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ESTABLISHMENT OF ADVENTITIOUS ROOT CULTURE FROM CELL SUSPENSIONS OF *WITHANIA SOMNIFERA* (L.) DUNAL: AN *IN VITRO* APPROACH FOR PRODUCTION OF WITHANOLIDES

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ABSTRACT

Withanolides are a group of naturally occurring steroidal lactones present in roots and leaves of *Withania somnifera* (L.) Dunal. Withanolides possesses a wide range of pharmacological activities including anticancer to treat various neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases. In recent years, several pharmacological studies have been conducted to utilize *W. somnifera* and several patents have been filed on the therapeutic values of withanolides. Under natural conditions, *W. somnifera* possesses low levels of withanolides and *in vitro* culture techniques along with metabolic engineering could be an attractive tool for solving this problem. In the present study, we have induced adventitious roots from cell suspension culture of *W. somnifera* for the production of withanolides. Half strength MS liquid medium containing 0.5 mg l^{-1} IBA in combination with 0.25 mg l^{-1} IAA showed higher production of adventitious roots from cell suspension culture after 4 weeks. Dark condition and 3 % sucrose favored biomass productivity. The outcome of the present study shows great potential of adventitious root culture for large scale production of withanolides.

KEYWORDS: **Ashwagandha, Callus, Adventitious roots, Withanolides.**

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INTRODUCTION

Ashwagandha (*Withania somnifera* (L.) Dunal) is an important medicinal plant, containing a number of medicinally important metabolites known as withanolides. They are a group of naturally occurring steroids with an ergostane type of skeleton having δ or γ lactone containing side chain of nine carbons attached at C-17 of main steroidal skeleton (Fig. 1). Withanolides possess a wide range of biological activities including anti-arthritic, anti-inflammatory, immunomodulatory control and various neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases can be treated. [1, 2, 3, 4, 5] The extracts of withanolides have been used for the treatment of various types of cancer and tumors. The antitumor potential of withanolides has been extensively investigated by the researchers all around the world. Such works led to the identification of diverse properties such as cytotoxicity, cell differentiation induction, cancer chemoprevention and COX-2 and quinine reductase enzymes inhibition potential of withanolides. Recently, Santagat *et al*. [6] evaluated more than 80,000 natural and synthetic compounds for their anticancer activity. They reported that many active compounds were natural products belonging to the five classes, including withanolides. In recent years, numerous pharmacological investigations have been carried out utilizing *W. somnifera* extracts and several patents have been filed on pharmacological and medicinal importance of withanolides. The traditional cultivation of *W. somnifera* with respect to withanolide production has been limited by a range of issues as it has long generation time, low seed viability, unpredictability of bioactive compounds and the difficulty of maintain *Withania* in the field. As an alternative, various *in vitro* methods including callus, hairy root and adventitious root culture system have been adapted for production of its therapeutically valuable compounds. [7, 8, 9, 10] Among these, adventitious root culture has been a promising alternate for production of withanolide, because the total withanolide content of the adventitious roots are comparable to those of field grown roots and are higher than those of callus and hairy root. *In vitro* cell suspension culture is more expedient for the large scale production of active compounds and contains a relatively homogenous cell population, allowing a rapid and uniform access to nutrition, precursors, growth hormones and signal compounds for the cells. [11] However, the undifferentiated cells in a suspension culture may lack the biosynthetic machinery required for the generation of diverse secondary metabolites of interest, as different cell types may be involved to complete pathway; therefore *in vitro* root culture is a preferable system. [12] Earlier reports have documented comparatively low yield of withanolides (Withanolide A and Withferin A) in cell suspension culture. [7, 8, 13, 14, 15] Considerable amount of withanolide content also has been recorded in adventitious root and multiple shoot culture system of *W. somnifera*. [16, 17] Among these two *in vitro* organ culture, root culture is more favorable than the shoot culture because of rapid root multiplication and the ability of cultivation in large scale bioreactors. In this regard, experiments were conducted in the authors' laboratory to optimize the most suitable explants and conditions of *in vitro* adventitious root production in *W. somnifera*. [18] The objective of the present study was to optimize the conditions for adventitious root production from cell suspension culture system. To our knowledge there have been a few reports on adventitious root production from leaf and callus explants of *W. somnifera* [10, 16, 18, 19] and this is the first time we optimized the culture condition for *in vitro* adventitious root production in *W. somnifera* through cell suspension culture. The present culture system would set a primary plat for large scale production of withanolides using bioreactors.

