



***IN VITRO* MICROPROPAGATION OF *WITHANIA SOMNIFERA* (L.) DUNAL
BY DIFFERENT CONCENTRATIONS OF GROWTH REGULATORS**

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ABSTRACT

Withania somnifera (L.) Dunal is an important ayurvedic medicinal plant known mainly for its rejuvenating properties. *In vitro* micropropagation or multiplication of *Withania somnifera* through apical shoot proliferation by using different concentrations of growth regulators in short period. Shoots were induced from the apical meristem of *Withania somnifera* on Murashige and Skoog (MS) basal media supplemented with different concentrations of 6- Benzylamino purine(BAP) (0.15, 0.20 and 0.25 mg/L). Apical meristem explants showed initiation of shoots within 6 –7 days of transfer, with optimum concentration of 0.25 mg/L and it is found to be most effective for multiple shoot generation within 1–2 weeks. The generated shoots were successfully rooted on Murashige and Skoog (MS) basal medium supplemented with Indole 3-butyric acid (IBA) (optimum concentration - 1mg/L) along with activated charcoal. Rooted plants were then transferred for primary and secondary hardening and grown in green house. These hardened plants have been successfully established in soil.

KEYWORDS: Apical meristem, BAP, IBA, Micropropagation, MS Media, *Withania somnifera*.



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INTRODUCTION

Medicinal plants are nature's gift, which has curative properties due to the presence of various complex chemical substances of different composition. They are found as secondary plant metabolites in one or more parts of these plants. These secondary plant metabolites are grouped as alkaloids, corticosteroids, essential oils etc. according to their composition¹. The use of herbs as medicinal plants due to the presence of various chemical substances is ever increasing. However, since most of these plants are taken from the wild, hundreds of species are now threatened with extinction². To overcome these problem medicinal plants can be raised through a technique called plant tissue culture. It is a technique of growing plant cells, tissues and organs in an artificially prepared nutrient medium under aseptic condition³. Plant tissue culture technology has been available to the plant breeders for nearly four decades and has been extensively employed for crop improvement in several crops⁴. *Withania somnifera* (L.) Dunal or Ashwagandha or Winter cherry or Indian ginseng belongs to family *Solanaceae* is an important medicinal plant with excellent export potential in an herbal drug trade. It is a well known for years as an important drug in the ayurvedic literature. The major biochemical constituents of Ashwagandha roots are steroidal alkaloids and steroidal lactones in a class of constituents called withanolides⁵. Both leaves and roots of the plant are used as the drug and steroidal lactones occur in both parts. Roots of the plant possess antistress, antitumor, immunomodulatory, antioxidant and antibacterial activity. Modern herbalists classify Ashwagandha as an adaptogen, a substance that increases the body's ability to withstand stress of all types. However, the evidence for an adaptogenic effect is limited to test tube and animal studies⁶. Other proposed uses of Ashwagandha, based on even weaker evidence, including preventing cancer⁷, improving immunity⁸, reversal of paclitaxel-induced neutropenia by *Withania somnifera* in mice, enhancing mental function and combating anxiety and depression.

Mainly *Withania somnifera* are propagated commercially by means of seeds because of the lack of natural ability for vegetative propagation⁹ but the seed viability is limited year-by-year¹⁰. Due to poor viability of stored seed, alternative procedure of propagation is essential for constant supply for industrial level. *In vitro* technology can be used as an alternative because of the advantage of tissue culture technology i.e. by producing high quality disease-free planting material irrespective of the season and weather. In modern conservation biotechnology, elite and over exploited plant germplasm conservation by *in vitro* method has been done using slow growth procedures or cryopreservation^{11, 12}. By reducing the culture temperature and by modifying culture media with supplements of osmotic agents, growth inhibitors or by removing growth promoters we can slow down the growth of plants. In the present study, we have investigated the ability of different growth hormones to develop a standard protocol to initiate multiple shoot and root culture of *Withania somnifera* that may provide a good source of pharmacologically active plant constituents.

MATERIALS AND METHODS

(i) *Plant Material Collection and Surface Sterilization*

Plants of *Withania somnifera* was collected locally from Pune University nursery. Apical buds and nodal sections were used as explants. The nodal explants were about 2-3cm in length cut from 3-month-old plants. These collected explants were then washed thoroughly with running tap water and immersed in 0.1% Bavistin (antifungal agent) solution for 5minutes, to this one drop of Tween 20 is added and kept for 5minutes. Inside the laminar the explants were subsequently treated with sodium hypochlorite for 4minutes and finally it is treated with mercuric chloride for 3minutes.

