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Enhanced Bioproduction of Withaferin A from Suspension Cultures of *Withania somnifera*

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Abstract

Callus cultures of Withania somnifera from leaves were established on MS (Murashige and Skoog) media supplemented with Dicamba (2 mg/l), Kinetin (0.1 mg/l) and Sucrose (3% w/v). Suspension cultures were established and the growth and production kinetics were studied. For growth kinetics MS media supplemented with Dicamba (2 mg/l), Kinetin (0.1 mg/l) and Sucrose (3% w/v) without agar was used which was found suitable for the initiation and maintenance of the suspension cultures cultures from the calli. Half B5 medium supplemented with 2,4-Dichlorophenylacetic acid(2,4-D) (1 mg/l), Kinetin (0.1 mg/l) and Sucrose (5% w/v) was employed. Effect of various precursors and elicitors on suspension cultures was studied. The addition of precursor's sodium acetate (50 mg/l) (~10 folds), Mevalanolactone (50 mg/l) (~14 folds), Squalene (50 mg/l) in colloidal form (~23 folds) and Cholesterol (25 mg/l) in colloidal form (~30.5 folds) have shown significant increase in bioproduction of withaferin A. Among the various biotic and abiotic elicitors used Verticillium dahliae (5% w/v cells extract) (~10 folds) and copper sulphate (100µm/l) (~2.5 folds) have shown moderate increase in the bioproduction of withaferin A. The effective precursors and elicitors were studied for optimization of day of addition and found that maximum bioproduction of withaferin A was seen in the cultures when precursors and elicitors were added to 3 day old suspension cultures.

Key Words: Elicitors, Precursors, Suspension cultures, Withaferin A.

INTRODUCTION

Bioproduction of secondary plant metabolites using elicitation of suspension cultures is well documented for plants producing minute quantities of useful phytochemicals [1]. Withaferin A, is a steroidal lactone belonging to withanolide group present in roots and leaves of *Withania somnifera* Dunal, commonly known as Ashwagandha (Sanskrit). The extracts of *Withania somnifera* and Withaferin A have antimicrobial [2], antitumor [3], immunomodulating [4], anti-inflammatory, antiarthritic, hepatoprotective, antioxidant, antifeedant and antistress [5] activities. The bioproduction of Withaferin A from *Withania somnifera* suffers from, plant to plant variation of active constitution in both quality and quantity as well as long gestation period of 4-5 years between planting and harvesting. Bioproduction of secondary plant metabolites can be enhanced alternatively, using plant cell cultures. It was reported [6] that multiple shoot cultures accumulated with withanolides from single shoot tip explant of *Withania somnifera.* Callus of *Withania somnifera* was induced [7] but the callus failed to synthesize withanolides. Vitali et al. [8] reported a method for initiation of callus with withanolides from *Withania somnifera.* The bioproduction of secondary metabolites can be enhanced by adding elicitors and precursors to cell cultures [9,10]. Sharada et al. [11] reported withanolide production in *in vitro* cultures of *Withania somnifera*. Veeresham Ciddi [12] reported 50 times increase in bioproduction of withaferin A from cell cultures of *Withania somnifera* using an elicitor salasin when compared to unelicited control. This tempted us to work with different precursors and elicitors which were not reported earlier to enhance the bioproduction of withaferin A from cell cultures of *Withania somnifera*.

MATERIALS AND METHODS

Initiation and maintenance of callus

The callus cultures were initiated from leaves collected from Kakatiya University campus, India. The leaves were surface sterilized by treatment with mercuric chloride (0.1% w/v) for 5 minutes and transferred aseptically onto MS (Murashige Skoog) [13] medium supplemented with Dicamba (2 mg/l), Kinetin (0.1 mg/l) and Sucrose (3% w/v). The callus initiation was observed after 4 days and callus was sufficiently formed by 3 weeks. The callus cultures were maintained on the same media at 4 week intervals.

Initiation and maintenance of suspension cultures

The fragile 8 weeks old callus (3g) was aseptically transferred into growth media composed of MS medium (50 ml) supplemented with Dicamba (2 mg/l), Kinetin (0.1 mg/l) and Sucrose (3% w/v) with out agar. The cultures were incubated in shaker incubator (New Brunswick Innova 4230, USA) at 26±2 °C and a rotation speed of 120 rpm. These suspension cultures are maintained by repeated sub culturing at an interval of 10 days.

Growth kinetics

From the suspension cultures growing in growth media the samples were withdrawn at the end of 3^{rd}, 5^{th}, 7^{th}, 9^{th}, 12^{th} and 15^{th} day of incubation. The cultures were filtered and weighed for the determination of the growth of the cells. This procedure was repeated thrice and the growth indices (G.I) were calculated.

