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ORIGINAL ARTICLE

In vitro propagation and enhancement of phytoconstituents in *Withania coagulans*, a rare medicinal plant

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ABSTRACT
Withania coagulans, belonging to family Solanaceae, is an important medicinal plant with restricted geographic distribution. Propagation of Withania coagulans is highly demanded in recent times due to its rich bioactive withanolides contentand its extinction from natural habitats. Shoot multiplication protocol of Withania coagulans was standardised by supplementing basal Murashige and Skoog's media with different concentrations of Benzylaminopurine (BAP), Kinetin and Thidiazuron (TDZ). Explants were transferred to fresh media with similar hormone constituents every three weeks. 1 mg/L BAP in combination with 0.5 mg/L Kin induced maximum shoots in this herb under in vitro conditions. Also among the 25 different combination of auxins (IAA and IBA) tested, MS media supplemented with 1 mg/L IAA and 4 mg/L IBA was found to be the best medium for in vitro adventitious root induction in Withania coagulans. The developed roots were then transferred for suspension cultures and mass cultivated in bioreactors successfully. These roots resulted in increased biomass and accumulations of active compounds. Culturing in vitro adventitious roots in bioreactors could also be used as a fast and efficient method of generating roots that would offer unique opportunities for producing root drugs without having to depend on field cultivation. Following this, the in vitro propagated Withania coagulans tissues were subjected to phytochemical analysis and were found to accumulate the constituents in high amount when compared to field grown roots. Also withanolides quantification using HPTLC proved high withaferin A and withanone accumulations when propagated under in vitro conditions.
Keywords : *Withania coagulans; in vitro; bioreactor; HPTLC.*

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INTRODUCTION

In vitro techniques have been found to be useful in the propagation of a large number of threatened and endangered plants [1]. The technology bears many advantages over conventional agricultural methods such as, production is independent of variation in crop quality or failure, yield of target compounds would be constant and geared to demand, there is no difficulty in applying good manufacturing practice to the early stages of production, production would be possible anywhere under strictly controlled conditions, independency of environmental problems, free from risk of contamination with pesticides, herbicides, agrochemicals or fertilizers and new methods of production could be patented [2].

Production of secondary metabolites in tissue cultures is usually higher when plant cells are organized into tissues/organs. The expression of secondary metabolic pathways in organized cultures is not surprising because it mimics exactly what the plant does. Root cultures are typical examples that can be used for production of phytochemicals. Root cultures have been used as a standard experimental system in studies of inorganic nutrition, nitrogen metabolism, plant growth regulation, and root development [3].

Among the twenty-three known species of *Withania*, only two (*Withania somnifera* (L.) Dunal and *Withania coagulans* Dunal) are economically significant and widely cultivated [4]. *Withania coagulans* Dunal belonging to the family *Solanaceae,* is a small bush which is spread across South Asia and is commercially important for its milk coagulating properties [5]. *Withania coagulans* is well known in the indigenous system of medicine for the treatment of ulcers, dyspepsia, rheumatism, drowsy, and consumption debility [6]. It received much attention in recent years due to the presence of a large number of steroidal alkaloids and lactones known as withanolides. Withanolides, chemically nomenclatured as

22- hydroxy ergostane-26-oic acid 26, 22-d-lactones, are C28-steroidal lactones based on an intact or rearranged ergostane frame with appropriate oxidations at C-22 and C-26 to form a d-lactone ring. Major Withanolides, like Withaferin A, Withanolide A and withanone of the plant have been demonstrated to possess significant therapeutic actions [7].

Withania plants were abundant until a few decades ago, but ruthless collection for medicinal purposes, habitat destruction and climate changes makes the species to become endangered in their natural habitats. Jain et al. (2009) reported that overexploitation and reproductive failures budge the species *W. coagulans* towards verge of extinction [8]. Therefore propagation and conservation of these plants is important to meet up with future demands. The conventional propagation of this species is performed through seeds and cuttings of stem since root is too slow and laborious to grow. *In vitro* propagation technique may be the best solution for its rapid multiplication and re-establishment in nature [9]. The *in vitro* cultures could provide as an alternative tool to field harvesting of this plant in order to produce 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 field grown plant [4]. Therefore, there is a massive need to develop an efficient protocol for the induction of *in vitro* adventitious roots and the shoot multiplication of *W. coagulans.*

Thus the present study was formulated to optimise the concentrations of growth hormones for root induction and shoot multiplication in *Withania coagulans* under *in vitro* conditions and was found that major phytochemicals and therapeutic withanolides excessively accumulated in *in vitro* roots of *W. coagulans* compared to the field grown roots.

