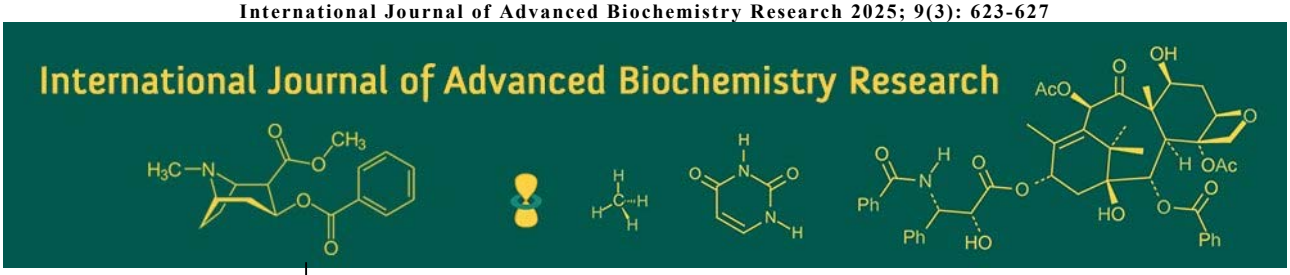

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Development of efficient *in vitro* propagation strategy for *Withania somnifera* (L.) Dunal (Ashwagandha)

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Abstract
Withania somnifera (L.) Dunal is a highly prized medicinal plant that has been valued for its extraordinary pharmaceutical potential since ancient times. Its demand has grown dramatically in both domestic and foreign markets as a result of scientific confirmation of its therapeutic efficacy against a wide range of diseases. Alternative methods to conventional cultivation are crucial to closing the significant gap between supply and demand. A practical method for creating large quantities of *W. somnifera* plants that are healthy, genetically stable, and phytochemically homogeneous is an *in vitro* propagation system. Both pre-clinical research and the herbal industry benefit from this approach. A significant obstacle in *in vitro* propagation, however, is maintaining a high rate of shoot multiplication in cytokinin-enriched MS medium for prolonged periods of time. In order to minimize the negative effects of cytokinin, the current study created a multi-step protocol that includes rotational subculturing of *Withania* shoots in auxin-cytokinin-enriched culture medium. Murashige and Skoog (MS) medium supplemented with different concentrations of BAP, IAA, 2,4-D, and IBA, as well as 0.8% agar and 3% sucrose, was used to start shoot cultures. Using BAP (5.0 mg/l) and IBA (3.0 mg/l) produced the best results, with the greatest number of shoots (56.71±24.91) after 60 days. Additionally, the highest shoot proliferation was obtained with a combination of BAP (5.0 mg/l) and IAA (3.0 mg/l); after 60 days, the average shoot length was 6.04±2.69 cm. Assuring the method's dependability, genetic homogeneity and the stability of pharmaceutically significant components in extended *in vitro* shoots were verified. *Withania somnifera* is becoming more and more in demand commercially, and this study provides a strong platform for its high-efficiency micropropagation.

Keywords: *Withania sominifera*, withanolide, shoot tip culture, *in vitro* shoot multiplication

Introduction
Withania somnifera, commonly known as Ashwagandha, is a vital medicinal plant belonging to the Solanaceae family and native to India. Known as Ashwagandha in Hindi and Sanskrit, and winter cherry in English, it is an excellent source of withanolides, particularly withaferin-A. Its roots, seeds, and leaves are widely used in Ayurvedic and Unani medicines for their therapeutic properties. Ashwagandha is effective in treating conditions such as arthritis, anxiety, insomnia, tumors, tuberculosis, asthma, leukoderma, and bronchitis, among others. It is also regarded as one of the best rejuvenative agents in Ayurveda. Ashwagandha roots are in high demand in the crude drug market, with a wholesale price of approximately Rs. 5000 per quintal. The plant primarily propagates through seeds, but seed viability declines significantly after one year, dropping to as low as 5%. Moreover, seeds are vulnerable to insect pests, and a chemical present in the fruit wall inhibits germination. These challenges make *in vitro* multiplication a more suitable method for mass propagation.
In vitro multiplication involves growing plant cells, tissues, or organs in an artificial nutrient medium, either static or liquid, under sterile conditions. This technique has an exceptionally high multiplication rate, enabling the production of up to 10^6 plants annually from a single explant. It is particularly well-suited for the rapid propagation of rare genotypes. The present study focused on *in vitro* shoot multiplication using shoot tip culture. The experiment was conducted under sterile conditions, employing Murashige and Skoog (MS) medium supplemented with various concentrations of auxins and cytokinins to optimize growth and proliferation.

