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ASSESSMENT OF CYTOMORPHOLOGICAL PARAMETERS AND CHEMICAL CONTENTS IN *IN VITRO* AND SEED PROPAGATED PLANTS OF ELITE GENOTYPES OF *WITHANIA SOMNIFERA* (L.) DUNAL

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ABSTRACT
Morphological parameters (plant height, total branches/plant, leaf and seed yield/plant, total plant weight, root length, root weight, and total branches/root) and chemical (from root) contents (amount of total alkaloids and withanolides; withaferin A-in root and also in leaf and withanolide A content estimated by HPLC) are assessed in tissue culture developed hardened matured (shoot tip regenerated plantlets, age is 280 days old from the day of inoculation) plants as well as field grown seed propagated plants of two high performing recommended varieties (Poshita and Jawahar 22) of *Withania somnifera* (L.) Dunal (Family: Solanaceae) following cultivation in experimental field plot of University of Kalyani (West Bengal plains-22° 99' N, 88° 45' E, elevation- 48 feet above mean sea level, sandy loamy soil, organic carbon 0.76%, soil pH 6.85) during the months of September to February at a spacing of 4.5 cm between lines and 30 cm between plants. Chromosome number (2n=48) is also being studied from root tip mitosis and PMC squash preparations in both genotypes (*in vitro* and *ex vitro* raised plants). Result obtained suggested fidelity of *in vitro* raised plants and also ensures the significance of biotechnological approaches in production of bioactive chemicals and planting materials of *W. somnifera*.
KEYWORDS: *Withania somnifera*, Poshita and Jawahar 22, cytomorphological and chemical fidelity, hardened and seed propagated plants.

INTRODUCTION

Withania somnifera (L.) Dunal (Family Solanaceae; commonly known as Ashwagandha; English name: Winter Cherry) is a perennial[1] plant species (herb: Gupta *et al.*[2], Khanna *et al.*[3]; shrub: Negi *et al.*[4]) with prodigious therapeutic applications in both traditional (Ayurvedic, Unani, Sidhdha) and modern system of medicine[5]. The species is also known as 'Indian ginseng' as its root possesses restorative properties similar to *Panax ginseng*[6]. The medicinal properties of *W. somnifera* are due to chemical constituents (alkaloids and withanolides) present primarily in roots, and also in leaves[7]. Patra *et al.*[8] reported that the species is a rainfed crop and grows well in semiarid subtropical areas receiving good rainfall (600-750 mm annual rainfall). Misra *et al.*[9] suggested that *W. somnifera* is a potential cash crop greening dry land zones and making waste land productive. The species is rarely reported (grows well in sandy loam and stony red clay soil with pH 7.5 to 8.0-Thomas *et al.*[10]; preference to acidic soil-Obidoska and Sadowska[11]) from Eastern India including West Bengal plains[12]; however, Das *et al.*[13] successfully cultivated two elite genotypes (Poshita and Jawahar 22, recommended varieties) in the experimental field plots of Bidhan Chandra Krishi Viswavidyalaya (West Bengal plain, Nadia-22°56'N latitude, 88° 32' E longitude and 9.75 m altitude, sandy loamy soil, organic carbon 0.76, pH 6.85) in two seasons (March to August-rain fed kharif crop; September to February- rabi crop).

Considering the upsurging demand and potential significance of *W. somnifera* (value added product as well as raw plant materials) in National and International markets, sustainable cultivation throughout India is an appreciable proposition; although, agro-climatic factor(s) are reported to play significant role in synthesis and accumulation of secondary metabolites[8, 11] and therefore it is rather difficult to optimize the maximum yield of chemical components under field grown conditions. *In vitro* tissue and organ culture technology are adopted in *W. somnifera* genotypes (Poshita and Jawahar 22) as an alternative strategy for steady production of secondary metabolites[14, 15]. The present communication describes morphological parameters and chemical contents in root (amount of total alkaloid and withanolide; withaferin A- also from leaf and withanolide A by HPLC) of *in vitro* (by exploring the totipotency of young shoot tips) derived plantlets of Poshita and Jawahar 22 of *W. somnifera* in comparison to seed propagated plants grown during the months of September to February. Cytological studies of hardened micropropagated plants are also assessed in comparison to field grown plants.