MATERIALS AND METHODS

Plant material: *Withania coagulans* seeds were obtained from Banaras Hindu University, Varanasi. The seeds were germinated *in vitro* after surface sterilization with Tween20 and 1% $HgCl_2$. The seedlings were maintained on MS basal medium with regular sub culturing. The nodes from two month old aseptic plantlets were used as explants for shoot multiplication and leaves excised were applied as explants for root induction.

Media and explants used: MS basal medium was prepared essentially based on the procedure described by Murashigae and Skoog [11]. The hormones and chemical were purchased from HiMedia Laboratories Ltd, Mumbai, India and Elix-3 water was used for the entire study. For root induction, MS basal media supplemented with varying concentrations and combinations of indole butyric acid (IBA) and indole acetic acid (IAA) and 3% sucrose were used. The leaves were trimmed into pieces of about $1cm^2$ and inoculated on to the respective media. *In vitro* induced roots were maintained in suspensions consisting of liquid basal MS media supplemented with respective hormone combinations. It was then transferred to air-lift bioreactor provided with liquid basal MS media for mass cultivation of roots. For shoot multiplication varying concentrations of hormones 6-Benzylaminopurine (BAP), Kinetin and Thidiazuron (TDZ) *w*ere used. The internodes from MS media grown plants were excised, trimmed at both the ends and were applied as explants. The inoculated explants were maintained in controlled temperature of 25ºC and observed regularly for their morphological growth. A photoperiod of 16 hrs was maintained using fluorescence lights (Philips India Ltd., Mumbai) followed by 8 hrs of dark period and a relative humidity of 60-70%.

Establishment of multiple shoots: All cultures were observed periodically and the shoots produced in various combinations were recorded on the basis of visual observation. The combination with increased shoot multiples was identified as the best. Subculturing was carried out at an interval of 3 wks and the experiment was repeated three times. The effect of different treatments was measured on the basis of number of shoots produced and the height achieved by the shoots.

Establishment of roots and mass cultivation: After a period of 30 days, the induced roots in various hormone combinations were analysed and the combination with more number of induced roots was identified as the best. The root of that particular combination was then transferred to suspension cultures with liquid MS basal media supplemented with and without the respective hormones. Thirty root tips or branch (1.5 gm) measuring in length 2-3 mm were cut under sterile conditions and transferred to conical flasks containing 30 mL liquid MS with the respective hormone combination. This study was taken to find out the growth pattern of roots in suspension culture under the influence of hormones. Fresh media were replaced regularly at 15 days intervals in culture bottles. After 30 days root growth and stabilization in suspension culture, a part of the well grown roots were transferred to air-lift bioreactor for their mass propagation. The bioreactor was provided with proper aeration and temperature was maintained at 22°C. The roots were harvested and their wet and dry weight was noted down. Increase in the root mass was calculated.

Preparation of extracts from roots: The *in vitro* grown plant parts and field grown roots of *W. coagulans* were ground thoroughly and one gram of powdered samples was extracted using 200ml methanol by repeated sonication and shaking at 104rpm at 22ºC. The extracts were then filtered using Whatmann no: 1 filter paper and was concentrated using flash evaporator maintained at 45ºC and 150 rpm. After complete solvent evaporation, the residue was dissolved using HPLC grade methanol for their estimations and quantifications [12].

Phytochemical estimations and withanolides quantification: The plant extracts were subjected to quantitative phytochemical screening of total flavonoids, steroids, alkaloids, phenols and saponins [13]. The withanolides, withaferin A and withanone was quantified using High Performance Thin Layer Chromatography (HPTLC). The sample extracts were applied on 20x10cm pre-coated silica gel aluminium $60F^{254}$ plates (E.MERCK, Germany) as 8mm band, by means of Semiautomatic CAMAG Linomat 5 device (Camag, Muttenz, Switzerland) fitted with 100 µL Hamilton syringe. Linear ascending development to a distance of 8 cm, was carried out in 20x10cm twin trough chamber saturated with 20 mL Toluene : Ethyl acetate: formic acid (5:5:1) for 30 mins at room temperature. Densitometric scanning was performed and withanolides were quantified using Camag winCATS software (1.4.4.6337).

Statistical analysis: In all the experiments, the experiment was repeated twice with 4 replicates and three explants in each. The mean ± SE of the results were determined. Data were statistically analysed by ANOVA and significant differences between means were assessed by Duncan's multiple range test using SAS.

