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Micropropagation of *Withania somnifera* Using Shoot Tip Explants

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Abstract

In the present investigation *in vitro* propagation of a rare medicinal plant, *Withania somnifera* was carried out. Direct regeneration achieved from shoot tip explants and shoot multiplication was obtained by using cytokinin BAP (2.0 μM /l) and combination of BAP (2.0 μM /l) + IBA (2.0 μM/l), respectively. MS media with shoot tip explants supplemented with BAP (2.0 μM/l) produced maximum average number of shoots (5 ± 0.53) and average shoot length was found to b 2.5 ± 0.15 cm. significant induced were sub cultured in shoot multiplication, developed shoot multiplication was obtained average number of shoots (5.3 ± 0.41) and average shoot length 6.5 ± 0.12 cm was shown on MS media in combination with BAP (4.0 μM) + IBA (2.0-8.0 μM/l). Maximum average number (6 ± 0.10) and average length 7.6 ± 0.18 cm of roots were observed on MS media supplemented with IBA (2.0 um/l). The MS medium supplemented with BAP and Kn was formed to be suitable for *in vitro* propagation of *W. somnifera*.

Introduction

Biotechnology plays a crucial role in conserving important plant species, especially as many valuable genetic resources are rapidly disappearing from natural habitats. Herbal medicines remain the primary healthcare choice for 75-80% of the global population due to their compatibility with the human body and fewer side effects (Sarkar and Modak 2023). The cultivation of high-value medicinal plants is gaining importance in agriculture and supporting the growing Indian herbal industry. However, challenges such as limited knowledge of seed biology and lack of efforts to identify and propagate elite plant specimens hinder large-scale cultivation (Mikulska et al. 2023).

W. somnifera (L.) Dunal, commonly known as Indian ginseng or Ashwagandha, is a medicinal shrub belonging to the Solanaceae family. It is widely distributed in drier regions of India, Pakistan, Afghanistan, and parts of Africa and the Middle East. In India, it is mainly cultivated in Madhya Pradesh, Uttar Pradesh, Punjab, Gujarat, and Rajasthan (Chattopadhyay et al. 2023). The plant grows up to 30-150 cm and holds significant value

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in Ayurvedic medicine. It possesses diverse therapeutic properties such as anti-inflammatory, anticancer, anti-stress, anti-aging, immunomodulatory, and antioxidant activities. Ashwagandha is traditionally used to treat ailments including tuberculosis, rheumatism, cardiac diseases, and inflammatory conditions. Its roots are particularly valued for their sedative effects and potential in managing senile disorders, such as Alzheimer's disease (Munir et al. 2022).

Ashwagandha roots are in high demand in the crude drug market. Although the plant is primarily propagated by seeds, seed viability declines sharply after a year and is further hampered by insect pests and germination inhibitors in the fruit wall. These limitations make *in vitro* propagation a preferred alternative. *In vitro* multiplication, conducted under sterile conditions using nutrient media, allows rapid and large-scale production of plants up to one million annually from a single explant (Taur et al. 2025). This study emphasized shoot tip culture using MS medium with different concentrations of auxins and cytokinins to Therefore, this investigation focused on developing an optimized protocol for *in vitro* propagation of *W. somnifera* by modifying key factors and implementing effective subculture strategies to ensure genetic fidelity, large-scale production for stable *in vitro* propagation of *W. somnifera,* and optimizing shoot multiplication (Połumackanycz et al. 2020).

Materials and Methods

Aseptic cultures of Ashwagandha were established using shoot tip segments from healthy, flowering mother plants obtained in the wild. Table .1 represents total surface sterilisation steps, Twigs (10 cm) were collected; leaves excised, and shoot tips washed with running water. In a laminar flow cabinet, explants were sterilised using 2% Bavistin, followed by 5% sodium hypochlorite, and rinsed with sterile distilled water. The sterilized explants were segmented and cultured on MS medium supplemented with sucrose and agar. Cultures were incubated at 25°C under a 16/8 h photoperiod. After 4 weeks, these aseptic cultures were used for further experimentation.