Production kinetics

The cell cultures (in growth media) after 3 passages were transferred into the production media composed of half strength B5 medium [14] supplemented with 2,4-D (1 mg/l), Kinetin (0.1 mg/l) and sucrose (5% w/v) with 50% v/v as inoculum in order to induce the bioproduction of withaferin A. The suspension cultures (10 ml) were aseptically transferred into each of pre-sterilized flasks (50 ml capacity) with pre-sterilized pipette (10 ml capacity) under laminar flow and incubated at 26±2 °C and 120 rpm in shaker incubator. The flasks were withdrawn at the end of 3rd, 5th, 7th, 9th, 12th, 15th and 20th day, extracted and analyzed for withaferin A content.

Initiation and preparation of fungal elicitors

Fungi *Fusarium solani* MTCC 350, *Alternaria alternate* MTCC 1779 and *Verticillium dahliae* MTCC 2063 were obtained from Institute of Microbial Technology (IMTEC, Chandigarh, India). The fungi were cultured on growth media prescribed in IMTEC catalogue, incubated for two weeks at room temperature in dark. Suspension cultures were initiated from two week old static cultures, using suitable liquid growth media and incubated in dark at 26±2 °C and 120 rpm in a gyratory shaker incubator for 72 hrs. The fungal cultures were autoclaved at 15 lb/in^2 and 121 °C for 15 minutes and filtered aseptically. The filtrate was denoted as culture filtrate (CF). Later the cell residues were washed thrice with sterile de-ionized water and homogenized in a motar and pestle with small amount of water. The volume was then made up to the original culture volume with water, autoclaved for 15 minutes at 121 °C and filtered aseptically. This filtrate was denoted as cell extract (CE) [15]. These CF and CE of different fungi were stored in refrigerator until use.

Addition of precursors and elicitors

After 3 passages in growth media the cell cultures were transferred aseptically into presterilized production media with 50% v/v as inoculum. 10ml of cell cultures were then aseptically transferred into each of the presterilized conical flasks (50ml capacity) with a sterile pipette (10ml capacity). The precursors solutions Sodium acetate (25 mg/l, 50 mg/l and 100 mg/l), Mevalanolactone (25 mg/l, 50 mg/l and 100 mg/l), Squalene in alcohol (25 mg/l, 50 mg/l and 100 mg/l), Squalene in colloidal form (in tween-80 (0.15% v/v)) (25 mg/l, 50 mg/l and 100 mg/l), Cholesterol in alcohol (25 mg/l, 50 mg/l and 100 mg/l) and Cholesterol in colloidal form (25 mg/l, 50 mg/l and 100 mg/l); the biotic elicitors solutions 1% and 5% v/v of fungal CF and CE of *Fusarium solani* MTCC 350, *Alternaria alternate* MTCC 1779 and *Verticillium dahliae* MTCC 2063 and the abiotic elicitors Calcium chloride (1 mg/l and 5 mg/l), Copper sulphate (100 µM/l and 500 µM/l) and Cinnamic acid (100 µM/l and500 µM/l) (sterilized by passing through 0.2 µ membrane filter) were added aseptically on day 0 to the suspension cultures in each of the cultured flasks and incubated at 26±2 °C and 120 rpm. The cultures were withdrawn at the end of day 12. Similarly the effective precursor and elicitors were further studied for optimum day of addition (day 0, 3, 5 and 7). Three culture flasks were used for each concentration, while running suitable control. Both the cells and media together were extracted for withaferin A and analyzed.

Extraction of Withaferin A

After 12 days of incubation with different precursors, elicitors and controls, the cell cultures were harvested and the contents of each culture flask were extracted with methanol (3x10 ml). To the methanolic extract, equal volume of distilled water was added and partitioned with dichloromethane (3x20 ml). The dichloromethane layers were pooled together and concentrated

under vaccum [16]. The residue was dissolved in 1 ml methanol (HPLC grade) and subjected to analysis by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

Analysis of extracts

Thin Layer Chromatography (TLC)

The methanolic extracts were co-chromatographed with authentic sample on precoated silica gel – G plates (Merck, Mumbai, India) using solvent system Hexane: Ethyl acetate: Methanol (2:7:1). The plates after developing were sprayed with vanillin-sulphuric acid and heated in an oven at 110 °C for 10-15 minutes.

