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# Enhanced Production of Secondary Metabolites Through Adventitious and Hairy Root Cultures of *Withania sominifera* cv. Poshita

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**Abstract:** A well-known herbal remedy for a variety of conditions is withania sominifera. Using a combination of indole butyric acid (IBA) at 1.0 mg/l and indole acetic acid (IAA) at 0.5 mg/L, adventitious roots were successfully initiated from leaf explants of in vitro propagated plantlets in MS medium in the current study. The best conditions for inducing hairy roots were found to be a co-culture period of 24 hours with Agrobacterium rhizogenes. Within 7 days of treatment, hairy roots started to appear. The addition of 50 M acetosyringone and a 1-hour infection time was found to increase the transformation frequency from 72.11 0.5% to 81.22 0.24%. Following a 30-day period of culture, the induced roots were moved to a shake flask system, and the growth index was discovered to be 33.35 for adventitious roots and 37.24 for hairy roots. The induced roots were moved to a shake flask culture system for a 30-day period of culture, and the growth indices for adventitious roots and hairy roots were found to be 33.35 and 37.24, respectively. The roots were extracted after being collected, and they were then screened for phytochemicals using GC-MS, TLC, and HPLC. Alkaloids, flavanoids, tannins, saponins, phenols, glycosides, terpenoids, reducing sugar, and anthraquinones were found in these analyses. The presence of Withaferin A and Withanolide A was confirmed by HPLC of the methanolic extracts of the roots. Withaferin A was present in transformed roots at a concentration of 69.21 g/g dry weight, which was two times greater than that of adventitious root cultures at 32.45 g/g dry weight. This work emphasises the importance of *Withania sominifera* poshita variety adventitious and hairy root culture, which may be utilised for systematic and reliable metabolite production that can be used for scientific and commercial applications.

**Keywords:** *Withania sominifera* Poshita Variety, Adventitious Roots, Hairy Roots

## 1. Introduction

Bioactive compounds from the medicinal plant, *Withania somnifera* (common name: ashwagandha), a member of the family *Solanaceae* has gathered a lot of interest due to its impressive pharmacological properties. This 'wonder herb' is known for its antioxidant [1, 2], neuroprotective [3], anti-mycobacterial [4, 5] and immunosuppressant properties. Reports suggest that ashwagandha root supplementation has led to significant increase in muscle mass and strength [6]. In addition, it has been used to treat ulcers, fever, cough, dyspnoea, impotence, rheumatism, toxicosis, leucoderma, sexual weakness, scrofula, anxiety, neurosis, generalized weakness, and spermatorhhoea [7-9]. It has also been used in unani medicine [10]. Furthermore the bioactive moieties of *W. somnifera* have been reported to have remarkable anti-tumor and anti-cancer effects [11]. Morphological and phytochemical variability have been observed between the wild and cultivated varieties of *Withania sominifera* [12]. the Central Institute of Medicinal and Aromatic plants, Lucknow, India had developed a cultivar named 'Poshita' that produced high root yield and better chemical quality, the poshita variety is known to have higher yield of withaferin A from the leaves [13]. It has been highlighted as a high-priority medicinal plant by the National Medicinal Plant Board of India, and is in great demand in the international market. A group of secondary metabolites called withanolides principle contributors of medicinal properties of these plants. the

withanolides are C-28 steroidal lactones [14, 15]. These bioactive moleules are primarily found in the roots of the plant. The immense therapeutic potentials of its roots has led to overexploitation especially for ayurvedic applications.

Adventitious roots of W. somnifera grown in vitro have been reported to produce withanolides [16, 17]. The *in vitro* method propagation offers high rates of root proliferation and active secondary metabolism in *Withania* varieties. A previous study indicated that the *in vitro* induced adventitious roots could be used effectively for the production of secondary metabolites [18]. Agrobacterium spp. are parasites known to infect plants and cause tumorous outgrowths. *Agrobacterium rhizogenes* infections lead to production of massive amounts of hairy roots [19]. Studies suggest that *RolA* genes present in the *Ri*-plasmid of *A. rhizogenes* could interact with nucleic acids and thereby regulate gene expression in plants [20, 21].

The yield of W. somnifera cv poshita roots are limited as they are seasonal plants with low germination and seed viability. In the current study, metabolite profiling of the extracts from adventitious and hairy roots of W. somnifera was performed to evaluate the yield potential of the economically viable therapeutic moieties synthesized by in vitro methods. Increase yield of the therapeutic moieties could pave way for increased exploitation of the hairy root culture method for extracting secondary metabolites. This can help overcome the restriction in the supply of W. somnifera roots.

