

In Vitro Biogenesis of Alkaloids and Withanolides in *Withania somnifera* (L.) Dunal (Solanaceae) var. 'Poshita' and 'Jawahar 22'

Ananya Das¹ • Shyamal Ghose² • Anjan Bhattacharyya³ • Animesh K. Datta^{1*}

¹ Department of Botany, Genetics and Plant Breeding Section, Kalyani University, Kalyani 741235, West Bengal, India

² Plant Cell and Tissue Culture Technology Laboratory, Department of Genetics, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, West Bengal, India

³ Regional Analytical Laboratory on MAP, Department of Agricultural Chemicals, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, West Bengal, India

Corresponding author: * dattaanimesh@gmail.com

ABSTRACT

Withania somnifera (L.) Dunal (Family: Solanaceae; common name-Aswagandha, a plant species with immense therapeutic uses) var. 'Poshita' and 'Jawahar 22' (recommended varieties) were assessed for their chemical contents (amount of total alkaloids and withanolide was estimated; withaferin A and withanolide A content were quantified by HPLC) under *in vitro* culture conditions in 3 different stages of callus development (stage I – undifferentiated callus, stages II and III – differentiated callus) on MS medium with different hormonal (BA, kin, 2,4-D, IAA, IBA) combinations using epicotyl, shoot tip and leaf explants with the objective of developing a suitable protocol which ensure production of specific stipulated amount of secondary metabolites in a quick span of time. Results obtained were discussed. Stage III of callus development yielded the maximum amount of secondary metabolites. The yield of alkaloids, withanolides and withaferin A were maximum in leaf explants while that of withanolide A was highest in cultures initiated from epicotyl explants, although the medium composition varied for each variety.

Keywords: Ashwagandha, callus culture, organogenesis, secondary metabolites

Abbreviations: ANOVA, analysis of variance; AR, analytical grade reagent; BA, 6-benzyladenine; BCKV, Bidhan Chandra Krishi Viswavidyalaya; CD, critical difference; CIMAP, Central Institute of Medicinal and Aromatic Plant Sciences; 2,4-D, 2,4-dichlorophenoxy acetic acid; HPLC, high pressure liquid chromatography; HPTLC, high performance thin layer chromatography; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; Kin, kinetin; MS, Murashige and Skoog; ppm, parts per million; TLC, thin layer chromatography

INTRODUCTION

Withania somnifera (L.) Dunal (Family: Solanaceae; commonly known as Aswagandha; English name – winter cherry) is an evergreen bushy shrub with immense therapeutic uses (Bhattacharya 2001; Leyon and Kutton 2004; Gupta and Rana 2007; Iqbal and Datta 2007a) in Ayurvedic (Nadkarni 1976; Gupta *et al.* 2004; Patra *et al.* 2004) and also in modern formulations possessing adaptogenic (Bhattacharya *et al.* 1987), rejuvenative (Kothari *et al.* 2003), antioxidant (Bhattacharya *et al.* 2001) and antitumorigenic (Sangwan *et al.* 2004) properties. For restorative properties, roots of the species are compared with *Panax ginseng* (Tripathi *et al.* 1996). The pharmacological importance of *W. somnifera* is due to the presence of secondary metabolites like alkaloids and withanolides (steroidal lactones; major groups of secondary metabolites) primarily in roots (Singh and Kumar 1998; Mirjalili *et al.* 2009); however, withaferin A (type of withanolide) is predominantly present in leaf (Gupta *et al.* 1996). Oh *et al.* (2008) reported induction of apoptosis by withaferin A in human leukemia U937 cells.

Since ancient times the plants of Ashwagandha were used indiscriminately from wild habitats, but presently attention is been paid to keep them under cultivation for sustainable use and subsequently high yielding varieties are been released ('WS 20'/'JA 20', 'WS 22' and 'Jawahar 22' by Jawaharlal Nehru Krishi Viswavidyalaya-Mandsore, Madhya Pradesh: Nigam *et al.* 1991; 'WSR' by the Regional Research Lab, Jammu and 'Rakshita' and 'Poshita' by the Central Institute of Medicinal and Aromatic Plant Sci-

ences - CIMAP, Lucknow; Misra *et al.* 2001) from India. Though cultivation of Aswagandha is an appreciable proposition, agro-climatic factors play a significant role in the synthesis and accumulation of secondary metabolites (Obidoska and Sadowska 2003; Patra *et al.* 2004). Thus, it would be a difficult task to optimize field growth conditions for maximum yield of the compounds. Further, genetic control of phytochemical compound production and its interaction with environment is rather not known in the species. Therefore, an alternative strategy for the exploitation of *W. somnifera* would be of utmost importance to obtain steady production of alkaloids and withanolides throughout the year in relatively quick span of time. With this in mind, *in vitro* tissue and organ culture technology towards biogenesis of alkaloids and withanolides under optimum culture conditions may be considered for its future exploitation and globalization of the crop and thereby maximizing trade. The present investigation is therefore an attempt to select suitable culture medium, explant nature and stages of callus growth to yield a maximum amount of alkaloids and withanolides (including withaferin A and withanolide A, both pharmacologically important) in 'Poshita' and 'Jawahar 22' (high-yielding varieties) of *W. somnifera*.

