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EFFECT OF EXOGENOUS INDOLE-3-BUTYRIC ACID AND INDOLE-3-ACETIC ACID ON BIOMASS AND LEGENDARY WITHANOLIDES FROM *IN VITRO* ROOT CULTURES OF *WITHANIA SOMNIFERA* – JAWAHAR 20 CULTIVAR

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ABSTRACT

"Withanolides", the legendary bioactive metabolite present in leaves and roots of *Withania somnifera,* is well known for its therapeutic activities since ancient times. Several efforts have been made to produce these metabolites under *in vitro* condition. In the present study, we have tested the effects of exogenous supplementation of IBA and IAA in *W. somnifera in vitro* root cultures with the aim of optimizing the production of biomass and major withanolides. Among the two auxins, IBA is an effective inducer of lateral root formation thereby increasing the biomass. Accumulation of withanolide A and the biomass increases as the concentration of IBA increased to 1mg/L (2576±0.37 µg/g DW and 12.89±0.25 g/dL respectively). IAA at lower concentration favours relatively high accumulation of withanolide A (1147±0.77 µg/g DW) and at higher concentration of 1mg/L helps in higher accumulation of withaferin A (624±0.87 µg/g DW).

KEYWORDS: **Withania somnifera, *in vitro* adventitious root, IBA, IAA, withanolides, HPTLC**

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INTRODUCTION

Withania somnifera (L.) Dunal (Family, Solanaceae) commonly known as Ashwgandha or winter cherry is one of the top ranking medicinal herbs used in Siddha and Ayurveda medicinal practices since ancient times. Ashwagandha is found to be a major ingredient of various adaptogenic and anti-stress tonics[1]. Its notorious pharmacological activities include physiologic and metabolic restoration, antiarthritic, anti aging, nerve tonic, cognitive function improved in the elderly and recovery from neurodegenerative disorders [2,3]. Various bioactive constituents of this plant have been isolated and reported to possess adaptogenic, anticancer, anticonvulsant, immunomodulatory, antioxidative and neurological effects [4]. Among the bioactive constituents, Withanolide A and Withaferin A were reported to be principal metabolites [5,6], distributed among various tissues [7]. Beside its therapeutic advantages, the annual production of this plant is not sufficient to meet the global requirement [8]. Therefore, *in vitro* culture system could be an alternative to native field grown plant for the production of medically valuable compounds. Outstandingly, adventitious root culture is the exceptional technique which delivers the secondary metabolites in enormous amount and it encounters the global demand. The advantage of using root cultures is that they grow rapidly, relatively easy to prepare and maintain, show a low level of variability and can be easily cloned to produce a large supply of experimental tissues. Root cultures can be used in many ways including studies of carbohydrate metabolism, mineral nutrients requirements, essential need for vitamins, growth regulators, differentiation of the root apex and gravitropism[9]. Further enhancement of biomass and secondary metabolite accumulation can also be achieved by manipulation of the medium composition [10] and also by the exogenous supply of auxins. IAA, IBA and NAA are responsible for rhizogenesis in various plant materials [11,12,13,14,15,16]. Auxin on exogenous supply, were reported to promote adventitious rooting [17] and the physiological stages of rooting have been correlated with changes in exogenous auxin concentration[18]. Hence, we have investigated the effects of Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA) on *in vitro* root cultures of *W. somnifera* in terms of biomass accumulation and withanolide production.

MATERIALS AND METHODS

(i) In vitro Adventitious Root Induction

Seeds of *W. somnifera* (L.) Dunal 'Jawahar 20' were obtained from Central Institute of Medicinal and Aromatic Plants (Lucknow), and germinated *in vitro* on Murashige and Skoog (MS) solid basal (MS) medium supplemented with 2% sucrose in dark at 25˚C. For adventitious root induction, young leaf explants measuring 2-3 cm were excised from *in vitro* germinated seedlings and were trimmed into pieces of about 1cm^2 and inoculated in MS medium supplemented with 1mg/L IBA and 1 mg/L IAA along with 4.5% sucrose [19]. The inoculated explants were incubated at 25ºC. A photoperiod of 16 / 8 h light was maintained throughout the culture period. *In vitro* induced root tips and branches were cultured in half strength liquid MS medium to establish suspension cultures.

(ii) Exogenous Auxin Supplementation

Root suspension cultures were established as described above. 500 mg of adventitious root were inoculated into half strength liquid MS medium supplemented with different concentration of IBA and IAA and also in combination of both the hormones along with 3% sucrose (Table 1). All cultures were kept under continuous agitation at 80 rpm in an orbital shaker (Orbitek) and incubated as described for the initiation of the root cultures. Root biomass growth and withanolide productivity were measured after 30 and 40 days of culture.

