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ORGANOGENIC TOTIPOTENCY IN *IN VITRO* CULTURE OF TWO ELITE GENOTYPES (*POSHITA* AND *JAWAHAR 22*) OF *WITHANIA SOMNIFERA* (L.) DUNAL AND QUANTIFICATION OF BIO-ACTIVE COMPOUNDS

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ABSTRACT

Multiple shoot induction (explants used: X-shoot tip ±2.0 cm of 18 days old petri plate grown seedlings; Y- shoot tip ±2.0 cm of 30 days old nursery grown seedlings; Z- shoot tip ±2.0 cm of 185 days old *in vitro* regenerated plantlets from callus masses), elongation of shoots and root induction protocol was developed in Poshita and Jawahar 22 (highly recommended varieties) of *Withania somnifera* (L.) Dunal (Family: Solanaceae) using Murashige and Skoog basal media with various concentration of benzyl adenine, kinetin, indole butyric acid, indole acetic acid and gibberelic acid in different combinations for each stages of development. Total alkaloids and withanolides (including withanolide A and withaferin A estimated by High Performance Liquid Chromatography) contents were also analyzed in multiple shoots (35 days and 65 days old) and *in vitro* rooted plants (40 days old). Results indicated better response of Poshita than Jawahar 22 and explant Z exhibited superiority to X and Y in relation to the production of secondary metabolites.

KEYWORDS: *Withania somnifera*, Poshita, Jawahar 22, organogenic and chemical totipotency, secondary metabolites, HPLC.

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INTRODUCTION

Withania somnifera (L.) Dunal (Family: Solanaceae, commonly known as Ashwagandha, English name: Winter cherry) is an important perennial plant species with immense therapeutic uses in traditional as well as modern system of medicine[1]. Due to restorative property of roots, the species is also known as 'Indian Ginseng'[2]. The pharmacological significance of *W. somnifera* is due to the presence of secondary metabolites like alkaloids and withanolides (steroidal lactones) primarily in roots[3]; however withaferin A (type of withanolide) is predominantly present in leaf[4]. Sabir *et al.*[5] reported that these bioactive chemicals possibly synthesized in leaves and accumulated in roots. Sangwan *et al.*[6] were of opinion that high quality phyto-pharmaceuticals can be harvested from plant parts those are uniform both qualitatively and quantitatively. Das *et al.*[7] reported that secondary metabolite contents of *W. somnifera* varied remarkably between seasons and genotypes under *ex vitro* condition. In this context it would be relevant to adopt tissue culture technology as *in vitro* studies provide an optimum culture condition for steady and quality production of bioactive chemicals throughout the year irrespective of agroclimatic parameter(s). With a view to it, the present study deals with 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).

MATERIALS AND METHODS

Plant Materials

Two elite genotypes (Poshita collected from Central Institute of Medicinal and Aromatic Plants, Lucknow-Misra *et al.*[8] and Jawahar 22 from Mandsaur, Madhya Pradesh- Nigam *et al.*[9]) were used as experimental

materials. Poshita and Jawahar 22 are designated by P and J respectively in the text.

Explants

For multiple shoot induction, three different types of explants (X-shoot tip ±2.0 cm of 18 days old petri plate grown seedlings; Y- shoot tip ±2.0 cm of 30 days old nursery grown seedlings; Z- shoot tip ±2.0 cm of 185 days old *in vitro* regenerated plantlets from callus masses) were inoculated.

Sterilization and Multiple Shoot Induction

Before collection of explant Y, young plants were sprayed with 1.0% Bavistin (fungicide-carbendazim 50%, BASS India) at 6:00 am for three consecutive days while explant X and Y were excised directly on the same day. Explants (only X and Y; Z is directly inoculated) were surface sterilized by immersing in 1.0% Teepol solution followed by repeated washings with distilled water (3 times) and subsequently treated with 1.0% Bavistin for 30 min on a rotary shaker (Lunar. Amalgamated supplier Pvt. Ltd. India) at 60 rpm. After through washing (3 times), explants were immersed in 0.1% $HgCl_2$ solution (Merck, India) for 2 minutes, finally washed with sterile distilled water (5 times) and graded into ±2.0 cm size prior to their inoculation on agar gelled (0.8%, Himedia, Type 1) Murashige and Skoog (MS)[10] basal medium with 3.0 % sucrose (Merck, lndia) on a laminar air flow hood (ESCD Airstream). MS basal medium supplemented with 27 different combinations of phytohormones [SRL trade name; Benzyl Adenine (BA) - 0.4, 0.6, 0.8, and 1.0 mg/l; Kinetin (Kin) - 0.2, 0.4, 0.6 mg/l and Indole butyric acid (IBA) - 0.4, 0.6, 0.8 mg/l] were tried out for induction of multiple shoots. The inoculated explants (10 replicas in culture bottles with 3 explants of each variety/ treatment) were incubated at 26 ± 1°C under florescent light of about 2500 lux for a 16 hours photoperiod per day. Observations on 65th day of inoculation were recorded in terms of number of multiple shoots developed from each type of explant and length of shoots was assessed.

