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SHORT COMMUNICATION

Enhanced production of withaferin-A in shoot cultures of *Withania somnifera* (L) Dunal

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Abstract *Withania somnifera* (L) Dunal, commonly known as ashwagandha or Indian ginseng, is the source of large number of pharmacologically active withanolides. Withaferin-A (WS-3), a major withanolide of *W. somnifera,* has been proven to be an effective anti-cancer molecule. In this study, a liquid culture system for shoot proliferation, biomass accumulation and withaferin-A production of an elite accession (AGB002) of *W. somnifera* was investigated. The nodal explants cultured on Murashige and Skoog (MS) semi-solid medium supplemented with various concentrations of 6-benzyl adenine (BA) and Kinetin (Kn) elicited varied responses. The highest number of regenerated shoots per explant (35±3.25) and the maximum average shoot length (5.0± 0.25 cm) were recorded on MS medium supplemented with BA (5.0 μM). The shoots were further proliferated in half and full strength MS liquid medium supplemented with the same concentration BA. It was interesting to note that shoots cultured on MS half strength liquid medium fortified with 4 gL-1 FW (fresh weight) shoot inoculum mass derived from 5 week old nodal explants of *W. somnifera* showed highest accumulation of biomass and withaferin A content in 5 weeks. Withaferin A was produced in relatively high amounts (1.30 % and 1.10 % DW) in shoots cultured in half and full strength MS liquid media respectively as compared to natural field grown plants (0.85 % DW). A considerable amount of the withaferin A was also excreted in the culture medium. Successful proliferation of shoots in liquid medium and the synthesis of withaferin A in vitro opens new avenues for bioreactor scale-up and the large-scale production of the compound.

Keywords *Withania somnifera* · Withaferin A · Shoot cultures · Growth regulators

Abbreviations

BA	6-Benzyladenine
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
Kn	Kinetin
MS	Murashige and Skoog medium
HPLC	High performance liquid chromatography
DW	Dry weight
FW	Fresh weight

Withania somnifera (L.) Dunal (Ashwagandha), also known as Indian ginseng is a high value medicinal plant. It is widely distributed throughout the tropical and subtropical regions of world including India (Singh and Kumar 1998). In India, the species grows wild in the North Western region extending up to an elevation of 1,500 m in the hill regions of Punjab, Himachal Pradesh and Jammu and Kashmir (Singh and Kumar 1998). It is an extensively used medicinal plant in India and other parts of the world. It is an essential constituent of over 100 traditional medicinal formulations (Tripathi et al. 1996). *W. somnifera*

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contains a large number of pharmacologically active secondary metabolites known as withanolides which are prospective high-value drug candidates. The plant is reported to have anti-oxidative, antitumor, immunomodulation, anti-stress, cardio-protective, anti-inflammatory and neurogenerative properties principally attributed to the steroidal lactone withanolides (Tuli et al. 2009). Withaferin-A (WS-3), a major withanolide of *W. somnifera,* has been proven to be an effective anti-cancer molecule (Kaileh et al. 2007; Yang et al. 2011).

The production of *Withania* drugs in India has been estimated about 9,127-tonnes per year far exceeding the annual plant production of 5,905-tonnes (Sivanandhan et al. 2012). Presently, whole plants are being harvested from wild for the production of *Withania*-based medicines to meet a growing demand of pharmaceutical industries. This random harvesting of ashwagandha germplasm for withanolide production is economically and environmentally unwise as it causes loss of genetic diversity and habitat destruction. An elegant alternative to these apparent hurdles would be to identify the genotype rich in bioactive withanolides, develop high yielding in vitro production methods and to and improve them genetically for cultivation to meet the demand of bioactive withanolides.

Plant cell and organ cultures offer an excellent opportunity for homogenous, controlled production of metabolites, throughout the year, especially when we take commercial demand into account. They not only facilitate the *de novo* synthesis of novel compounds, but also are able to produce metabolites, even in higher amounts than in the intact plants. Over recent decades, several attempts have been made to improve withanolide production by tissue culture (Ray and Jha 2001; Sharada et al. 2007; Dewir et al. 2010). Production of bioactive molecules by in-vitro liquid culturing techniques has gained considerable attention in recent years, and such methods are increasingly attractive alternatives to whole plant cultivation for production of high value phytochemicals (Pati et al. 2010; Gawde and Paratkar 2012).

