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Research Article

INFLUENCE OF AUXIN ON BIOMASS PRODUCTION AND WITHANOLIDE ACCUMULATION IN ADVENTITIOUS ROOT CULTURE OF INDIAN RENNET: *WITHANIA COAGULANS*

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ABSTRACT

Objective: Withanolides are the biologically active, principle compound present in *Withania coagulans*, which is having a high medicinal value and possesses potent therapeutic activity. The present study was attempted with an objective to investigate a biomass growth and withanolide production in *in vitro* root tissues of *W. coagulans*.

Methods: High-performance thin layer chromatography (HPTLC) often serves as a method for quantification of major withanolides. In the present study, methanolic withanolide extract of *in vitro* cultured *W. coagulans* root tissue were carried out using HPTLC. The HPTLC analysis was performed using precoated silica gel aluminum plate (20 cm × 20 cm) 60F254 (E.MERCK, Germany) with mobile phase toluene: ethyl acetate: formic acid (5:5:1).

Results: The optimization of different combination and concentration of plant growth regulators (indole-3-butyric acid [IBA] and indole-acetic acid) was used to stimulate the biomass growth and withanolide production. The maximum biomass growth (7.48±0.25 g/dL) was observed in medium with 4.93 μM^{-1} IBA. The higher amount of withanolide A (204.98±0.87 µg/L DW) and withaferin A (227.15±0.57 µg/L DW) accumulation was recorded in culture grown on half Murashiga-Skoog media supplemented with 4.93 μM^{-1} and 2.46 μM^{-1} IBA.

Conclusion: We concluded that *in vitro*-cultured system could provide unique opportunities for large-scale production of pharmaceutically important compound than field grown plants.

Keywords: Adventitious root culture, High-performance thin layer chromatography, *Withania coagulans*, Withanolides.

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INTRODUCTION

The genus *Withania* (Family: Solanaceae) is a highly acclaimed genus of medicinal plants in the Indian ayurvedic system of medicine because of its magnificent pharmaceutical and nutraceutical properties [1]. *Withania* is a small genus of shrubs which are distributed in the east of the Mediterranean region and extend to South Asia. Among the 23 known species of *Withania,* only two, *Withania somnifera* and *Withania coagulans,* are economically considerable and widely cultivated [2,3]. *W. coagulans* Dunal is one of the vital medicinal plants. It is commercially important for its milk coagulating properties [4] and is well known in the indigenous system of medicine for the treatment of ulcers, dyspepsia, rheumatism, dropsy, consumption, and senile debility. It has received much attention in recent years due to the presence of a large number of steroidal alkaloids and lactones known as withanolides [5].

The major biochemical constituent of this plant is withanolides. They are a group of naturally occurring C-28 steroidal lactones built on an intact or rearranged ergostane agenda, in which C-22 and C-26 are appropriately oxidized to form a 6-membered lactone ring [6]. Biogenetic transformations of the steroidal skeleton and side chain have diversified the structure of withanolides. It have attracted attention due to their extensive range of biological activities, including antitumor, anti-inflammatory, antimicrobial, cytotoxic, immunomodulating, and cancer chemopreventive activities [7,8]. Withaferin A, withanolide A, and withanone are the major withanolides present in W. *somnifera* and *W. coagulans*.

The *in vitro* propagation technique may be the greatest solution for its rapid multiplication and reestablishment in nature [9]. It also provides an alternative to field grown plant harvesting for the production of therapeutically valuable compounds [10] and is reported that the withanolide contents of the *in vitro* hairy root cultures of *W. coagulans* were higher than in the root of the plant [11]. Recently, phytohormones specifically auxin plays an essential role in regulating root development and it has been shown to be intimately involved in the production of adventitious root growth and withanolide accumulation in *W. somnifera* [12]. Auxin, indole-acetic acid (IAA) was shown to be involved in the rooting process by Thimann and Went as far back as 1934, and a second "synthetic" auxin indole-3-butyric acid (IBA) also promoted rooting [13]. Adventitious root formation has many practical implications in horticulture and agronomy and there is a lot of commercial interest because of the many plant species that are difficult to root [14,15]. Therefore, there is a need to develop an efficient protocol for the induction of *in vitro* adventitious roots of *W. coagulans.*