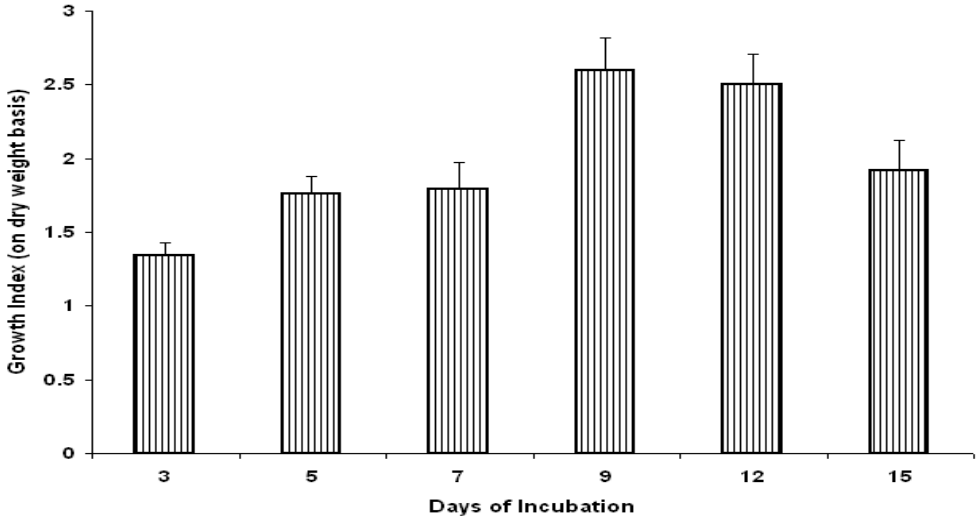
High Performance Liquid Chromatography (HPLC)

After detection of withaferin A by TLC, HPLC analysis was carried out using a modified procedure reported [17]. The system consists of Shimadzu LC-10AT pump with photo diode array (SPD-M 10A *VP* model) detector. The column used is C-18 (Tracer Analitica, Nucleosil-100, 25 cm x 0.4 i.d, 5µl). The mobile phase consists of Methanol: Water (53:47), pumped isocratically at ambient temperature with a flow rate 1 ml/min and withaferin A was detected at a λmax of 229nm. Initially a standard graph was plotted with the authentic sample and was used for the estimation of withaferin A in cultured flasks.

RESULTS AND DISCUSSION

The callus cultures of *Withania somnifera* were initiated and maintained on MS media supplemented with Dicamba (2 mg/l), Kinetin (0.1 mg/l) and Sucrose (3% w/v). Suspension cultures were initiated from 8 week old callus cultures in the same media without agar, which was found to be suitable for initiation and maintenance of suspension cultures from calli. The growth kinetics of suspension cultures is shown in Fig. 1. Maximum growth of cells was found to be on 9th day. A decline in growth was observed on day 15.

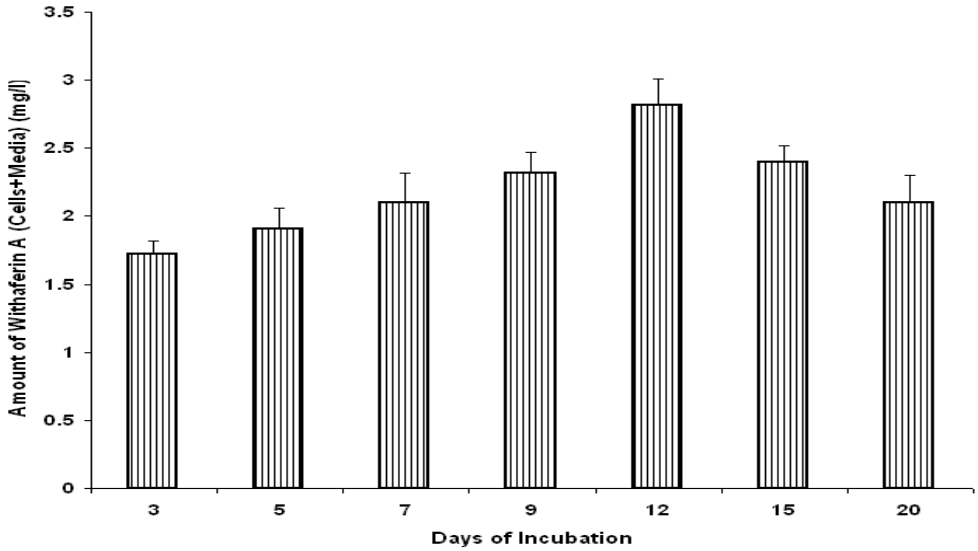
Fig. 1. Growth kinetics of suspension cultures of *Withania somnifera.*



On co-chromatography with authentic sample, the extracts obtained from the cultures revealed the presence of withaferin A. The extracted sample has the same retention factor (R_f) (0.52) as that of pure sample and showed a violet spot up on treatment with vanillin-sulphuric acid and heating the plates in oven at 110 °C for 10-15 min. on HPLC analysis, the extracts obtained from the suspension cultures showed a peak at retention time (14.6 min) same as that of authentic sample. The peak showed λmax and U.V spectrum as that of authentic sample using diode array detection.