MATERIALS AND METHODS

Callus induction

Leaf segments (0.5 cm) from one month old *in vitro* plantlets were cultured on MS [20] medium containing IBA (0.5, 1.0, 2.0 and 3.0 mg l^{-1}) alone or a combination of IBA (2.0 mg l^{-1}) with

2,4-D (0.25, 0.5 mg l^{-1}) or IAA (0.25, 0.5 mg l^{-1}). The media were solidified with 0.8 % agar powder and 3 % sucrose was added as a carbon source (Himedia®, Mumbai, India). The pH of the medium was adjusted to 5.8 before autoclaving at 121 ºC and 104 K Pa for 15 min. Cultures were maintained at 25 ± 2 ºC, 16 h photoperiod under 40 l mol m^{-2} s^{-1} light intensity provided by white fluorescent tubes and a relative humidity of 55-65 %. Twenty five explants were used per treatment with three replicates. The callus induction percentage and callus type were evaluated after one month's culture.

Adventitious root culture via cell suspension

For initiation of adventitious roots, 100 mg FW of white, friable callus was transferred in 150 ml Erlenmeyer flask containing 30 ml MS liquid medium supplemented with different concentration (0.1, 0.25, 0.5, 0.75, 1.0 mg l^{-1}) of IBA alone or IAA combination. The suspension cultures were kept at 90 rpm on a rotary shaker and maintained at 25 ± 2 ºC in darkness. Fifteen Erlenmeyer flasks containing white, friable callus were used per treatment with three replicates. Percentage of adventitious root formation, root biomass and growth ratio were evaluated after two weeks of culture.

Inoculum density, medium strength, sucrose concentrations and light or dark on adventitious root cultures

To determine the optimal inoculum density, various amounts (25, 50, 75 and 100 mg FW) of white friable callus were transferred in MS liquid medium containing 0.5 mg l^{-1} IBA and 0.25 mg l^{-1} IAA. In this study we standardized the effect of medium strength (1/4, 1/2, 3/4 and full MS), sucrose concentrations (1, 2, 3, 4, 5 and 6 %) in 1/ 2 strength MS medium supplemented with 0.5 mg l^{-1} IBA and 0.25 mg l^{-1} IAA on adventitious root formation. Incubation condition of light versus dark in incubating orbital shaker was also analyzed. Each experiment consisted of three replicates of fifteen cultures

Light versus dark condition and data analysis

The statistical analysis was performed according to the V 16.0 SPSS system. Mean and standard errors were used throughout, and the statistical significance between the mean values was assessed applying a Duncan's multiple range tests. A probability of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Initiation of callus and establishment of cell suspension cultures

In this study, callus was obtained from leaf explants that were grown on MS medium supplemented IBA (0.5-3.0 mg l^{-1}) alone or 2.0 mg l^{-1} IBA in combination with 2, 4-D (0.25-0.5 mg l^{-1}) and IAA (0.2-0.5 mg l^{-1}). Among the two combinations of growth regulators, the highest (92.65 %) ratio of white friable callus initiation was observed in 2.0 mg l^{-1} IBA in combination with 0.25 mg l^{-1} IAA (Table 1; Fig. 2a). In this study, freshly initiated white, friable callus was selected for subculture on MS liquid medium containing 0.1-1.0 mg l^{-1} IBA alone or 0.5 mg l^{-1} IBA in combination with 0.1-1.0 mg l^{-1} IAA. The type and concentration of auxin alter dramatically both the growth and product formation in cultures. [16] The present study showed that IBA in combination IAA was suitable for adventitious root formation from cell suspension culture. The highest rate of adventitious root formation (93.65 %) has been observed with the cell suspension cultures supplemented with 0.5 mg l^{-1} IBA in combination with 0.25 mg l^{-1} IAA (Table 2; Fig. 2b-d). Sivanandhan *et al*. [10] reported the formation of adventitious root from callus explants in MS medium containing 0.5 mg l^{-1} IBA and 0.1 mg l^{-1} IAA.