Root cultures can be used as standard experimental system in studies of carbohydrate metabolism, mineral nutrient requirements, essentiality of vitamins and other growth regulators, differentiation of the root apex and gravitropism. 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 tissue [16]. Further improvement of biomass and withanolide accumulation can also be achieved by medium containing manipulation of the exogenous supplement of auxins. IBA and IAA were responsible for the adventitious root culture of *W. somnifera* and also the combination of IBA and IAA was promoted adventitious root induction in various plant materials [17]. Internally, macronutrients such as nitrogen play an important role not only in the growth of tissue cell lines but also in the production of biomass and withanolide in root suspension cultures [18].

To the best of our knowledge, there have been no reports on the effect of auxin combination on the biomass and withanolide accumulation in root

suspension cultures of *W. coagulans*. Therefore, we have investigated the effects of different auxin combination on root suspension culture of *W. coagulans* in terms of biomass and withanolide accumulation.

METHODS

Plant materials

The seeds of *W. coagulans* were obtained from Banaras Hindu University, Varanasi. Surface-sterilized seeds were then germinated *in vitro* in Murashige-Skoog (MS) media supplemented with 2% sucrose and seedlings produced were maintained on MS basal medium.

In vitro adventitious root induction

The young or mature leaves from 2 months old seedlings were used as explant for the present study. The leaves were cut into 0.5 cm^2 and inoculated on MS [19] medium with 3% (w/v) sucrose, 0.8% (w/v) agar, and supplemented with concentration of auxins such as (5.71 μM^{-1}) IAA and (19.7 μM^{-1}) IBA were added. The cultures were kept under 16-hrs photoperiod (40 µmol/m s^{-1}) provided by 40 W fluorescent lamps (Philips, Kolkata, India) at 25±2°C. The growth of adventitious root was recorded at weekly intervals. The roots were transferred into half strength MS suspension medium for further studies [17].

Effect of auxin combination on adventitious root growth and withanolide production

After a period of 30 days, the root tips (about 25-30) from root induction medium were cut under sterile condition and transferred into half strength MS liquid medium containing different concentration of IBA and IAA and also in combination of both the IBA and IAA along with 3% sucrose (Table 1). All the culture vessels were kept under continuous agitation at 110 rpm in an orbital shaker (Orbritek, Scigenics, and Chennai, India) and incubated at 25±2°C, with a 16 hrs photoperiod. The biomass growth and withanolides production were measured after 4 weeks of culture. The roots were harvested and their wet and dry weight were recorded after that the roots were dried to a constant weight at 60°C for 24 hrs.

$$GI=\frac{\text{Fresh weight of harvested biomass - fresh weight of the inoculums}}{\text{Fresh weight of the inoculums}}$$

Extraction of secondary metabolites

The dried root samples were ground thoroughly using mortar pestle and root powder was obtained. Initially, 1 g of root powder was weighed and treated with 1 ml ammonia for 20 minutes at room temperature, followed by sonication for 20 minutes with 50 ml of methanol and placed in a shaker for 2 hrs at 150 rpm at 22°C. At the end of 2 hrs, the extract was filtered using whatmann no.1 filter paper and the residues were again treated with 50 ml methanol. This step was repeated four times to obtain 200 ml of the extract. The extract was then concentrated by evaporation using flash evaporator maintained at 45°C and 150 rpm. After complete evaporation, the residue was dissolved using high-performance liquid chromatography grade methanol [20].

Quantification of major withanolides

High-performance thin layer chromatography (HPTLC) was performed on precoated silica gel aluminum plate (20 cm × 20 cm) 60F254 (E.MERCK, Germany). The methanolic extract of *W. coagulans* was loaded to the plates as 6 mm bands, under a stream of nitrogen using the Camag (Switzerland) Linomat V semiautomatic sample applicator fitted with a 100 µl of Hamilton HPTLC syringe. The HPTLC plates were developed up to 80 mm using the mobile phase toluene: ethyl acetate: Formic acid in the ratio of 5:5:1, respectively, in a Camag Twin trough glass tank. It was presaturated with the mobile phase solvents for 30 minutes at room temperature (25±2°C). The developed plate was air dried and the image was captured at 245 nm and 366 nm. Densitometric scanning was performed at 235 nm for withanolide A and 530 nm for withaferin A using Camag TLC scanner III controlled by Camag CATS 4 integration software. The Rf values of the resolved spots were noted. The peak areas were evaluated using linear regression [21].