Materials and Methods

Materials

A well-characterized genotype of *Withania somnifera*, maintained at Shri Shivaji College of Agricultural Biotechnology, affiliated with Dr. PDKV, Akola (M.S.), was utilized for this experiment. Young, healthy *Withania somnifera* plants were used as the source of explants. Shoot tips of *Withania somnifera* have been used as the explants (Fig.1).

Methods

Shoot tips from the mother plant have been picked carefully and the explants were carefully excised using a sterile scalpel blade to prevent damage. The explant should have only stem and all the leaves should be removed. The explants should contain nodal part which appear as T-shaped explant. They were then washed with running tap water and then surface sterilized using 0.1% mercuric chloride ($HgCl_2$) for 1 minute and then washed with autoclaved distilled water twice. Then again, 1% Bavistin was used to ensure aseptic conditions and treated for 2 minutes followed by washing with autoclaved distilled water. The sterilized explants were then inoculated onto shoot initiation medium with full-strength MS medium enriched with 3% sucrose and 0.8% agar (w/v) which was supplemented with varying concentrations of growth hormones, including IAA, NAA, BAP, 2,4-D and IBA. The medium was sterilized by autoclaving at 15 psi and 121 °C for 20 min and the cultures were incubated in a growth chamber under a 16:8 h photoperiod, illuminated with cool white fluorescent (F 40 T 12/CW/EG) lamp at a photon flux density of 100 µ mol m−2 s−1 at 25±2 °C and 65-70% relative humidity. The cultures were incubated at a controlled temperature of 25±2 °C, 16-hour photoperiod and 60% relative humidity). The details of procedure has been depicted in flowchart in figure 2.

For different stages (shoot initiation, shoot multiplication and rooting), culture media consisted of Murashige and Skoog (1962) [23] basal medium solidified with 0.8% (w/v) agar, supplemented with 3% (w/v) sucrose and plant growth regulators (PGRs) of various concentrations. All PGRs were purchased from Himedia company. The pH of the medium was adjusted to 5.8-5.9 prior to the addition of 0.8% agar (w/v). Regenerated shoots were transferred to half-strength MS medium containing auxins for root induction. The root cultures were maintained in darkness. Rooted plantlets were hardened in a greenhouse and subsequently transferred to soil for further growth and evaluation.

Results and Discussion

In the face of indiscriminate exploitation for bioactive withanolides an effective propagation protocol of *W. somnifera* for conservation, usage in traditional medicine, and for commercial exploitation was developed. In this study, shoot tips of an elite genotype of *W. ashwagandha* (WA02) were inoculated on MS medium supplemented with various combinations and concentration of PGRs. Shoot multiplication was observed at all the concentrations of PGRs after 30 and 60 days intervals. However, the response of the shoot tips varied across different treatment combinations for shoot initiation. The observations recorded included no. of shoots, shoot length aged 30 days and 60 days. A total of 3 replicates with 30 explants have been selected for the current experiment. The most effective treatment for number of shoots per explants was treatment comprising BAP (5.0 mg/l) and IBA (3.0 mg/l), which yielded 26.61±10.81 shoots (Table 1) after 30 days and 56.71±24.91 shoots after 60 days (Fig. 1). This was followed by treatment with BAP 4.0 mg/l and NAA 2.5 mg/l), which resulted in 23.00±10.28 shoots after 30 days and 53.00±23.69 shoots after 60 days, and treatment with BAP 4 mg/L and 2,4-D 5 mg/L produced 47.17±21.02 shoots after 60 days (Table 2). These findings suggest that BAP (5.0 mg/l) + IBA (3.0 mg/l) is the optimal combination for multiple shoot initiation and can be recommended for this purpose.

