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Dual Elicitation for Improved Production of Withaferin A by Cell Suspension Cultures of *Withania somnifera*

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Abstract High yielding transformed callus culture of *W. somnifera* was established by infecting hypocotyls with *Agrobacterium tumefaciens* MTCC-2250. Maximum withaferin A content of 0.0875 mg/g dry cell weight and transformation efficiency of 80% were obtained. Confirmation of transformation was done on the basis of the presence of the *ags* gene by using polymerase chain reaction. Various abiotic elicitors (arachidonic acid, methyl jasmonate, calcium chloride, and copper sulfate) and biotic elicitors (cell extracts and culture filtrates of *Alternia alternata*, *Fusarium solani*, and *Verticilium dahaliae*) were tested at different concentrations to enhance withaferin A production in suspension culture of transformed cells. Maximum enhancements of 5.4 times and 9.7 times, respectively, were obtained when copper sulfate (100 μM) and the cell extract of *V. dahaliae* (5% *v*/*v*) were added separately to suspension cultures. The dual elicitation strategy by the combined addition of these two elicitors resulted in 13.8-fold enhancement of withaferin A content in comparison to control cultures (2.65 mg/L). The present study indicates the potential of this biotechnology-based methodology for the large-scale production of withaferin A.

Keywords *Agrobacterium* · Biotic elicitor · Elicitation · Suspension culture · Withaferin A · *Withania somnifera*

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Introduction

Withania somnifera, also known as ashwagandha, Indian ginseng, and winter cherry, has been an important herb in the Ayurvedic and indigenous medical systems for more than 3,000 years [1]. It has received much attention in recent years due to the presence of a large number of steroidal alkaloids and lactones known as withanolides. At present, 12 alkaloids, 35 withanolides, and several sitoindosides from this plant have been isolated and studied. The principle withanolide in the Indian variety of the plant is withaferin A. This drug is known to have anti-inflammatory [2], antitumor [3], antioxidant [4], anticonvulsive [5], and immunosuppressive properties [6]. Presently, withanolides have been commercially obtained by solvent extraction of roots and leaves of the plant. Low yield from the natural source, genotypic and chemotypic variations, heterogeneity in content, long gestation period (4–5 years) between planting and harvesting, and uneconomical chemical synthesis are major constrains in industrial withanolide production. To enhance the commercial prospects for the production of withanolides, an alternative choice could be the use of plant cell cultures [7]. Plant cell cultures remain a favored methodology for biotechnological production of nature-based therapeutics. Transformed cultures, obtained from *A. tumefaciens*-mediated infection, are one of the important tools for the enhanced production of secondary metabolites. Untransformed cultures generally require exogenously added phytohormones and have a tendency to loose their biosynthetic capability after a short period. On the other hand, *Agrobacterium* transformed cultures have been comparatively fast growing and genetically more stable and hence more suitable for large-scale production of plant-based products.

Low content of withanolides and slow growth rate of untransformed cell cultures of *W. somnifera* in earlier reports [8–12] lead us to design the present study to develop fast-growing and high-producing transformed cell lines of the Indian variety of *W. somnifera* and enhance withaferin A production by the addition of elicitors.

Elicitors are chemicals or biofactors from various sources that can trigger physiological responses and phytoalexin production. It may include abiotic elicitors such as metal ions and inorganic compounds, and biotic elicitors from a microbiological origin or plant cell wall components. It is well known that upon treatment with elicitors, an array of defense reactions, including the accumulation of a range of plant defensive secondary metabolites in plants or in cell cultures, occurs [13]. Hence, the combined addition of abiotic and biotic elicitors was tried as yield enhancement strategy in present study.