# 2. Materials and Methods

### *2.1. Adventitious Root Induction*

*Withania sominifera* Poshita variety seeds were purchased from the Central Institute of Aromatic and Medicinal Plants (CIMAP), Hyderabad. The seeds were soaked for 72 h in distilled water, surface sterilized with tween 20 (HiMedia, Mumbai, India), followed by treatment with 70% ethanol (HiMedia, Mumbai, India) and 0.1% $HgCl_2$ (HiMedia, Mumbai, India). The seeds were repeatedly washed in sterile distilled water, and then inoculated in 50 mL of half-strength MS medium (HiMedia, Mumbai, India) for germination [22]. Multiple shoots were induced for the propagation of the plant. The basal MS media was supplemented with 2 mg/L Kinetin (HiMedia, Mumbai, India) and 1 mg/L BAP (HiMedia, Mumbai, India). Shoot buds were elongated on MS media without any hormone and transferred to rooting medium for further propagation. The three-week-old leaves from *in vitro* grown plantlets of *Withania sominifera* cv. Poshita were taken as explants for the investigation. The leaves were cut into two halves, wound were induced in the veins and inoculated on MS solid medium (7.5% agar (HiMedia, Mumbai, India)) supplemented with varying concentrations of IBA (HiMedia, Mumbai, India), IAA (HiMedia, Mumbai, India) and NAA (HiMedia, Mumbai, India) (0.25 to 4 mg/L). The cultures were kept at 25±2°C, at a relative humidity of 80±10%. The combinations of IBA, IAA and NAA which showed the best root induction was selected [23].

### *2.2. Hairy Root Transformation*

*Agrobacterium rhizogenes* strain A4 was obtained from IIT, Guwahati, India. The culture was maintained in Yeast Extract Peptone (YEP) agar medium (HiMedia, Mumbai, India). From the mother culture, 0.1 mL of *A. rhizogenes* was inoculated in 50 mL of YEP broth (HiMedia, Mumbai, India) and incubated at 28°C in an orbital shaker at 80 rpm. After 2 days, the bacterial cells were collected by centrifugation at 3000 rpm, when the absorbance of the culture reached 0.5 at 600 nm. The supernatant was discarded and the pellet was dissolved in half-strength MS medium. The bacterial suspension thus obtained was used for infection. *In vitro* grown disinfected leaves were taken as explants, cut into small pieces 5 mm x 5 mm, pricked, and dipped in 24 h old culture of *A. rhizogenes*. The cultures were then placed in an incubator shaker at 25°C under shaking condition for different time periods (0–1 h). These were then co-cultivated on plant growth regulator free solid MS medium containing acetosyringone (50–150 µM) and placed in culture room at 25°C ± 2 in dark for 48 h. The control experiment was carried out using acetosyringone (HiMedia, Mumbai, India) and without co-cultivation. The leaves were then inoculated into MS media containing 500 mg/L cefotaxime (HiMedia, Mumbai, India). Hairy roots were sub cultured every three weeks on MS medium supplemented with cefotaxime and in subsequent passages the antibiotic was eliminated [24].

Transgenic roots were confirmed using polymerase chain reaction (PCR) (Eppendorf Mastercycler PCR machine). Transformation was confirmed by the presence of *rol* gene located on the T-DNA by using a set of *rol* A specific primers (5'GAATTAGCCGGACTAAACGT and 5'-TTGTTTGGATGCCCTAATT, synthesized by Eurofins Scientific, Bangalore, India [25].

### *2.3. Scale up of Root Cultures for Biomass Production*

After 3 weeks of root initiation, the roots from the media which showed best root induction were selected for further studies. These roots were then separated from the explants aseptically and were sub cultured into 100 mL aliquots of MS medium. The cultures were maintained on an orbital shaker under continuous agitation at 120 rpm at 25±2°C. The growth of adventitious roots and hairy roots were recorded separately at weekly intervals. After 30 days of root cultures in suspension, the fresh weight of the root was noted and the growth index was calculated [26].

$$Growth\ Index = \frac{(HFW - IFW)}{IFW} \quad (1)$$

Here, HFW is the harvested fresh and IFW represents the inoculum fresh weight.

The media was changed every 15 days in order to maintain adequate nutrients for growth. Large scale cultures were established in 5 L balloon type bubble reactors. The roots

were transferred to 2.5 L full-strength MS medium supplemented with the selected hormone combination for adventitious root cultures and in plant growth regulator (PGR) free medium for transformed roots. The cultures were maintained for 28 days at 25±2°C. The roots were harvested and growth index was determined as per equation (1).