Half B5 media supplemented with 2,4-D (1 mg/l), Kinetin (0.1 mg/l) and sucrose (5% w/v) was found to be suitable as production medium. The production kinetics of withaferin A is shown in Fig. 2. The maximum amount of withaferin A was found to be on day 12.

Fig. 2. Production kinetics of Withaferin A in suspension cultures of *Withania somnifera.*

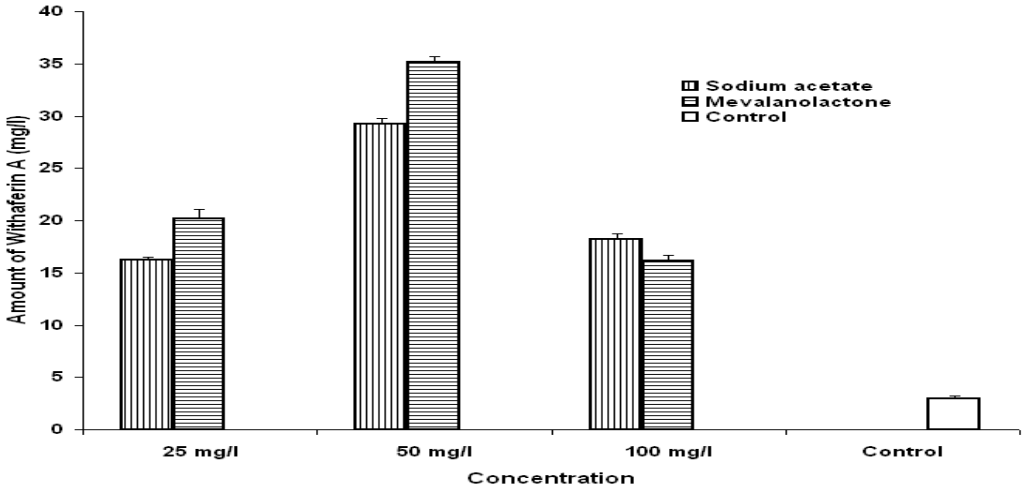


The content of withaferin A in cells and media was found to be 2.29±0.72 mg/l and 0.52±0.02 mg/l respectively. This result suggests that majority of the withaferin produced was intracellular.

The addition of Sodium acetate 25 mg/l, 50 mg/l and 100 mg/l, increased the withaferin A content to ~5, ~10 and ~6 folds (16.19±0.32, 29.21±0.58 and 18.24±0.48 mg/l) respectively over the control cultures (2.96±0.23 mg/l) Fig. 3.

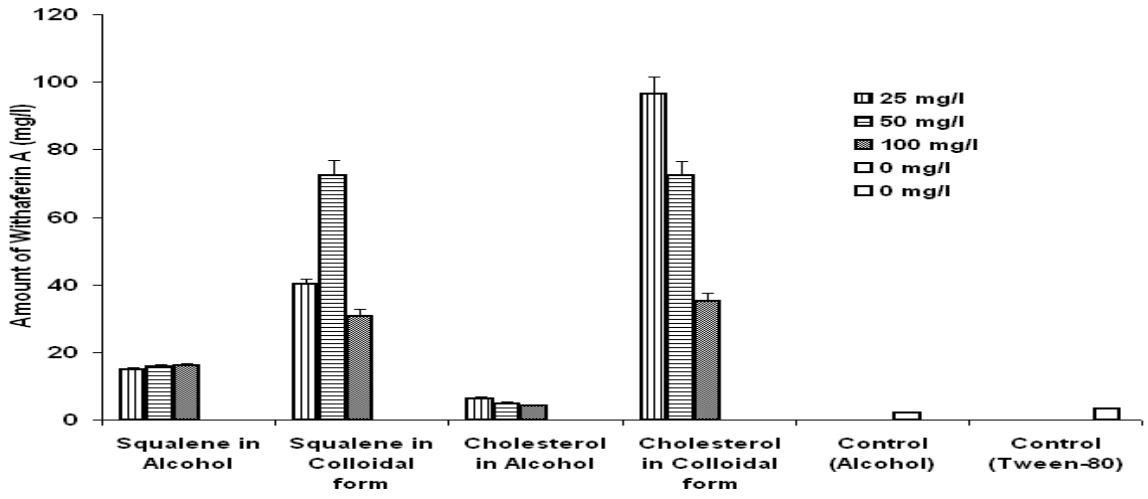
Mevalanolactone 25 mg/l, 50 mg/l and 100 mg/l supplementation to the suspension cultures, increased withaferin A content to ~7, ~14 and ~5 folds (20.16±0.90, 35.17±0.53 and 16.12±0.61 mg/l) respectively over the control cultures (2.96±0.23 mg/l) Fig. 3.