Cytokinins are a group of plant hormones that play a significant role in cell division and various physiological processes, including cell growth and differentiation (Mahesh & Jeyachandran, 2013) [13]. The ability of BAP, either alone or in combination with Kn, to stimulate bud break and promote *in vitro* multiple shoot formation has been documented in *Withania somnifera* (Ray & Jha, 2001; Sharada *et al*., 2007; Ahuja *et al*., 2009; Dewir *et al*., 2010; Mir *et al*., 2014a) [16, 17, 2, 5, 14], as well as in *Trichodesma indicum* (Mahesh & Jeyachandran, 2013) [13] and *Drimia robusta* (Baskaran *et al*., 2013) [3], among other species (Kumar *et al*., 2005; Baskaran *et al*., 2012) [2, 3]. The beneficial effects of BAP were noticeable up to a concentration threshold of 5.0 µM, beyond which no significant improvement was observed in the studied parameters. Excessive BA levels, whether applied alone or with Kn, did not enhance the response further. Additionally, higher BA concentrations favored callus formation, with minimal and sporadic callusing occurring at the shoot base during the initial culture stages (first two subcultures). Similar findings have been reported for *Withania somnifera* (Sangwan *et al*., 2007a, b) [22] and other related genera (Baskaran *et al*., 2013) [3]. Explants derived from *in vitro* shoot tips demonstrated high regeneration capacity, maintaining their potential even after 8-10 subcultures for large-scale propagation. Only treatments in which shoot tip explants exhibited a positive response were further examined for shoot proliferation. The highest average shoot length of 2.62±1.17 cm at 30 days and 6.04±2.69 cm at 60 days was recorded with the application of BAP (5.0 mg/L) in combination with IAA (3.0 mg/L) (Fig. 3, 4, 5; Table 3). Thus, the combination of BAP (5.0 mg/L) and IAA (3.0 mg/L) is recommended for optimal shoot proliferation (Table 1).

Root induction and acclimatization of *in vitro*-derived plantlets are critical stages in the micropropagation process. After 60 days in culture, some *W. somnifera* shoots initiated root development naturally. The remaining unrooted shoots were transferred to half-strength and full-strength MS media supplemented with different concentrations of 2,4-D, NAA, IAA, and IBA (Table 2). Rooting performance was superior in half-strength MS liquid medium in terms of root number and length, although the overall rooting percentage remained similar across treatments, aligning with previous findings in *W. somnifera* (Mir *et al*., 2014a) [14]. Consequently, only half-strength media compositions were emphasized in Table 2. Among the auxins tested, 2,4-D proved to be the most effective in inducing adventitious roots, yielding the highest percentage of root formation and average root number per shoot (Ahuja *et al*., 2009; Baskaran *et al*., 2013) [2, 3]. Conversely, both NAA and IAA induced callus formation before rooting commenced

Table 1: Effect of different concentration of BAP and IAA on shoot length of *Withania somnifera*

