Biochemical study of root extract of *Withania somnifera* (L.) plant through HPLC analysis

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ABSTRACT

Secondary metabolite contents of *W. somnifera* varied remarkably between seasons and genotypes under *ex vitro* condition. *In vitro* studies provide an optimum culture condition for steady and quality production of bioactive chemicals throughout the year without involvement of environmental stresses. Mass production of micro-shoots and plantlets by exploring the organogenic totipotency of shoot tip explants (*ex vitro* and *in vitro* grown) considering two elite genotypes (Poshita and Jawahar 22) of *W. somnifera*, and assessment of their capability in production and accumulation of bioactive metabolites (total alkaloid and withanolides amount were quantified; withanolide A and withaferin A contents were estimated by High Performance Liquid Chromatography-HPLC).

Key words: Bioactive chemicals, Culture condition, *Ex vitro*, *In vitro*, Mass production, Micro-shoots, Secondary metabolite, *W. somnifera*.

INTRODUCTION

*Withania somnifera*, common name as Ashwagandha, is an very important medicinal plant in Indian tradition system normally for over 3000 years it has been used in indigenous medicine in Ayurveda. Now various scientific researches carried out with *Withania* due to its high different types of therapeutic potential. *Withania* consists of withanolides (Kirson, *et al.*, 1971; Chaurasiya, *et al.*, 2008; Lal, *et al.*, 2006) as their major constituents which is 28 carbon containing steroidal lactone in which C-26 and C-22 are normally oxidized and form a 6-membered of lactone ring. Now due to several pharmacological investigations it is proved that *Withania* extract contains several important contents (Mirjalili, *et al.*, 2009). In the present study, we report indirect organogenesis through leaf and nodal segment. Further, the methanol extract prepared from the roots (Khan, *et al.*, 2014) of *Withania* were subjected to RP-HPLC for Withanolides or withaferin (Dalavayi, *et al.*, 2006). Rana, *et al.*, (2012) worked on the valuable medicinal property of Ashwagandha plant, to produce natural objects artificially with a wide series of pharmacologically active secondary metabolities used as study of drugs known as withanolides. Organogenic calli were induced on Murashige and Skoog medium containing 2 mgl⁻¹ kinetin and 1 mgl⁻¹ indole-3-butyric acid. High-performance liquid chromatography was used for measurable by quantity to determine by the major withanolides in the somaclones.

The major part of *Withania* used in medicine in traditional medical field is the dry root or tuber. Because of their starchy nature Indian cultivar and farmers is preferred drug manufacturers through it. A protocol of *in vitro* cultivation developed for improve production of Ashwagandha by Amirkhani, *et al.*, (2010).

MATERIAL AND METHODS

The seeds of the cultivated variety of *Withania somnifera* L. (Dunal) were obtained from the local nurseries while the seeds of wild varieties obtained from several research institutes like National Botanical Research Institute, Forest Research Institute, Dehradun and Central Institute of Medicinal and Aromatic plants, Luchnow. The seeds were rinsed with 90% ethanol for 10-20 second followed by 0.1% Mercuric chloride for 10-15 min. Then seeds were washed with 4-6 times in sterile double distilled water or autoclaved water to remove all traces quantities of MgCl₂. Five to seven (5-7) seeds were inoculated in a culture tube having 20ml of MS medium (Murashige and Skoog, 1962) which contains 3% sucrose and 0.8% agar (Himedia, India). The optimum pH of the medium was taken to be 5.8 before the autoclaving at 15 lbs pressure and 121°C for 20min. Here all the given cultures were incubated at 25±2°C with 16hrs photoperiod provided by cool while fluorescent tubes. After 10-15 days young leaves of cultivated and wild variety of withania have been collected from growing young plants and surface sterized. Cotyledon explants (size 1.5cm) were inoculated...
in full strength MS medium supplemented with 0.8% agar-agar and same concentration (1mg/l - 3mg/l) of 2, 4-D, NAA and IBA. Maximum callus formation in cultivated and wild explants occurs in MS medium supplemented with 1mg/l IBA, 1mg/l NAA and 2mg/l 2,4-D. When the well developed callus forms it has been transferred to shoot induction media it may contain BAP alone, Kn alone and combination of BAP + Kn. After three to four weeks shoots are forms from the callus. MS medium with BAP and Kn alone show maximum formation of shoot from callus with concentration of 3mg/l BAP and 2mg/l Kn while maximum frequency of shoot initiation in cultivated as well as wild variety takes placed with BAP. When the well developed shoots forms (after 3-4 weeks) it has been transferred to root induction media it may contain 0.5mg/l BAP + 1.0mg/l IBA. In MS medium maximum rooting takes placed with 0.5mg/l BAP and 2mg/l IBA in both plants. For hardening-off, 7 to 8 weeks old rooted shoots were withdrawn from the culture flasks. After giving the washing treatment to Agar with water the rooted plantlets obtained from shoots were transferred to bags of polythene or small pots that contains vermicompost fertilizer, red soil and sand in the mixture ratio of 1:2:2 and transferred in a mist house for acclimatization. After successful acclimatization in the mist house for 2-months successfully grown plants are transferred to greenhouse. Highest hardening and surviving frequency also appears in those in vitro plants, which was rooted in MS medium supplemented with 2mg/l IBA and 0.5mg/l BAP. After this plants are subjected to extraction. Root extract of *Withania somnifera* were analyzed for the presence of glycosides, steroids, phenolic compounds and flavonoids. One gram of sample was weighed and dissolved with various solvents such as ethanol, methanol and water. Then the sample was allowed to stay overnight for 24 hours. After overnight incubation the sample was filtered by Whatman filter paper then the filtrate was centrifuged at 25,000 rpm for 10-15 minutes, and the supernatant was used for HPLC and Phyto-chemical screening.