MATERIALS AND METHODS

Plant Materials

Two elite genotypes (Poshita collected from Central Institute of Medicinal and Aromatic Plants, Lucknow- Misra *et al.*[9] and Jawahar 22 from Mandsaur, Madhya Pradesh- Nigam *et al.*[16]) are used as experimental material. Poshita and Jawahar 22 are designated as P and J respectively in the text.

Explant Sources

Well rooted elongated plantlets (145 days age from the day of inoculation) derived by shoot multiplication from shoot tips are used as explants source (X-Shoot tip ±2.0 cm of 18 days old seedling; Y-shoot tip ±2.0 cm of 30 days old seedling; Z-shoot ±2.0 cm of 185 days old *in vitro* regenerated callus mass).

Acclimatization Procedure

Individual plantlets with well differentiated roots **(Fig. 1)** were taken out from culture bottles, washed with sterilized distilled water, dipped in rooting solution (Indole butyric acid-IBA 10 mg/l) for 20 min and transferred to sterilized substrates (soil:sand-1:1 and sand) in polythene cups (3-4 perforations at the bottom) placed on petri plates containing little amount of sterilized distilled water. The polythene cups are then covered by polythene bags. Inside the bags sterilized distilled water is sprayed twice a day and continued upto ten days to maintain the moist environment. Bavistin (0.1%) solution is sprayed (at every three days interval) on young plants to prevent fungal contamination. Urea (0.05%) solution is also added in petri dishes. Plantlets under hardening were kept in two conditions in a day (i) shade (18 hours) (ii) diffused sunlight (6 hours). After ten days the polythene covers are cut open at one end, kept nine more days before removal of coverings and the healthy young plants **(Fig. 2)** are transferred to earthen pots (filled with garden soil), kept for 15 days and finally transferred to the Experimental field plots of University of Kalyani. Survivability percentage is recorded on 19th day (1st stage-in polythene cups) as well as 34th day (2nd stage-in the field condition-**Fig. 3**) of acclimatization experiment.

***Ex Vitro* Propagation of the Variety**

Mother seed stock of Poshita (moisture content 4.62%) and Jawahar 22 (moisture content 3.75%) are given in petri plates (300 seeds for each variety) and the germinating seedlings (Poshita: 46.0%, Jawahar 22: 57.0%) are raised in seed beds (survivability: Poshita-85.23%; Jawahar 22- 88.21%) and subsequently grown in lines (spacing of 45 cm between lines and 30 cm between plants) in field plots of University of Kalyani.

Chromosome Studies

Mitotic analysis: Seeds of Poshita and Jawahar 22 are germinated in petri plates lined with moist filter papers (28±1°C) and roots measuring 1-2 mm are cut, pretreated (4 hours in aqueous solution of para-dichlorobenzene and asculine mixture) at 16 ±1°C, fixed in propionoalcohol (1:3 v/v), hydrolyzed and stained in 1 N HCl-orcein mixture (9:1), squashed in 45% acetic acid and observed under the microscope. Properly condensed metaphase plates are only scored for determination of chromosome number.

Meiotic Analysis: For studying male meiosis, suitably sized flower buds of Poshita and Jawahar 22 (assessed from five randomly selected plants in both genotypes and in both categories) are fixed (5:30 to 6:30 a.m.) in Carnoy's fluid (absolute alcohol: chloroform: glacial acetic acid- 6:3:1) for 72 hours (at least 2 changes are given in Carnoy's fluid at an interval of 24 hours) with 1-2 drops of iron alum (for proper staining) and preserved in 70% alcohol. Anther squash preparations are made in 1% propionocarmine solution and well scattered metaphase I (MI) and anaphase I (AI) plates are only scored. Data obtained is pooled over the plants. Pollen fertility is also assessed in the plant types of both varieties by staining pollen grains in 1.0% propionocarmine solution and fully stained pollen grains are considered fertile as was suggested by Marks[17]. Photomicrographs are taken from suitable temporary squash preparations.