(ii) Culture Media and Culture Conditions

After complete washing with sterile distilled water, these explants were then inoculated on prepared solidified basal media containing MS salts¹³, 3.0% sucrose as carbon source and 0.8% agar as solidifying agent. The medium was supplemented with different hormonal concentration of BAP 0.15, 0.20 and 0.25 mg/L. The pH of the medium was adjusted to 5.8±0.02 before autoclaving at 15 pounds per square inch (15 psi) for 20 minutes. The inoculated culture bottles were maintained at 25±1°C, 55±5% relative humidity, under a 16-hour photoperiod in the growth chamber at 2000-2500 lux light intensity of cool, white and fluorescent light and 50±5% relative humidity.

(iii) Shoot induction and In vitro morphogenesis

After some weeks the regenerated shoots & apical meristem were then aseptically excised & inoculated (under LAF) onto MS medium supplemented with different hormones to initiate a new cycle of multiplication with different hormones [MS1 (1 mg/L BAP + 0.3 mg/L IAA), MS2 (0.5 mg/L Kinetin + 0.1 mg/L IAA), MS3 (0.5 BAP mg/L + 0.1mg IAA), MS4 (1 mg/L Kinetin + 0.3 mg/L IAA)]. The healthy shoot buds were excised & transferred onto a MS basal medium to leaching out the residual cytokine. After one week, the shoot buds were taken off from MS basal media & transferred onto various rooting media i.e., MS-A media (1mg/L IBA) , MS-B media (0.5mg/L NAA), MS-C media (1mg/L IBA + 500mg/L Activated charcoal) & MS-D Media (0.5 mg/L

NAA + 500mg activated charcoal). After complete rooting the left out agar was washed off and the plants were transferred to pots having a mixture of sterilized sand and clay in 1:1 ratio. After two weeks, the acclimatized plants were transfer to pots containing normal garden soil and maintained in greenhouse under natural day length conditions. After 3-4weeks, the beakers were gradually removed and the pots containing plant exposed to partial shade for a short time, this time of exposure in the natural condition increase simultaneously. Finally, the plants were transplanted in the natural environment.

RESULTS**1. Initiation**

The explants (apical meristem & nodal section) of *Withania somnifera* (L.) Dunal were cultured on MS basal medium supplemented with different concentrations of BAP (0.15, 0.20 and 0.25 mg/L). They were observed regularly for 1–2 weeks for their development by measuring their length. Shoots started generating after 5-6 days of inoculation and the best results (optimum conc. for maximum shoot generation in minimum time) were obtained with the BAP conc. 0.25 mg/L. Other concentrations showed initiation after some time (0.20 mg/L BAP), but less effective responses were obtained. Apical meristem & nodal section of *W.somnifera* were then inoculated on MS basal media supplemented with BAP (0.25 mg/L) (Table 1; Figure 1 & 2).

Table 1
Shoot regeneration from apical meristem & nodal section

Source of Explants	No. of cultures	Total initiation After 10 days	Fungal Contamination	Dead explants	Live explants	Success rate
Apical meristem	12	10	No	-	10	83.33 %
Nodal explants	12	8	No	4	8	66.6 %



Figure 1
Inoculation and initiation from apical meristem & nodal section.



Figure 2
Effects of various BAP hormone concentrations on explants (after 12 days)

2. Subculture

After 15 days, the healthy shoot buds were excised from initiation media and transferred onto MS multiplication medium (MS1, MS2, MS3, MS4) supplemented with different concentration of hormones (MS1 (1 mg/L BAP + 0.3 mg/L IAA), MS2 (0.5 mg/L Kinetin + 0.1 mg/L IAA), MS3 (0.5 BAP mg/L + 0.1mg IAA), MS4 (1 mg/L Kinetin + 0.3 mg/L IAA). MS3 media showed the maximum number of shoot regeneration (Table 2 & Figure 3).

Table 2
Effect of various hormone concentrations on shoot regeneration.

Multiplication Media	No. of explants Inoculated	No. of shoots proliferation / explants	No. of contamination	Survival rate
MS1 media	8	3	1	37.5%
MS2 media	8	4	-	50%
MS3 media	8	7	-	87.5%
MS4 media	8	5	2	62.5%

Figure 3
Effects of various hormone concentration on shoot regeneration



1. (MS1 (1 mg/L BAP + 0.3 mg/L IAA), 2. MS2 (0.5 mg/L Kinetin + 0.1 mg/L IAA), 3. MS3 (0.5 BAP mg/L + 0.1mg IAA), 4. MS4 (1 mg/L Kinetin + 0.3 mg/L IAA)