Fig. 3. Effect of Sodium acetate and Mevalanolactone on the bioproduction of Withaferin A.



Squalene 25 mg/l, 50 mg/l and 100 mg/l in alcohol increased withaferin A content to ~7.5, ~8 and ~6 folds (14.93±0.61, 15.76±0.73 and 11.82±0.52 mg/l) respectively over the control cultures (1.97±0.33 mg/l) and as colloidal form in Tween-80 same concentrations of squalene increased withaferin A content significantly to ~12.5, ~23 and ~9.5 folds (40.31±1.50, 72.44±4.32 and 30.73±2.14 mg/l) respectively over the control cultures (3.16±0.43 mg/l) Fig. 4.

Fig. 4. Effect of Squalene and Cholesterol on the bioproduction of Withaferin A.

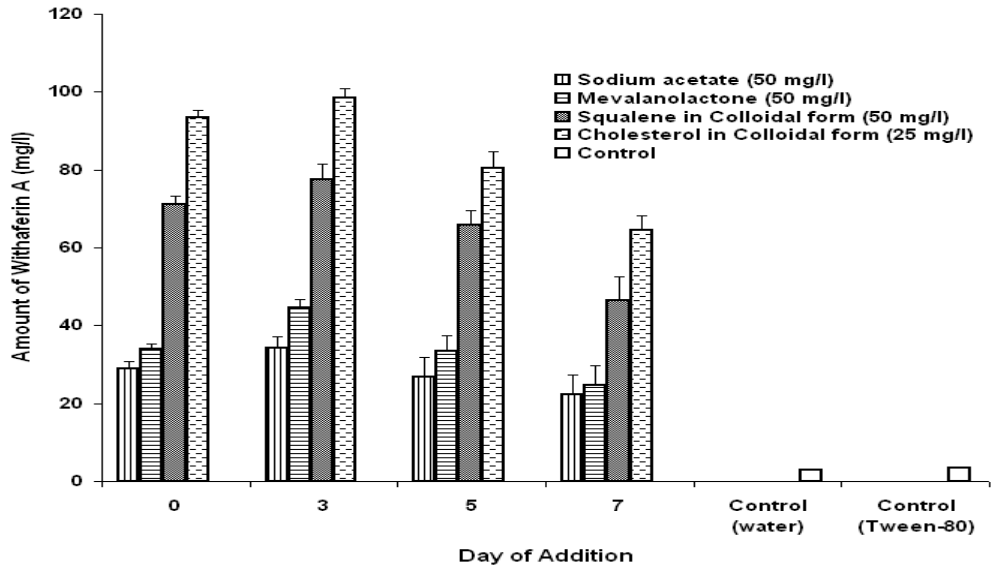


Addition of Cholesterol 25 mg/l, 50 mg/l and 100 mg/l in alcohol increased withaferin A content to ~3, ~2.5 and ~2 folds (6.18±0.52, 4.91±0.44 and 4.11±0.45 mg/l) respectively over the control cultures (1.97±0.33 mg/l) and as colloidal form in Tween-80 same concentrations of cholesterol increased withaferin A content significantly to ~30.5, ~23 and ~11 folds (96.51±5.12, 72.45±4.21 and 35.22±2.32 mg/l) respectively over the control cultures (3.16±0.43 mg/l) Fig. 4.

Withaferin A is a steroidal lactone and is biosynthesized from acetate via, mevalonic acid pathway [18]. The studies with precursors have shown a positive effect on bioproduction of withaferin A. Progressively higher yields of withaferin A were obtained starting with Sodium acetate (Acetate), Mevalanolactone (Mevalonic acid) to Squalene and Cholesterol. This is because of the utilization of the precursors by the cells in cultures may become easier as we move up in the biosynthetic chain whereby a significant increase in bioproduction was observed with squalene and cholesterol. The availability of the precursor to the cell is also an important factor to be considered for the improved production of withaferin A, which was proved by the addition of colloidal form of squalene and cholesterol. Addition of Cholesterol has shown enhancement in solasodine content in suspension cultures of *Solanum xanthocarpum* and *Solanum aviculare* [19].

Precursors (Sodium acetate 50 mg/l, Mevalanolactone 50 mg/l, Squalene in colloidal form 50 mg/l and Cholesterol 25 mg/l) which have significant effect on the improvement of bioproduction of withaferin A were studied for optimization of day of addition. All these have shown appreciable enhancement in yield of withaferin A when added on day 3 (34.38±1.40, 44.68±2.10, 77.61±3.90 and 98.61±6.10) to the cell cultures of *Withania somnifera*. This could be due to the reason that the biogenetic process has positively accelerated leading to increased production of withaferin A Fig. 5.