The pure compound of WS-3 is commercially obtained from the aerial parts of *W. somnifera* plants but the production is not sufficient for the current market demand. The chemical synthesis of withaferin A is complex and is not economically viable. Therefore, enhanced production of withaferin A via in vitro cell culturing is highly desirable. Here, we report the successful establishment of *W. somnifera* multiple shoot cultures and the standardization of a liquid shoot culture system for rapid proliferation of multiple shoots, as a cost effective system for the production of withaferin A.

Plant material for the present study was selected from Ashwagandha Gene Bank stocks at the Indian Institute of Integrative Medicine (IIIM), Jammu in the form of 55 populations collected from different parts of India and characterized through morphochemical and molecular markers. An elite WS-3 rich accession (AGB002) of *W. somnifera* collected from Bikaner, Rajasthan was used as a source of explants. The leaves of this accession were rich in WS-3 (0.85 % of DW). The nodal explants were washed thoroughly under running tap water for 30 min and soaked in 1 % (v/v) solution of *Tween-20* (Sigma-Aldrich, St. Louis, USA) for 15 min followed by washing under running tap water for 1 h to remove dust particles. The cleaned tissues were cut into nodal segments (1 cm) and aseptically treated with Bavastine (1 % w/v) for 15 min followed by treatment with 0.25 % (w/v) $HgCl_2$ for 3 min. The explants finally washed four times with sterile distilled water were cultured on Murashige and Skoog (1962) (MS) media supplemented with 3 % (w/v) sucrose and various combinations and concentrations of the cytokinins, 6-benzyladenine (BA) (0.5, 1, 2, 5, 7 and 10 μM) and Kinetin (Kn) (1, 2 and 5 μM). The pH of the medium was adjusted to 5.8–5.9 prior to the addition of 0.8 % agar (w/v). The medium was sterilized by autoclaving at 15 psi and 121ºC for 20 min. Static (semi-solid) cultures and liquid cultures were maintained under a 16 h photoperiod, illuminated with a cool white fluorescent (F 40 T 12/CW/EG) lamp at a photon flux density of 100 μ mol m^{-2} s^{-1} at 25±2ºC. Relative humidity was maintained at 60–70 %. The proliferative shoot culture lines established on MS medium supplemented with BA (5.0 μM) were maintained by regular subculture at five weekly intervals. Liquid cultures were shaken continuously at 85 rpm.

Cultures were established with an optimized shoot inoculum mass of 4 g l^{-1} FW shoot cluster. After 4 weeks of culture, the shoot number, length, number of nodes, fresh weight, dry weight and withaferin A content were recorded in static cultures. WS-3 production and biomass accumulation was monitored for 5 weeks after every week in case of liquid cultures. After every week shoots from the flasks were harvested, washed under running water to remove the media adhered, oven dried at 45–50ºC for 48–50 h, powdered and weighed. Each experiment consisted of ten culture flasks and was repeated a minimum of three times.

WS-3 was exhaustively extracted from dried shoot powder with water: ethanol (1:1 v/v) with stirring at room temperature (25±2ºC) for 6 h. The extract was filtered through Whatman No.1 filter paper to remove debris and evaporated to dryness under vacuum. The dried residue was weighed and re-dissolved in a known volume of HPLC grade methanol for quantification. WS-3 was extracted from liquid cultures using methods similar to those used for the plant tissues. WS-3 was quantified using a Shimadzu HPLC system consisting of a LC-10AT*VP* pump, SIL-10AD*VP* auto-sampler, CTO-10ASVP column oven, a SPD-M10A*VP* diode array detector and SCL-10A*VP* Version 5.40 (Shimadzu). A C_{18} phenomenex column (5 μm, 250×4.0 mm I.D., PN 00G-4252-EO) was used with UV detection at 237 nm as described previously. Samples were eluted with MeOH-H_2O (60: 40,

v/v) at a flow rate of 0.7 ml min^{-1}. Class *VP* software (Version 6.10) was used for data analysis and processing. The identity of WS-3 was confirmed by co-injection of an authentic standard and quantified by integrating peak areas as previously described. Comparative quantitative estimations in the native plant (AGB002) and its cultures were made in this study using the same method as described here.

