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Improved production of withanolides in adventitious root cultures of *Withania somnifera* by suspension culture method

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ABSTRACT

Jawahar Ashwagandha-20 (JA-20) and Arka Ashwagandha (AA) seeds were raised in *in vitro* conditions and phenotypic differences between the plants was recorded. The adventitious roots were derived from leaves of the two varieties in *in vitro* conditions. The effects of the strength of media and concentration and the combination of auxins (IAA and IBA) for adventitious roots multiplication using suspension culture were studied. After 30 days of suspension culture, root biomass was measured and HPLC analysis of major withanolides in leaves and adventitious roots was conducted. Arka Ashwagandha variety had higher total withanolide content of 1.621 mg/g, Withaferin A content of 1.362 mg/g and root yield of 4.066 g from 0.1g inoculum in 30 days compared to JA-20 which had total withanolide content of 1.156 mg/g, Withaferin A content of 0.930 mg/g on dry weight basis and root mass of 3.71g from 0.1g of inoculum in 30 days. The present study thus helps in the identification of an elite cultivar of Ashwagandha and development of a standard protocol for mass multiplication of adventitious root in hormone-free media. This is beneficial in the preparation of health supplements in terms of human health issues due to negligible residual effects of hormones in the final product.

KEY WORDS: ADVENTITIOUS ROOT CULTURE, ASHWAGANDHA, JA 20, IAA- INDOLE ACETIC ACID, IBA- INDOLE-3-BUTYRIC ACID

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INTRODUCTION

Ashwagandha (*Withania somnifera Dunal*) is a medicinal crop of commercial importance, belongs to family *Solanacea*e, and is considered as an alternate to *Panax ginseng* in its therapeutic values (CSIR.,1976). It is used for curing a wide range of diseases in Ayurveda and other indigenous systems of medicine for over 5000 years (Akram *et al*., 2011). The herb has the highest value for its pharmacological activity in preparation of various Ayurvedic formulations. It is anti-stress, anti aging, and aids in recovering from neurodegenerative disorders (Bhattacharya *et al.,* 2002). It is a small, erect, branched, woody shrub that grows up to 1.50m tall. It is cultivated under rainfed condition in marginal soils by small and marginal farmers of Madhya Pradesh, Rajasthan, Gujarat, Andhra Pradesh, Karnataka, and other states. Apart from chemical constituents like alkaloids and withanolides, it also contains a variety of amino acids including aspartic acid, proline, tryptophan, tyrosine, cysteine, alanine, glycine, and a high amount of iron.

Withanolides are C-28 steroidal lactones (Alfonso *et al*., 1993). Major withanolides identified include Withanolide A, Withanoside IV and VI (Tohda *et al*., 2005), Withaferin A (Oh *et al*., 2008), Withanosides IV and V (Matsuda *et al*., 2001) and Withanoside B (Pramanick *et al*., 2008). Ashwagandha is available in the Western world as a dietary supplement. Its also known as “Indian Ginseng” and “Winter Cherry”. Withanolide A has strong neuro pharmacological properties of promoting outgrowth, synaptic reconstruction, and a potential to reconstruct neural networks (Kuboyama *et a*l., 2005; Tohda *et al*., 2005 a,b). Withaferin A inhibits angiogenesis (Mohan *et al*., 2004), metastasis (Misico *et al*., 2002). The major pharmacological activity of *Withania somnifera* is contributed to two major withanolides, Withaferin and Withaferin D (Gupta *et al.,* 2007, Sindhu *et al*., 2018).

Arka Ashwagandha is a variety identified at ICAR-Indian Institute of Horticulture Research, for high dry root yield and high total withanolide content. The variety has double the dry root yield (10 q/ha) than JA-20 (5.27q/ha). The other significant features are early vigor, field tolerance to bacterial wilt, late blight, leaf spot diseases and pests (*Epilachna* beetle, mites and aphids). It matures in 180 days and is characterized by desired root thickness and depth. The distinguishing features of the variety are lengthy tertiary branch, thick stem which has dense curved pubescence, lanceolate leaves with obtuse leaf tip, bigger fruit capsules and fruits. JA-20 is a released variety from MPKVV, Mandsaur, Madhya Pradesh used as a check in AICRP National trials and it yields about 5 q/ha.

