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Callus induction and in vivo and *in vitro* comparative study of primary metabolites of *Withania Somnifera*

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ABSTRACT

In present study callus was raised from the leaf explant of Withania somnifera. Maximum callus was obtained on MS medium supplemented with 2, 4- D 1.0 mg/liter and IBA 0.5 mg/liter. The callus and different plant parts were used for primary metabolite quantification and antioxidant activity. Maximum soluble sugars found in callus, maximum amount of starch, protein and phenolic contents were found in stem and maximum lipid found in leaf.

Keywords *Withania somnifera*, Callus culture, 2,4-D, Primary metabolites.

INTRODUCTION

Withania somnifera (Solanaceae) is known for its varied therapeutic uses in Ayurvedic and Unani medical practices throughout Indian subcontinent. Within Ayurvedic System of Medicine, this plant concoction is highly reputed for being able to impart long life, youthful vigour and intellectual power. The plant had been reported to grow in wild and is also cultivated in selective areas of India. Their pharmacological properties are diverse ranging from anti-inflammatory, anti-tumor, anti-stress, anti-oxidant, immunomodulatory, hemopoetic and cardio-protective effects [1, 2, 3].

Tissue culture tool are considered expensive than conventional mass multiplication method when it comes to commercial and large scale production needs. Tissue culture media accounts to large proportion of any *in vitro* mass propagation protocol. In present study, callus was raised from leaf explants of *Withania somnifera* and primary metabolites of callus as well as plant parts were compared.

MATERIALS AND METHODS

Plant material
Healthy plants of *Withania somnifera* were collected from herbal garden of Singhania University, Pacheri Bari, Jhunjhunju Rajasthan (India). Colleted plants were authenticated from Herbarium, University of Rajasthan, Jaipur.

Chemicals
All the chemicals and growth regulators were used are analytical grade and purchased from Hi Media Pvt. Ltd. Mumbai, India.

Callus induction
Leaves were surface sterilized by 1 % Teepol for 2-3 min followed by immersion in 70 % ethanol for 1 minute and in 0.1 % mercuric chloride for ½ minute and then rinsed thoroughly with sterile distilled water. The seeds were inoculated in the MS medium [4] fortified with different concentrations of IBA and 2, 4-D. The pH of the media was adjusted to 5.8 before autoclaving. All media were autoclaved at 1.06 kg cm^{-2} and 121ºC for 15 min. The cultures were incubated in growth room at temperature of 25±2°C and 16-h photoperiod. 20 replicate cultures were established and each experiment was repeated twice and the cultures were observed at regular intervals. 8 week old callus was used for the primary metabolite studies.

Primary metabolite estimation
Estimation of carbohydrates:
(A) Total soluble sugars: The dried and milled test sample 50 mg each was macerated in a grinder with 20 ml of ethanol and left for 12 hrs. and mixtures was centrifuged (1200 rpm) for 15 min, the supernatants were removed and was concentrated on a water-bath. The volume of these aqueous concentrates was raised to 50 ml with distilled water (Ext. A) and processed further by following the method of Loomis and Shull [5] for soluble sugars. However, the residual pellet obtained by centrifugation was used for the estimation of starch.

(B) Starch: The above residue of each test sample was suspended in a mixture of 5 ml of 52% perchloric acid solution and 6.5 ml of distilled water, shaken vigorously (5 min) and centrifuged (2500 rpm). This step was repeated three times and the supernatants of each sample was pooled and the volume was raised to 100 ml with distilled water (Ext B). Out of this (Ext. B), 1 ml aliquot was taken separately to estimate starch quantitatively [6].

Quantification of carbohydrates:
Aliquot (1 ml) of each of the test sample from Ext. A and B were used to quantifying the total levels of carbohydrates using phenols-sulphuric acid method [7]. A regression curve for standard sugar (glucose) was also prepared. A stock solution of glucose (100 µg/ ml) was prepared in distilled water, out of which 0.1 to 0.9 ml was transferred to test tube and the volume was raised to 1 ml with distilled water. To each of these, 1 ml of 5% aqueous phenol was added rapidly having kept in an ice chest and shaken gently. Later 5 ml of Conc. H_2SO_4 was rapidly added by agitating gently during the addition of the acid subsequently, the tube was kept on a water-bath (26º– 30ºC) for 20 min, and the optical density (ODs) of the yellow orange colors thus developed were taken at 490 nm in a Spectrophotometer after having set it for 100% transmission against the blank. Four replicates of each sample were run and there mean values were calculated. A regression was computed between its known concentrations and their respective ODs. This was based on Beer's Law. The concentration (mg/gdw) of the total soluble sugars was directly worked out from the regression curve of the standard glucose.