Table 1
Supplementation of IBA and IAA in half strength liquid MS medium

S.No.	Concentration of IBA (mg/L)	Concentration of IAA (mg/L)
A0	0	0
A1	0	0.25
A2	0	0.5
A3	0	1
A4	0.25	0
A5	0.5	0
A6	1	0
A7	1	0.25
A8	1	0.5
A9	1	1
A10	0.25	1
A11	0.5	1

(iii) Determination of growth index

Adventitious roots from different treatments were separated from the medium by passing through a 1mm stainless steel sieve. Root fresh weight (FW) was measured after rinsing once with sterile water, and root dry weight (DW) was recorded after roots were dried to a constant weight at 60ºC for 2 days. The growth index was calculated using following equation [20].

$$\text{Growth Index} = \frac{\text{Harvested fresh weight (g)} - \text{Inoculated fresh weight (g)}}{\text{Inoculated fresh weight (g)}}$$

(iv) Preparation of the extracts

In vitro root material harvested after 30 and 40 days of culture was air dried and powdered using sterile mortar and pestle. The required amount of powder (1gm) was used for extraction. The samples were extracted with methanol. A ratio of 1:50 sample to solvent was maintained throughout extraction. The extraction was carried out four times. Each time the extract was sonicated for 20mins and kept in a shaker for 2hrs at 100rpm and filtered using whatmann No. 1 filter paper. All fractions were then pooled, filtered and evaporated to dryness using a rotary vacuum evaporator at 125rpm in a water bath at 40°C. The residue was dissolved in 10ml of HPLC grade methanol and stored at -20°C until further analysis.

(v) Preparation of standards

Withanolide A and Withaferin A standards were obtained from chromodex (USA). Standard stock solutions of withanolide A and withaferin A (1.0mg/ml) were prepared using HPLC grade methanol and stored in a refrigerator at 4°C. From the stock solutions, working solutions (0.1mg/ml) were prepared by dilution with HPLC grade Methanol.

(vi) Analysis of Withanolides by HPTLC

High Performance Thin layer chromatography (HPTLC) studies were carried out using the optimized solvent system containing three different solvents namely, toluene: ethyl acetate: formic acid in the ratio 5:5:1v/v [21].Chromatography was performed at 25±2°C on precoated aluminium plates as mentioned above of size 20x10cm/10x10cm and 0.2mm thickness were used. The standards withanolide-A and withaferin-A at a concentration 0.1mg/ml were applied in the concentration ranging from 200 to 1000 ng per band for quantification. A volume of 20µl of samples dissolved in HPLC grade methanol along with the standard were applied to the plates as 6/8mm bands, 8mm from the bottom, 15mm from the side, under a stream of nitrogen, by means of a CAMAG (Switzerland) Linomat V semiautomatic sample applicator fixed with a 100µl Hamilton HPTLC syringe. The spraying rate was 150nLs^{-1}. Linear ascending development to a distance of 80mm was carried out on 10x10cm/20x20cm twin trough chamber saturated with the mobile phase, pre-saturated with the solvent for 30min. After run, the plates were removed from the chamber, air dried and visualized at 254 and 366nm. Densitometric scanning was

performed with Camag TLC scanner III controlled by CAMAG CATS 4 integration software at 235nm for withanolide A and withaferin A. The slit dimensions were 4x0.3/6x0.3mm and the scanning speed was 20 mm s^{-1}. The R_f values of the resolved spots were noted. Evaluation was by peak areas with linear regression. The amount of withaferin A and withanolide A was computed from peak areas in all samples. The plates were derivatized in Anisaldehyde: sulphuric acid reagent (conc. sulfuric acid: methanol: glacial acetic acid: anisaldehyde in the ratio of 5:85:10:0.5) for two seconds and kept in hot-air oven for 10min at 110°C for detection of spots.