Recent advances in tissue culture methodologies have improved secondary metabolite production across various medicinal plants. Selection of high bioactive producing lines, optimization of culture conditions, metabolic engineering, elicitation strategies and use of bioreactor culture systems has made the production of useful metabolites *in vitro* at a shorter duration of time (Sarin *et al*., 2005). Plant-specific metabolites can be effectively obtained from organ and plant culture systems (Verpoorte *et al*., 2002). Plant cell and organ culture systems are promising methodologies as they aid in rapid proliferation of cells/organs, condensed biosynthetic cycles in comparison to field grown plants (Ramachandra Rao and Ravishankar, 2002, Thanh Tam et al., 2019).

Adventitious roots suspension cultures are found to be ideal for biomass accumulation in *Echinacea purpurea* (Wu *et al*., 2007) and *P. notoginseng* (Gao *et al*., 2005). Recent studies indicate that explant type and genotype affect the accumulation of bioactive compounds in adventitious root cultures of *Polygonum multiflorum* (Ho *et al.,*2019) Lack of post-harvest storage technology for roots (Govil *et al*., 1993; Singh and Kumar., 1998), excessive exploitation of natural resources, problems in field cultivation as it is dependent on monsoon, time consuming and laborious are reasons enough to multiply adventitious roots of *Withania somnifera* in suspension culture which meet the global market requirement of Ashwagandha.

MATERIALS AND METHODS

Seeds of *Withania somnifera* like JA-20 and Arka Ashwagandha were selected. Seed pretreatment was conducted as per our earlier reports (Sindhu *et al*., 2018), morphological and phenotypic examination of 30-day old plants was done. The adventitious roots were induced in these two varieties, which were then harvested from 10-day old leaf culture bottles supplemented with auxins. These roots were washed with sterile distilled water two times to remove the small traces of agar followed by treatment for 1 minute with 3% sodium hypochloride, then the traces of sodium hypochlorite from roots were removed by washing again with sterile distilled water. The roots were dried by blotting with sterile tissue paper. The known quantity of roots (100 mg) were weighed and transferred into 100 ml of full strength MS liquid media supplemented with different concentration and a combination of auxins in conical flasks with 3% sucrose concentration under 16 hours of photoperiod and placed in an orbital shaker at 90 rpm at 25°C. The mass of multiplied roots was observed after 30 days of inoculation and was compared with the control.

Extraction of bioactive principles from *W. somnifera:* The adventitious roots were extracted from the liquid medium and the *in vitro* leaves were taken washed using distilled water to remove the traces of medium, dried and powdered using pestle and mortar. Adventitious roots were

assessed for total withanolides and also different components that contribute to total withanolides whereas the leaves were assessed only for Withaferin A content. The analysis of bio actives was done using High Performance Liquid Chromatography method. One gram of dry root powder was extracted with methanol at 80^{0}C on a water bath and the residue was re-extracted twice with methanol, till the extract was colorless. The extracts were pooled and filtered through sample clarification kit and were subjected to analysis by HPLC with Photo Diode Array. Seven standards such as Withanoside IV, Withanoside V, Withaferin A, Withanolide A, Withanolide B, Withanone and Withanostramolide from Natural Remedies Pvt. Ltd, Bangalore were used to quantify the total amount of withaferin A present in leaf samples and withanolides in root samples. The chromatogram was recorded at 227 nm and later the contents of various withanolides were added to estimate the total withanolide and expressed as mg/g on a dry weight basis.

$$\frac{\text{Area of the sample x Standard Wt. (mg) x Sample dilution x Purity of standard}}{\text{Area of the standard x Standard dilution x sample weight (mg) x 100}} \text{ X 100}$$

Data Analysis:

All experiments were repeated thrice. Mean values of treatments were subjected to ANOVA and significant differences were separated by Duncan's Multiple Range Test. To determine significance at P<0.05SPSS (Windows version 75.1, SPSS Inc., Chicago) was used.