Table 1. Surface sterilisation.

Chemical	Concentration	Time duration
Tap water	100 ml	25 min
Bavistin	1 gm	10 min (3 wash s.w) 5 min
Mercury chloride ($HgCl_2$)	0.1 gm	5 min (3 wash s.w) 5 min
Sodium hypocloride	5 ml + 5 ml (H_2O)	10 min (3 wash s.w) 5 min
Cefotaxime	100 µl	10 min (3 wash s.w) 5 min

Shoot tips were extracted from aseptic nodal segments cultures by carefully removing leaves and aligned using a millimeter grid for precision. Shoot tips were inoculated in MS medium supplemented with various cytokinin concentrations, including BAP and Kn. Media were supplemented with 3% sucrose, pH 5.8, and

autoclaved. After 4 weeks, induced shoot clumps were subcultured onto multiplication media. While multiplication medium supported growth in *W. somnifera*. Subcultures were performed every 4 weeks, and multiplication rates were visually assessed and calculated after every subculture cycles.

Shoots (2-3 cm tall with 4-5 leaves) were subcultured to root on MS medium supplemented with BAP, Kn, and IBA (2.0 μM/l). A fresh basal cut was made on each shoot before culturing on root induction medium. Rooting was performed in a controlled growth cabinet at 25°C, with a 16/8 h light/dark cycle and 75% humidity.

The acclimatization of rooted *W. somnifera* plants showed significant results, due to the development of strong shoots and healthy roots. Plants were gently transferred to pots with coco pit and incubated for 10 days, misted three times daily. After that, bags were gradually opened, followed by another 6-day incubation before moving to a glasshouse for final acclimatisation.

Data from the study were analysed using ANOVA. Based on the distribution, either an independent samples t-test or-Whitney U test was used for two-group comparisons. ANOVA followed by an HSD test was applied to determine significant differences between means.

Results and Discussion

During the establishment of cultures, it was observed that nodal explants placed on MS medium without any cytokinin (plant growth hormone) did not produce shoots. These nodal segments were taken from field-grown *W. somnifera* plants and placed on MS medium with varying concentrations of BAP and Kn (0.5, 1.0, 1.5, and 2.0 μM) as mentioned in Table no. 2. Shoots started to appear between 12 to 14 days after culturing.

Table 2. Effects of BAP and Kn on shoot initiation from nodal explant.

Sl. No	Treatment of MS medium + BAP + Kn μM/l	Average no. of shoot initiated	Average no. of shoot length
1.	MS medium + 0.0 (control)	-	-
2.	MS + BAP (0.5 μM) + Kn (0.5 μM)	1.0 ± 0.28	1.0 ± 0.26
3.	MS + BAP (1.0 μM) + Kn (1.0μM)	1.3 ± 0.06	1.4 ± 0.34
4.	MS + BAP (1.5 μM) + Kn (1.5 μM)	1.0 ± 0.20	1.4 ± 0.25
5.	MS + BAP (2.0 μM) + Kn (2.0 μM)	2.0 ± 0.37	2.8 ± 0.15
6.	MS + BAP (2.5 μM) + Kn (2.5 μM)	1.3 ± 0.41	1.2 ± 0.20

The shoots that developed from the nodal explants were transferred to a medium designed for shoot multiplication. In this study, the combined influence of cytokinins specifically (BAP and Kn) on shoot multiplication and elongation in *W. somnifera* was examined. Different concentrations of these cytokinins had a notable impact on shoot development. Among the various combinations tested, MS medium containing 4.0 μM

BAP along with 4.0 μM Kn was found to be the most effective for shoot proliferation. This treatment resulted in the highest number of shoots per explant (8.03 ± 0.13) and the greatest average shoot length (6 ± 0.26 cm), compared to other treatments.

There were significant differences in the rate of shoot multiplication and the number of shoots across the different cytokinin concentrations. Increasing the concentrations of BAP and Kn up to 10 μM initially boosted shoot production, but further increases led to a decline in shoot numbers. At lower concentrations (4 μM), the shoots appeared healthy, vibrant green, and free of vitrification symptoms. In contrast, higher concentrations (10.0 μM) resulted in vitrified, abnormal shoots. The combination of Kn with 4 μM BAP proved to be the most beneficial for promoting healthy shoot proliferation.