Materials and Methods

Germination of Seeds

Seeds of *W. somnifera* were washed in 1% Savlon and then treated with 0.1% Bavestin and rinsed five to six times with sterile double-distilled water (SDDW). Surface sterilization was performed using 70% *v*/*v* ethanol treatment for 30 s and rinsed thrice with SDDW. This was followed by treatment with 0.01% *w*/*v* mercuric chloride for 5 min and rinsing with SDDW for four to five times. For aseptic germination, sterilized seeds were then placed on Murashige and Skoog (MS) medium [14] with 30 g/L sucrose and 7 g/L agar at 25±2°C in a 16/8-h light/dark cycle with a light intensity of 1,200 lx.

Initiation of Cultures

Hypocotyls were used as explants for callus initiation by *Agrobacterium*-mediated transformation. Explants from 20–25-day-old in vitro germinated plants were used for culture initiation studies. Transformed cultures were initiated by infecting explants with the *A. tumefacienes* strain (MTCC 2250). The *Agrobacterium* strain used was procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. For this, bacterial colonies were cultured for 2 days on solid yeast mannitol broth medium (YMB) at 25±2°C. Ten loopfuls of bacteria were then allowed to grow on liquid YMB medium for 24 h at 25±2°C. The inoculum (2% *v*/*v*) from this culture was reinoculated in liquid YMB medium and grown till they achieved an optical density at 600 nm of ~1.0. The suspension was then centrifuged at 6,000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in 5 mL of the fresh liquid YMB media. This concentrated culture was used further for the infection of plant materials.

Forty explants were kept in a sterile Petri plate, pricked manually with a 24-gauge metal needle (~5 wounds per cm^2), dipped in *Agrobacterium* culture, and incubated for 5 min. The liquid YMB medium without bacteria was applied to the explants as a control. The infected explants were preincubated for cocultivation at 25±2°C for 48 h on sterile MS medium, solidified with 10 g/L agar. The infected explants were then transferred to antibiotic (Cefotaxime, 1 g/L) containing MS medium to check the overgrowth of bacteria and were incubated at 25±2°C in 16/8-h light/dark regime. The transformed cultures were transferred to fresh MS medium containing 1 g/L cefotaxime. Axenic cultures were obtained by subsequent subculture to fresh MS medium for every 7 days containing the antibiotic. The transformed cultures were checked for *Agrobacterium* contamination by culturing samples on YMB medium after every subculture.

Genetic Confirmation of Transformed Cultures

Total genomic deoxyribonucleic acid was isolated from the transformed cell line and untransformed hypocotyls of in vitro grown plants using the AuPrep Kit (Life Technologies, India). The integration of the *ags* gene from the bacterial plasmid was confirmed by polymerase chain reaction (PCR) analysis. The Presence of the *ags* gene was confirmed by using 5′-CGGAAATTGTGGCTCGTTGTGGAC-3′ and 5′-AATCGTTCA GAGAGCGTCC GAAGTT-3′ as primers. All primers were obtained from Microsynth (Switzerland). Amplification by PCR was performed under the following conditions: initial denaturation at 94°C for 4 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min for 35 cycles, with a final extension at 72°C for 5 min. The amplicons were analyzed by electrophoresis on 1% *w*/*v* agarose gel. For the positive control, plasmid from the *Agrobacterium* strain was used during the PCR.

Growth and Production Kinetics in Suspension Cultures

For the establishment of growth and production kinetics, the suspension cultures were initiated by transferring a friable fraction of transformed callus equivalent to 5 g/L dry cell weight into 250-mL Erlenmeyer flask containing 50 mL of liquid MS media supplemented with 50 g/L sucrose. Cultures were incubated on a gyratory shaker at 125 rpm and 25±2°C under a 16/8-h light/dark regime. Individual flasks, in duplicate, were harvested at a regular interval of 2 days and analyzed for dry cell weight, residual sugar, and withaferin A content.

Measurement of Dry Cell Weight and Residual Sugar

To determine dry cell weight, cells were collected by centrifugation at 5,000 rpm for 15 min and dried at 25±2°C until constant weight was achieved. Residual sucrose estimation in the spent medium was done by the phenol/sulfuric acid method [15].