RESULTS

1. Effect of exogenous Auxins on biomass and withanolide accumulation

Well, clearly, our data showed that the type and concentration of auxins strongly influenced the formation of lateral root, thereby increasing the growth index (Table 2 & Figure 1) and also further indicated that IBA is an effective inducer of lateral root formation. Lateral roots were initiated within ten days after exposure to both the auxins, whereas, in control root, no such formation occurs. Lateral roots elongated further and gave a bushy appearance. *In vitro* roots inoculated in IBA supplemented medium exhibited rapid lateral root growth than IAA supplemented medium. Methanolic extracts of *in vitro* root samples along with mature field grown root extract were subjected to HPTLC analysis. The mobile phase used in the method gave good separation and resolution between peaks of two withanolide standards, withanolide A and of withaferin A. The R_f value for withanolide A and withaferin A were 0.46 and 0.32 respectively. Both the auxins at higher concentration (1mg/L) individually favour significantly greater biomass after 40 days of culture. But IAA at lower concentration (0.25mg/L) stimulates relatively high accumulation of withanolide A (1147±0.77 µg/g DW). Quite interestingly, higher concentration of IAA (1mg/L) helps in higher accumulation of withaferin A (624±0.87 µg/g DW) which is nearly 17.3 fold increase than the control root samples (Graph 2). On the contrary, accumulation of withanolide A (2576±0.37 µg/g DW) and the biomass (12.89±0.25 g/DL) increases as the concentration of IBA increased to 1mg/L. When the accumulation pattern was compared with the matured field grown root (623±0.12 µg/g DW) sample, *in vitro* root supplemented with 1mg/L IBA showed comparably more accumulation, which is a nearly fourfold increase (Graph 1 & Lane A6 of Figure 2). Auxins when supplemented in combination suppress the biomass as well as secondary metabolite accumulation. Biomass of *in vitro* root cultures was increased when the half strength liquid MS medium supplemented with 1mg/L IBA and 0.25mg/L IAA when compared to other combinations. Similarly, both withanolide A and withaferin A accumulated preferentially higher at this combination.

Table 2
Effect of IBA and IAA on growth index of in Vitro root after 40 days of culture

Different Auxin treatments	Harvested fresh weight (g/dL)	Growth Index
A0	6.2±0.17	11.4
A1	8.45±0.41	15.9
A2	9.14±0.37	17.28
A3	11.57±0.84	22.14
A4	7.63±0.43	14.26
A5	10.57±0.29	20.14
A6	12.89±0.25	24.78
A7	9.32±0.67	17.64
A8	8.65±0.46	16.3
A9	8.54±0.74	16.08
A10	7.86±0.39	14.72
A11	7.52±0.26	14.04

Values represents mean ± SE of five replicates of three independent experiments A0 – A11: As indicated in Table 1.