**Used for tissue culture:** Medium used for tissue culture for *in vitro* growth and regeneration of Ashwagandha was the standard MS medium (Murashige and Skoog, 1962) containing macronutrient salts, vitamins, Fe-EDTA, 0.01% (w/v) myo-inositol along with 3% (w/v) sucrose. The media composition is listed as below:

For MS media, four stock solution were prepared as follows:

| Stock I  | Macronutrients | 10x  |
| Stock II | Macronutrients | 100x |
| Stock III| Fe-EDTA        | 100x |
| Stock IV | Vit and AA     | 100x |

High-performance liquid chromatography (sometimes referred to as high-pressure liquid chromatography), HPLC, is a chromatographic technique used in analytical chemistry and analytical recent biochemistry to separate a mixture of compounds for the purpose to identify, quantify and purify the individual specific substances of the complex mixture.

In HPLC sample mixture injected in a sampler, pumps sample mechanically for pushing sample in liquid form by a tubing system to a separation column and concentration of components in a mixture detected by a digital analyte detector which may be a UV/Vis, or a photodiode array (PDA) for quantitative analysis and qualitative analysis of the separation having a digital microprocessor to control HPLC (Shashi, *et al.*, 2004) components and user software (Khajuria, *et al.*, 2004).

**RESULTS AND DISCUSSION**

**HPLC analysis of root extract of *Withania***: The principle chemical constituents of these plants, are withanolides, mainly present in leaves (also found in shoots and roots at low concentration) and their optimum concentration usually may range from 0.001 to 0.5% dry weight (DW). The productivity and isolation of withanolide-A in the *in vitro* cultures varied and fluctuates between 0.014 - 0.14 mg/gram of fresh weight of *Withania* plant with the alternatively change in the growth hormone composition and concentration of the nutrient culture basal media as well as its genotype used as important source of the excised explants. After which at different growth periods Withaferin-A contents of roots were analysed using HPLC, in order to evaluate the secondary metabolite contents from plants of different phonological stages. HPLC which are designed to provide high accuracy and high sensitivity analysis. The HPLC analysis of root extract determine the 0.022% and 0.023% of withanolide-A shows that these plants grow in high percentage of BAP (i.e. 1.00 ppm) and kinetin (i.e. 2.00 ppm) as shown in Table 1.

The knowledge of various chemical constituents of species of *Withania* has been extensively and successfully studied and large numbers of chemical constituents such as steroidal lactones, tannin, alkaloids, flavonoids, etc. have been identified, extracted, and isolated (Kapoor, 2001; Atta-ur-Rahman, *et al.*, 1991, 1993; Choudary, *et al.*, 1996; Rastogi and Mehrotra, 1998; Bandypadhyay and Jha, 2007). At present, knowledge indicated that more than twelve (12) alkaloids, forty (40) withanolides, and several different sitoindosides (a withanolide having a glucose molecule occur at carbon 27) have been obtained and identified.

**Table 1:** Differentiation of qualitative traits (W & C) by cotyledons leaf explants and their alkaloids percentages with conc. 2.0 mg/l Kn +1.0 mg/l BAP

<table>
<thead>
<tr>
<th>No. of explants</th>
<th>Shoots formation (%)</th>
<th>Cultivars</th>
<th>Alkaloids % withanoside IV + withanolide</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>89.56±2.24</td>
<td>Wild</td>
<td>0.023 % w/w</td>
</tr>
<tr>
<td>90</td>
<td>87.00±1.31</td>
<td>Cultivated</td>
<td>0.022 % w/w</td>
</tr>
</tbody>
</table>

(Mean ± or ‑ Standard error).
will be noted from aerial parts, roots and fruits of *Withania* species. The principle chemical constituents of these plants, are withanolides, mainly present in leaves (also found in shoots and roots at low concentration) and their optimum concentration usually may be ranges from 0.001 to 0.5% dry weight (DW) (Kapoor, 2001; Anonymous, 2004).

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**REFERENCES**