Extraction and Quantitative Analysis of Withaferin A

For the extraction of withaferin A, dried and powdered cells (100 mg) were sonicated with 5 mL methanol for 15 min at 4–6°C. Cells were then allowed for complete extraction in methanol at 24 h for 4–6°C. The supernatant was removed after centrifugation (5,000 rpm, 10 min) and evaporated to dryness. The extract was redissolved in high-performance liquid chromatography (HPLC)-grade methanol, filtered through a 0.22-μm filter and then subjected to analysis by HPLC. Quantitative estimation was done by using a Nova Pak RP-C_{18} column (Waters, USA; 250×4.6 mm) in the Agilent 1100 series (Agilent Technologies, USA) HPLC system equipped with a quarternary pump (G1311A), diode array detector (G1315B), and temperature controller (G1316A). Separation was carried out by elution with the mobile phase methanol/water (53:47 *v*/*v*) at a 1.0-mL/min flow rate. Column temperature was maintained at 30°C. Withaferin A was detected at 229 nm. Commercially available withaferin A (Life Technologies) was used as the standard.

Improvement in Withaferin A Production by Elicitation

Elicitation by Abiotic Agents

Some known abiotic elicitors for plant-based secondary metabolites such as signaling molecules (methyl jasmonate [MJ], arachidonic acid [AA]) and metal ions (calcium as calcium chloride [CC] and copper as copper sulfate [CS]) were tested for their effect on withaferin A accumulation in cell suspension cultures. Stock solutions of CC and CS were prepared by dissolving them in SDDW and adjusting the pH to 5.8. MJ and AA were dissolved in 95% *v*/*v* ethanol. Abiotic elicitors were aseptically added to the cultivation medium at the following concentrations: MJ—50, 100, and 250 μM, AA—5, 10, 20, and 50 μM, CC—0.5, 1, and 2 mM, and CS—50, 100, 250, and 500 μM. The elicitor solutions were vortexed thoroughly before addition to the flasks.

Elicitation by Biotic Agents

Initiation of Fungal Cultures

The fungal cultures tested, *A. alternata* (MTCC-1779), *F. solani* (MTCC-350), and *V. dahliae* (MTCC-2063), were obtained from the Institute of Microbial Technology, Chandigarh, India. These fungal cultures were grown on a liquid potato dextrose growth medium for 2 weeks under static conditions at 25±2°C in the dark by transferring the 1-cm^2 piece of grown fungi on the solid potato dextrose medium. Suspension cultures were initiated with 5% *v*/*v* inoculum from 2-week-old static cultures in 50 mL of the same medium in a 250-mL Erlenemeyer flask and were grown in the dark at 25±2°C and 120 rpm in a gyratory shaker incubator for 96 h.

Preparation of Elicitors

After growth for 96 h, the fungal suspension cultures were autoclaved at 15 lb/in.^{2} and 121°C for 15 min. The autoclaved cultures were filtered, and the filtrate was denoted as culture filtrate (CF). The cell residues were washed thrice with SDDW and homogenized in a mortar and pestle with 20 mL SDDW under aseptic conditions. The volume was then made up to 50 mL with SDDW. This preparation was denoted as the cell extract (CE). These CFs and CEs of different fungi were kept in refrigerator at 4–6°C until further use. Biotic elicitors (CE and CF of *A. alternata*, *F. solani*, and *V. dahliae*) were tested at 1, 2.5, and 5% *v*/*v* concentrations.

All the elicitors, abiotic and biotic, were added aseptically on the eighth day of cultivation. Following the elicitor treatment separately, cultures were harvested on the 12th day, in duplicate, and analyzed for biomass and withaferin A content.

Dual Elicitation Strategy for Productivity Enhancement

Results from both yield enhancement strategies *viz.* addition of abiotic and biotic elicitors were integrated in order to develop a bioprocess for synergistic enhancement of withaferin A productivity in suspension cultures of *W. somnifera*. Cultures were cultivated at 125 rpm in 50 mL of liquid MS medium with 30 g/L sucrose in 250-mL Erlenemeyer flasks at 27± 2°C with a photoperiod of 16/8-h light/dark cycle. Selected abiotic elicitor (CS at 100 μM) and biotic elicitor (CE of *V. dahliae* at 5% *v*/*v*) were added on the eighth day of cultivation. Cultures were harvested in duplicate on the 12th day and analyzed for biomass and withaferin A production.