MATERIALS AND METHODS

Seeds of 'Poshita' and 'Jawahar 22' were collected from CIMAP and Mandsaur, respectively, and were placed separately on Petri dishes lined with moist filter paper (33 ± 1°C) for germination. The germinated seedlings were transferred to a seed bed and sub-

sequently to field plots of the Medicinal and Aromatic Plant Garden at Bidhan Chandra Krishi Vishwavidyalaya - BCKV, Mohanpur, West Bengal to raise mature plants as a mother source for *in vitro* culture.

Before collection of explants, seedlings or plants were sprayed with 1.0% Bavistin (fungicide-carbendazim 50%, BASS India) for three consecutive days (in the morning) to check for fungal contamination. Explants (epicotyls, young shoot tips and young growing leaves) from the mother sources were excised on the same day, surface sterilized [thoroughly washed with 2% solution of Teepol followed by washing with distilled water and subsequently treated with 1% Bavistin for 30 min in a rotary shaker (Lunar, Amalgamated supplier Pvt. Ltd., India) and 0.1% HgCl₂ (Merck, India) for 3 min] and finally washed with sterile distilled water 3-4 times under aseptic conditions prior to their inoculation on agar (Hi-media, Type 1) gelled (0.8%) Murashige and Skoog (1962) (MS) basal medium (pH 5.8) with 3% sucrose (Merck, India). MS medium was supplemented with different phytohormonal combinations. The phytohormones (all in mg/L; SRL trade name) used were: 6-benzyladenine (BA; 1.5, 2.0, 2.5), Kinetin (Kin; 0.5, 1.0), 2,4-dichlorophenoxy acetic acid (2,4-D; 1.0, 1.5, 2.0, 2.5), indole-3-acetic acid (IAA; 1.0, 2.0, 3.0) and indole-3-butyric acid (IBA; 0.5, 1.0). IBA was used for shoot tips, 2,4-D for epicotyls and IAA for leaves (after repeated trials for the species) in addition to BA and kinetin in different combinations for callus growth and regeneration (per media composition 20 replications were made for each type of explant). Sterilized explants were excised into size grades (epicotyls, 15-days-old, 1-2 cm; young shoot tips, 2.0-3.0 cm in length and young growing leaves of ~100 mm² were taken from 3 months-old plants) and inoculated onto medium on a laminar air flow (ESCD Airstream) and incubated at 26 ± 1°C under florescent light of about 2500 lux for a 16-18-h photoperiod. Among different stages of callus development, the present study focused on the following three (Fig. 1A-D): stage I, creamish callus growing from the base of the explant (Fig. 1A); however, leaf callus was greenish and the entire leaf turned into callus; stage II, callus with shoot bud initiation and development (Fig. 1B, 1C); stage III, callus with 1.0 to 2.0 cm shoots (Fig. 1D). Days to stages of callus development varied with explants (Tables 1, 2). Chemical analysis (amount of total alkaloids and withanolide was estimated on the basis of dry weight; withaferin A and withanolide A content were quantified by high pressure liquid chromatography - HPLC) was conducted on three stages (3 replications/stage) of 'Poshita' and 'Jawahar22' callus development from the best media (Tables 1-4) combinations (based on callus weight, volume of enlargement and growth). Results obtained were statistically analyzed (mean value was calculated from randomly selected three

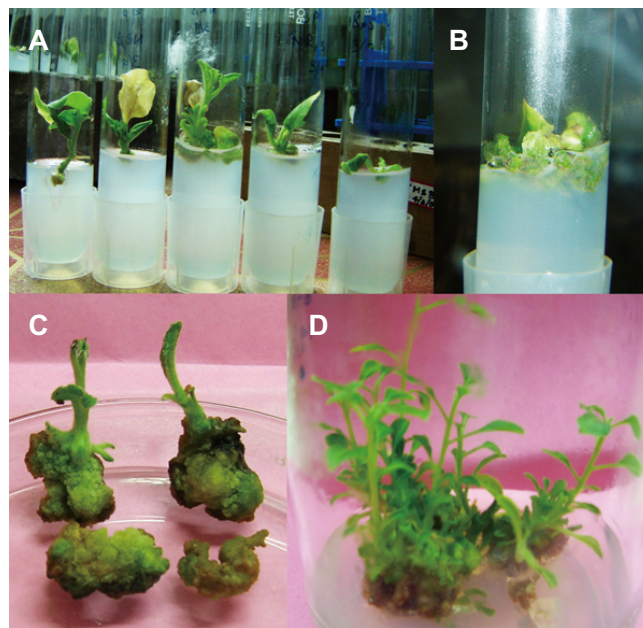


Fig. 1 (A) Base callus with shoot tip explant (stage I). (B) Organogenic callus (stage II). (C) Onset of regeneration in leaf callus (stage II). (D) Regenerated callus (stage III).

replicas; CD at 5% level was performed using analysis of variance - ANOVA). Significant variations (from critical difference - CD) for chemical contents were assessed among the stages for a particular medium and among the media for a particular stage to determine the best stage and media composition.