RESULTS

Shoot multiplication of Withania coagulans

The nodal explants developed different responses in different media combinations. Shoots remained fresh and green but failed to induce multiple shoots in growth regulator free basal MS media. All nodal explants cultured on growth regulator supplemented MS media grew length wise and began to proliferate multiple shoots in the 2nd week. Combinations of media consisting of BAP, Kin and TDZ were formed. Fig. 1 shows the formation of multiple shoots from nodal explants of *in vitro Withania coagulans* with different concentrations of hormone regulators. BAP and Kin alone discretely did not induce remarkable shoot formations and the explants cultured on combination media of BAP+Kin induced more axillary shoot proliferation when compared to the TDZ. The mean number and length of shoot is represented in Table 1. Maximal shoot generation was achieved in MS media containing 1BAP+0.5 Kin followed by 1BAP+0.1Kin and 0.5BAP+0.5Kin. It was also evident that among these three combinations, 1BAP+0.5 Kin resulted in the highest number of shoots and mean shoot length. Abnormal callusing was observed in the explants incorporated even in the lowest concentration of TZD. Complete shoot inhibition and excessive callusing was observed in the media supplemented with 0.8TDZ.

In vitro rhizogenesis in *Withania coagulans*

The results of the present investigation showed that increasing concentration of IBA resulted in positive effect on root induction in *W. coagulans*. The results are presented in Fig.2 where different combinations of IAA and IBA gave different responses. The results exhibited that the presence of auxin plays an important role in inducing roots as there was no sign of roots in MS media without auxin supplementation. 20 days of culture in media supplemented with auxins produced maximum induced roots and upon increasing the culture period to 30 days, no significant result was observed. Increasing concentrations of IAA has also shown to have a positive effect on root induction but a concentration higher than optimal displayed a negative impact.

The present finding suggest a maximum root induction in MS media supplemented with 1.0 mg/L IAA and 4.0 mg/L IBA with 100% root induction response as shown in Table 2. Followed by MS media supplemented with IBA alone *i.e.* 4.0 mg/L showed 94.4% root induction. A combination of 6.0mg/L IBA with 0.5, 1.0, 2.0, 4.0 mg/L of IAA were also carried out in the present study and was observed that a higher percentage of roots were induced on increasing medium concentration of IBA to 6.0mg/L compared to that supplemented with 4.0 mg/L IBA (data not shown). But the roots mainly aroused from the high amount of callus induced in response to the increased concentration of IBA which was found to be insignificant and thus excluded.

Mass propagation of roots in suspension and bioreactor

The mass proliferation of the induced roots were further carried out using suspension cultures and bioreactors (Fig. 3). For the establishment of root cultures in suspension, the roots obtained from MS media supplemented with 4.0 mg/L IBA and 1.0 mg/L IAA with 3% sucrose were considered, which was selected as the best auxin combination for root induction. After the culture period, the fresh weight of the root was noted and finally growth index was calculated (Wu et al. 2008) (Table 3). Media change was given every 15 days in order to supply adequate nutrients for the culture growth. At the end of 45th day,

the growth index was found to be 69.0, indicating an increase in root mass. The roots appeared healthy, thin and white in colour.

The stabilised roots were then mass cultivated in bioreactors consisting liquid MS media without auxin supplements. The results showed an increase in root mass by 41 fold after a period of 30 days.

Quantitative analysis of essential phytoconstituents

The quantitative estimation of important phytoconstituents such as flavonoids, saponins, steroids, alkaloids and phenols in the methanolic extracts of *W. coagulans* are presented in Table. 4. The phytoconstituents present in the sample is expressed as mg/g. All the phytoconstituents were quantified highest in *in vitro* extracts of *W. coagulans* than field grown root extract.

Withanolide quantification using HPTLC

The extracts of field grown roots and *in vitro* propagated tissues of *W. coagulans* were subjected to HPTLC profiling for quantification of major withanolides, withaferin A and withanone (Fig. 4a). Multi wavelength scanning was carried out for withaferin A and withanone to determine their absorption spectra (λmax) as 223nm and 231nm respectively. The detection was then performed using the respective λmax and a distinct fingerprint profile was developed for withanolide standards and crude samples. The amounts of these withanolides in the extracts were quantified from the spotted standards and the linear regression generated.

The quantitative evaluation of the plates was determined in three independent experiments. The developed plates were then derivatized with 10% sulphuric acid reagent. Characteristic fluorescence for withanolides were observed under UV light at 366nm which could be applied to identify them among other separated compounds of the crude extracts. Linear correlation between the applied concentration and the peak area obtained was found. The Rf values were identified and the quantification of withanolides were determined with respect to the peak area obtained and is represented in the Fig. 4b respectively. The concentration withaferin A was found to accumulate in similar manner in *W. coagulans* roots with a higher content in the aerial parts when grown in *in vitro* conditions whereas the withanone quantity was found to increase profoundly in all the *in vitro* tissues of *W. coagulans* when compared to the commercially utilised field grown roots.