Assessment of Morphological Characters

Morphological traits **(Table 1)** are analyzed from randomly selected 5 *in vitro* propagated hardened (280 days old matured plants and the age was calculated from the date of inoculation for induction of multiple shoots) plants and seed propagated plants scored from 5 lines (one plant from each line randomly, border plants are excluded) at maturity (September-February).

Chemical Analysis

Secondary metabolite content (amount of total alkaloid and withanolide; withaferin A- also from leaf and withanolide A- by HPLC; **Figs. 10, 11,12,13**) in roots of microprogated and seed propagated plants of Poshita and Jawahar 22 are assessed and extraction and estimation of secondary metabolites are performed following the methodology suggested by Das *et al.*[13] . HPLC studies revealed a good linearity between the range of 1.0 µg/ml and 10.0 µg/ml with a correlation coefficient of 0.9978 (withaferin A) and 0.9899 (withanolide A). The limit of detection are recoded to be 0.30 µg/ml and 0.25 µg/ml for withaferin A and withanolide A respectively based on signal to noise ratio 3:1. Amount of the chemical contents are computed from chromatograms using the formula suggested by Scott[18].

RESULTS AND DISCUSSION

Survivability percentage of tissue culture derived hardened plant is found to be relatively higher in Jawahar 22 (95.0%- 1st stage, 96.0%-2nd stage) than Poshita (93.0%- 1st stage, 93.0%-2nd stage). Various percentage of survivability of regenerated plantlets are reported in *W. Somnifera* ranging from 60.0 %[19], 70.0%[20] to 100.0%[21]. Such variation signifies the possible impact of genotypes, explants, rooting media and condition of acclimatization on survivability of plantlets.

Morphological parameters as well as chemical attributes of *in vitro* propagated hardened matured plants (derived from X, Y and Z explants) in comparison to seed propagated plant of Poshita and Jawahar 22 are presented in **Table 1.** In both genotypes, *in vitro* raised hardened plants from Z explant demonstrated relative betterment for all the studied attributes in comparison to plantlets derived from X and Y explants. Z explants derived hardened plants also showed enhancement in chemical contents mostly (excepting: withaferin A content in leaf in Poshita) in comparison to seed propagated plants; while morphometric traits are nearly identical *in vitro* as well as *ex vitro* raised plants. Ray and Jha[22] reported that six month old and one year old tissue culture derived plants yielded 0.066 and 1.6 % withaferin A respectively, and six month old seed derived plants showed a higher accumulation of withaferin A than tissue culture plants of same age. Vitali *et al.*[23] reported low contents of withanolides and the presence of withanolide J in micropropagated plants of *W. somnifera*. Das *et al.*[14] suggested that differentiated callus with multiple shoot yielded significantly higher amount of alkaloid (P-0.61%, J-0.53%) and withanolides (P-2.32%, J-2.10%) including withaferin A (P-1.60 mg/gm, J-1.40 mg/gm) and withanolide A (P-2.42 mg/gm, J-2.19 mg/gm) irrespective of the explants used in *W. somnifera*. Das *et al.*[15] reported trace amount (P-0.04 to 0.09%; J-0.03 to 0.07%) of only alkaloids in 35 days old multiple shoots in *in vitro*, while both alkaloids (P-0.11 to 0.15 %; J-0.10 to 0.14%) and withanolides (P-0.12 to 0.17 %; J-0.13 to 0.15%; withaferin A: P-0.21 to 0.31%; J-0.20 to 0.24%; withanolide A: P-0.12 to 0.28%; J-0.13 to 0.24%) are detected from 65 days old shoots in both Poshita and Jawahar 22 of *W. somnifera.* Thus, it seems that differentiated callus is best for biogeneration of alkaloids and withanolides.