RESULTS AND DISCUSSION

1. Morphological comparison between two varieties of Ashwagandha.

In vitro plants were assessed for plant height using 1 mm ruler from base of the stem to apical meristem. Leaf shape was also observed.

Table 1. Phenotypic differences between Arka Ashwagandha and JA-20 in *in vitro* conditions.

Traits	Arka Ashwagandha	JA -20
Plant height (cm)	6.30	5.50
Number of roots	17	16
Leaf shape	Lanceolate	Ovate
Leaf base	Concave	Concave
Leaf tip	Obtuse	Acute

2. Influence of hormone supplementation on the proliferation of *Withania somnifera* adventitious roots in suspension culture

For large scale production of useful bioactive metabolites, the use of cell suspension cultures is favored due to its rapid growth cycles over other kinds of cell culture methods. Qualitative and quantitative analysis requires a considerable quantity of cells to determine growth responses and metabolism of phytochemicals, for these studies cell suspension cultures are found to be best suited (Vanishree *et al*., 2004).

In the current study, the establishment of adventitious root suspension culture of *Withania somnifera* was done for two varieties Arka Ashwagandha and JA-20 with different hormone supplementation on media and further the biomass accumulation and total withanolides were estimated. Significant phenotypic differences were recorded in the proliferation of root cultures in MS medium, supplemented with hormones and without hormones. In the hormone supplemented medium, the root biomass was higher, but there was no elongation of lateral roots. The suspension culture media with hormones gave rise to fluffy roots, with callus like exudates formed around the senescent root tissues and subsequently released into the medium which could not be further multiplied in suspension cultures whereas media without supplementation of hormones had normal roots

Table 2. Data represent mean ± standard error of five replications in three independent experiments, each with one explant per treatment. Values followed by the different letters are significant P<0.05 according to Duncan's Range Multiple Test. Data were scored after 30 days of culture. Growth Conditions: Media- Full MS liquid medium supplement with different hormone combinations, 3% sucrose, 16 hours photoperiod at 25±2°C.

Treatment	Combination of auxins mg/l		The quantity of root obtained in gram/100 ml of media after 30 days of culture		Remarks
	IAA	IBA	AA	JA-20	
Control	0.0	0.0	4.066±0.118f	3.718±0.150f	Normal roots
T1	0.25	0.75	8.340±0.098b	7.254±0.135b	Fluffy roots
T2	0.50	0.50	6.400±0.139c	6.248±0.072c	Fluffy roots
T3	0.75	0.25	5.640±0.083d	5.364±0.068d	Fluffy roots
T4	1.0	0.0	4.682±0.086e	4.098±0.114e	Fluffy roots
T5	0.0	1.0	9.612±0.088a	8.314±0.138a	Fluffy roots

Table 3. Data represent mean ± standard error of five replications in three independent experiments, each with one explant per treatment. Values followed by the different letters are significant P<0.05 according to Duncan's Range Multiple Test. Data were scored after 30 days of culture. Growth Conditions- Media- Full MS liquid medium supplement with different hormone combinations, 3% sucrose, 16 hours photoperiod at 25±2°C.

Treatment	Arka Ashwagandha	JA-20
Half MS	3.684±0.074b	3.520±0.073b
Full MS	4.066±0.066a	3.718±0.054a

with important factors of proliferation like lateral root formation, elongation of lateral roots which favored further multiplication of adventitious roots. Lateral root formation was essential for rapid growth and higher biomass production in *Rauwolfia serpentine L* (Pandey *et al*., 2010), thus indicating that phytohormone supplementation does not always enhance the regeneration frequency (Roychowdhury *et al*., 2013).