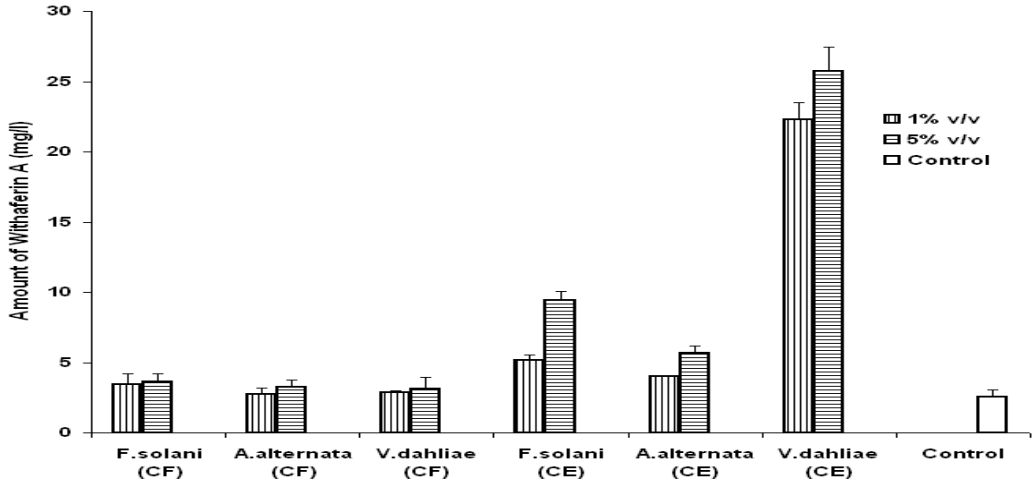
Fig. 5. Effect of effective precursors on different days of addition to suspension cultures of *Withania somnifera.*



Addition of 1% and 5% v/v CF solutions of *Fusarium solani* MTCC 350 (3.45±0.76 and 3.65±0.53 mg/l), *Alternaria alternate* MTCC 1779 (2.76±0.41 and 3.28±0.46 mg/l) and *Verticillium dahliae* MTCC 2063 (2.86±0.12 and 3.14±0.78 mg/l) to cell cultures of *Withania somnifera* had no significant effect on the improvement of withaferin A over control culture (2.54±0.53 mg/l). 1% and 5% v/v CE solutions of *Fusarium solani* MTCC 350 (5.16±0.38 and 9.41±0.64 mg/l), *Alternaria alternate* MTCC 1779 (3.99±0.10 and 5.66±0.54 mg/l) showed moderate increase in withaferin A content whereas addition of 1% and 5% CE solutions of

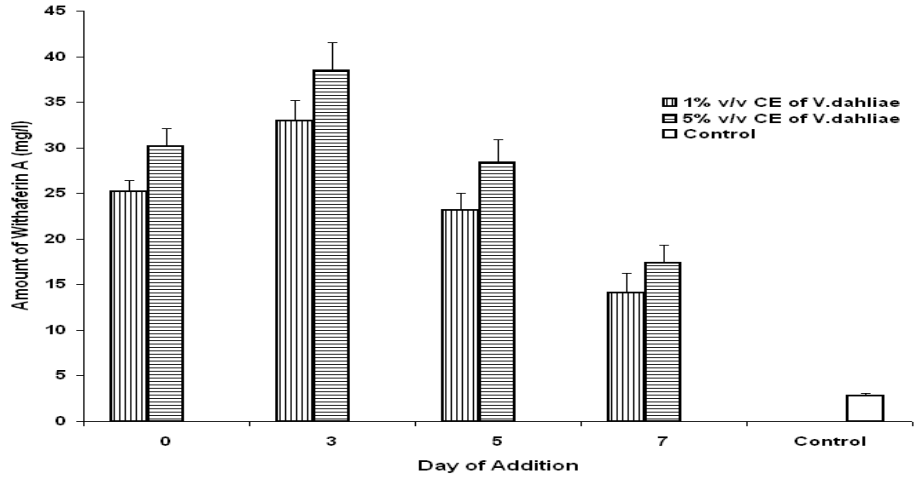
Verticillium dahliae MTCC 2063 has increased withaferin A content significantly to ~9 and ~10 folds (22.29±1.20 and 25.72±1.71 mg/l) over control cultures (2.54±0.53 mg/l) Fig. 6.

Fig. 6. Effect of biotic elicitors on bioproduction of Withaferin A.