### *2.4. Phytochemical Screening and Thin Layer Chromatography*

Harvested roots were shadow dried, ground and extracted with different solvents namely, petroleum ether, chloroform, ethyl acetate and methanol (HiMedia, Mumbai, India). All the extracts were subjected to phytochemical screening by standard test procedures. The extracted samples were also analyzed by thin layer chromatography using 7 cm X 3 cm sized silica gel 60 F254 plates, with the solvent system Toluene: Ethyl acetate: Formic acid in 1:1:0.5 ratio as the mobile phase. The mobile phase ratio was developed by the lead author. 5 µL of the extract was loaded on precoated silica gel 60 F254 plates. The bands were analyzed by heating at 100°C for 15 min after spraying with 10% sulphuric acid (HiMedia, Mumbai, India).

### *2.5. GC-MS Analysis*

Phytochemical composition of methanol extract of *Withania sominifera* poshita was analyzed using GC MS and HPLC. The methanol extract was dried and the residue was dissolved in 1 mL methanol and 20 µL was used for HPLC analysis. The standards of withaferin-A and withanolide A purchased from Chromadex US were used as reference for HPLC analysis. HPLC analysis was performed at 225 nm in C18 column.

Gas chromatography-mass spectrometry (GC–MS) analysis was carried using Shimadzu GC-MS System model number QP 2010S. The methanolic extract was dried and the residue was dissolved in 1 mL methanol and from this, 1 µL of the extract was injected into the GC system provided with 30 m × 250 µm × 0.25 µm Rxi-5Sil MS column. Helium was used as the carrier gas with a column flow rate of 1.00 mL/min. The GC oven was held at 80°C and then ramped from 80 to 260°C at 5°C/min. The chromatogram and mass spectra were recorded and analysed [27].

## 3. Results and Discussion

The seeds showed 100% germination after 3 days of inoculation. Multiple shoots were established in MS media fortified with 2 mg/L Kinetin and 1 mg/L BAP. *In vitro* raised shoots were rooted in half strength MS with 1.0 mg/L IBA + 0.5 mg/L IAA added as a supplement.

The leaves derived from 3-week old plantlets were found to be excellent explants source for adventitious root induction. Root induction was observed from the cut ends and veins within 2 weeks. Highest frequency of adventitious root induction was observed in MS media supplemented with hormone combination of 1 mg/L IBA + 0.5 mg/L IAA (Figure 2). IBA and IAA are known to promote adventitious root induction in *Withania sominifera* cv. Poshita. A study reported that the IBA may directly influence the expression of genes that play an important role in the adventitious root induction, this was in agreement with our findings [28]. Transformation frequency was determined to be 72% for *Withania sominifera* Poshita variety.

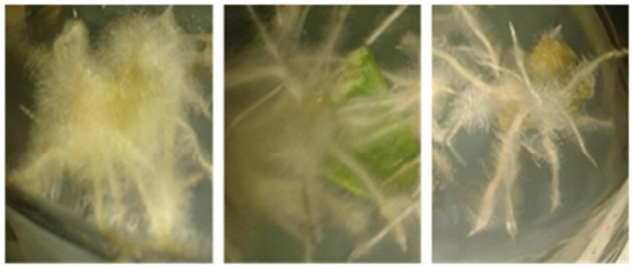


***Figure 1.*** *Effect of Phytohormones on adventitious root induction.*

Upon treatment with *A. rhizogenes*, the production of hairy roots was significantly enhanced (Figure 3a & 3b). The supplementation of the co-cultivation medium with acetosyringone significantly decreased the time required for root initiation by 15 days (Figure 3c). The presence of *rol* A gene in the hairy root lines was detected by PCR analysis. All transformants showed the presence of 436 bp *rol* A product amplification (Figure 3f).

Shake flask culture system notably increased the root biomass. A 33-fold increase in root biomass was recorded after 30 days of culture period (Figure 3d). The growth index was measured to be 33.35 which further increased to 52.42 by scaling up the culture to 5 L culture medium (Figure 3e).