Total alkaloid and withanolide contents were extracted following the methodology reported by Gupta *et al.* (1996) with minor modifications. Samples (3 g of each replicate taken from 3 separate randomly selected calli which were kept at -20°C after harvest and powdered using mortar and pestle) were extracted with 50 ml methanol (J.T. Baker, analytical grade reagent - AR) in a sonicator (3.5 L Ultrasonic bath, PCI Mumbai) (60 to 70°C) for 2 h, filtered (using Whatman filter paper 41) and the residues (other than the filtrate) were again re-extracted twice with 2 × 50 ml methanol. The pooled methanolic extract (from three fractions) was dried in a rotary vacuum evaporator (BUCHI Rotavapor RII) at 40°C and the resultant crude extract was suspended in 100 ml distilled water and defatted with 50 ml hexane (J.T. Baker, AR) by

Table 1 Total alkaloid and withanolide contents (absolute %) in callus of shoot tip and epicotyl explant of *W. somnifera*. (Hormones in mg/L)

Media/explant	Stages of callus development							
	Alkaloid content (%)				Withanolide content (%)			
	I	II	III	CD	I	II	III	CD
Shoot tip								
P- MS+ BA 2.0	0.31	0.35	0.40	0.02	0.80	1.00	1.26	0.07
P- MS+ BA 1.5 + Kin 0.5	0.29	0.31	0.38	0.02	0.76	0.89	1.00	0.05
P- MS+ BA 1.0 + IBA 0.5	0.25	0.41	0.44	0.03	0.55	0.60	0.61	0.06
CD	0.02	0.03	0.01	--	0.07	0.07	0.05	--
J- MS+ BA 1.5 + Kin 0.5	0.29	0.31	0.36	0.02	0.50	0.85	1.00	0.10
J- MS+ BA 2.5	0.27	0.27	0.32	0.03	0.46	0.81	0.96	0.03
J- MS+ BA 1.0 + IBA 0.5	0.22	0.39	0.41	0.01	0.31	0.52	0.64	0.04
CD	0.03	0.02	0.01	--	0.02	0.02	0.01	--
Epicotyl								
P- MS+ BA 2.5	0.37	0.42	0.48	0.01	0.91	1.20	1.35	0.10
P- MS+ BA 1.0 + Kin 0.2	0.32	0.35	0.41	0.01	0.81	1.30	1.25	0.03
P- MS+ Kin 0.2 + 2,4-D 1.0	0.28	0.45	0.49	0.05	0.62	0.64	0.68	0.06
CD	0.01	0.05	0.01	--	0.04	0.12	0.08	--
J- MS+ BA 1.0 + Kin 0.5	0.34	0.38	0.42	0.03	0.65	0.93	1.12	0.01
J- MS+ BA 2.0	0.32	0.33	0.37	0.07	0.61	0.90	1.00	0.06
J- MS+ Kin 0.2 + 2,4-D 1.0	0.29	0.41	0.45	0.04	0.51	0.53	0.58	0.01
CD	0.03	0.02	0.08	--	0.03	0.01	0.05	--

MS, Murashige and Skoog; BA, 6-benzyl adenine; Kin, kinetin; 2,4-D, 2,4-dichlorophenoxy acetic acid; P, 'Poshita'; J, 'Jawahar'; CD, critical difference

I- Creamish callus developed after 25 days (shoot tip), 20 days (epicotyl) of inoculation

II- Callus with green bulbil-like buds obtained after 35 days (shoot tip), 30 days (epicotyl) of 1st inoculation

III- Callus with 1 to 1.5 cm shoots obtained after 65 days (shoot tip), 60 days (epicotyl) of 2nd inoculation

Table 2 Total alkaloid and withanolide content (absolute %) in callus of leaf explant of *W. somnifera*. (Hormones in mg/L)