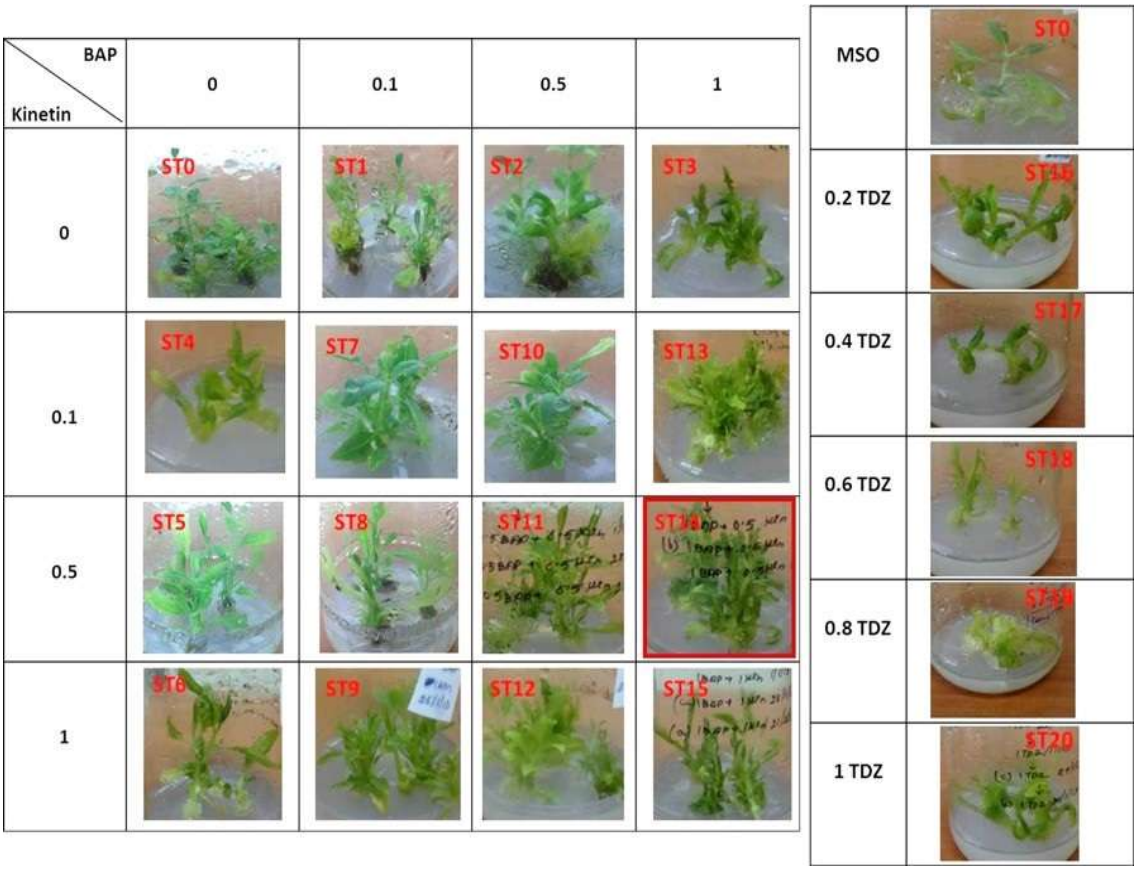

Fig. 1: Influence of growth regulators on *in vitro* shoot multiplication of *W. coagulans*. ST14 (1.0 BAP+0.5Kin) was determined to be the best media combination for producing multiple shoots with appreciable shoot length and leaf counts.