The effective biotic elicitor *Verticillium dahliae* MTCC 2063 CE (1% and 5% v/v) was studied for day optimization and found that the addition of 1% and 5% CE of *Verticillium dahliae* on day 3 showed ~12 and ~14 folds (32.94±2.30 and 38.42±3.12 mg/l) increase in the bioproduction of withaferin A content over control cultures (2.78±0.31 mg/l) of *Withania somnifera* Fig. 7. Kaveti et al. [1] reported a moderate increase in the bioproduction of Azadirachtin by the addition of biotic elicitor *Fusarium solani* to the suspension cultures of *Azadirachta indica*.

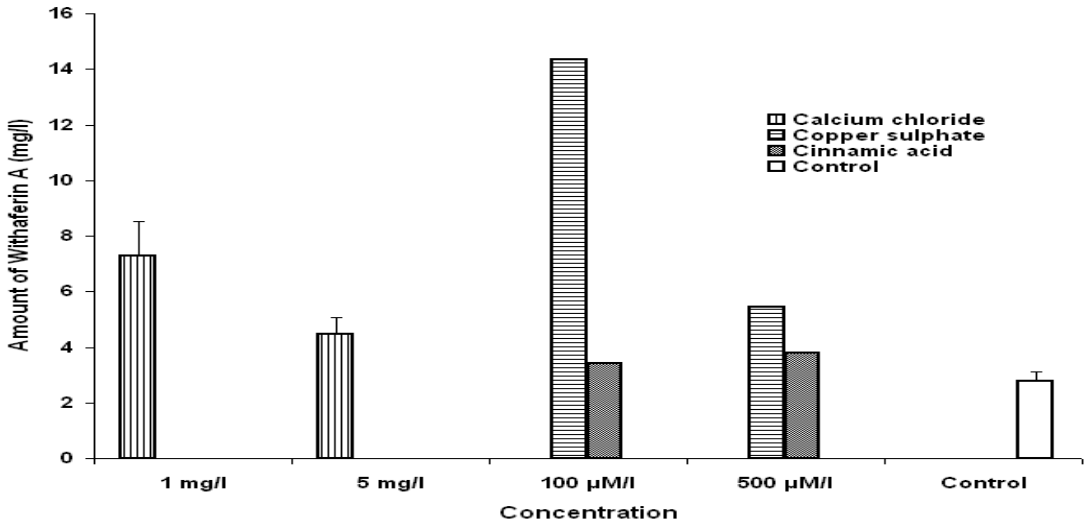
Fig. 7. Effect of *Verticillium dahliae* CE (cell extract) on different days of addition to suspension cultures of *Withania somnifera*.



The increase in the bioproduction of withaferin A by CE of *Verticillium dahliae* may be due to the fact that the extract used may have the compound capable of stimulating the plant defensive system at appropriate site to enhance the yield of secondary metabolites.

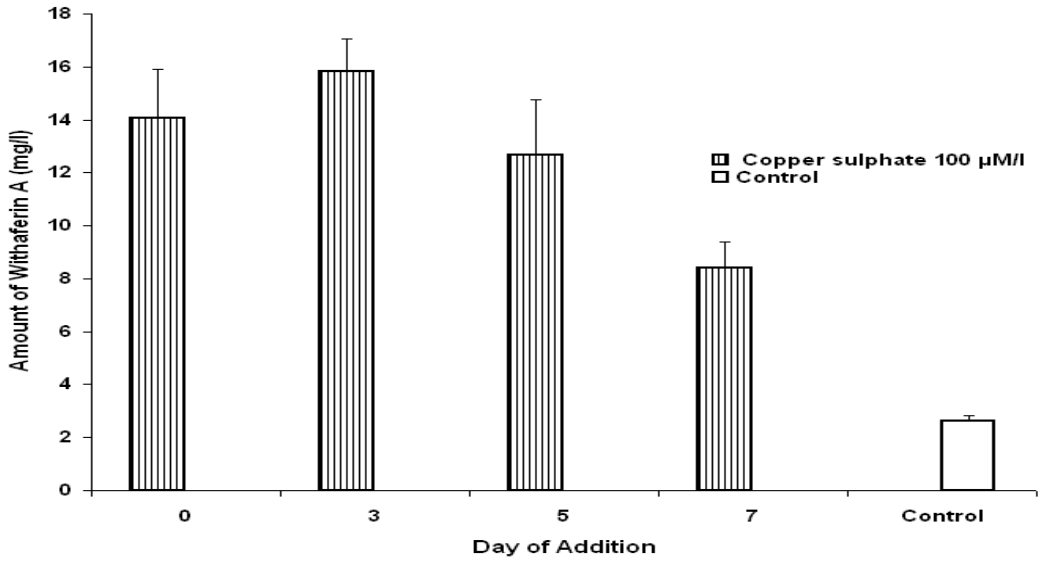
Addition of abiotic elicitors Calcium chloride 1mg/l and 5 mg/l, Copper sulphate 100 µM/l and 500 µM/l and Cinnamic acid 100 µM/l and 500 µM/l showed ~2.5 and ~1.5 folds (7.30±1.21 and 4.48±0.59 mg/l), ~5 and ~2 folds (14.33±2.10 and 5.44±0.69 mg/l) and ~1 and ~1.5 folds (3.41±0.19 and 3.79±0.63 mg/l) increase in the bioproduction of withaferin A content over control cultures (2.79±0.3l mg/l) of *Withania somnifera* Fig. 8.