Table 1: Hormonal combination

Treatments	IBA (μM^{-1})	IAA (μM^{-1})
T0 (MS0)	0	0
T1	1.23	0
T2	2.46	0
T3	4.93	0
T4	0	0.35
T5	0	0.71
T6	0	1.43
T7	4.93	0.35
T8	4.93	0.71
T9	4.93	1.43
T10	1.23	1.43
T11	2.46	1.43

IBA: Indole-3-butyric acid, IAA: Indole-acetic acid, T0 - Control, T1 - Half strength liquid MS medium supplemented with 0.25 mg/L IAA, T2-0.5 mg/L IAA, T3-1 mg/L IAA, T4-0.25 mg/L IBA, T5-0.5 mg/L IBA, T6-1 mg/L IBA, T7-0.25 mg/L IAA and 1 mg/L IBA, T8-0.5 mg/L IAA and 1 mg/L IBA, T9-1 mg/L IAA and IBA, T10-0.25 mg/L IBA and 1 mg/L IAA, T11-0.5 mg/L IBA and 1 mg/L IAA

The amount of withaferin A and withanolide A in the samples was quantified using peak area. The plates were derivatized using anisaldehyde sulfuric acid (85 mL methanol: 10 mL glacial acetic acid: 5 mL sulfuric acid: 0.5 mL anisaldehyde) reagent and placed in hot air oven for 2 minutes at 110°C for detection of spots.

RESULTS AND DISCUSSION

Influence of auxins on lateral root growth and withanolides production

Plant growth regulators (PGRs) are organic substance which is used to regulate the plant growth and also modify the plant physiological processes. They may act as either biostimulants (or) bioinhibitors, act inside plant cells to stimulate (or) inhibit specific enzymes or enzyme system, and help regulate plant metabolism [22]. The early characterization of auxins as "root-forming substance of plants" due to established a long-standing relationship between this group of small molecules and lateral root development [23,24]. The aerial portion of the plant body, a series of iterative modules produces the overall root architecture; the root which is established during embryogenesis and gives rise to new lateral roots branches in a continuous, indeterminate manner. Proof from many research has been indicate the vital role of auxins in orchestrating the final root architecture. The major role of auxins as a component of endogenous developmental programs as well as in mediating environmental stimuli to shape the final root architecture remains at the heart of several active research programs [25].

Lateral roots are initiated from root pericycle cells adjacent to the protoxylem poles of the parent root [26]. Although there remains a paucity of data that defines the molecular machinery responsible for generating a lateral root, current observations point out that the process progresses through at least four recognizable phases: Priming, initiation, patterning, and emergence [27,28]. Each of these phases is controlled or at least influenced by auxin [25]. Since they are control the growth of stem, roots, fruits, and convert stem into flowers. It acts to inhibit the growth of buds and also promote the biomass growth and secondary metabolites production [29].

Among the dynamic auxins, IBA and IAA are the most frequently used Plant Growth Regulator because it is not rapidly degraded by the plant and is not translocated from the site of application. However, the high concentrations of auxins are toxic to plants. They are most toxic to dicots than monocots. Because of this property, synthetic auxin herbicides including 2,4-D and 2,4,5-T have been developed and used for weed control. In this regard, lower concentration of auxins is highly peripheral for lateral root growth and plant-derived secondary metabolites production [30].