Results and Discussion

Establishment of Cultures

A. tumefaciens transformed cultures of *W. somnifera* were successfully induced from hypocotyls of in vitro grown plants with 80% transformation efficiency. The confirmation of genetic transformation was done by checking the integration of the *ags* gene in the plant chromosome using PCR and gel electrophoresis and is presented in Fig. 1.

Growth and Production Kinetics

Complete growth, production, and substrate consumption profiles of *W. somnifera* cells in the suspension culture were established and given in Fig. 2. A relatively very short log phase of 2 days was observed in batch kinetics. Maximum biomass (26.2 g/L dry weight) and withaferin A accumulation (2.65 mg/L) were obtained on the 12th day of cultivation. Withaferin A production was mostly occurred during the growth phase inside the cells. Nearly complete consumption of sugar was observed on the 12th day when maximum growth and production were achieved. Therefore, it may be speculated that the onset of the stationary phase was due to the limitation of sucrose in the medium.

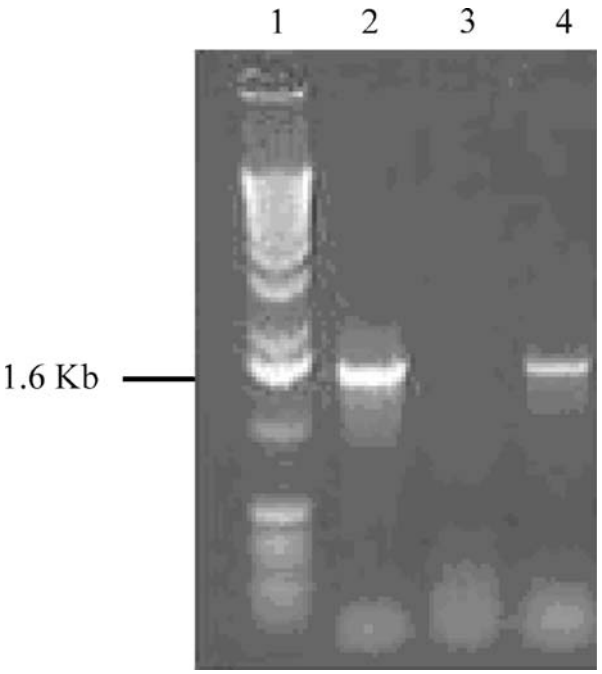

Fig. 1 PCR analysis showing the presence or absence of the *ags* gene in transformed cultures of *W. somnifera*; *Lane 1*: 500-bp ladder, *Lane 2*: positive control-I (pTi-2250), *Lane 3*: negative control (Stem), *Lane 4*: transformed cell culture

Improvement in Withaferin A Production by Elicitation

To enhance withaferin A production in cell suspension cultures, various abiotic elicitors (AA, MJ, CC, and CS) and fungal preparations of *A. alternata*, *F. solani*, and *V. dahliae* as biotic elicitors were tested at different concentrations.

Elicitation by Abiotic Agents

The effect of abiotic elicitors on withaferin A production is given in Fig. 3. All chemical agents tested had resulted in the significant improvement of withaferin A production, but cell growth was significantly reduced by the addition of various abiotic elicitors except CC.