Media/explant	Stages of callus development							
	Alkaloid content (%)				Withanolide content (%)			
	I	II	III	CD	I	II	III	CD
Leaf								
P-MS+ BA 3.0 (I)	0.48	0.50	0.61	0.06	1.24	1.61	2.32	0.03
MS+ BA 3.0 + Kin 1.0 +IAA 2.0 (II and III)								
P-MS+ Kin 3.0 (I)	0.40	0.42	0.54	0.03	1.20	1.55	2.10	0.05
MS+ BA 3.0 + Kin 1.0 +IAA 1.0 (II and III)								
P-MS+ Kin 3.5 (I)	0.38	0.48	0.51	0.05	1.10	1.40	1.41	0.07
MS+ BA 3.0 + Kin2.0+ IAA1.0 (II and III)								
CD	0.04	0.04	0.06	----	0.03	0.10	0.10	----
J-MS+ Kin 3.0 (I)	0.45	0.47	0.52	0.03	1.20	1.54	2.10	0.04
MS+ BA1.0 + Kin 3.0 +IAA 2.0 (II and III)								
J-MS+ Kin 3.5 (I)	0.41	0.44	0.46	0.05	1.13	1.45	1.87	0.06
MS+ BA1.0+ Kin 3.0 + IAA 1.0 (II and III)								
J-MS+ BA3.0 (I)	0.38	0.50	0.53	0.06	1.08	1.38	1.40	0.07
MS+ BA2.0 + Kin 3.0 + IAA1.0 (II and III)								
CD	0.06	0.05	0.06	----	0.05	0.06	0.03	----

MS, Murarshige and Skoog; BA, 6-benzyl adenine; Kin, kinetin; 2,4-D, 2,4-dichlorophenoxy acetic acid; P, 'Poshita'; J, 'Jawahar'; CD, critical difference

I- Creamish callus developed after 40 days (young leaf) of inoculation

II- Callus with green bulbil-like buds obtained after 65 days (young leaf) of 1st inoculation

III- Callus with 1 to 1.5 cm shoots obtained after 125 days (young leaf) of 2nd inoculation

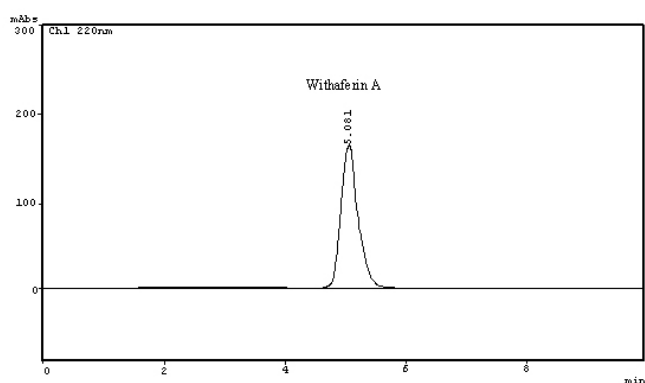


Fig. 2 Chromatogram of withaferin A - analytical standard.

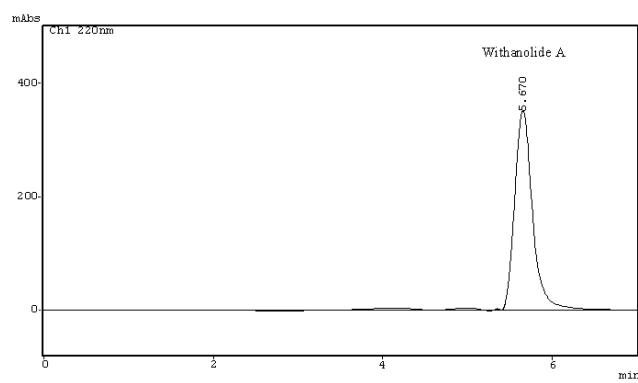


Fig. 3 Chromatogram of withanolide A - analytical standard.

partitioning. The aqueous extract was acidified with 1 N H₂SO₄ (Merck, 98%, AR) (1% with respect to the volume of the extract), extracted with diethyl ether (Merck, AR), passed through anhydrous Na₂SO₄ (Merck, AR) and evaporated to dryness to obtain the crude total withanolide content which was then dissolved in methanol (J.T. Baker, HPLC grade) and subsequently filtered through a nylon 6.6-membrane filter (pore size 0.2 μm, P.A.L.L. Corp.) to make it ready for HPLC analysis. The remaining part of the aqueous extract was basified with ammonia (25%-0.91 purity, Merck; ammonia solution was added drop by drop until pH 8.2 was obtained and pH was measured in a digital pH meter Electronic Measurement Pvt. Ltd., India), partitioned in (3 × 50 ml) chloroform (Merck, AR), passed through anhydrous Na₂SO₄ and evaporated in a rotary vacuum evaporator (40°C) to obtain total alkaloid content.