Withaferin A marker used in the present study was isolated in the natural product chemistry division, IIIM using chromatography techniques. Purity of these markers was confirmed by various spectral data. Growth hormones used were purchased from Sigma Aldrich. Data were statistically analyzed using analysis of variance (ANOVA) using SPSS software. Data are presented as mean ± standard error (SE).

In this study, multiple shoots were initiated on MS medium supplemented with different concentration of BA and Kinetin (Kn). Shoot multiplication was observed at all the concentrations of BA alone or in combination with Kn. However, the average number of shoots per explants and ex-plant responses varied significantly between treatments (Table S1). Among different plant growth regulators, BA proved to be effective in inducing multiple shoots on MS medium. BA is thought to shorten the duration of S-phase of cell division by recruitment of latent DNA replication processes in both in vitro and in vivo systems (Francis and Sorell 2001). The present finding was in harmony of the previous reports on induction of multiple shoots in *W. somnifera* (Ray and Jha 2001; Sharada et al. 2007; Ahuja et al. 2009; Dewir et al. 2010). The highest shoot multiplication of 35±3.25 shoots per ex-plant with an average shoot length of 5.0±0.25 cm was observed on MS medium supplemented with BA (5.0 μM) (Table S1 & Fig. 1a). However, further increase in the concentration of BA alone or in combination with Kn did not elicit any greater response in the parameters studied (Table S1). It was also interesting to note that shoot cultures obtained with BA (5.0 μM) and Kn (1.0 μM) flowered profusely with 60 % in vitro flowering after 8 weeks and the flowers were fully functional.

The shoots (4 gL^{-1} FW) derived from 4 week old nodal explants of *W. somnifera* were further cultured in 250 ml flasks containing 50 ml liquid half and full strength MS media supplemented with the same concentration of BA. Liquid medium has been selected for mass production of shoots as liquid cultures are considered beneficial for the production of withanolides of uniformity. Figure 1b–c shows the representative shoot multiplication in liquid medium. Both shoot biomass and WS-3 (Fig. 1d) content was monitored weekly for 6 weeks in both half and full strength MS media. A steady