Four replicates of each experimental sample were taken and their mean values recorded. The sugar content in terms of glucose equivalent and the use of conversion factor (0.9 to convert the values of glucose to starch) was made in each case.

Extraction of Proteins: A 60 mg of the dried test sample was macerated [8] in 10 ml of cold TCA (10%) for 30 min kept at low temperature 4º C for 24 hr and then centrifuged. Each of the supernatants was discarded and the resultant pellet was re-suspended in 5% TCA (10 ml) and heated on a water bath at 80º C for 30 min. Each of these samples was cooled, re-centrifuged and each time the supernatant discarded. Later the pellet was washed with distilled water, centrifuged and each of the residues was dissolved in 1N NaOH (10 ml) and left overnight at room temperature.

Quantification of Proteins: In each of 1 ml extract, total protein content was estimated using the protocol of [9]. A stock solution (1mg/ml) of bovine serum albumin (Sigma Chemicals) was prepared in 1 N NaOH, from which 0.1 to 0.9 ml of the solution was dispensed separately in a test tube. After this, the volume of each was raised to 1 ml by adding distilled water. To each test sample, 5ml of freshly prepared alkaline solution (prepared by mixing 50 ml of 2% Na_2CO_3 in 0.1 N NaOH and 1 ml of 0.5%CuSO4. $5H_2O$ in 1% sodium potassium tartrate) was added at room temperature and left undisturbed for a period of 10 min.

Subsequently, to each of these mixture tubes 0.5 ml of Folin-Ciocalteau reagent (CSIR centre for Bio-chemicals, Delhi: diluted with equal volume of distilled water just before use) was rapidly added and after half an hr, the OD of each was measured at 750 nm using a spectrophotometer against the blank. Three replicates of each concentration were taken and there mean values were used to compute a regression curve. The total protein content in each sample was calculated by referring the ODs of test sample with the standard curve of BSA.

Three replicates were examined in each case and their mean values were recorded.

Extraction of Lipids: One g of each of the dried and milled test sample was macerated with 10ml distilled water [10]. To this, 30 ml of chloroform-methanol (2: 1, v/v) was added and mixed thoroughly. Each mixture was left overnight at room temperature, 20 ml of chloroform and the equal volume of distilled water was added and centrifuged.

Out of the three layers, a clear lower layer of chloroform containing all lipids was collected in pre-weighted beaker, the solvent evaporated completely and weighed, which was taken as the weight of total lipids/g of the dried tissue sample.

Extraction of Phenols: Each of 200 mg dried and milled test samples was homogenized in 80% ethanol (10 ml) for 2 hrs and left over night at room temperature. It was centrifuged, the supernatants were collected individually and the volume of each was raised to 40 ml with 80% ethanol.

Quantification of Phenol: To estimate total phenols in each of the test sample, the protocol of Bray and Thorpe [11] was followed, wherein a standard curve of caffeic acid (a phenol) was Prepared A stock solution (100 µg/ml) of caffeic acid was prepared in 80% ethanol, from which 0.1 to 0.9 ml was transferred into test-tubes separately and the volume in each case was raised to 1 ml with 80% ethanol. To each of these tubes, 1 ml of Folin–Ciocalteau reagent (prepared by diluting the reagent with distilled water in 1:2 ratio just before use) accompanied by 2 ml of 20% Na_2CO_3 solution was added and the mixture was shaken vigorously.

Each of these were boiled on a water bath (1 min), cooled and diluted to 25 ml with distilled water. The OD was taken at 750 nm using a spectrophotometer against a blank. Three such replicates were taken for each concentration and the average OD was plotted against the respective concentration to compute a regression curve.

Each test sample was processed in this similar manner, ODs were measured and the total level of phenols was calculated from the mean values (with reference to caffeic acid) by referring the OD of the test sample with the regression curve of the standard.

RESULTS

Callus induction

MS medium supplemented with different concentrations of IBA and 2, 4 - D for callus induction. Leaf showed maximum callus formation on MS medium with IBA at the concentration of 1.5 mg/liter. Callus was compact and greenish yellow colored. But on 2, 4- D at 1.0 mg/liter µM/liter concentration it was fragile and yellowish. Callus obtained after 8 weeks of culture from MS medium supplemented with IBA (1.5 mg/liter) was further evaluated for primary metabolites (Figure: 1).