For HPLC analysis (Shimadzu-LC 10AT equipped with a Photodiode array detector SPD-M10A; reverse phase C18 column Phenomenex, size 250 × 4.60 mm) the following parameters were fixed for the entire analysis. The mobile phase consisted of methanol (HPLC grade): water (Millipore) (9: 1 v/v) with a 0.6 ml/min flow rate. The detector was set at λ_{max} = 227 nm. The injection volume of the samples was 20 μl. The peak of withaferin A and withanolide A was assigned by comparing the UV-spectra and retention time (RT) of the analytical standards (ChromaDex; withaferin A - ID No. ASB-00023250-005, withanolide A - ID No. ASB-00023251-005) (Figs. 2, 3). Quantification was accomplished using a 5-point calibration curve prepared by diluting the stock solution of the analytical standards in methanol (HPLC grade). Good linearity was achieved between 1.0 and 10.0 μg/ml with a correlation coefficient of 0.9978 (withaferin A) and 0.9899 (withanolide A). The limit of detection was estimated to be 0.30 and 0.25 μg/ml for withaferin A and withanolide A, respectively

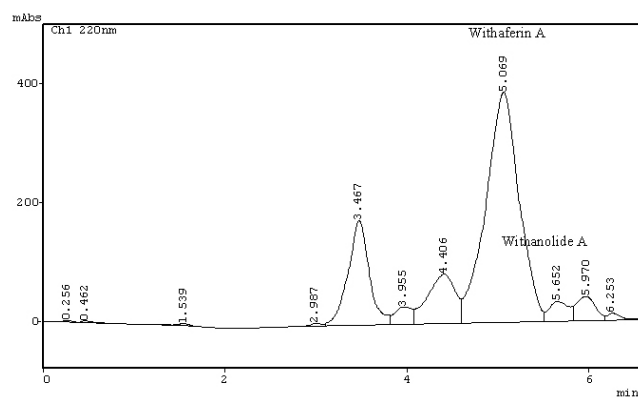


Fig. 4 Chromatogram of sample (leaf callus at stage II).

based on a signal to noise ratio = 3: 1. The amount of compounds (Fig. 4) was computed from chromatograms using the formula suggested by Scott (1996). Recoveries of withaferin A and withanolide A at different fortification levels (5, 10, 20 ppm) were determined in triplicate to validate and evaluate the accuracy of the method. The average recoveries were 97.84% for withaferin A and 95.35% for withanolide A.

RESULTS AND DISCUSSION

Results obtained from chemical analysis of 'Poshita' and 'Jawahar22' of *W. somnifera* are presented in Tables 1-4, which indicate that both varieties responded more or less identically and that callus stage III (differentiated callus

Table 3 Quantification of withaferin A and withanolide A (mg/g) in callus of shoot tip and epicotyl explant of *W. somnifera*. (Hormones in mg/L)

Media/explant	Chemical content at three stages of callus development							
	Withaferin A (mg/g)				Withanolide A (mg/g)			
	I	II	III	CD	I	II	III	CD
Shoot tip								
P- MS+ BA 2.0	0.00	0.32	0.20	0.01	0.00	1.69	2.21	0.03
P- MS+ BA 1.5 + Kin 0.5	0.00	0.26	0.17	0.01	0.00	1.58	2.13	0.05
P- MS+ BA 1.0 + IBA 0.5	0.00	0.00	0.00	--	0.00	1.49	1.77	0.01
CD	--	0.01	0.01	--	--	0.04	0.02	--
J- MS+ BA 1.5 + Kin 0.5	0.00	0.30	0.10	0.01	0.00	1.62	2.00	0.03
J- MS+ BA 2.5	0.00	0.24	0.07	0.03	0.00	1.53	1.89	0.03
J- MS+ BA 1.0 + IBA 0.5	0.00	0.00	0.00	--	0.00	1.45	1.65	0.02
CD	--	0.01	0.01	--	--	0.03	0.03	--
Epicotyl								
P- MS+ BA 2.5	0.00	0.35	0.13	0.02	0.00	1.85	2.42	0.02
P- MS+ BA 1.0 + Kin 0.2	0.00	0.29	0.09	0.01	0.00	1.79	2.28	0.02
P- MS+ Kin 0.2 + 2-4-D 1.0	0.00	0.00	0.00	--	0.00	1.65	1.85	0.02
CD	--	0.02	0.01	--	--	0.04	0.02	--
J- MS+ BA 1.0 + Kin 0.5	0.00	0.32	0.10	0.02	0.00	1.71	2.19	0.05
J- MS+ BA 2.0	0.00	0.27	0.07	0.03	0.00	1.68	2.01	0.02
J- MS+ Kin 0.2 +2-4-D 1.0	0.00	0.01	0.01	--	0.00	1.64	1.76	0.06
CD	--	0.01	0.03	--	--	0.05	0.06	--

MS, Murarshige and Skoog; BA, 6-benzyl adenine; Kin, kinetin; 2,4-D, 2,4-dichlorophenoxy acetic acid; P, 'Poshita'; J, 'Jawahar'; CD, critical difference