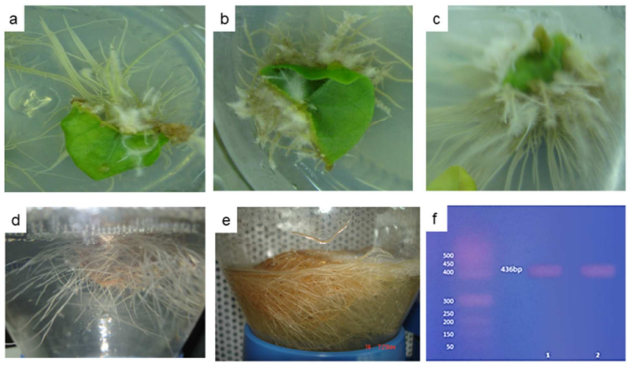


***Figure 2.*** *Treatment with A. rhizogenes a) root development from leaf segment after 30 minutes of coculture. b) root development after 1 hour of co-culture, c) root induction after the addition of acetosyringone, d) Root culture initiation in bioreactor, e) growth of induced roots at the harvesting period, and f) confirmation of the presence of rol A gene by PCR amplification and electrophoresis: Lanes 1 and 2 represent the amplified genomic DNA from the hairy root cultures of W. somnifera cv. poshita.*

Phytochemical analysis of root extracts of *Withania sominifera* poshita variety revealed the presence of alkaloids, flavanoids, tannins, saponins, phenols, glycosides, terpenoids, reducing sugar and anthraquinones. Chief phytoconstituents were identified in order to relate their presence with

bioactivities of the root metabolites. The alkaloids, tannins and saponins isolated from plants are known for their various biological activities including pharmacological benefits, [29-31] which justify the significance of our findings in the current scenario.

TLC profiling of all the root extracts showed divergent polarities of the phytochemical. Rf values ranging from 0.95 – 0.20 were observed. The presence of Withaferin A and Withanolide A were confirmed in all the tested extracts. TLC screening revealed that the presence of wide range of phytoconstituents present in the root extracts. The retention factors of all the tested extracts are detailed below (Table 1, Figure 4). Hence, the *in vitro* derived root extracts could be highly explored for therapeutic efficacy against various ailments data from various cell culture experiments and rodent models have revealed that the withaferin-A can be used to combat carcinogenesis [32].

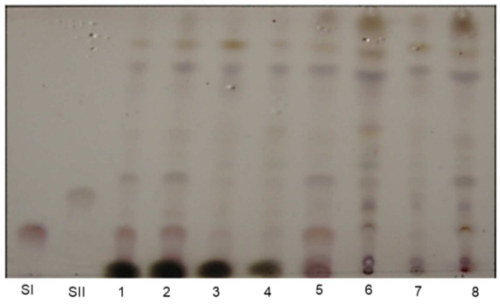


***Figure 3.*** *TLC chromatogram of the root extract of in vitro propogated W. somnifera CV. Poshita. Lane SI – Withaferin A; lane SII- Withanolide A; lanes 1 -4: adventitious root extract in methanol, ethyl acetate, chloroform, and petroleum ether respectively; lanes 5-8: hairy root extract in methanol, ethyl acetate, chloroform, and petroleum ether respectively.*

***Table 1.*** *Retention factors for in vitro propagated root extracts of Withania sominifera Poshita variety.*

| $R_f$ Values | Adventitious root extracts | | | | Hairy root extracts | | | |
|---|---|---|---|---|---|---|---|---|
| | Methanol | Ethyl acetate | Chloroform | Petroleum ether | Methanol | Ethyl acetate | Chloroform | Petroleum ether |
| 0.95 | + | + | + | + | + | + | + | + |
| 0.84 | - | + | - | + | - | + | - | + |
| 0.80 | + | _ | + | - | + | _ | + | - |
| 0.76 | + | + | + | + | + | + | + | + |
| 0.70 | + | + | - | - | + | + | - | - |
| 0.64 | + | + | + | + | + | + | + | + |
| 0.60 | + | - | - | - | + | - | - | - |
| 0.56 | + | + | + | - | + | + | + | - |
| 0.52 Withanolide A | + | + | + | + | + | + | + | + |
| 0.42 | + | - | - | - | + | - | - | - |
| 0.36 | + | + | + | + | + | + | + | + |
| 0.32 Withaferin A | + | + | + | + | + | + | + | + |
| 0.26 | - | + | + | + | - | + | + | + |
| 0.24 | - | + | + | + | - | + | + | - |
| 0.13 | + | + | + | + | + | + | + | - |
| 0.04 | + | + | + | + | + | + | + | + |