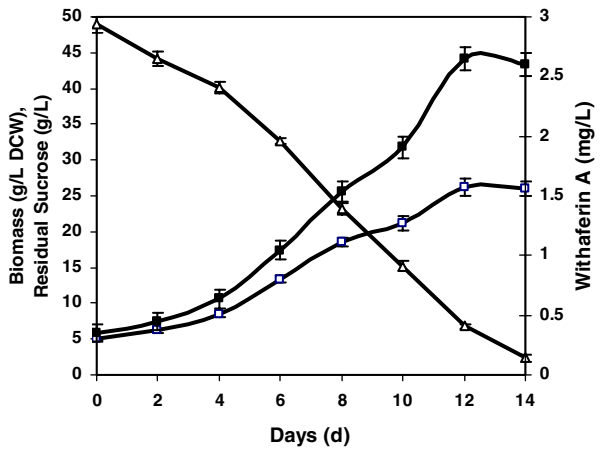

Fig. 2 Growth and production kinetics of *W. somnifera* cells in suspension cultures. *Empty squares*, biomass; *filled squares*, withaferin A; *triangles*, residual sucrose (average values are given; error bars are represented as *vertical lines*)

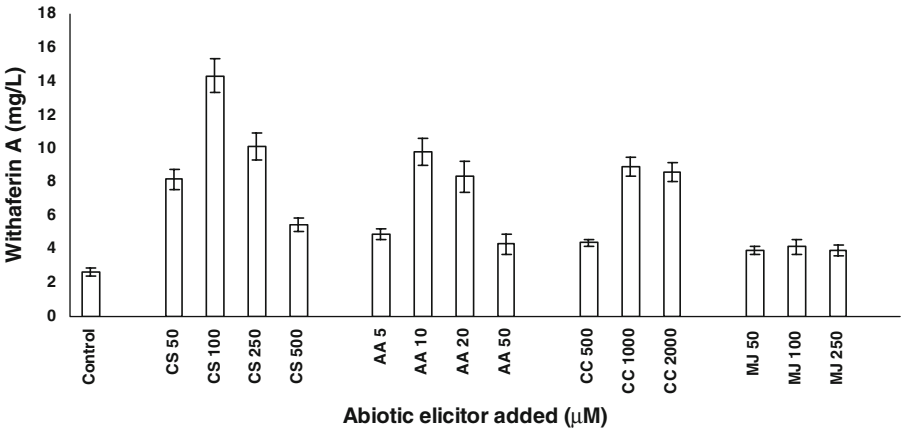

Fig. 3 Effect of abiotic elicitors on withaferin A production by suspension cultures of *W. somnifera* (average values are given; error bars are represented as *vertical lines*)

A maximum withaferin A accumulation of 14.33 mg/L was obtained when 100 μM CS was added to cell suspension cultures of *W. sominfera*, which was 5.4 times higher in comparison to control cultures (2.65 mg/L). Heavy metals like copper had been known to influence plant cells through the induction of signaling pathways connected at least partially with jasmonic acid. This generally happens due to the synthesis of proteins similar to pathogen-related and other hypersensitive responses [16, 17]. The addition of copper compounds had increased production of coumarins in *Helianthus tuberosus* [18], isoflavonoid accumulation in *Pueraria lobata* [19], coumarin biosynthesis in sunflower [20], phenolics induction and compartmentation in *Phyllanthus tenellus* [16], and jasmonic acid production in *Arabidopsis thaliana* and *Phaseolus coccineus* plants [17].

CC had resulted in maximum withaferin A production of 8.92 mg/L at a concentration of 1 mM. Almost similar levels of withanolide were obtained upon further increase in CC concentration. Calcium ions had been reported to involve in ion channels and is a key second messenger for many diverse physiological changes and cellular processes. Increased calcium ion flux to cells is also known to be a very important step in induced accumulation of plant secondary metabolites [13].

The addition of MJ at 100 μM concentration resulted in an increased withaferin A production of 4.15 mg/L. Exogenous application of jasmonates to the plant cell culture or intact plant is known to stimulate the biosynthesis of secondary metabolites [21]. The chemical structure of the jasmonic acid-related molecule is also known to play an important role in elicitation, which is highly specific in nature [22]. Jasmonates are reported to act as signal molecules for induced indole alkaloids accumulation in *C. roseus* [23], phytoalexin biosynthesis in rice [24, 25], indole glucosinolates production in *Arabidopsis* [26], *h*-thujaplicin formation in Mexican cypress cell culture [27], and artemisinin production in *Artemisia annua* cell suspension cultures [28].