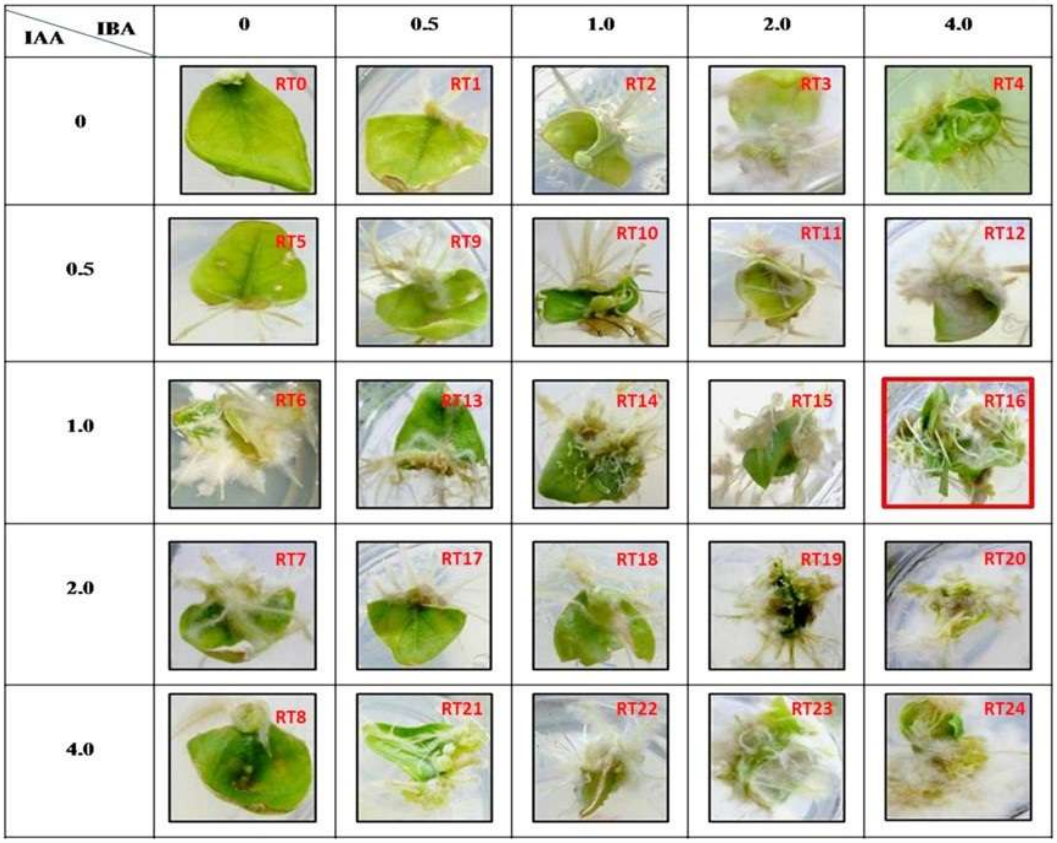

Fig. 2: Influence of Auxins on *in vitro* root induction of *Withania coagulans*. Among the 25 different combinations of root induction media, 1.0 IAA+4.0 IBA induced maximum number of root hairs.

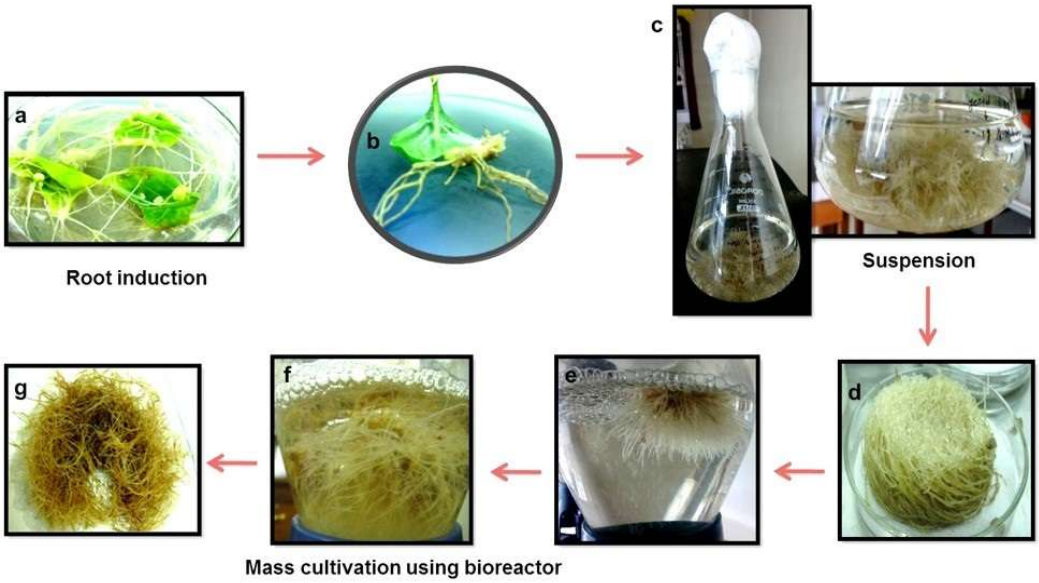

Fig. 3: Mass cultivation of adventitious roots of *W. coagulans*. (a, b) 0.5 gm of induced adventitious roots were separated and transferred to suspension media (c, d) roots were grown in suspension culture of half strength MS media without agar (e, f) 1 gm of suspension grown roots were then transferred to bioreactor with 1 L of half strength MS media. Complete proliferation was achieved in 30 days (g) the roots from the bioreactor were harvested