Table 4 Quantification of withaferin A and withanolide A (mg/g) in callus of leaf explant of *W. somnifera*. (Hormones in mg/L)

Media/explant	Chemical content at three stages of callus development							
	Withaferin A (mg/g)				Withanolide A (mg/g)			
	I	II	III	CD	I	II	III	CD
Leaf								
P-MS+ BA3.0 (I)	0.00	1.30	1.60	0.06	0.00	0.00	0.27	0.03
MS+ BA 3.0 + Kin1.0 + IAA2.0 (II and III)								
P-MS+ Kin 3.0 (I)	0.00	1.00	1.40	0.03	0.00	0.00	0.21	0.06
MS+ BA 3.0 + Kin1.0 + IAA1.0 (II and III)								
P-MS+ Kin 3.5 (I)	0.00	0.00	0.09	0.03	0.00	0.00	0.06	0.07
MS+ BA 3.0 + Kin2.0 + IAA1.0 (II and III)								
CD	----	0.05	0.06	----	----	----	0.02	----
J-MS+ Kin 3.0 (I)	0.00	1.00	1.40	0.04	0.00	0.00	0.21	0.03
MS+BA1.0 + Kin 3.0 +IAA 2.0 (II and III)								
J-MS+ Kin 3.5 (I)	0.00	1.20	1.22	0.04	0.00	0.00	0.18	0.04
MS+ BA1.0 + Kin 3.0 +IAA 1.0 (II and III)								
J-MS+ BA3.0 (I)	0.00	0.00	0.00	----	0.00	0.00	0.04	0.03
MS+ BA2.0+ Kin 3.0 + IAA1.0 (II and III)								
CD	----	0.06	0.05	----	----	----	0.02	----

MS, Murarshige and Skoog; BA, 6-benzyl adenine; Kin, kinetin; 2,4-D, 2,4-dichlorophenoxy acetic acid; P, 'Poshita'; J, 'Jawahar'; CD, critical difference

with multiple shoots) yielded a significantly higher amount of alkaloids and withanolides, including withaferin A and withanolide A, irrespective of the nature of the explant used. However, quantification of withaferin A was maximum in stage II with epicotyl and shoot tips and stage III with leaf explants. Thus, a shift towards organ development resulted in improved potential of alkaloid and withanolide production. Sharada *et al.* (2007) suggested that organogenesis might be a key regulatory factor stimulating the production of withanolides *in vitro* and also pointed out that the enzymes responsible for biogenesis of such compounds were possibly optimally operative in morphologically differentiated cultures. In the present investigation, the yield of alkaloid ('Poshita', 0.61% and 'Jawahar22', 0.53%), withanolides ('Poshita', 2.32% and 'Jawahar22', 2.10%) and withaferin A ('Poshita', 1.60 mg/g and 'Jawahar22', 1.40 mg/g) were estimated to be maximum in samples from leaf explants ('Poshita' medium: MS + BA 3.0 mg/L + kinetin 1.0 mg/L + IAA 2.0 mg/L; 'Jawahar22' medium: MS + BA 1.0 mg/L + kinetin 3.0 mg/L + IAA 2.0 mg/L). Withanolide A, in contrast, was highest in cultures initiated from epicotyl explants ('Poshita', 2.42 mg/g, medium: MS + BA 2.5 mg/L; 'Jawahar22', 2.19 mg/g, medium: MS + BA 1.0 mg/L + kinetin 0.5 mg/L). Results therefore indicated that in both varieties of *W. somnifera* differentiation of callus, media composition and the source of the explant were useful criteria for biogenesis of the studied secondary meta-

bolites. Wiermann (1981) attributed varied capacity to synthesized secondary metabolites *in vitro* to the morphological nature of explants utilized to initiate tissue culture lines. *In vitro* shoot culture was reported to be beneficial for withanolide A (Sangwan *et al.* 2007) and withaferin A (Ray and Jha 2001; Jha *et al.* 2005; Sharada *et al.* 2007) production. Sabir *et al.* (2008) suggested that multiple shoot cultures exhibited an increase in withanolide A accumulation compared to shoots of the mother plant. *In vitro*-generated root cultures as well as callus and suspension cultures also produced withanolides albeit at a lower level. Gupta *et al.* (1996) performed quantitative analysis of Indian chemotypes of *W. somnifera* by TLC densitometry and observed that withaferin A is totally absent in roots, stems and persistent calyx of intact plants but present in leaves (1.6%). In the present investigation withaferin A and withanolide A could not be detected by HPLC analysis from undifferentiated callus (stage I) thereby corroborating Yu *et al.* (1974), Heble (1985), Roja *et al.* (1991), amongst others. HPLC (Dalavayi *et al.* 2006; Chaurasia *et al.* 2008) and HPTLC (Srivastava *et al.* 2008; Nayak *et al.* 2009) methods were used successfully to quantify the amounts of different withanolides in Ashwagandha plant samples. Murthy *et al.* (2008) detected withanolide A in extracts of transformed roots and non-transformed root cultures using HPLC analysis and reported that its amount was 2.7-fold greater in transformed than in non-transformed roots.