S. No	Code	Treatment	Average no. of shoot initiated		Average shoot length	
			After 30 days (Mean + S.E)	After 60 days (Mean + S.E)	After 30 days (Mean + S.E)	After 60 days (Mean + S.E)
1	T_1	MS + BAP (1.0 mg/l) + IAA (1.0 mg/l)	12.42±5.52	28.62±12.72	1.11±0.49	2.57±1.15
2	T_2	MS + BAP (2.0 mg/l) + IAA (1.5 mg/l)	12.42±5.52	28.62±12.72	0.63±0.28	1.46±0.65
3	T_3	MS + BAP (3.0 mg/l) + IAA (2.0 mg/l)	4.60±1.84	10.60±4.24	0.95±0.42	2.19±0.98
4	T_4	MS + BAP (4.0 mg/l) + IAA (2.5 mg/l)	19.09±8.50	43.99±19.61	0.87±0.39	2.01±0.90
5	T_5	MS + BAP (5.0 mg/l) + IAA (3.0 mg/l)	10.12±4.37	23.32±10.07	2.62±1.17	6.04±2.69
6	T6	MS + 2,4-D (1.0 mg/l) + NAA (1.0 mg/l)	No response	No response	No response	No response
7	T_7	MS + 2,4-D (2.0 mg/l) + NAA (1.5 mg/l)	No response	No response	No response	No response
8	T_8	MS + 2,4-D (3.0 mg/l) + NAA (2.0 mg/l)	No response	No response	No response	No response
9	T_9	MS + 2,4-D (4.0 mg/l) + NAA (2.5 mg/l)	No response	No response	No response	No response
10	T_{10}	MS + 2,4-D (5.0 mg/l) + NAA (3.0 mg/l)	12.58±5.61	28.94±12.93	0.63±0.28	1.46±0.65
11	T_{11}	MS + 2,4-D (2.0 mg/l) + NAA (1.0 mg/l)	No response	No response	No response	No response
12	T_{12}	MS + 2,4-D (4.0 mg/l) + NAA (2.0 mg/l)	No response	No response	No response	No response
13	T_{13}	MS + 2,4-D (6.0 mg/l) + NAA (3.0 mg/l)	10.12±04.06	23.32±10.01	0.81±0.36	1.88±0.84
14	T_{14}	MS + 2,4-D (1.0 mg/l) + BAP (0.0 mg/l)	No response	No response	No response	No response
15	T_{15}	MS + 2,4-D (2.0 mg/l) + BAP (1.0 mg/l)	10.12±4.37	23.32±10.07	1.36±0.60	3.14±1.40
16	T_{16}	MS + 2,4-D (3.0 mg/l) + BAP (2.0 mg/l)	12.42±5.52	28.62±12.72	0.66±0.29	1.52±0.67
17	T_{17}	MS + 2,4-D (4.0 mg/l) + BAP (3.0 mg/l)	12.42±5.52	28.62±12.72	0.88±0.39	2.03±0.90
18	T_{18}	MS + 2,4-D (5.0 mg/l) + BAP (4.0 mg/l)	20.47±9.20	47.17±21.02	0.86±0.38	1.99±0.89
19	T_{19}	MS + BAP (1.0 mg/l) + IBA (1.0 mg/l)	12.58±5.61	28.99±12.93	0.70±0.31	1.63±0.72
20	T_{20}	MS + BAP (2.0 mg/l) + IBA (1.5 mg/l)	10.28±4.57	23.69±10.54	0.91±0.40	2.09±0.93
21	T_{21}	MS + BAP (3.0 mg/l) + IBA (2.0 mg/l)	12.58±5.61	28.99±12.93	1.00±0.45	2.32±1.03
22	T_{22}	MS + BAP (1.0 mg/l) + IBA (1.0 mg/l)	12.58±5.61	28.99±12.93	1.02±0.45	2.36±1.05
23	T_{23}	MS + BAP (2.0 mg/l) + IBA (1.5 mg/l)	12.85±5.72	29.62±13.19	0.76±0.34	1.77±0.78
24	T_{24}	MS + BAP (3.0 mg/l) + IBA (2.0 mg/l)	19.22±8.57	44.30±19.76	0.99±0.44	2.28±1.02
25	T_{25}	MS + BAP (4.0 mg/l) + IBA (2.5 mg/l)	12.58±5.61	28.99±12.93	1.92±0.85	4.43±1.97
26	T_{26}	MS + BAP (5.0 mg/l) + IBA (3.0 mg/l)	26.61±10.81	56.71±24.91	2.02±0.89	4.66±2.06
27	T_{27}	MS + BAP (1.0 mg/l) + NAA (1.0 mg/l)	19.22±8.57	44.03±19.76	1.25±0.56	2.89±1.29
28	T_{28}	MS + BAP (2.0 mg/l) + NAA (1.5 mg/l)	15.41±6.87	35.51±15.87	1.92±0.85	4.43±1.97
29	T_{29}	MS + BAP (3.0 mg/l) + NAA (2.0 mg/l)	16.26±7.26	37.47±16.74	1.92±0.85	4.43±1.97
30	T_{30}	MS + BAP (4.0 mg/l) + NAA (2.5 mg/l)	23.00±10.28	53.00±23.69	1.92±0.85	4.43±1.97
31	T_{31}	MS + BAP (5.0 mg/l) + NAA (3.0 mg/l)	10.28±4.57	23.69±10.54	1.90±0.85	4.39±1.96
Note :-All values are mean of three replicates.						