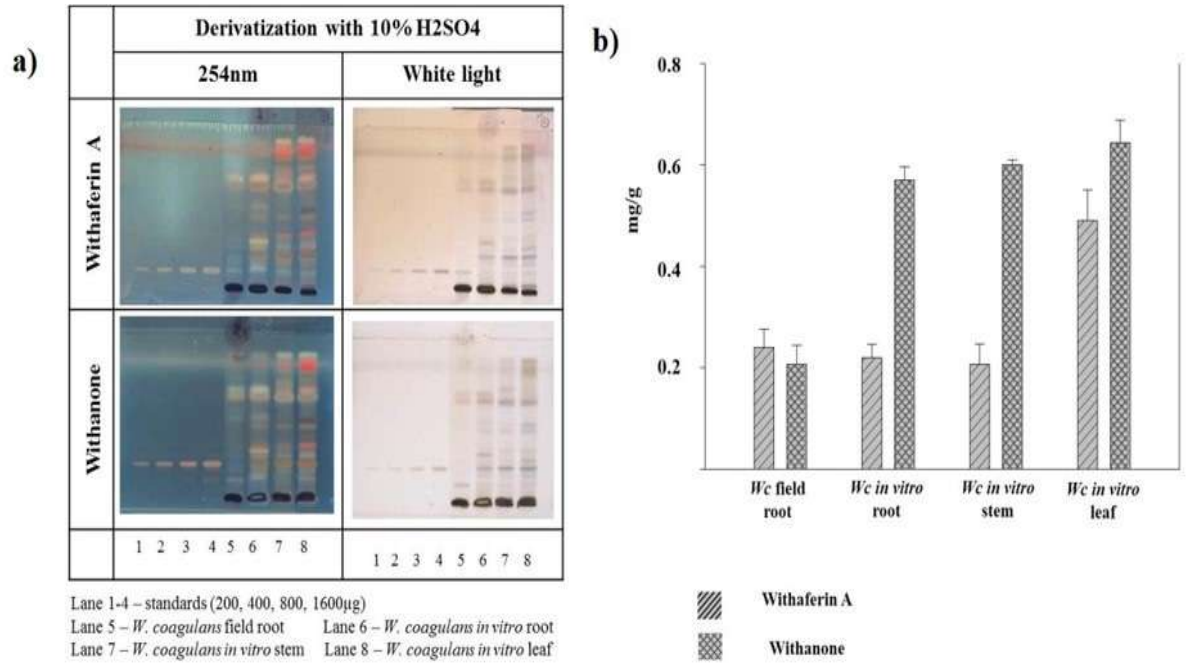

Fig. 4: HPTLC quantification of withanolides. (a) Chromatograms of field root and *in vitro* tissue extracts of *W. coagulans* plotted against the respective withanolides. (b) The Withaferin A and withanone in all the extracts of *W. coagulans* was quantified and withaferin A was analysed to accumulate in a similar pattern in all *W. coagulans* tissues. The withanone quantity was found to profoundly accumulate in all the *in vitro* tissues of *W. coagulans* when compared to the field grown roots.

Table 1: Effect of growth regulators on shoot multiplication of *Withania coagulans* under *in vitro* conditions

S.No	BAP mg/L	Kinetin mg/L	TDZ mg/L	Mean number of shoots	Mean shoot length
ST0	0	0	-	1.18±0.1^{e}	4.05±0.4^{a}
ST1	0.1	-	-	1.26±0.07^{e}	4.0±0.08^{a}
ST2	0.5	-	-	2.18±0.1^{c}	3.05±0.1^{b}
ST3	1.0	-	-	2.37±0.3^{b}	2.81±0.2^{b}
ST4	-	0.1	-	1.39±0.1^{d}	4.0±0.2^{a}
ST5	-	0.5	-	1.5±0.2^{d}	3.01±0.1^{b}
ST6	-	1.0	-	1.96±0.3^{c}	2.64±0.1^{c}
ST7	0.1	0.1	-	2.2±0.3^{c}	3.53±0.9^{a}
ST8	0.1	0.5	-	2.68±0.5^{b}	1.99±0.1^{d}
ST9	0.1	1.0	-	2.41±0.6^{b}	2.78±0.3^{c}
ST10	0.5	0.1	-	2.14±0.5^{c}	1.91±0.1^{d}
ST11	0.5	0.5	-	2.74±0.06^{b}	1.95±0.1^{d}
ST12	0.5	1.0	-	3.33±0.5^{a}	1.70±0.3^{d}
ST13	1.0	0.1	-	3.16±0.8^{a}	2.0±0.3^{d}
ST14	**1.0**	**0.5**	**-**	**3.37±0.6^{a}**	**2.12±0.2^{d}**
ST15	1.0	1.0	-	2.56±0.1^{b}	2.43±0.4^{c}
ST16	-	-	0.2	1.35±0.1^{d}	3.24±0.9^{b}
ST17	-	-	0.4	1.15±0.4^{e}	2.94±0.3^{b}
ST18	-	-	0.6	1.97±0.6^{c}	2.79±0.4^{c}
ST19	-	-	0.8	1.26±0.35^{e}	0.9±0.8^{e}
ST20	-	-	1.0	1.89±0.1^{c}	2.22±0.2^{c}

*Means followed by the same letters within a column are not significantly different (P≤0.05).