Elongation of Shoots

Single shoots (>1.0 cm in height) from the bunch were separately picked out and placed on fresh MS media supplemented with different concentrations of Gibberelic acid (GA_3 - 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5 mg/l) to elongate (in 10 replicas in bottles/ treatment) them and observation (in terms of shoot length) was recorded at 40th day of incubation.

Root Induction

Elongated shoots (7.0 to 11.5 cm in height) were transferred to the rooting media- full MS (6 combinations), ½ MS (6) and ¼ MS (6) supplemented with different concentrations of IBA (0.5, 1.0, 1.5 and 2.0 mg/l) and Indole acetic acid (IAA - 0.5, 1.0, 1.5 and 2.0 mg/l) in 10 replicas, in bottles/ treatment. Observations were made at 40th day of incubation on the basis of number of roots/plant and length of roots in *in vitro* culture vessels.

Chemical Analysis

Secondary metabolite content (total alkaloid and withanolides amount were quantified; withanolide A and withaferin A contents were estimated by HPLC) in multiple shoots (harvested at 35th and 65th days of inoculation); root and shoot part of plantlet (40th day of inoculation to rooting media) were estimated on fresh weight basis. Extraction and estimation of secondary metabolites was performed following the methodology suggested by Das *et al.*[11]. HPLC studies suggests a good linearity between the range of 1.0 µg/ml and 10.0 µg/ml with a correlation coefficient of 0.9899 (withanolide A) and 0.9978 (withaferin A). The limit of detection were estimated to be 0.25 µg/ml and 0.30µg/ml for withanolide A and withaferin A respectively based on signal to noise ratio 3:1. Amount of the metabolites were computed from chromatograms using the formula suggested by Scott[12].

RESULT AND DISCUSSION

Three different types of explants (X, Y and Z) responded differentially for induction of multiple shoot **(Fig. 1)** and the best response irrespective of explants types was noted in MS + BA 1.0 mg/l + IBA 0.4 mg/l in Poshita (average number of shoots: X-27.2 ± 0.95; Y-18.5 ± 0.99; Z-24.8 ± 0.33; shoot length in cm: X-2.3 ± 0.34; Y-2.1 ± 0.65; Z-2.2 ± 0.35) as well as in Jawahar 22 (average number of shoots: X-25.5 ± 0.51; Y-17.6 ± 0.98; Z-23.8 ± 0.36; shoot length in cm: X-2.1 ± 0.21; Y-1.9 ± 0.32; Z-2.0 ± 0.32). Combination of BA and Kin increased the number of shoots to an appreciable level but the trend was gradually reduced with an increase in BA (beyond 0.6 mg/l) with Kin, whereas addition of IBA reflected an increase in number even in 1.0 mg/l concentration of BA. Results demonstrated better response of explant X in relation to shoot attributes in both the genotypes. Thus, it seems that selection of explants plays a key role for shoot multiplication. Sen and Sharma[13] observed shoot multiplication of *W. somnifera* with shoot tip explant using lower concentrations of BA (2.2, 4.4 and 8.9 µM) and recorded maximum number of shoots (100-150 after 60 days) using 2.3 µM 2,4-D or 2.5 µM IBA in combination with 4.4 µM BA during the initial stages of shoot multiplication, and highlighted that germinating seeds can directly yield multiple shoot in presence of BA alone. Deka *et al.*[14] suggested that the combined effect of 0.3 mg/l benzyl amino purine (BAP) and 0.2 mg/l Kin

showed a good response in shoot proliferation (mean of 19 shoots/explant) and regeneration (85% in 60 days) from axillary buds and shoot tips of *W. somnifera*. Sivanesan and Murugesan[15] also observed maximum number of multiple shoots regeneration in BAP and IAA each (1.5 mg/l) using axillary shoots and concluded that for shoot multiplication BA, Kin and IAA and their addition in proper ratio at proper stages are responsible.