Elongated shoots of *W. somnifera* were subcultured onto (MS) medium supplemented with various concentrations of auxins, namely indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), to evaluate their efficacy in promoting root induction. Root initiation was observed within 10-12 days of transfer to the rooting medium containing 2.0 μM IBA.

Among the tested auxins, IBA at 2.0 μM demonstrated the most effective response, producing the highest average root length (8.6 ± 0.28 cm) and number of roots (18 ± 0.16). IBA at the same concentration yielded a moderate response, with a maximum root length of 6.0 ± 0.16 cm and an average of 12 ± 0.13 roots per explant. Notably, roots induced on IBA-supplemented medium were thicker and exhibited more extensive lateral branching. In contrast, IBA (2.0 μM) resulted in significantly lower rooting efficiency, with an average root length of 2.8 ± 0.15 cm and 3.7 ± 0.16 roots. A decline in both root number and length was observed with increasing concentrations of IAA and IBA beyond the optimal level. Based on these findings, IBA at 2.0 μM proved to be the most suitable auxin for efficient *in vitro* rooting in *W. somnifera*. Here, is the table no 3 given for the same reference.

Table 3. IBA (μM) number of roots per shoot root length (cm).

Sl. No	MS medium + IBA (μM)	Avg. root number (mean ± SE)	Avg. root length (cm) (mean ± SE)
1.	MS medium + IBA 1.0	6.6 ± 0.37	5.3 ± 0.20
2.	MS medium + IBA2.0	12.0 ± 0.20	9.8 ± 0.26
3.	MS medium + IBA3.0	5.3 ± 0.15	4.7 ± 0.43
4.	MS medium + IBA4.0	3.6 ± 0.25	4.0 ± 0.32
5.	MS medium + IBA5.0	3.3 ± 0.36	3.7 ± 0.28

Nodles were sterilised under the laminar flow hood. use sterile forceps to transfer the sterilized note onto sterile paper or a petri dish. Trim away any dead or damaged tissue from the nodes using a sterile scalpel. Carefully place the node into sterile culture medium (e.g., MS medium supplemented with appropriate hormones such as BAP, Kn and IBA).