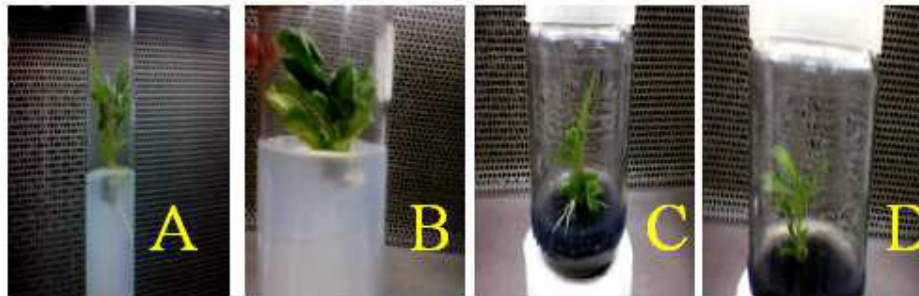
3. Root culture

After 15 days, generated shoots were inoculated on MS medium containing different hormones i.e., MS-A media (1mg/L IBA), MS-B media (0.5mg/L NAA), MS-C media (1mg/L IBA + 500mg/L Activated charcoal) & MS-D Media (0.5 mg/L NAA + 500mg activated charcoal), respectively. The roots started developing after 1 – 2 weeks at the base of the shoots. Maximum root initiations were obtained in MS-C media (Table 3 & Figure 4).

Table 3
Effects of various Auxins on root regeneration

Media	No. of explants inoculated	After 1 week	After 2 weeks	Final success %
MS-A(IBA)	5	Root initiation shown in 1 culture tube	Root initiation shown in 2 tubes	40 %
MS-B (NAA)	5	Roots yet to be developed in all tubes	Roots yet to be developed in all tubes	10%
MS-C(IBA + Activated charcoal)	5	Root initiation shown in 2 culture bottle.	Root initiation shown in 3 tubes	80%
MS-D(NAA + Activated charcoal)	5	Roots yet to develop	Root initiation started in 2 culture bottle.	10%

Figure 4
Effects of various Auxins on root regeneration (After 1 week)



1. MS-A media (1mg/L IBA), 2. MS-B media (0.5mg/L NAA), 3. MS-C media (1mg/L IBA + 500mg/L Activated charcoal) & 4. MS-D Media (0.5 mg/L NAA + 500mg activated charcoal)

DISCUSSION

Micropropagation holds tremendous potential as a tool for the production of high quality plant-based medicines. Attempts to multiple shoot regeneration from nodal explants of *Withania somnifera* (L.) Dunal, on MS media supplemented with different concentrations of cytokine (BAP) alone or in combination with auxin (IAA). The result showed that the nodal explants cultured on MS basal medium without growth regulators failed to induce shoot proliferation. The results obtained were agreed according to data reported by^{14, 15, 16,17,18,19}. Among different combinations of media tested, the MS medium supplemented with 0.5 mg/L BAP + 0.1mg IAA (Table 2 & Figure 3) proved the best optimized medium with a maximum shooting (87.5%). The

results were supported by previous observations^{14, 20, 10} who found that BAP and IAA are the best growth regulators for multiple shoot induction in *Withania* tissue culture. This may be due to the synergetic effect of IAA in combination with BAP on the enhancement of shoot multiplication²¹. The combination of auxin and cytokinin has been reported for callus induction from leaf explants^{22, 23, 11}. Activated charcoal was used for effective rooting within a short time period. The optimum root induction was obtained on MS medium supplemented with IBA (1mg/l) (Table 3 & Figure 4). Similar results were obtained by²⁴. The rooted plantlets were taken out from the culture vials and washed with distilled water delicately to remove adhering

agar medium to reduce the chances of contamination. Then, these plantlets were transferred to the polycups containing garden soil and vermicompost. They were hardened, acclimatized, and finally transplanted to natural surroundings²⁵. We hope that continuation and intensification efforts in this field will lead to controllable and successful biotechnological production of specific, valuable, and yet unknown endangered medicinal plants⁴.

CONCLUSION

Withania somnifera (L) Dunal., commonly known as Ashwagandha, mentioned as an

important ancient Ayurvedic drug of India. Ashwagandha contains Withanolides, which has anti-bacterial, anti-inflammatory & enhance the body's defence against infections & tumors. Due to lack of optimum protocol for *in vitro* multiplication of *Withania somnifera*, the present study was undertaken to develop a method of high multiplication rate using different concentrations of growth regulators in short period of time and enhancing the production of rare and endangered medicinally important ashwagandha plant. This regeneration system could be used in mass propagation of Ashwagandha (*Withania somnifera* (L.) Dunal).

ABBREVIATIONS

BAP- 6- Benzylamino purine, IAA-, Indole-3-acetic acid, IBA-Indole 3-butyric acid, Medium-Murashige and Skoog medium, NAA- α -Naphthalene acetic acid.

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