Elongation details showed that average shoot length (4.1-11.5 cm) varied amongst media compositions as well as for explants. MS medium supplemented with GA_3 (0.3 mg/l) showed highest shoot elongation (P: X-10.8 cm ± 0.29, Y-9.7 cm ± 0.35, Z-11.5 cm ± 0.85; J: X-10.2 cm ± 0.88, Y-9.1 cm ± 0.65, Z-10.9 cm ± 0.95) in both the genotypes. Response (in terms of shoot length) of Poshita was better than Jawahar 22 and explant Z (P : 6.2cm ±0.25 to 11.5 cm ± 0.85 and J : 5.4 cm ± 0.31 to 10.9cm ±0.95) derived shoots showed higher shoot length than other explants (X and Y: P - 4.8cm ±1.21 to 10.8 cm ± 0.29 and J - 4.1cm ±0.37 to 10.2cm ±0.88). Elongation of shoots is an important step towards production of micropropagated plantlets. Shoot induction and elongation were also studied in *W. somnifera* by different authors (Kulkarni *et al.*[16], Govindraju *et al.*[17], Supe *et al.*[18]).

Half MS supplemented with IBA (2.0 mg/l) showed higher average number of roots (P: 26.3 ± 0.21 and J-26.0 ± 0.26) and root length (P-6.7 cm ± 1.11 and J- 6.4 cm ± 1.54) on Z explants. IBA responded better for root induction **(Fig. 2)** than IAA in all strength of MS. One fourth strength of MS and higher concentration of IAA (1.5 mg/l and 2.0 mg/l) cause death of shoots. The pattern of root growth was variable in different media compositions for both the genotypes whereas shoots maintained the same trend irrespective of media composition. Pawar *et al.*[19] observed root induction with a frequency of 90 ± 5 on MS media containing IAA at 4 µM and Napthalene acetic acid (NAA) at 8 µM. Govindaraju *et al.*[17] also observed rhizogenesis of the elongated plantlets successfully in half strength MS media (both liquid and solid) with IBA (0.5-1.0 mg/l) alone and in combination with IAA (0.5 mg/l).

Chemical analysis **(Table 1)** revealed trace amount of only alkaloids in 35 days old multiple shoots, while both alkaloids and withanolides were detected from 65 days old shoots in both genotypes. Results indicated that fully differentiated *in vitro* grown plantlets (40^{th} day of inoculation to rooting media; with both shoots and roots) of Poshita and Jawahar 22 showed higher amount of withanolide A **(Fig. 3 and 4)** in roots but withaferin A in shoots was low. The yield of secondary metabolites (withanolide A) was better in differentiated organs of Poshita than Jawahar 22 and the explant type Z was more competent in secondary metabolite accumulation than others. *Ex vitro* (P: withanolide A – 0.65 mg/gm ± 0.21, withaferin A – 0.56 mg/gm ± 0.43 and J: withanolide A – 0.62 mg/gm ± 0.25, withaferin A – 0.51 mg/gm ± 0.15 harvested from root of 180 days old plant- Das *et al.*[7]) and *in vitro* [withanolide A: P - 2.42 mg/gm and J - 2.19 mg/gm harvested from 180 days old callus developed with petri dish grown seedling shoot of age 15 days; withaferin A: P – 1.60 mg/gm and J: 1.40 mg/gm harvested from 250 days old callus developed with young leaves (± 1.5 cm) from apical region of 60 days old nursery grown plants- Das *et al.*[11]] studies performed earlier revealed that the amount of withanolide A is significant in relation to time of harvest**.** **C**omparative results indicated that the differentiated callus yielded higher amount of bioactive chemicals. Sangwan *et al.*[20] reported that biogeneration of withanolide A in *W. somnifera* is associated with the stages of differentiation. Ray and Jha[21] reported induction of multiple shoots from single shoot-tip explants of *W. somnifera* in MS medium supplemented with BA, and detected withanolide D and withaferin A in the regenerated shoots. They also reported that supplementation of the solid regeneration medium with 4% sucrose increased accumulation of both withaferin A and withanolide D, while culture in liquid regeneration medium containing 10% coconut milk favoured not only an increase in the number of microshoots per explant but also withaferin A accumulation. Sharada *et al.*[22] observed presence of withanolide A (2.59 µg/gm-dry mass) and withaferin A (0.16 µg/gm-dry mass) in multiple shoots derived from shoot tip explants of *in vitro* cultured plants, and mentioned that the regeneration potentialities of explants is dependent on donor tissues of the mother plants and the degree of withanolide synthesis and accumulation is closely associated with morphological differentiation. Kaul *et al.*[23] suggested that chemotypic fidelity could be achieved in *in vitro* propagated plants of elite genotypes and stability in production of bioactive compounds particularly withaferin A could be maintained.

The present investigation highlighted the variable response of explants and genotypes for organogenic as well as chemical totipotency in *in vitro* culture conditions providing scope for selection of genotype(s) (Poshita seems to be better than Jawahar 22) as well as explant type (Z) at specific stages of differentiation to improve withanolide A production. Enhancement of withanolide A content and its steady production is of utmost necessity in the species to meet up the upsurging demand of phyto-medicinal components, and its utilization in human welfare.

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TABLE 1. SECONDARY METABOLITE CONTENT IN POSHITA (P) AND JAWAHAR 22 (J).