Lateral root growth formation in different auxin supplementation

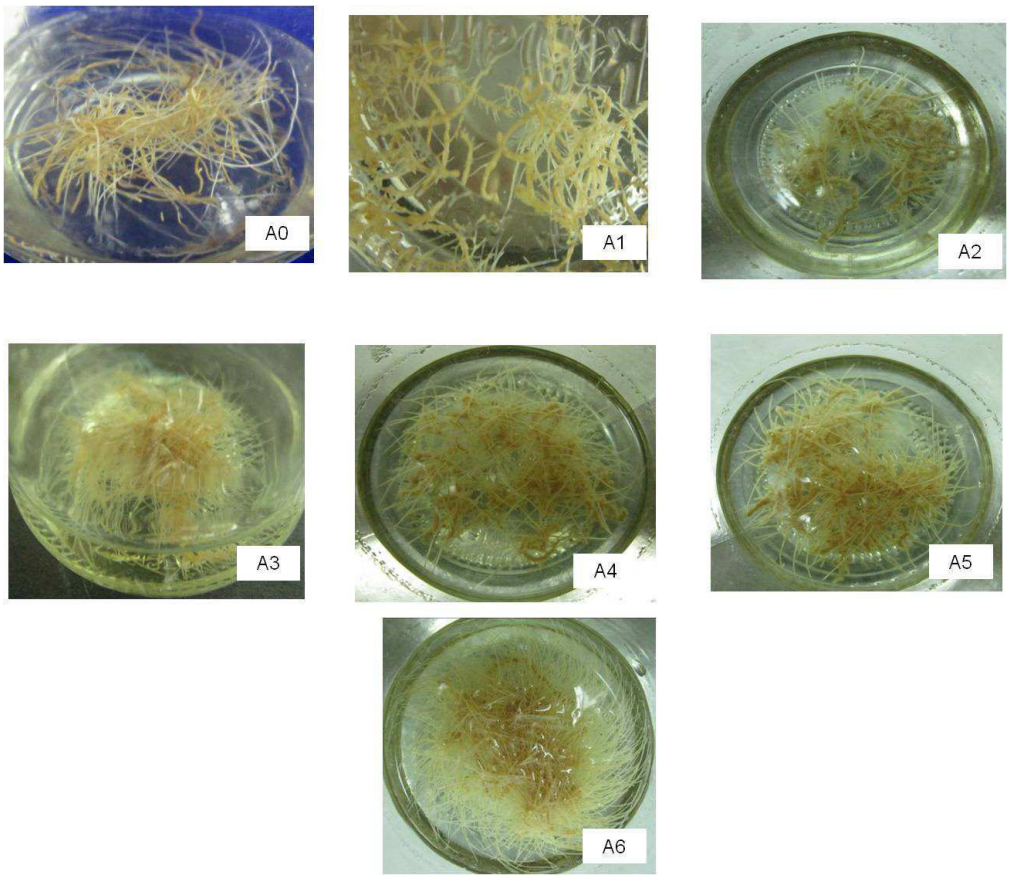


Figure 1
A0 – A6: As indicated in Table

Accumulation pattern of Withanolide A after 40 days of culture in auxin treated in vitro root samples

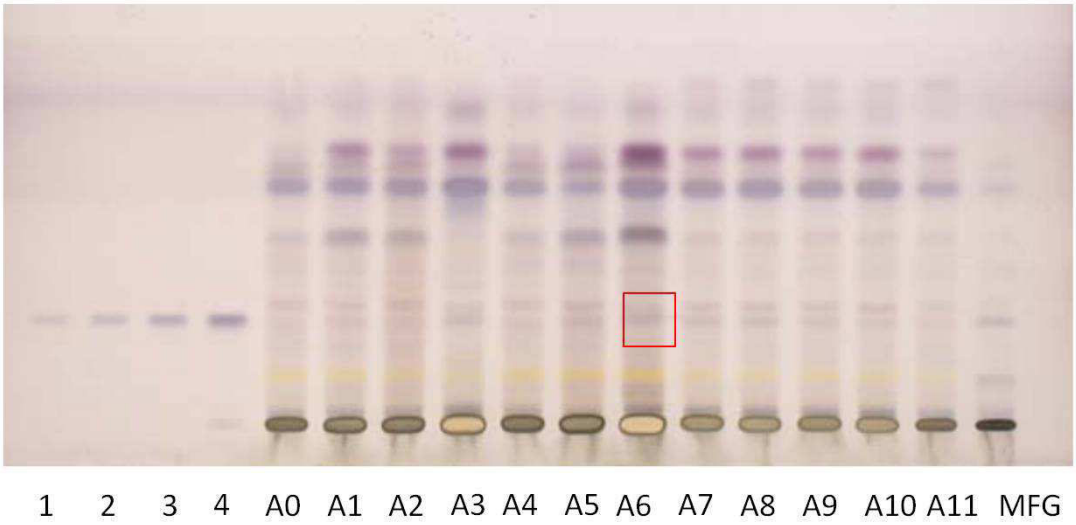
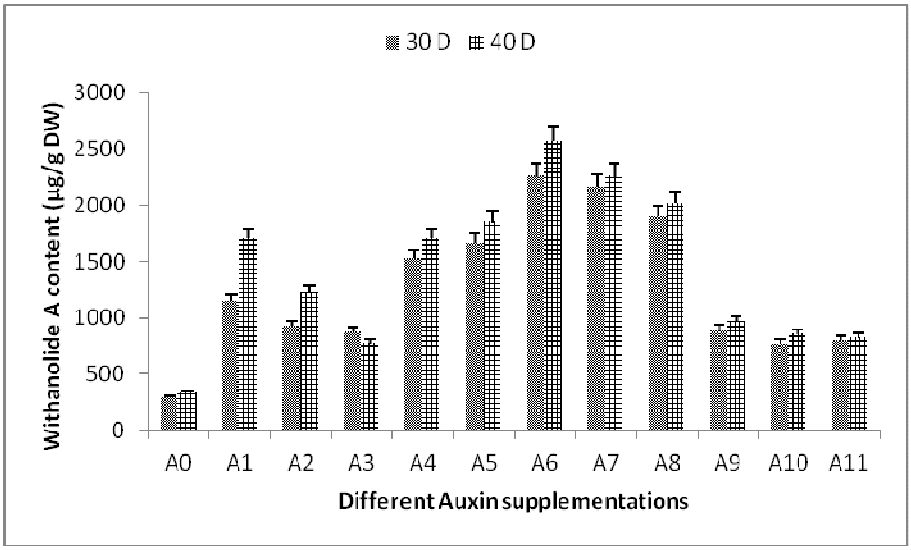