Mitotic chromosome number in micropropagated hardened **(Fig. 4)** and seed grown plants of both the genotypes is found to be 2n=48, thereby corroborating previous reports[24-29]. Iqbal and Datta[27] and Das *et al.*[29] reported polysomatomy (2n=6,12, 18, 24, 36, 48 and 72) in the *W. somnifera* from root tip mitosis with predominance of 2n=48 chromosomes. Meiotic analysis also revealed 2n=48 chromosomes **(Fig 5, 6 and 7)** in both Poshita and Jawahar 22 (*in vitro* raised hardened plants: P- 23.7II+0.52I/cell, 207 cells scored; J: 23.68II+0.60I/cell, 89 cells studied, all AI cells were cytologically balanced with 24/24 separation; seed propagated plants: P-23.67II+0.67I/cell, 177 PMCs scored; J: 23.69II+0.62I/cell, 438 meiocytes analyzed; AI cells were mostly cytologically balanced, 24/24 separation – **Fig. 8**, at AI-87.91% cells in P and 90.30% in J with 364 and 874 cells scored respectively; rare often AI cells in both cultivars showed bridge formation- **Fig. 9,** and occurrence of laggards). Pollen fertility is recorded to be nearly same in both plant types (*in vitro* generated: P-59.96%, J-71.05%; *ex vitro*: P-62.25%, J-73.27%). Ray and Jha[22] reported 21 pairs of bivalents in regenerated plants of *W. somnifera*. Assessment of morphological and cytological parameters suggested stability and uniformity of *in vitro* raised plants in relation to seed propagated plants.

Present investigation therefore confirms the suitability of tissue and organ culture biotechnology (considering the poor germinability of cultivars in West Bengal plain- Das *et al.*, 2009) for improvement (mass propagation through biotechnology) of the valuable medicinal plant *W. somnifera.*

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Table 1. Morphological and Chemical attributes of Matured hardened and Seed Propagated plants of Poshita and Jawahar 22 varieties of *W. somnifera*

Attributes	Plant types							
	Poshita				Jawahar 22			
	In vitro propagated, hardened mature plant			Seed propagated plants	*In vitro* propagated, hardened mature plant			Seed propagated plants
	X	Y	Z		X	Y	Z	
Morphological								
Plant height (cm)	72.15±0.96	68.45±0.56	73.21±0.45	68.74±0.25	75.12±0.65	69.85±0.74	72.15±0.96	68.45±0.56
Total branches/plant	7.89±0.42	4.00±0.15	7.45±0.65	4.21±0.25	8.21±0.36	4.54±0.65	7.89±0.42	4.00±0.15
Leaf yield (gm)	5.98±0.27	5.52±0.54	5.78±0.65	5.45±0.21	6.12±0.32	5.98 ±0.87	5.98±0.27	5.52±0.54
Seed yield (gm)	49.58±0.65	38.74±0.35	50.12±0.65	39.54±0.58	51.25±0.54	40.12±0.35	49.58±0.65	38.74±0.35
Total plant weight (gm)	3.19±0.54	3.22±0.54	3.26±0.35	3.29±0.25	3.38±0.51	3.46 ±0.41	3.19±0.54	3.22±0.54
Root length (cm)	44.21±0.34	38.97±0.45	45.74±0.21	39.25±0.12	46.07±0.52	41.24±0.22	44.21±0.34	38.97±0.45
Total branches/root	19.20±0.43	17.03±0.21	19.31±0.62	17.21±0.41	20.12±0.54	17.95±0.54	19.20±0.43	17.03±0.21
Root weight (gm)	4.21±0.54	3.31±0.41	4.36±0.28	3.57±0.28	4.65±0.14	3.74±0.52	4.21±0.54	3.31±0.41
Chemical								
Total alkaloid- in root (%)	0.54±0.21	0.53±0.25	0.58±0.31	0.54±21	0.30±0.23	0.29±0.21	0.34±0.22	0.27±0.28
Total withanolides -root (%)	0..36±0.25	0.38±0.41	0.41±0.32	0.39±0.21	0.32±0.23	0.31±0.52	0.34±0.34	0.31±0.28
Withanolide A -root (mg/gm)	0..63±0.45	0.63±0.71	0.67±0.45	0.64±0.21	0.62±0.51	0.61±0.14	0.63±0.37	0.60±0.25
Withaferin A -root (mg/gm)	0.55±0.61	0.53±0.52	0.57±0.51	0.55±0.43	0.49±0.36	0.46±0.25	0.52±0.81	0.50±0.15
Withaferin A (%)-leaf	1.51±0.47	1.49±0.61	1.54±0.55	1.75±0.18	1.26±0.54	1.24±0.33	1.30±0.45	1.28±0.19