Fig. 8. Effect of abiotic elicitors on bioproduction of Withaferin A.



There was a significant improvement (~6 folds) (15.81±1.24 mg/l) in the bioproduction of withaferin A at low concentration (100 µM/l) over control (2.61±0.22 mg/l) on day 3 addition of Copper sulphate Fig. 9.

Fig. 9. Effect of Copper sulphate on different days of addition to suspension cultures of *Withania somnifera.*



The increase in the bioproduction of withaferin A was found to be marginal by the addition of abiotic elicitors except Copper sulphate which showed a potential influence, this may be due to its ability to induce the enzymes involved in the biogenetic pathway of withaferin A. It was reported that, Copper (II) ions were optimal for inducing the accumulation of high levels of sesquiterpenoid phytoalexins in fruit cavities of thorn apple (*Datura stramonium*) cell cultures (White head et al. 1990).

CONCLUSION

The precursors enhanced the productivity of withaferin A in suspension cultures of *Withania somnifera*. Among the elicitors Copper sulphate and *Verticilium dahalie* cell extract showed significant effect on the production of withaferin A. The maximum productivity of cell cultures observed was 98 mg/l of withaferin A. The productivity of the cultures varied from 1.97±0.33 mg/l to 98.61±5.20 mg/l through out the study.

REFERENCES

[1] B. Kaveti, V. Ciddi, S. Keshetty, C. K. Kokate, *J. Plant Biotechnol*., **2003,** 5, 121.
[2] S. Chatterjee, S. K. Chakraborti, *Antonie van leuwenhoek*, **1980,** 46, 59.
[3] P.U. Devi, *Ind. J. Exp. Biol*., **1996,** 34, 927.
[4] M. Ziauddin, N. Phansalkar, P. Patki, S. Diwanay, B. Patwardhan, *J. Ethanopharmacol*., **1996,** 50, 69.
[5] R. D. Budhiraja, S. Sudhir, *J. Scientific and Ind. Res*., **1987,** 46, 488.
[6] S. Ray, S. Jha, *Planta Medica*, **2001,** 67, 432.
[7] G. Roja, M. R. Heble, A. T. Siphaimalani, *Phytotherapy Res*., **1991,** 5, 185.
[8] G. Vitali, L. Conte, M. Nicoletti, *Planta Medica*, **1996,** 62, 287.
[9] H. Dornenburg, D. Knorr, *Enz. Microbiol. Technol*., **1995,** 17, 674.
[10] C. A. Hay, L. A. Anderson, M. F. Roberts, J. D. Phillipson, *Plant Cell Rep*., **1986,** 5, 1.
[11] M. Sharada, A. Ahuja, K. Suri, S. Vij, R. Khajuria, V. Verma, A. Kumar, *Biologia Plantarum*, **2007**, 51, 161.
[12] V. Ciddi, *Ind. J. Pharm. Sci*., **2006,** 68, 490.
[13] T. Murashige, F. Skoog, *Physiol. Plant*, **1961,** 15: 473-497.
[14] O. L. Gamborg, R. A. Miller, K. Ojima, *Exper. Cell Res*., **1968,** 50, 151.
[15] G. Suvarnalatha, L. Rajendran, G. A. Ravishanker, C. Venkataraman, *Biotechnol. Lett*., **1994,**16, 1275.
[16] L. Dinan, J. Harmatha, R. Lafont, *J. Chromtography A*, **2001**, 935, 105.
[17] R. Beselle, D. Lavie, *J. Chromatography*, **1987,** 389, 195.
[18] W. J. S. Lockley, H. H. Rees, T. W. Goodwin, *Phytochemistry*, **1976,** 15, 437.
[19] P. Khanna, Aminuddin, G. I. Sharma, S. K. Manoj, A. K. Rathore, *Ind. J. Exp. Biol*., **1976,** 14, 694.
[20] M. I. Whitehead, L. A. Atkinson, R. D. Threfall, *Planta*, **1990**,182, 81.