Table 2: Response of explants to variation in auxins concentration

S.No	IBAmg/L	IAAmg/L	Mean roots ± SE (30 days)	Percentage of root response (30 days)	Percentage of root induction (30 days)
RT0	0	0	0.333±0.21^{h}	33.33	0.58
RT1	0.5	0	10.167±0.79^{e}	100	17.68
RT2	1.0	0	15.667±0.67^{d}	100	28.99
RT3	2.0	0	18.667±0.49^{d}	100	32.46
RT4	4.0	0	56.667±1.05^{a}	100	98.55
RT5	0	0.5	7.167±0.54^{f}	100	12.46
RT6	0	1.0	3.333±0.21^{g}	100	5.79
RT7	0	2.0	2.167±1.38^{g}	33.33	3.77
RT8	0	4.0	3.833±0.98^{g}	83.33	6.67
RT9	0.5	0.5	18.0±0.58^{d}	100	31.3
RT10	1.0	0.5	24.333±0.33^{c}	100	42.32
RT11	2.0	0.5	28.333±0.67^{c}	100	49.28
RT12	4.0	0.5	28.833±0.54^{c}	100	50.14
RT13	0.5	1.0	9.333±0.76^{e}	100	16.23
RT14	1.0	1.0	16.0±0.68^{d}	100	27.83
RT15	2.0	1.0	22.333±4.51^{d}	83.33	38.84
RT16	**4.0**	**1.0**	**57.5**±1.12^{a}	**100**	**100**
RT17	0.5	2.0	2.333±0.56^{g}	83.33	4.06
RT18	1.0	2.0	10.333±0.76^{e}	100	17.97
RT19	2.0	2.0	16.333±0.50^{d}	100	28.40
RT20	4.0	2.0	40.5±0.34^{b}	100	70.43
RT21	0.5	4.0	9.0±2.9^{f}	66.67	15.65
RT22	1.0	4.0	11.167±0.6^{e}	100	19.42
RT23	2.0	4.0	8.167±2.6^{f}	66.67	14.20
RT24	4.0	4.0	12.833±0.75^{e}	100	22.32

***** Means followed by the same letters within a column are not significantly different (P≤0.05)

Table 3: Growth of *W. coagulans* adventitious roots in suspension culture and bioreactor

S. No.	Culture	Incubation period (days)	Fresh wt of the inoculum (gm)	Fresh wt of the harvested biomass (gm)	Growth index	Fold increase
1	Suspension	45	0.5	35	69	70
2	Bioreactor	30	1.0	41	40	41

Table 4: Quantitative phytochemical analysis of field root and in vitro tissue extracts of *W. coagulans*

Sample	Total flavonoids	Total steroids	Total phenols	Alkaloids	Saponins
Wc field root	0.78±0	4.13±0.36	1.10±0	34.55±0	0.78±0
***In vitro* propagated tissue extracts**					
Wc root	9.32±0	7.71±0.35	0.93±0	55.03±2.93	**26.28±0.08**
Wc stem	6.37±0	**28.08±0**	**2.28±0.04**	40.40±0	18.47±0
Wc leaf	**16.55±0**	26.29±0.35	2.19±0.04	**116.47±0**	17.46±0.07

DISCUSSION

In vitro propagation is crucially recommended when the metabolite of interest is produced only in specialized plant tissues or glands in the parent plant. The objective of the present study was to induce high frequency withanolide rich adventitious roots under *in vitro* conditions by normalising the supplementation of growth regulator in the culture media. Among phytohormones, auxin plays an essential role in regulating roots development and it has been shown to be intimately involved in the process of adventitious rooting. Among auxins IAA was first used for stimulating roots of cuttings [14] and soon after another auxin which also promoted rooting; IBA was discovered and was considered even more effective [15]. Wadegaonkar et al. (2006) reported that combinations of IAA and IBA were effective in inducing adventitious roots from leaf explants of *W. somnifera* [16]. Thus, different combinations of IAA

and IBA were checked for *Withania coagulans* in the present study. Differences in the ability to form adventitious roots have been attributed to differences in auxin metabolism [17].

The result showed 94.4% root induction in MS media supplemented with 4mg/L of IBA. This showsbetter performance of IBA over IAA. The reason for these differences in root inducing ability may be higher stability of IBA and slow but continuous release of IAA from IBA [18, 19] and release of IBA through hydrolysis of conjugates results in better metabolism and transport [17]. But the results suggested that IBA alone could not bring 100% root induction, since a better result was obtained in the combination of both IAA and IBA. This may be due to the fact that IBA is a very simple 'conjugate' of IAA and must be converted to IAA by β-oxidation to have an auxin effect as suggested by Muller et al. (2005) and that either IBA itself is active or that it modulates the activity of IAA [20, 21].

It was observed that there was differences in pattern of root numbers induced per explant *i.e*. percentage of root induction in each combination. Though many of the explants gave fast response in presence of varying proportions of auxin supplemented, the number of roots induced differed. 100% of root induction was observed in the combination 1.0 IAA and 4.0 IBA, followed by 94.4% in 4.0 IBA and 70.0% in 2.0 IAA and 4.0 IBA.