Primary metabolites:

Maximum levels of soluble sugars (73.5±0.18 mg/gdw) was found in callus and minimum level of soluble sugar (42.1±0.32 mg/gdw) and Phenol (19.5±0.32 mg/gdw) was found in roots. Highest concentration of Phenol (38.1±0.22 mg/gdw) was in stem and lowest concentration of starch (19.1 ±1.25 mg/gdw) in callus. Maximum level of lipid (42.7±1.18 mg/gdw) and starch (44.2±1.32 mg/gdw) in leaves while minimum lipids (20.3±0.54 mg/gdw) and phenolic contents (19.5±0.32 mg/gdw) in root were found. (Shown in table 2 and graph 1).

DISCUSSION

The plant is an important medicinal plant and is used in large number of Ayurvedic preparations to treat variety of diseases. Identification and preclinical/clinical development of novel antiangiogenic agents continues to be a topic of intense research [12, 13]. *In vitro* raised callus are being widely used. The callus was raised from explants of cotyledonary leaf and root segments of *Carthamus tinctorius* [14] and *Allium sativum* [15] were efficient for differentiation. Callus formation from axillary meristem explants of *Withania somnifera* in MS medium with IBA 1.5 mg/liter.

Table-1 Percentage of the callus induction from *Withania somnifera* seeds under different levels of 2, 4-D and IAA after 8 weeks of culture.

S. No	Growth regulators	Concentration (µM/liter)	Percentage of the callus induction	Nature of callus
1	IBA	0.5 1.0 **1.5** 2.0	25±1.1 80±1.6 **95±0.5** 70±1.3	The callus was friable, prolific in growth.
2	2,4-D	0.5 1.0 1.5 2.0	25±0.4 85±1.3 79±1.5 54±1.3	Fast growing, light yellow to light brown colored callus.

Graph: 1 Primary metabolites of *Withania somnifera* in mg/ gram dry weight

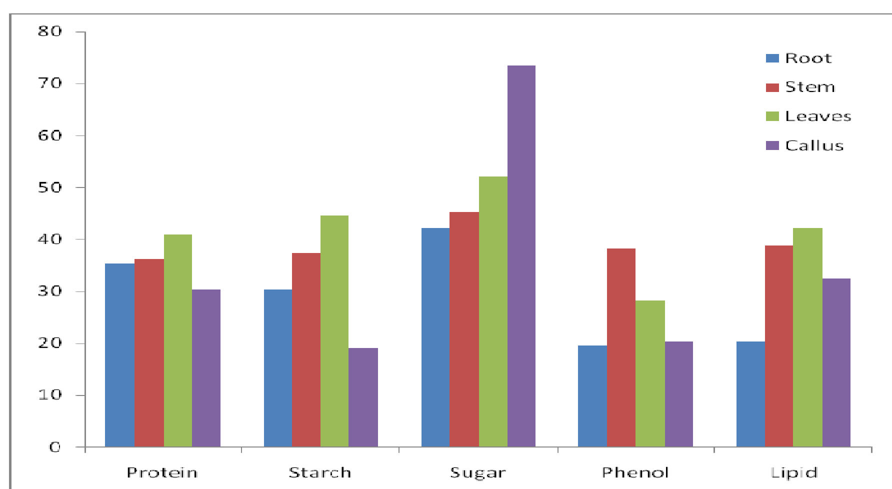

Figure 1 Callus stages in Leaf derived callus A. inoculation B. after 2week 3. After 4 week and 4. After 8 week

In our study IBA 1.5 mg/liter and 2, 4- D 1.0 mg/liter both auxin concentrations showed callus formation but IBA 1.5 mg/liter concentration showed maximum yield. *Nerium indicum* Mill. and *Alangium salviifolium* Linn. have investigated for their primary metabolites [16, 17]. Comparative *in vitro* and *in vivo* biochemical performance has been evaluated in *Adhatoda vasica* [18]. In present study callus was showed highest soluble sugars but less phenolic contents starch lipids and proteins than *in vivo* plant parts. *In vitro* cells accumulate more sugar due to its

easy availability in culture medium and these cells are in highly proliferating stage so they accumulate more primary metabolites than storage metabolites (starch, lipid) and secondary metabolites (phenolic contents). In our study also different plant parts as well as callus had showed superoxide radical scavenging activity according to their phenolic contents since presence of phenolic contents supports antioxidant status of the callus as also reported in Indian herbal tea [19].

Table: 2 Primary metabolites in *Withania somnifera.*

	Root	Stem	Leaves	Callus
Protein	35.3±1.23	36.2±0.52	40.8±1.15	30.2±1.62
Starch	30.2±1.15	37.6±1.24	44.2±1.32	19.1 ±1.25
Sugar	42.1±0.32	45.2±1.12	52.1±1.33	73.5±0.18
Phenol	19.5±0.32	38.1±0.22	28.1±1.16	20.2±0.32
Lipid	20.3±0.54	38.2±0.18	42.7±1.18	32.4±1.26

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