Exogenous supplement of auxin is an important factor for adventitious root formation. However, endogenous auxins may also play a role in

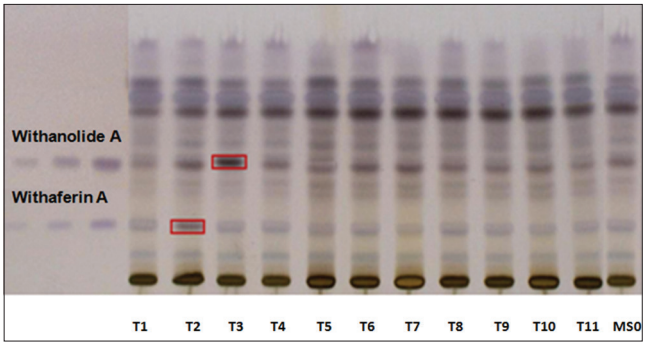


Fig. 1: Analysis of Withanolide A and Withaferin A on auxin treated *in vitro* roots of *W. coagulans*. Lane 1-3: Standards withanolide A and withaferin A, T1 - Half strength liquid Murashigae-Skoog medium supplemented with 0.25 mg/L Indole-acetic acid (IAA), T2 - 0.5 mg/L IAA, T3 - 1 mg/L IAA, T4 - 0.25 mg/L Indole-3-butyric acid (IBA), T5 - 0.5 mg/L IBA, T6 - 1 mg/L IBA, T7 - 0.25 mg/L IAA and 1 mg/L IBA, T8 - 0.5 mg/L IAA and 1 mg/L IBA, T9 - 1 mg/L IAA and IBA, T10 - 0.25 mg/L IBA and 1 mg/L IAA, T11 - 0.5 mg/L IBA and 1 mg/L IAA, MS0 (Control)

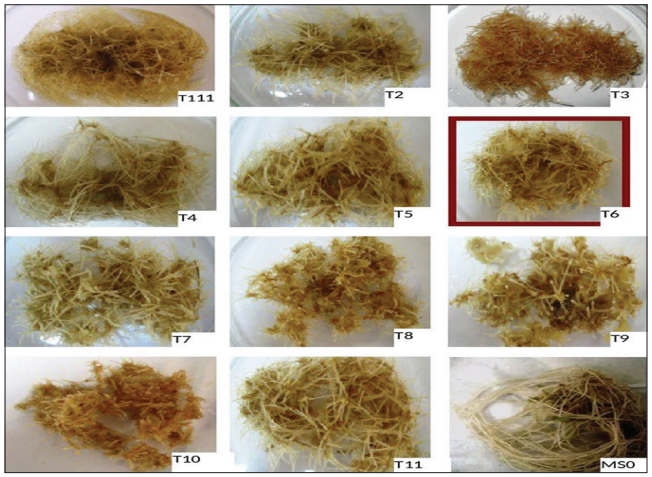
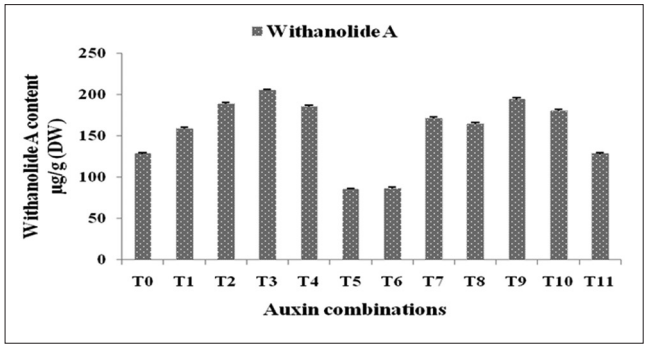


Fig. 2: Lateral root branch formation in different auxin concentrations

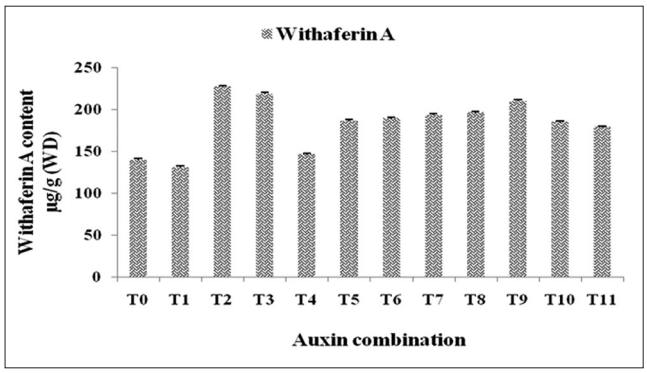
the rooting process. Therefore, medium with altered auxin levels was investigated for their ability to form adventitious roots mass and plant metabolites production [31]. To our knowledge, well clearly indicated that type and concentration of auxins strongly influence the formation of lateral root, thereby increasing the growth index was represented in Table 2 and Figs. 1 and 2.