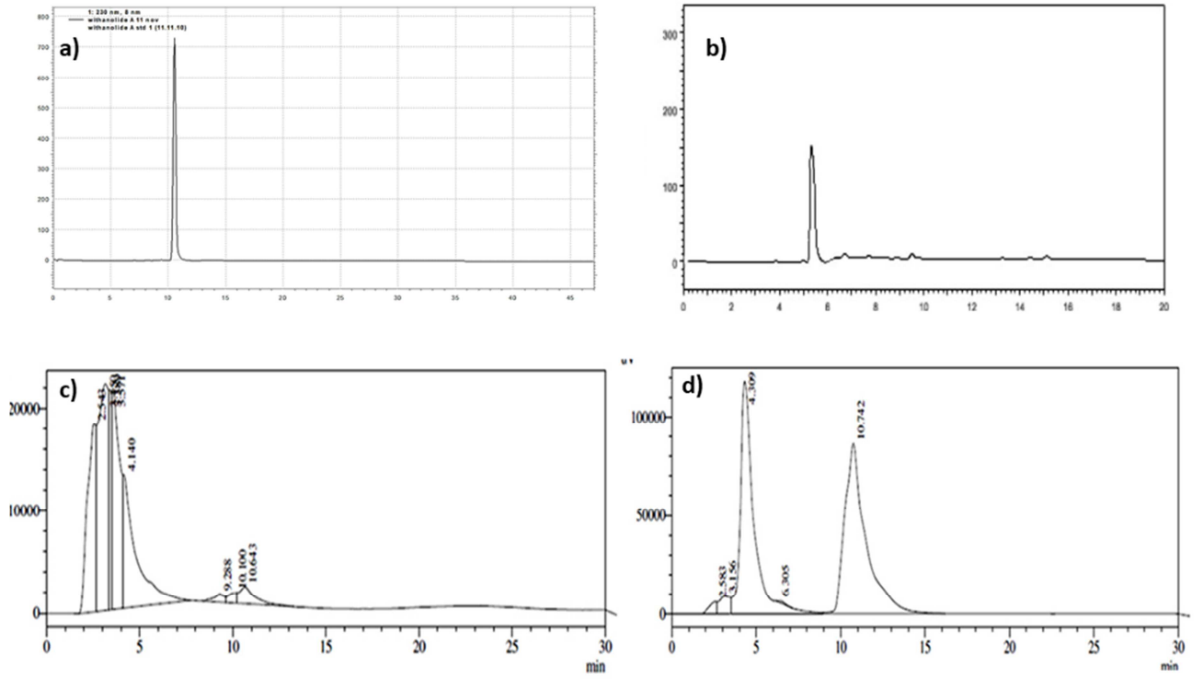


***Figure 4.*** *HPLC of a) withanolide A - standard b) withaferin A -standard, c) extracts of hairy roots, d) extracts of adventitious roots.*

HPLC analysis was effective in quantifying bioactive steroidal lactones. It was observed that cultures transformed with *Agrobacterium rhizogenes* showed significantly high levels of withaferin A content, which was determined to be 69.21 µg/g dry weight. Withaferin A and Withanolide A content in adventitious root cultures were 32.45 µg/g dry weight and 114 µg/g dry weight respectively. HPLC chromatogram of the adventitious and hairy root cultures has been depicted below in Figure 4.

The GC-MS analysis of methanol extract of the adventitious roots revealed the presence of various phytochemical constituents that contribute to the medicinal properties of the plant (Figure 5). The presence of the phytochemical compounds was confirmed based on the peak area, retention time and molecular formula.