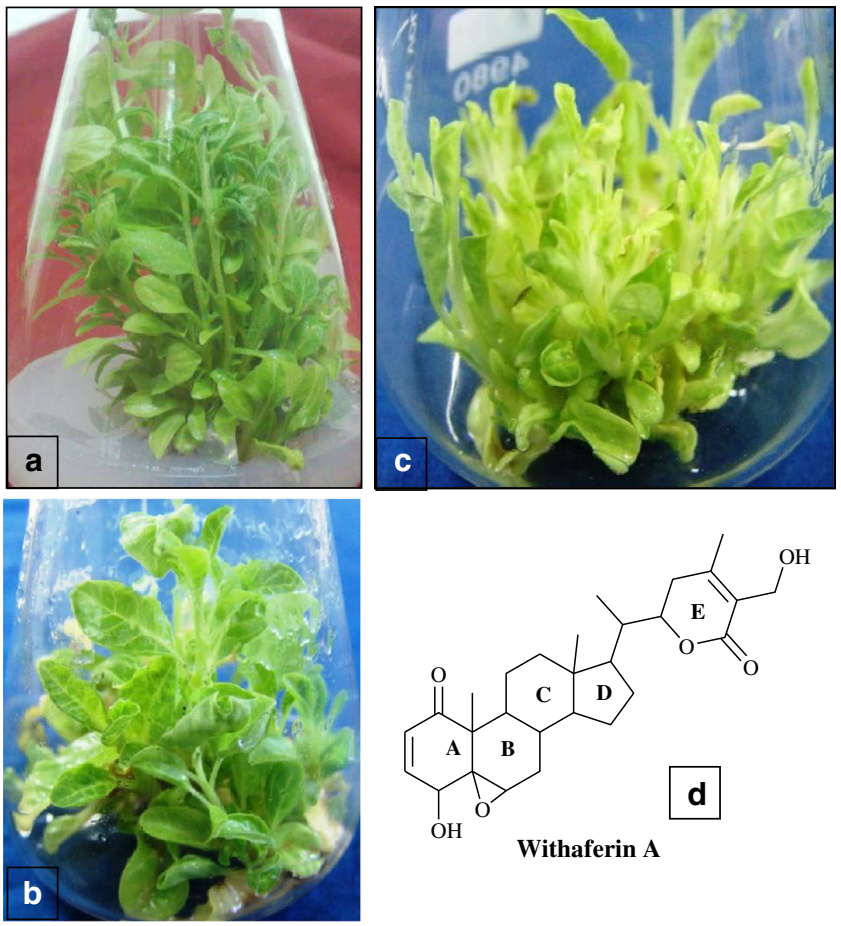

Fig. 1 **a** Shoot multiplication from nodal explants of *W. somnifera* on MS static medium supplemented with 5.0 μM BA after 4 weeks, (**b & c**) Shoot multiplication after 5 weeks of culture in half strength MS liquid medium supplemented with 5.0 μM BA, (**d**) structure of withaferin A

increase in the biomass accumulation and WS-3 content in both the treatments was observed from the first week of inoculation of explants to the fifth week, after which a slight decrease in the WS-3 percentage was observed (Fig. S1). The possible reason for this decline may be the inter-conversion or degradation of withanolides and leaching in to the medium. The maximum accumulation of WS-3 was recorded by the end of fifth week in both the treatments however, it was 1.3 % of DW in the half strength MS medium and 1.10 % of DW in case of full strength medium (Table S2). The biomass accumulation and WS-3 content in MS half strength medium reached a maximum of 25.5±2.15 g FW (2.5±0.08 g DW) and 1.3 % DW biomass respectively by the end of fifth week (Fig. S1). Biomass accumulation and shoot multiplication levels did not show any significant variations. In both media, shoots showed fast proliferation with an observable increase in leaf size, shoot length, intermodal distance, and shoot numbers (Fig. 1b). The number of shoots per ex-plant in liquid culture was higher 38± 4.5 as compared to 35±3.25 shoots on static media (Fig. c). With the high growth rate of shoots, almost 80 % of the medium was utilized at the end of the fifth week culturing cycle.

WS-3 production was significantly higher in tissue culture raised shoots, as compared to the mother donor plant (Table S2; Fig. S2). This finding was consistent with previous reports of withanolide production by shoot cultures of *W. somnifera* (Ray and Jha 2001; Sharada et al. 2007; Ahuja et al. 2009). Ray and Jha (2001) showed the accumulation of withanolides such as withaferin A and withanolide D in micro shoots in MS liquid medium supplemented with BA and coconut water. Sharada et al. (2007) observed the importance of ex-plant selection in the production of withanolides. They recorded that the culture lines established from leaf explants accumulated a higher level of withanolides and those from shoot tips of fully developed plants produced lower level of withanolides when compared to field-grown parent plant. Sivanandhan et al. (2011) recorded that the polyamines along with plant growth regulators enhanced the withanolides production in in vitro-raised plants when compared to field-grown parent plants. Ahuja et al. (2009) studied the accumulation of glycowithanolides. In the present study, influence of half and full strength liquid MS media on the withanolide production was also studied. It was very interesting that both the combinations were found effective in terms of WS-3 production and biomass accumulation however, half strength medium showed approximately 0.20 % (DW basis) higher WS-3 accumulation compared to full strength liquid MS medium (Table S2). This study has successfully demonstrated an enhancement of WS-3 production by in-vitro cultures grown on liquid MS media, and reports the optimization of culture conditions. A further important benefit of using a liquid culture medium is the leaching of WS-3 into the extracellular liquid phase, at concentrations as high as 0.35 mgL^{-1} (data not shown).

Interestingly, half-strength liquid MS medium resulted in greater enhancement, it is therefore, anticipated that half-strength liquid MS medium would serve as a cost-effective alternative for the production WS-3. This study thus, enhances the prospects for establishment of cost effective bioreactor for the large scale in vitro synthesis of the compound in *W. somnifera*.

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