When AA was added at a concentration of 10 μM, a 3.69-fold enhancement of withaferin A accumulation (9.79 mg/L) was achieved. AA is known to enhance the production of various plant metabolites [29–33]. The possible mechanism for this might involve oxidative burst, which results in the generation of reactive oxygen species and H_2O_2 [33].

Elicitation by Biotic Agents

CFs of all fungi tested, *A. alternata*, *F. solani*, and *V. dahliae*, had not found to enhance withaferin A accumulation in cell suspension cultures. On the other hand, CE of these fungi significantly improved its production. The effect of the addition of CEs of these fungi is represented as Fig. 4.

The addition of *V. dahilae* CE at a concentration of 5% *v*/*v* had resulted in the maximum increment of withaferin A production by 9.7 times in comparison to control cultures (2.65 mg/L). Although the addition of CE resulted in a slight decrease in biomass (22.4 g/L) in comparison to control cultures (26.2 g/L), then also a significantly higher production level of 25.7 mg/L was achieved by this strategy. The possible hypothesis for enhanced production might be that the extracellular enzymes from *W. sominfera* cell cultures hydrolyze the walls of autoclaved fungi to generate the real elicitor moieties. The variation in response due to the addition of different fungal preparations might be due to the difference of elicitor moieties generated in quantity and quality, depending on the fungal species employed. The addition of elicitor preparations of *Verticillium* species had increased the accumulation of tessaric acid production in *Tessaria absinthioides* cell suspension cultures [34]. The significant enhancement of various plant secondary metabolite production by the addition of fungal preparations as biotic elicitors had been already listed [13].

Dual Elicitation Strategy for Productivity Enhancement

The addition of abiotic and biotic elicitors, CS (100 μM) and *V. dahilae* CE (5% *v*/*v*), had resulted in the maximum enhancement of wihaferin A production by 5.4 and 9.7 times, respectively, compared to the control culture. Based on these results, the dual elicitation strategy was developed and experimentally implemented by simultaneous addition of both elicitors at optimum concentrations to see synergistic enhancement of withaferin A production in cell suspension cultures of *W. somnifera*. The proposed yield improvement strategy had resulted in withaferin A production of 36.57 mg/L, which was about 13.8 times higher than control cultures (2.65 mg/L).

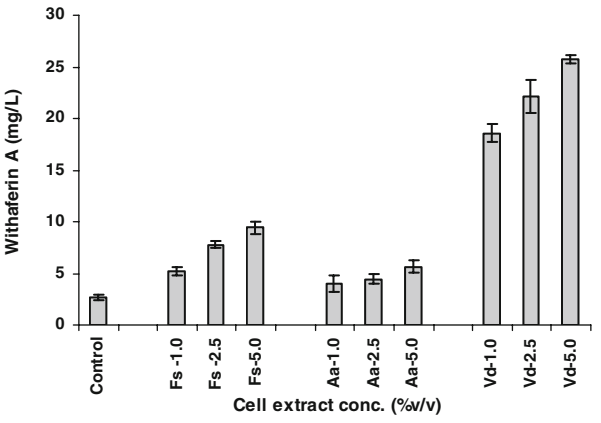

Fig. 4 Effect of fungal CEs on withaferin A production by suspension cultures of *W. somnifera* (average values are given; error bars are represented as *vertical lines*)

Conclusions

This study represents a successful approach for the production of withaferin A by transformed cell suspension cultures from *W. somnifera* of Indian variety. Significantly higher withaferin A accumulation was obtained in comparison to that reported earlier. Synergistic improvement of the withanolide level was achieved by a dual elicitation strategy, comprising of the addition of selected abiotic and biotic elicitors, to overcome the problem of low content in plant cell cultures. This yield enhancement could generally be applied for the development of cell culture-based bioprocesses for mass-scale production of commercially important phytochemicals. Further research work in this direction is under progress.

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