Table 2: Effect of different concentration of 2,4-D and NAA on rooting of *Withania somnifera.*

S. No.	Treatments	Average growth (mm) after 30 days Mean±S.E	Average growth (mm) after 60 days Mean±S.E
1	FMS + 3% sucrose + 2,4-D (1.0 mg/l) + NAA (1.0 mg/l)	No response	No response
2	FMS + 3% sucrose + 2,4-D (2.0 mg/l) + NAA (1.5 mg/l)	No response	No response
3	FMS + 3% sucrose + 2,4-D (3.0 mg/l) + NAA (2.0 mg/l)	No response	No response
4	HMS + 3% sucrose + 2,4-D (4.0 mg/l) + NAA (2.5 mg/l)	No response	No response
5	HMS + 3% sucrose + 2,4-D (5.0 mg/l) + NAA (3.0 mg/l)	12.58±5.61	28.94±12.93

Fig 1: *Withania sominifera* herb

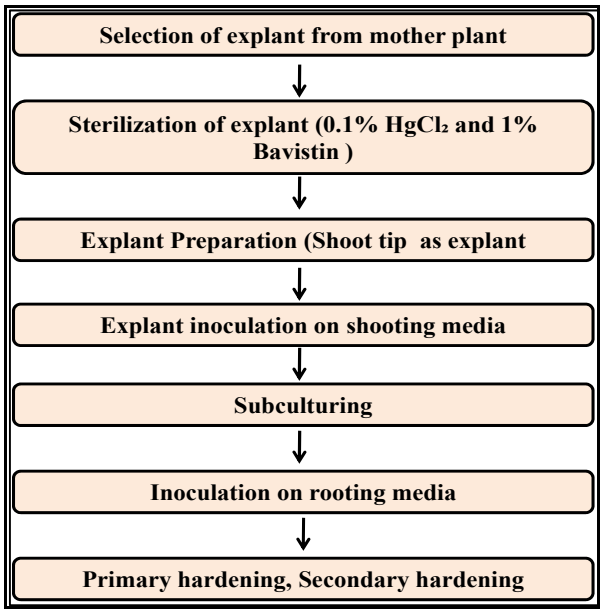

Fig 2: Procedure flow chart

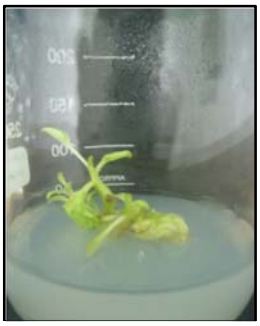

Fig 3: Multiple shoot induction after 30 days on BAP 5 mg/l + IBA 3 mg/l

Fig 4: Multiple shoot induction after 30 days on BAP (5.0 mg/l) + IAA (3.0 mg/l)

Fig 5: No response on 2,4-D 5 mg/l + NAA 3 mg/l

Fig 6: Shoot tip explants after sub-culturing on MS medium with BAP 3 mg/l + IBA 2 mg/l

Conclusion

The micropropagation of *Withania somnifera* has emerged as an effective technique for preserving its germplasm. This method is straightforward, highly efficient, and saves considerable time. A well-optimized protocol for *in vitro* culture was developed using different concentrations and combinations of cytokinins (BAP) and auxins (IAA, NAA, IBA, 2,4-D), with shoot tips serving as explants. Among the tested treatments, the highest number of shoots was obtained using a combination of BAP (5.0 mg/L) and IBA (3.0 mg/L), producing 24.61±10.81 shoots within 30 days and 56.71±24.91 shoots after 60 days. The second most effective treatment involved BAP (4.0 mg/L) and NAA (2.5 mg/L), generating 23.00±10.28 shoots at 30 days and 53.00±23.69 shoots at 60 days. Another treatment also resulted in 47.17±21.02 shoots after 60 days. Regarding shoot elongation, the treatment with BAP (5.0 mg/L) and IAA (3.0 mg/L) led to the longest shoots, reaching an average length of 6.04±2.69 cm after 60 days, followed by another treatment with a shoot length of 4.66±2.06 cm. These results indicate that MS medium supplemented with BAP (5.0 mg/L) and IBA (3.0 mg/L) is the most effective combination for promoting multiple shoot formation, yielding a maximum of 56.71±24.91 shoots per culture. This study establishes a rapid and efficient propagation system for *Withania somnifera*, providing a dependable approach for large-scale cultivation and conservation of this important medicinal plant.

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