From this study it is suggested that adventitious root cultures of *W. coagulans* are promising for large-scale biomass production in suspension cultures and that it can be successfully taken to bioreactors for mass proliferation. Similarly, adventitious root suspension cultures are proved to be efficient for biomass accumulation in *P. notoginseng* [22] and *Echinacea purpurea* [23]. Since the roots contain a number of therapeutically applicable withanolides, *in vitro* mass cultivation of roots will be an effective technique for the large scale production of these secondary metabolites. The development of a fast growing root culturing system would offer unique opportunities for producing root drugs under laboratory conditions without having to depend on field cultivation. Therefore, this study proposes that mass cultivation of roots of *in vitro W. coagulans* could be successfully preceded and would offer unique opportunities for producing drugs from roots. Also the withanolide contents of the hairy root cultures of *W. coagulans* were higher than in the root of the plant. In the hairy root cultures all the withanolides were accumulated in the root tissues and withaferin A or withanolide A were not detected in the culture medium samples [4].

The effect of cytokine on shoot multiplication of *Withania coagulans* was observed and analysed taking the shoot number and their attained length into consideration. The media free of cytokinen produced only single shoot from the nodal explants along with rhizogenesis. TDZ resulted in excessive callusing and retarded the growth and proliferation of shoots from the explants. Similar callusing problems in the nodal explants with TDZ media have been previously reported in many studies [24, 25, 26, 27]. In the present study, among different combinations of media investigated, 1BAP+0.5 Kin was more effective and shoot multiplication was attained at its best levels. The frequency of shoot proliferation dropped with increasing concentration of Kinetin (1BAP+1Kin). Also excessive Kin concentration in the media resulted early maturation and yellowing of leaves in the proliferated shoots. Taking the shoot length into consideration, it was observed that maximum length was attained in the media supplemented with no growth regulator and it gradually decreased with increase in the hormone supplementation, indicating the shoot proliferation and the shoot length are conflict to each other.

Suitable growth regulator type and concentration influences the growth of plants and plant tissues and varies upon different plant species. This study supports the rapid root induction and shoots multiplication of this important medicinal plant under *in vitro* conditions which can be further used in the transformation studies.

Estimation of biochemical attributes is informative for finding the therapeutic potential of a plant/plant part. Field roots are the sole part of the *W. coagulans* consumed orally to cure various diseases and therefore phytochemical estimations of the i*n vitro* cultured *W. coagulans* were quantified in comparison to the field grown root extracts. Steroids, alkaloids and saponins being the important constituent of withnolides, the major bioactive principle of genera *Withania* is reported to accumulate high in *in vitro* cultures of *W. coagulans* than the field roots. Hence high content of these compounds in the *in vitro* cultures exhibits great deal of pharmacological importance.

A rapid, precise and accurate identification and estimations of active marker componds as the qualitative and quantitative target to measure the authenticity quality is essential [28]. HPTLC is one such efficient, fast and reliable alternative to determinate and quantify natural single compounds. Using HPTLC, exact quantification of the compounds can be achieved by scanning the bands with absorbance after separation is achieved [29, 30].

In the present study, a densitometric HPTLC analysis was performed for the development of characteristic finger print profile to quantify the therapeutic withanolides, withaferin A and withanone in the methanolic extracts of *W. coagulans*. The extracts of *W. coagulans* showed the equal presence of withaferin A in all the samples whereas the *in vitro* tissues of *W. coagulans* accumulated withanone in extravagant amounts.

Withaferin A and withanone belong to a class of compounds from Withania known as withanolides have been identified as prominent phytocompounds with active pharmacological properties [31, 32, 33]. This is the first report of increased withanone accumulations in *in vitro* cultures of *W. coagulans*. The production of these secondary metabolites from plant under cultivation or grown in nature is constantly not satisfactory and is activated only during a particular growth or developmental stage, stress or nutrient availability or in specific season. Therefore, plant cell and tissue cultures have been a possible production method for secondary metabolites [34, 35]. Production of useful compounds independent of soil profile and climatic changes under controlled conditions, free of insects and microbes, improve productivity due to automated cell growth control, rational regulation of metabolite productive process and extractable organic substances from cell cultures [36]. *W. coagulans* root cultures should be explored as a potential efficient method for producing useful withanolides owing to their enormous commercial value, the plants scarcity in the natural environments and costly synthetic process of the secondary metabolites. In this regard, adventitious root cultures have been used as a more stable and fast growing technique for withanolides production. Based on the analytical assays, *in vitro* cultures of *W. coagulans* was found to be a valuable reservoir of bioactive compounds with high medicinal values because of its higher phytoconstituents, especially the steroids, alkaloids and saponins quantitatively compared to field grown roots.

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