Attributes	Explants	Multiple shoots				Plantlets of 40 days age			
		35 days		65 days		Aerial part		Root	
		P	J	P	J	P	J	P	J
Alkaloid (%)	X	0.04	0.03	0.11	0.10	0.17	0.17	0.19	0.17
	Y	0.09	0.07	0.11	0.11	0.15	0.15	0.17	0.17
	Z	0.07	0.07	0.15	0.14	0.19	0.19	0.21	0.19
Withanolide (%)	X	---	---	0.14	0.13	0.15	0.15	0.15	0.14
	Y	---	---	0.12	0.13	0.12	0.11	0.13	0.13
	Z	---	---	0.17	0.15	0.22	0.19	0.22	0.20
Withanolide A (mg/gm)	X	---	---	0.14	0.13	0.12	0.13	0.98	0.92
	Y	---	---	0.12	0.13	0.09	0.09	0.85	0.81
	Z	---	---	0.28	0.24	0.14	0.13	1.11	1.08
Withaferin A (mg/gm)	X	---	---	0.21	0.20	0.09	0.08	---	---
	Y	---	---	0.24	0.21	0.07	0.07	---	---
	Z	---	---	0.31	0.24	0.10	0.10	---	---

Explant X: Shoot tip ±2.0 cm of 18 days old petri plate grown seedlings; **Explant Y:** Shoot tip ±2.0 cm of 30 days old nursery grown seedlings; **Explant Z:** Shoot tip ±2.0 cm of 185 days old *in vitro* regenerated plantlets from callus masses. ---: Not detectable

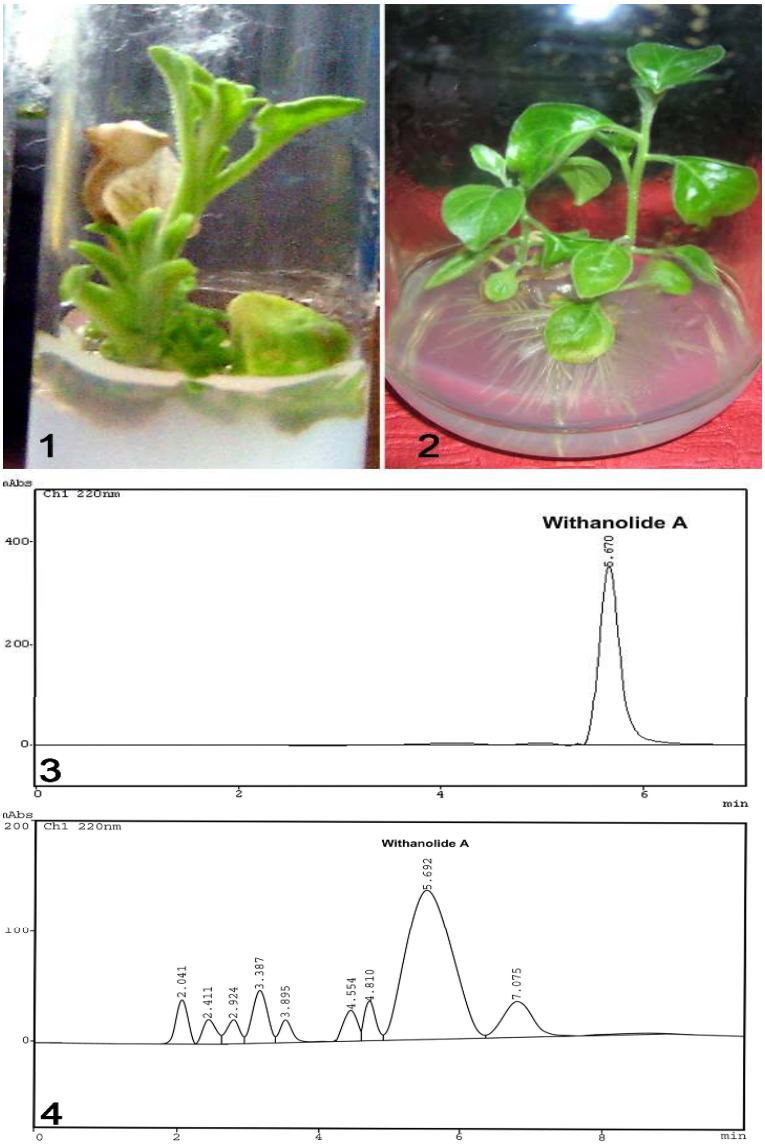

Fig. 1. Multiple shoot induction with shoot tip explants.
Fig. 2. *In vitro* rooted plantlet.
Fig. 3. Chromatogram of withanolide A-analytical standard.
Fig. 4. Chromatogram of sample (root of *in vitro* rooted plantlet).

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