomass in control and treated sample, **b** MDA production per gm of cell biomass in control and treated sample. Asterisks indicate a significant difference from the control (Student's *t* test; \* $P < 0.05$  \*\* $P < 0.01$ )



filtrate and mycelia extract also significantly induced the accumulation of  $H_2O_2$  within suspension up to 48H of treatment.

*Arabidopsis* leaves were treated with 1 and 5 mM SA for 8H enhanced  $H_2O_2$  levels by 59% and 194%, respectively, compared to control leaves. However, when SA (5 mM) is treated along with 10 mM  $H_2O_2$  for 8H, it resulted in enhanced in vivo  $H_2O_2$  levels by 367% compared to control leaves [56]. Also, the application of SA in suspension culture of *Salvia miltiorrhiza* showed significantly enhanced rosmarinic acid (RA) production,  $H_2O_2$  production, as well as phenylalanine ammonia-lyase (PAL) activity [58]. Moreover, the treatment of SA to tobacco suspension culture cell (*Nicotiana tabacum*, cell line BY-2) triggered different type of reactive oxygen species (ROS) like superoxide, hydroxyl radical and hydrogen peroxide [59]. The accumulation of hydrogen peroxide triggered MDA production in BY-2 cell-line, which was indirectly induced by SA [59].

### Lipid peroxidation

The peroxidation of lipids is considered as the most damaging process known to occur in every living organism. During lipid peroxidation (LPO), products are formed from polyunsaturated precursors that include small hydrocarbon fragments such as ketones, malondialdehyde (MDA), etc., and compounds related to them [60]. Generally, all kinds of abiotic and biotic stresses lead to reactive oxygen species generation, which inferred to oxidation of unsaturated membrane lipids called lipid peroxidation. Here, we estimated the lipid peroxidation by indirect method to the quantification of MDA production at 48H treatment. MDA content was significantly increased during application of SA, culture filtrate and mycelia extract to suspension cell culture (Fig. 7b). The MDA amount was  $0.28 \pm 0.014$ ,  $0.30 \pm 0.04$  and  $0.25 \pm 0.007$   $\mu\text{M}/\text{g}$  FCB in SA, culture filtrate and mycelia extract treated respectively than control ( $0.21 \pm 0.007$   $\mu\text{M}/\text{g}$  FCB).

Previous studies reported the application of SA to BY-2 suspension culture leading to the accumulation of MDA through induction of hydrogen peroxide [59]. Toxic effect of aluminum salt has been studied on soybean cell suspension cultures, which showed induction of lipid peroxidation, leading to MDA accumulation [61]. The osmotic stresses are also known to induce MDA production in Palm embryonic suspension culture. Palm embryogenic suspension culture cell were subjected to osmotic stress with Polyethylene glycol 6000 (0–20%). Raising PEG levels in suspension culture induces malondialdehyde (MDA) concentration, and was the highest at 10% PEG, and thereafter, MDA content decreased with increasing the PEG concentration [62]. Beside to known SA, culture filtrate and mycelia extract of *A. terreus* significantly induced the MDA content at 48H of treatment.

### Conclusions

In this study, we studied the ability of innate endophyte of *W. somnifera*, *Aspergillus terreus* 2aWF to act as biotic elicitor, and significantly induce withanolide A accumulation in root cell suspension cultures. 5% of culture filtrate and only 1% mycelia extract could serve as an elicitor for maximum induction of withanolide A. This is the first report on exploration of *W. somnifera* plant's native fungal endophyte culture filtrate and mycelia extract as an elicitor in their host cell suspension culture. Apart from conventional methods, a sustainable step of recruiting native endophytes of important medicinal plants as elicitors to induce secondary metabolites in suspension cultures must potentially be unveiled.

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**Author contributions** RKK performed all the experiments and CSV analyzed and prepared the manuscript. All authors read and approved the manuscript.

**Conflict of interest** The authors declare that they have no conflict of interest.

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