We also estimated the amount of alkaloids ('Poshita', 0.54%; 'Jawahar22', 0.27%) and withanolides ('Poshita', 0.39%; 'Jawahar22', 0.31%), including withaferin A ('Poshita', 0.55%; 'Jawahar22', 0.50%) and withanolide A ('Poshita', 0.64%; 'Jawahar22', 0.60%) content from the roots of fully matured plants (plants were uprooted for collection of roots thereby affecting the population as the species is perennial in nature) under field conditions of West Bengal plains, India (data unpublished). Nigam *et al.* (1991) in 'Jawahar22' and Iqbal and Datta (2007b) in 'Poshita' reported 0.27% alkaloids and 0.5 to 0.8% alkaloids and 0.7 to 0.8% withanolides, respectively from roots under *in vivo* conditions. Comparative analysis of the chemical content (*in vitro* and *in vivo*) indicated an enhancement – mostly in *in vitro* culture conditions – with proper recommendations. Therefore, the present study is a positive endeavour providing a suitable protocol ensuring production of a specific, stipulated amount of secondary metabolites.

ACKNOWLEDGEMENTS

Authors readily acknowledge Excel Industries Ltd. for their financial assistance throughout the study.

REFERENCES

- Bhattacharya SK, Goel RK, Kaur R, Ghosal S (1987) Anti-stress activity of sitoinosides VII and VIII, new acylsteryl glucosides from *Withania somnifera*. *Phytotherapy Research* **1**, 32-37
- Bhattacharya A, Ghosal S, Bhattacharya SK (2001) Anti-oxidant effect of *Withania somnifera* glycowithanolides in chronic footshock stress-induced perturbation of oxidative free radical scavenging enzyme and lipid peroxidation in rat frontal cortex and striatum. *Journal of Ethnopharmacology* **74**, 1-6
- Chaurasia ND, Uniyal GC, Lal P, Misra L, Sangwan NS, Tuli R, Sangwan RS (2008) Analysis of withanolides in root and leaf of *Withania somnifera* by HPLC with photodiode array and evaporative light scattering detection. *Phytochemical Analysis* **19**, 148-154
- Dalavayi S, Kulkarni SM, Itikala RL, Itikala S (2006) Determination of withaferin A in two *Withania* species by RP-HPLC method. *Indian Journal of Pharmaceutical Sciences* **68**, 253-256
- Gupta AP, Verma RK, Misra HO, Gupta MM (1996) Quantitative determination of withaferin-A in different plant parts of *Withania somnifera* by TLC densitometry. *Journal of Medicinal and Aromatic Plant Sciences* **18**, 788-790
- Gupta GL, Rana AC (2007) *Withania somnifera* (Ashwagandha): a review. *Pharmacognosy Reviews* **1**, 129-136
- Gupta ML, Misra AK, Khanuja SPS (2004) Root-rot and wilt: a new disease of ashwagandha (*Withania somnifera*) caused by *Fusarium solani*. *Journal of Medicinal and Aromatic Plant Sciences* **26**, 285-287
- Heble MR (1985) Multiple shoot cultures: a viable alternative *in vitro* system for the production of known and new biologically active plant constituents. In: Neumann KH, Barz W, Reinhard E (Eds) *Primary and Secondary Metabolism of Plant Cell Cultures*, Springer-Verlag, Berlin, pp 281-289
- Iqbal M, Datta AK (2007a) Ashwagandha (*Withania somnifera*) – the elixir of life. *Plant Archives* **7**, 449-455
- Iqbal M, Datta AK (2007b) High yielding morphological variation in *Withania somnifera* (L.) Dun. *Plant Archives* **7**, 553-554
- Jha S, Bandyopadhyay M, Chaudhuri KN (2005) Biotechnological approaches for the production of forskolin, withanolides, colchicine and tylophorine. *Plant Genetic Resources* **3**, 101-115
- Kothari SK, Singh CP, Vijay KY, Singh K (2003) Morphology, yield and quality of ashwagandha (*Withania somnifera* L. Dunal) roots and its cultivation economics as influenced by tillage depth and plant population density. *Journal of Horticultural Science and Biotechnology* **78**, 422-425
- Leyon PV, Kuttan G (2004) Effect of *Withania somnifera* on B16F-10 melanoma induced metastasis in mice. *Phytotherapy Research* **18**, 118-122
- Mirjalili MH, Moyano E, Bonfill M, Cusido RM, Palazón J (2009) Steroidal lactones from *Withania somnifera*, an ancient plant for novel medicine. *Molecules* **14**, 2373-2393