Genes involved in the synthesis of auxins are expressed in roots, which contribute to normal root growth and maintenance (Petersson *et al*., 2009). Auxin biosynthesis genes are expressed at root stem cell nice to increase the level of auxins (Stepanova *et al.,* 2008). Signaling pathways for other plant hormones also influence the auxin response in roots (Kuppusamy *et al*., 2008). Though there was higher root mass in media supplemented with hormones, considering lateral root formation, proliferation and morphology of roots, MS media without hormone supplementation was considered best.

3. Influence of media strength on proliferation of *Withania somnifera* adventitious roots in suspension cultures

Both half strength MS medium and full strength MS medium were found to be suitable for biomass production of adventitious roots in *Panax ginseng*, but highest secondary metabolite content was induced in full strength MS medium (Yu et al., 2000). Full strength MS medium supplemented with 2.0mg/l IBA under continuous agitation increased the biomass of root tissue in *Vernonia amygdalina* (Khalaffala *et al*., 2009). Several researchers have also reported cell suspension culture studies in *Withania somnifera* (Sivanandan *et al*., 2012b and Praveen *et al*., 2011), hence full strength media without hormone supplementation was considered best suited for adventitious root multiplication in suspension culture as it provides more nutrients and reduces the frequency of subculturing in both varieties of Ashwagandha.

Different types of roots obtained by hormone treatments in *Withania somnifera*.

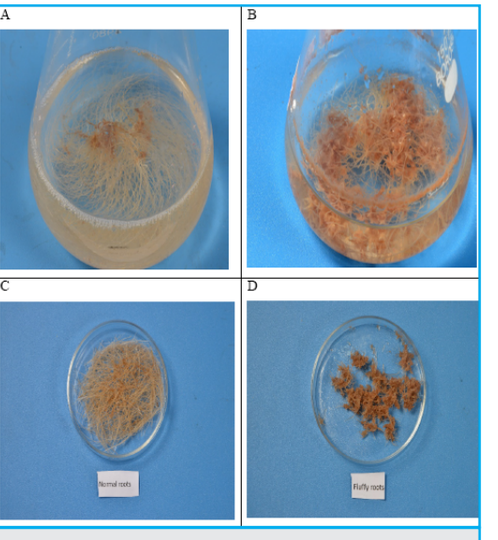

FIGURE 1. Different types of roots obtained in Arka Ashwagandha. A-Control, B-Hormone treated roots, C-Normal roots obtained after suspension culture in hormone-free medium, D-Fluffy roots obtained after suspension culture in hormone media.

4. Withanolides identified and total withanolide content in leaf and adventitious roots by HPLC analysis

HPLC analysis of *Withania somnifera* using methanolic extraction was reported by many researchers like Ganzera *et al*., (2003), Sangwan *et al*., (2004). The present study indicated that the roots had higher total withanolide content i.e., 1.621mg/g on a dry weight basis in Arka ashwagandha, compared to the total withanolide content in JA-20 root was 1.156 mg/g on a dry weight basis. Withanolide A which has a biological activity of sedative and hypnotic was 0.38mg/g on a dry weight basis in Arka Ashwagandha in comparison to JA 20

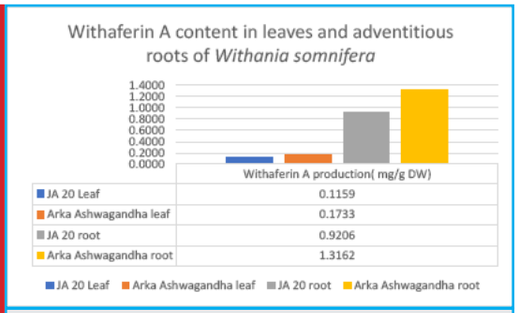

FIGURE 2. Withaferin A production in leaves and roots of *Withania somnifera* analyzed by HPLC.