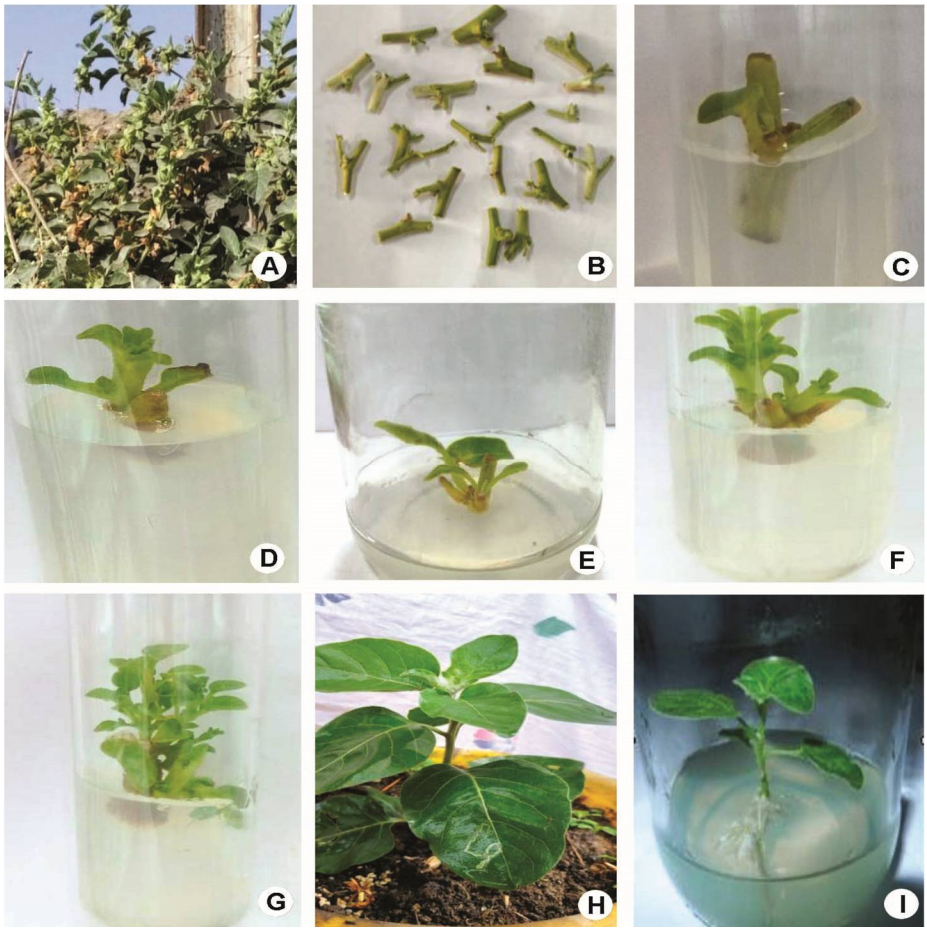

Fig. 1. Plant regeneration of *W. Somnifera* from nodal explants: (A) *W. somnifera* Plant, (B) nodal explants, (C) shoot Initiation from nodal ex plant after 1 week, (D-E) shoot differentiation after 2 weeks, (F) shoot proliferation, (G) shoot elongation, (H) rooting on MS medium containing IBA 2.0 μM and (I) hardening of plant in pot after 5 weeks.

The present study demonstrates the successful *in vitro* micropropagation of *Withania somnifera* using shoot tip explants, contributing to the conservation and mass propagation of this valuable medicinal plant (Singh et al. 2011). Fig. D and E represents the initiation and proliferation of shoot cultures, which were effectively influenced by the type and concentration of plant growth regulators (PGRs), particularly cytokinin's. Among the PGRs tested, benzylaminopurine (BAP) alone or in combination with Indole-3-butyric acid (IBA) showed significant results in inducing shoot proliferation. This aligns with previous studies that reported BAP as the most effective cytokinin for shoot multiplication in *W. somnifera* (Baskaran et al. 2013). The rooting of regenerated shoots was achieved on half-strength MS medium supplemented with indole-3-butyric acid (IBA), with the best rooting response observed at a concentration of 2.0 uM/l (shown in

Fig. I). This supports findings in earlier literature that IBA is more effective than other auxins like IAA for root induction in *W. somnifera* (Dar et al. 2016). Rooted plantlets showed high survival rates during the acclimatization phase, suggesting that the developed protocol can produce viable plants for field transfer. Genetic stability was not evaluated in the current study, but is a critical aspect for future work, especially considering the pharmaceutical importance of withanolides produced by *W. somnifera*. Previous reports have suggested that prolonged *in vitro* culture may induce somaclonal variation, which could affect the phytochemical consistency of the propagated plants. (Rahman et al. 2020). The production of Ashwagandha roots through conventional methods of cultivation (seed) is less than the requirement due to numerous reasons, viz. poor yield, takes a long time, poor viability of seeds, susceptibility of the seeds and seedlings to fungal infections like seedling mortality and blight, leaf blight, and seed rotting. This medicinally significant plant species has been depleted from its natural habitat and is now included in the list of endangered species (Kanung et al. 2011). Our investigations are useful to conserve this medicinal plant and also enhance the medicinal drug.

The micropropagation protocol established here offers a reliable and efficient method for the large-scale production of *W. somnifera*. It also provides a platform for future genetic transformation studies and conservation strategies for this endangered medicinal plant. At elevated concentrations of IBA (2.0 μM), callus development was observed at the base of the shoots, completely inhibiting root formation. The regenerated plantlets exhibited a survival rate of 85% upon transfer to plastic pots filled with a sterilized mixture of soil and sand (1 : 1 v/v) supplemented with 15% vermiculite. These plants were subsequently maintained under greenhouse conditions (Taur et al. 2025).

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