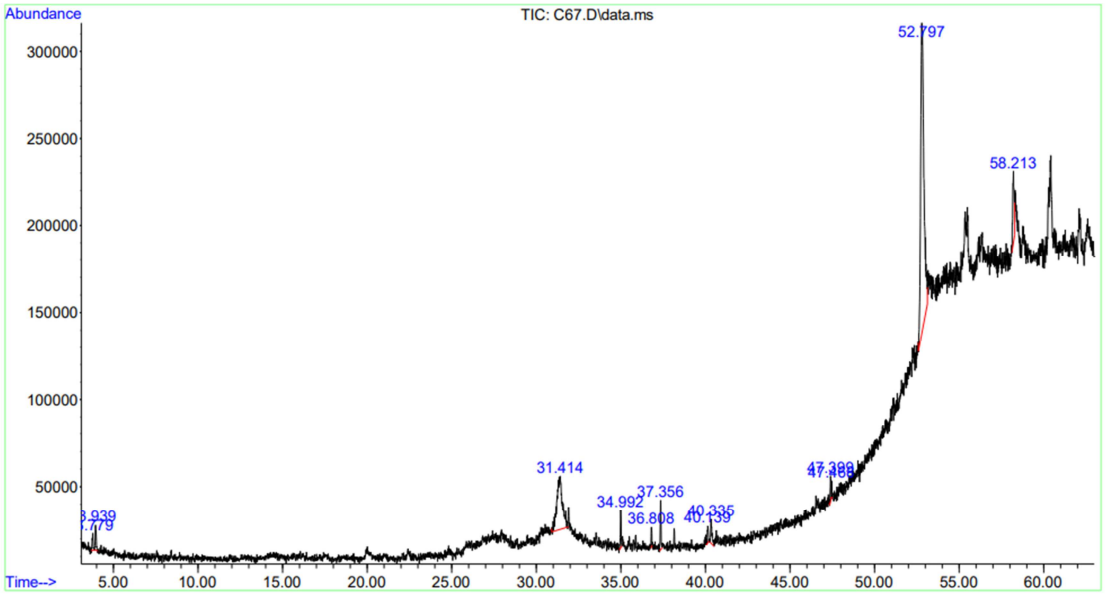


***Figure 5.*** *GCMS chromatogram of W. somnifera cv. Poshita methanolic extract.*

Results imply that cis-13-Octadecenoic acid, 9-Octadecenoic acid (Z)-, 2, 3-dihydroxypropyl ester and 9 and 12-Octadecadienoic acid (Z, Z) were the major components constituting about 16 -10% of the extract. Cis-13-Octadecenoic acid has numerous biological effects including and not limited to anti-inflammatory and antiarthritic properties. In recent years, anti-carcinogenic properties have also been studied. The extract also showed phytochemicals such as Ethanone, Campesterol 1,4,7-Androstatrien-3,17-dione, Olean-13(18)-ene Hexadecanoic acid, 2,3-Dihydroxypropyl elaidate, 11-Bromoundecanoic acid, DL-Homatropine, trimethylsilyl ether, and l-Gala-l-ido-octose. L-gala-l-ido-octose has effects on learning and memory, particularly to prevent cognitive deficits.

The results from this investigation emphasizes the efficiency of plant tissue culture for augmented agronomic applications, which transcends the problems encountered in conventional propagation and harvesting strategies [33-35]. The results of the HPLC analysis indicated the significantly higher yield of the metabolite Withaferin A when compared to adventitious roots. Therefore, these promising results suggest that propagating adventitious roots and hairy roots *in vitro* is a quicker method of cloning and harvesting secondary metabolites than cuttings, particularly for seasonal plants. Lower cost and less labour-intensive clonal propagation through the use of modified bubble column bioreactors was found to enhance large-scale production of secondary metabolites in *Withania sominifera* Poshita variety roots. Genetically transformed hairy root cultures could produce high levels of secondary metabolites comparable than intact plants. These secondary metabolites can be used as pharmaceuticals, pigments and flavors.

## 4. Conclusion

In the current study, metabolite profiling of the extracts from adventitious and hairy roots of *W. somnifera* was performed to evaluate the yield potential of the economically viable therapeutic moieties synthesized by in vitro methods. The results from this investigation emphasize the efficiency of plant tissue culture for augmented agronomic applications, which transcends the problems encountered in conventional propagation and harvesting strategies [33-35]. Phytochemical analysis of root extracts of *Withania sominifera* poshita variety revealed the presence of alkaloids, flavanoids, tannins, saponins, phenols, glycosides, terpenoids, reducing sugar and anthraquinones and also the HPLC analysis indicated the significantly higher yield of the metabolite Withaferin A in transformed roots when compared to adventitious roots. Therefore, these promising results suggest that propagating adventitious roots and hairy roots in vitro is a quicker method of cloning and harvesting secondary metabolites than cuttings, particularly for seasonal plants.

## Abbreviations Used

IAA: Indole Acetic Acid
IBA: Indole Butyric Acid
NAA: Naphthalene acetic acid
M S: Murashige and Skoog
YEP: Yeast Extract Peptone
MH: Muller Hinton
PDA: Potato Dextrose Agar

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## Declarations

### *Conflict of Interest*

The authors declare that they have no conflict of interest.

### *Ethics Approval and Consent to Participate*

This article does not contain any studies with human participants or animals performed by any of the authors.

### *Consent for Publication*

Not required.

### *Availability of Data and Material*

The data analysed during the present investigation are available from the corresponding author on reasonable request.

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