Lateral roots were initiated within 2 weeks of culture, after exposure to the auxins; whereas without auxins, there was no lateral roots formation. The lateral root elongated further and become a bristle appearance. The root supplemented with IBA at 4.93 μM^{-1} concentration invoked better biomass growth than compared to other combinations and concentrations. The HPTLC analysis of *in vitro* root samples of *W. coagulans* was performed using the solvent system tolene: ethyl acetate:formic acid (5:5:2) to check the accumulation of withanolide A and withaferin A. The result revealed that among the different combination and concentrations of auxins (IBA and IAA).

IBA at high concentration 4.93 μM^{-1} individually stimulates the lateral root branches (7.48±0.25 g/dL) and withanolide A (204.98±0.87 µg/L DW) production after 30 days of root suspension culture (Table 2, Graph 1, and Fig. 1). Whereas medium with 2.46 μM^{-1} IBA favors relatively high accumulation of withaferin A (227.15±0.57) (Graph 2).



Graph 1: Influence of auxins on withanolide A accumulation after 30 days of root suspension culture



Graph 2: Influence of auxins on withaferin A accumulation after 30 days of root suspension culture

Table 2: Effect of auxins on growth index

Different auxin treatments	Harvested fresh weight (g/dL)	Growth index
MS0	2.18±0.17	9.9
T1	4.38±0.41	20.9
T2	5.30±0.37	25.5
T3	7.48±0.25	36.4
T4	3.37±0.43	15.85
T5	6.45±0.29	31.25
T6	6.85±0.84	33.25
T7	5.52±0.67	26.6
T8	5.0±0.46	24.0
T9	4.70±0.74	22.50
T10	3.53±0.39	16.65
T11	2.93±0.26	13.65

Values represents mean±SE of five replicates of three independent experiments. T0 - Control, T1 - Half strength liquid MS medium supplemented with 0.25 mg/L IAA, T2-0.5 mg/L IAA, T3-1 mg/L IAA, T4-0.25 mg/L IBA, T5-0.5 mg/L IBA, T6-1 mg/L IBA, T7-0.25 mg/L IAA and 1 mg/L IBA, T8-0.5 mg/L IAA and 1 mg/L IBA, T9-1 mg/L IAA and IBA, T10-0.25 mg/L IBA and 1 mg/L IAA, T11-0.5 mg/L IBA and 1 mg/L IAA, IBA: Indole-3-butyric acid, IAA: Indole-acetic acid

Approximately 5-fold increase in withanolide A and withaferin A accumulation and 4-fold increase in biomass growth index (36.4 g/dl) were recorded than the control root cultures when the medium was supplemented with 4.93 μM^{-1} IBA.

Auxins when supplemented in combinations with IBA and IAA, suppress the biomass growth and secondary metabolites production [12]. From the result, it was concluded that the increasing concentration of IBA alone was highly influence for lateral root formation and withanolide production. IBA is an important factor for lateral rooting and gives a better performance than IAA due to higher stability, differences in metabolism, and transport [32]. There is now a great deal of evidence

that IBA occurs naturally in plants [17] and also suggests that IBA was the most potent auxin for the development of adventitious roots for enhanced production of secondary metabolites from *W. somnifera.* Though production of secondary metabolites in adventitious root metabolites was reported to be *de novo* synthesized with in root tissue. Hence, studies were conducted on tissue-specific synthesis of withanolide under *in vitro* condition [33].

CONCLUSION

Our results demonstrated that influence of auxins (IBA and IAA) on the lateral adventitious root growth and withanolide production of *W. coagulans* root suspension culture was reported for the first time. This may be a valuable alternative approach for producing therapeutically important bioactive principle compounds under *in vitro* condition. The above results are useful for the large-scale cultivation of *W. coagulans* root suspension culture for the production of withanolides.

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