Effect of inoculum density on biomass accumulation

Among the various inoculums densities (25, 50, 75 and 100 mg) studied, 50mg was most suitable for optimum production of root biomass. Low (25 mg) and high (75 and 100 mg) level of inoculums resulted in low production of adventitious roots (Fig. 2e, 3a). This is in

accordance with the previous study showing that the 10 g l^{-1} DW favored the total cell biomass production in *W.somnifera*). [13]

Effect of medium salt strength

The optimum nutrient concentration is a critical determinant in controlling the growth of adventitious roots. [21] The strength of the MS medium was tested to determine the optical salt concentration for *in vitro* adventitious root formation. The best for the production of adventitious roots was half strength (Fig. 3b). Higher MS strength inhibited adventitious root formation. In contrast to our results, Yu *et al*. [22] reported that both half and full strength MS are suitable for root biomass production in *Panax ginseng*.

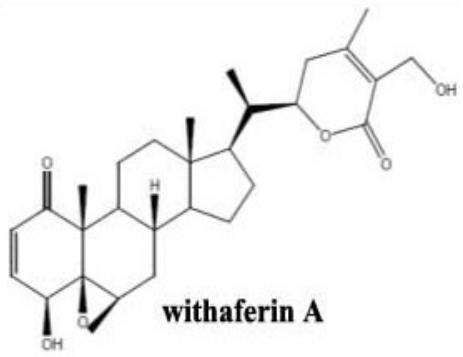
Effect of sucrose concentration

Sugar supplement is an important carbon source for adventitious root cultures and its concentration can affect several *in vitro* parameters. [21] In order to determine the optimum concentration of sucrose, cell suspension was grown under different concentrations of sucrose in half strength MS liquid medium for 3 weeks. The fresh weight of adventitious roots increased when the medium containing 3 % sucrose concentration (Fig. 2f, 3c). Yu *et al*. [22] have been used 5 % sucrose concentration for optimum root biomass production in *P. ginseng*. In *Gynura procumbens*, 2 % sucrose concentration was most suitable for the adventitious root formation. [23]

Light versus Dark

In general, *in vitro* root cultures are incubated in the dark its natural environment, [12] however light may have a pronounced effect on *in vitro* root production in several plant species. In *Ipomoea aquatica* hairy root produced higher rate of biomass in the higher condition than in the dark. [24] In *G. procumbens*, light or dark condition did not significantly affect the root biomass production. [23] In the present study, we found that the light and dark conditions strongly affected root biomass production. The best performance was obtained in dark condition (Fig. 3d).