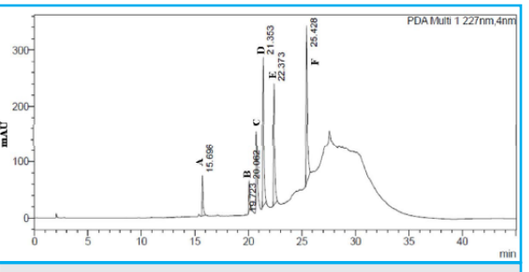

FIGURE 3. Standard HPLC chromatogram of *W. somnifera* root extracts. A. Withanoside IV, B. Withanoside V, C. Withaferin A, D. 12-Deoxy Withastramonolide, E. Withanolide A and F. Withanolide B.

which has 0.28mg/g on a dry weight basis. Madhavi *et al* (2012) reported a Withanolide A content of 136 µg/g dry weight in 120 days old hairy root culture and 13µg/g dry weight in 210 days old hairy root culture of *Withania somnifera,* Dewin *et al.,* (2010) reported Withanolide A content of 0.019 mg/g in *in vitro* roots of *Withania somnifera* which is much lesser than the Withanolide A content reported in the present study. The methanolic extract of *Withania somnifera* has GABA mimetic activity, anti-inflammatory and anti-stress properties (Mir *et al*., 2012).

Withaferin A contributes to most of the pharmacological activity of *Withania somnifera* with its antibacterial, antifungal, antiarthritic, antitumor and antibiotic properties. Dalavayi *et al*., (2006) identified Withaferin A in roots and leaves of Ashwagandha. The Withaferin A content in Arka Ashwagandha roots was identified to be 1.316mg/g on a dry weight basis compared to JA- 20 with a content of 0.926mg/g on a dry weight basis. The Withaferin A content in Arka Ashwagandha leaves was 0.1733mg/g on a dry weight basis compared to JA-20 leaves 0.116 mg/g on a dry weight basis.

Madhavi *et al* (2012) reported that Withaferin A was not detected in 120 day old hairy root cultures, whereas it was 136µg /g dry weight in 210 day old hairy root cultures of *Withania somnifera,* Sivanandan *et al* (2012) reported 0.85mg/g in 40 day old callus cultures of *Withania somnifera,* which is much lesser than the Withaferin A content reported in the present study. Dewir *et al* (2010) reported a Withaferin A content of 0.013 mg/g dry weight in *in vitro* roots, the present study indicates 100 times increased Withaferin A content.

CONCLUSION

This current study helps in the identification of an elite cultivar of *Withania somnifera* for mass production of withaferin A and total withanolides. Field cultivation of *Withania somnifera* is a laborious task, as the crop is prone to diseases like seed rot and blight, harvesting of roots is a tedious task. The provision for alternative sources of Ashwagandha through cell cultures and micropropagation must be encouraged as it reduces the heavy dependence on the wild population to fulfill the global demand of *Withania somnifera*. Adventitious root multiplication through suspension cultures in hormone free medium provide an easy way for mass production of useful withanolides and can be used by neutraceutical and pharmaceutical industries as there would not be traces of hormones in the end product.

Comparison of Withaferin A content between adventitious roots and *in vitro* leaves in *Withania somnifera*.

Table 4. Analysis of Withanolides in adventitious roots of *Withania somnifera* by HPLC Data represents mean ± standard error of three replications. Values followed by the different letters are significant P<0.05 according to Duncan's Range Multiple Test. Data were scored after 30 days of culture.

Variety	Withanoside IV	Withanoside V	Withaferin A	12-Deoxy withastromalide	Withanolide A	Withanolide B	Total Withanolides
	mg/g on dry weight basis						
Arka ashwagandha root	0.013±0.000[a]	0.025±0.001[b]	1.362±0.032[a]	0.181±0.004[a]	0.038±0.001[a]	0.003±0.000[a]	1.621±0.069[a]
JA-20 root	0.011±0.001[b]	0.037±0.003[a]	0.930±0.005[b]	0.173±0.004[b]	0.028±0.001[b]	0.002±0.000[b]	1.1558±0.013[b]

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