Figure 2

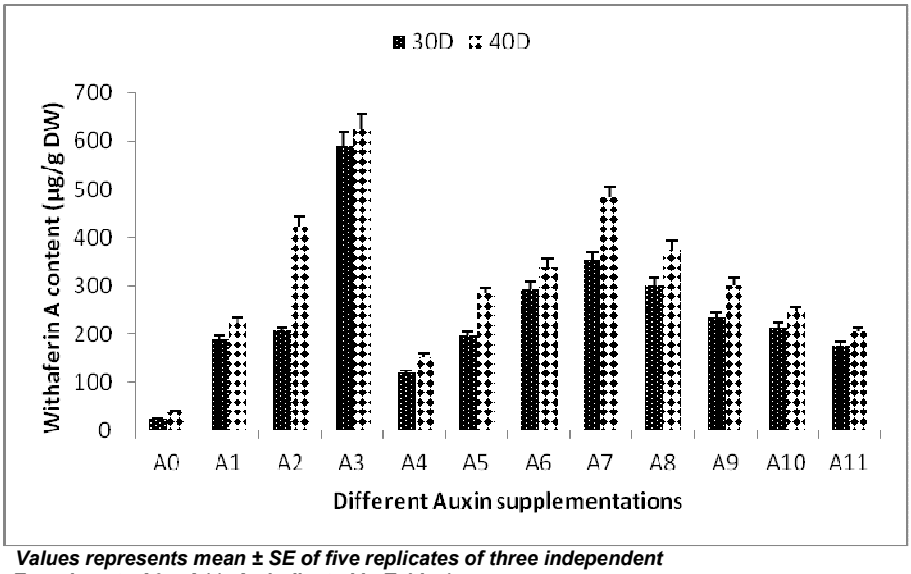
Lane 1-4 : Standard Withanolide A,
Lane A0 – A11: As indicated in Table 1
Lane MFG : Mature Field Grown Root.

Graph 1
Impact of auxins on withanolide A accumulation after 30 and 40 days of culture



Values represents mean ± SE of five replicates of three independent experiments A0 – A11: As indicated in Table 1

Graph 2
Impact of auxins on withaferin A accumulation after 30 and 40 days of culture



Values represents mean ± SE of five replicates of three independent Experiments A0 – A11: As indicated in Table 1

DISCUSSION

Plant growth regulators are a significant factor in influencing biomass and secondary metabolite biosynthesis in plant cell cultures. The successful formation of adventitious roots is a necessary step of vegetative propagation of many plants. Alterations in the types and concentrations of auxin or cytokinin, as well as the auxin/cytokinin ratio have dramatic effects on both growth and metabolite formation in plants [22]. Auxin is one of the major endogenous hormones known to be intimately involved in the process of adventitious rooting [23]. Adventitious roots induced by *in vitro* methods showed high rate active secondary metabolism [24, 25]. Clearly, our data revealed that the concentration of auxins strongly influenced the accumulation of major withanolides and also further indicated that

IBA alone than in combination with IAA was found to enhance the biomass. Nearly, 2 fold increase in biomass and 7 fold increase in Withanolide A accumulation were observed than the control untreated root samples when the medium was supplemented with higher concentration of IBA (1mg/L). The present finding was in harmony of the previous report of [26] who reported that IBA was the most efficient plant growth regulator for direct rhizogenesis. Further, IBA was concluded to be the most potent auxin for the development of adventitious root from *W. somnifera*[27]. The presence of IBA in the rooting medium enhances the number of roots per shoot and reduces the time required to obtain the maximum percentage of rooting[28]. Many of the previous results suggested that withaferin A was found to be low in concentration in *in vitro* raised root tissues[27], similarly our results also showed that concentration of withaferin A was low in *in vitro* root when compared to withanolide A. IAA was found to be less effective compared to IBA in inducing lateral root formation. Moreover, low concentration of IAA favours high accumulation of withanolide A as well as biomass. Lower efficiency in lateral rooting of IAA could possibly be explained by the more energy used up for other activity such as synthesis, metabolism and transport which subsequently may lead to insufficient energy for cell growth and development during root formation [27]. Many authors also suggested that high IAA concentration has an inhibitory effect on root elongation[29, 30]. Poor performance of IAA in formation of lateral roots could also be due to rapid photo-oxidation than IBA[31].

CONCLUSION

It can be summarized that the type of auxin significantly influences the lateral root growth formation. Our results indicate that exogenous supplementation of IBA can enhance growth and withanolide A production in *W.somnifera.* As a result, *in vitro* root cultures of *W.somnifera* may be a valuable alternative approach for producing therapeutically valuable secondary metabolites. The above results will therefore be useful in designing systems for the large-scale cultivation of Withania *in vitro* root suspension cultures for the production of withanolide A.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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