Figure 1
Structure of withaferin A and withanolide A



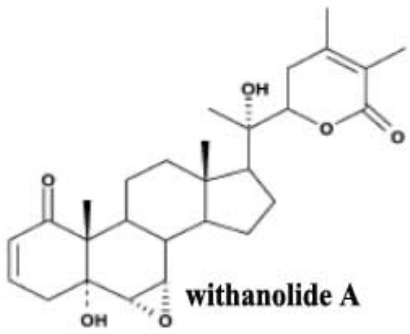
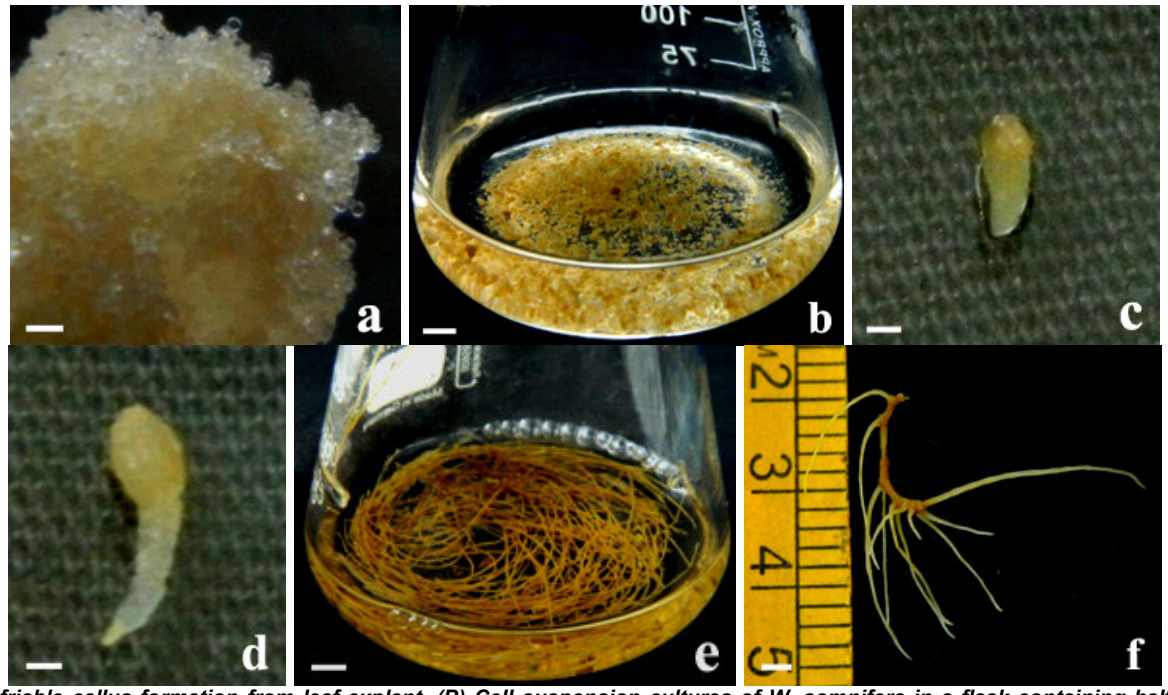


Table 1
Effect of auxins on callus formation

PGRs	% of callus formation	Observation result
IBA		
0.5	61.61	White, non friable callus
1.0	70.08	White, non friable callus
2.0	76.56	White, friable callus
3.0	84.23	Yellow, friable callus
IBA+2,4-D		
2.0+0.25	87.82	White, friable callus
2.0+0.5	85.46	Yellow, non friable callus
IBA+IAA		
2.0+0.25	92.65	White, friable callus
2.0+0.5	89.26	White, non friable callus

Data represents mean ± SE of three replicates; each experiment was repeated thrice.
Means separation within column by Dunkan's multiple range test at P < 0.05

Figure 2
Establishment of adventitious root culture from cell suspension of W. somnifera.