- Misra HO, Sharma JR, Lal RK, Gupta MM, Shukla N, Singh NK, Singh N (2001) Registration of a new variety Poshita of *Withania somnifera*. *Journal of Medicinal and Aromatic Plant Sciences* **23**, 97-98
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497
- Murthy HN, Dijkstra C, Anthony P, White DA, Davey MR, Power JB, Hahn EJ, Paek KY (2008) Establishment of *Withania somnifera* hairy root cultures for the production of withanolide A. *Journal of Integrative Plant Biology* **50**, 975-981
- Nadkarni KM (1976) *Indian Materia Medica*, Popular Prakashan Ltd., Bombay, India, 1291 pp
- Nayak PS, Upadhyaya SD, Upadhyaya A (2009) HPTLC method for analysis of withaferin-A in Ashwagandha (*Withania somnifera*). *Journal of Planar Chromatography* **22**, 197-200
- Nigam KB, Patidar H, Kandalkar VS, Pathan MA (1991) A new, promising, pre-release variety, 'WS20', of ashwagandha (*Withania somnifera*). *Indian Journal of Agricultural Sciences* **61**, 581-582
- Obidoska G, Sadowska A (2003) Effects of pH and nitrogen content of the substrate on the yield of *Withania somnifera* (L.) Dun. *Biuletyn Instytutu Hodowli i Aklimatyzacji Roslin* **288**, 373-381
- Oh JH, Lee TJ, Kim SH, Choi YH, Lee SH, Lee JM, Kim YH, Park JW, Kwon TK (2008) Induction of apoptosis by withaferin A in human leukemia U937 cells through down-regulation of Akt phosphorylation. *Apoptosis* **13**, 1494-1504
- Patra DD, Singh K, Misra HO, Gupta AK, Singh J, Singh SC, Khanuja SPS (2004) Agrotechnologies of Ashwagandha. *Journal of Medicinal and Aromatic Plant Sciences* **26**, 332-335
- Roja G, Heble MR, Sipahimalini AT (1991) Tissue cultures of *Withania somnifera*: morphogenesis and withanolide synthesis. *Phytotherapy Research* **5**, 185-187
- Ray S, Jha S (2001) Production of withaferin A in shoot cultures of *Withania somnifera*. *Planta Medica* **67**, 432-436
- Sabir F, Sangwan NS, Chaurasiya ND, Misra LN, Sangwan RS (2008) *In vitro* withanolide production by *Withania somnifera* L. cultures. *Physiologia Plantarum* **63**, 409-412
- Sangwan RS, Chaurasia ND, Lal P, Misra LN, Uniyal GC, Tuli R, Sangwan NS (2007) Withanolide A biogenesis in *in vitro* shoot culture of Ashwagandha (*Withania somnifera* Dunal), a main medicinal plant in Ayurveda. *Chemical and Pharmaceutical Bulletin* **55**, 1371-1375
- Sangwan RS, Chaurasia ND, Misra LN, Lal P, Uniyal GC, Sharma R, Sangwan NS, Suri KA, Qazi GN, Tuli R (2004) Phytochemical variability in commercial herbal products and preparations of *Withania somnifera* (Ashwagandha). *Current Science* **86**, 461-465
- Scott RPW (1996) *Chromatographic Detectors: Design, Function, and Operation* (Vol 70), Marcel Dekker Inc., New York, 495 pp
- Sharada M, Ahuja A, Suri KA, Vij SP, Khajuria RK, Verma V, Kumar A (2007) Withanolide production by *in vitro* cultures of *Withania somnifera* and its association with differentiation. *Biologia Plantarum* **51**, 161-164
- Singh S, Kumar S (1998) *Withania somnifera* The Indian Ginseng Ashwagandha, Central Institute of Medicinal and Aromatic Plants, Lucknow, India, 131 pp
- Srivastava P, Tiwari N, Yadav AK, Kumar V, Shanker K, Verma RK, Gupta MM, Gupta AK, Khanuja SP (2008) Simultaneous quantification of withanolides in *Withania somnifera* by a validated high performance thin-layer chromatographic method. *Journal of AOAC International* **5**, 1154-1161
- Tripathi AK, Shukla YN, Kumar S, Kumar S (1996) Ashwagandha (*Withania somnifera* Dunal (Solanaceae): a status report. *Journal of Medicinal and Aromatic Plant Sciences* **18**, 46-62
- Wiermann R (1981) Secondary plant products cell and tissue differentiation. In: Stumpf PK, Conn EE (Eds) *The Biochemistry of Plants - A Comprehensive Treatise* (Vol. VII), Academic Press, New York, pp 86-116
- Yu PLC, EI Oley MM, Stohs, STA (1974) Phytochemical investigation of *Withania somnifera* tissue culture. *Journal of Natural Products* **37**, 593-597