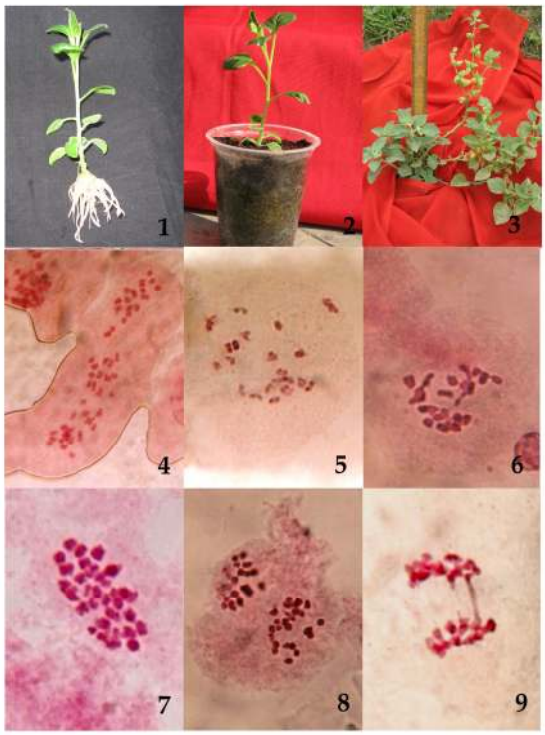

PHOTO BOARD 1:

Fig. 1. *In vitro* rooted plantlet of Poshita.
Fig.2. Poshita plant under acclimatization in polythene cup.
Fig.3. Matured, hardened plant of Poshita.
Fig.4. Mitotic cell in Jawahar 22 with 2n=48 chromosomes.
Figs. 5-7. 24II formation in diakinesis (Fig. 5) and metaphase I (Figs. 6 and 7) in Poshita and Jawahar 22 respectively.
Fig. 8. Anaphase I with equal (24/24) segregation of chromosomes in Poshita.
Fig. 9. Bridge formation at anaphase I of Jawahar 22

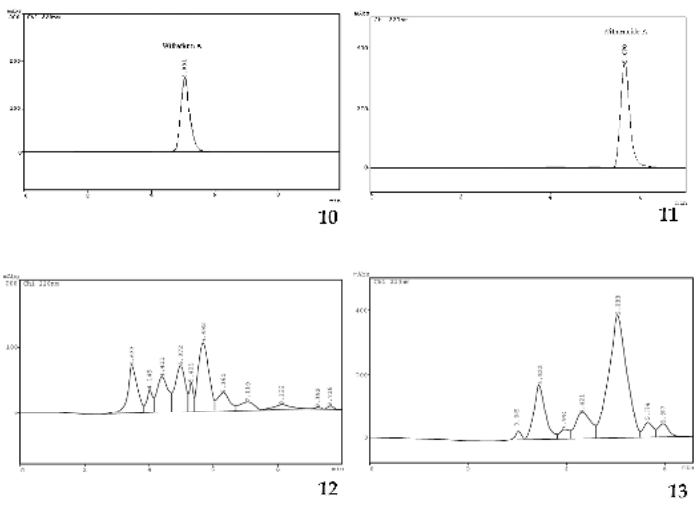

PHOTO BOARD 2

Figs. 10-11. Chromatograms showing analytical standard of withaferin A and withanolide A respectively.
Figs. 12-13. Chromatograms exhibiting root (Fig. 12) and leaf (Fig. 13) samples of *in vitro* propagated hardened plant of Poshita.

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