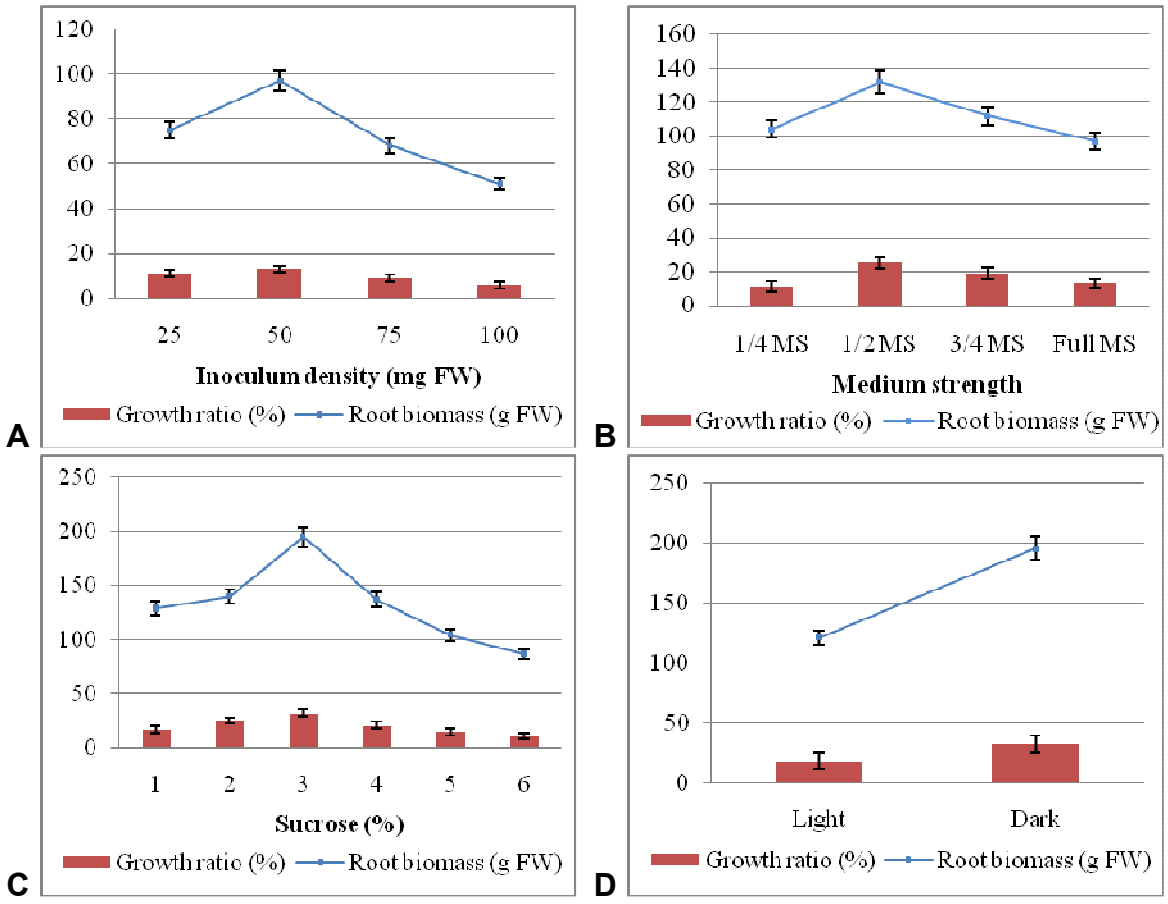
(A) Soft and friable callus formation from leaf explant, (B) Cell suspension cultures of W. somnifera in a flask containing half strength MS liquid medium supplemented with 0.5 mg l^{-1} IBA and 0.25 mg l^{-1} IAA, (C, D) Cell suspension derived adventitious root formation, (E, F) Root elongation and biomass production.

Table 2
Effect of auxins on adventitious root formation

PGRs	% of responses	Root biomass (g FW)	Growth ratio (%)
IBA			
0.1	20.23	17.66 ± 0.33	3.51
0.25	59.12	28.00 ± 0.57	3.43
0.5	76.45	32.66 ± 0.66	4.65
0.75	41.62	45.80 ± 0.57	6.22
1.0	37.40	39.00 ± 0.57	5.41
IBA + IAA			
0.5 + 0.1	81.32	47.66 ± 0.33	7.56
0.5 + 0.25	93.65	51.88 ± 0.57	7.93
0.5 + 0.5	70.71	42.00 ± 0.00	6.45
0.5 + 0.75	39.21	35.66 ± 1.15	4.56
0.5 + 1.0	17.10	21.80 ± 0.57	3.22

Data represents mean ± SE of three replicates; each experiment was repeated thrice.
Means separation within column by Dunkan's multiple range test at P < 0.05

Figure 3
Adventitious root culture of W. somnifera



(A) Effect of inoculum density, (B) Medium strength, (C) Sucrose concentration, (D) Light versus dark on adventitious root cultures.
Means separation within column by Dunkan's multiple range test at P < 0.05

CONCLUSION

In conclusion, cell suspension culture mediated adventitious root production is a promising approach to obtain withanolides. To the best of our knowledge, this is the first report of adventitious root production from cell suspension culture in *W. somnifera*. The system established in the present study is useful for the large scale production of withanolide from homogenous cell population, allowing uniform access to nutrition, precursors, growth hormones and signal compounds for the cells.

ABBREVIATIONS

MS Murashige and Skoog medium (1962)
IBA Indole-3-butyric acid
IAA Indole-3-acetic acid
2,4-D 2,4-Dichlorophenoxy acetic acid
FW Fresh Weight

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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