Optimization of culture conditions for *in vitro* propagation of medicinally important plant *Withania somnifera* (Ashwagandha)

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Abstract
Withania somnifera Dunal is an evergreen, upright, perennial herb commonly named Indian ginseng which falls in Solanaceae family. The plant is recognized worldwide for its therapeutic properties, it contains secondary metabolites of flavonoids class and many active ingredients like withanolide, withaferin etc. Effort was made to optimize the rejuvenation process for this medicinally important plant. MS media were adjusted for leaf regeneration and for callus induction of this plant.

In vitro rejuvenation of leaf sprouts was accomplished from nodal segments cultivated on media complemented with BAP (1.0-3.0 mg/L) alone and in synergy with KN i.e. BAP (1.5-4.0 mg/L) + KN (1.0-2.0mg/L). The quantity of shoots per explants was recorded highest in MS media complemented with BAP (2.0 mg/L) + KN (2.0 mg/L) with 85% shooting response and average shoot length approximately 3.6 cm. was promoted by BAP (4.0 mg/L)+KN (2.0 mg/L). Different explants (leaf and nodal segments) were used to establish callus. Induction of callus was detected best in MS media complemented with 2,4- D 2.0 mg/L after 20-30 days (75%).

Keywords: *Withania somnifera*, 2,4-Dichlorophenoxy acetic acid, Benzylaminopurine, MS -Murashige and Skoog medium.

Introduction

Withania somnifera (L.) Dunal is a vertical, perennial herb commonly called "Indian ginseng" or "Indian winter cherry" which falls in Solanaceae family, rises up to an elevation of 0.30-150 m. In Indian Ayurvedic system of medicine as a Rasayana, ashawagandha is considered very valued herb. Being a medicinal plant, it is used for various kinds of disease treatments and specially in various nervous disorders as nervine tonic.[18] *W. somnifera* grows all over the arid parts in India and is widely grown in areas of Uttar Pradesh, Madhya Pradesh, northwestern parts of the India like Gujarat and Rajasthan and plains of Punjab. *Withania somnifera* has various medicinal properties.

It contains secondary metabolites of flavonoids class and many active ingredients like withanolide, withaferins etc. Numerous earlier studies indicate that it has anti-stress, anti-cancerous, antioxidant, anti-inflammatory and revitalizing properties.[9]

The utmost significant secondary metabolites are Withanolides which are present in rhizome in Ashwagandha. They are utilized in therapy of various disorders like cardiac diseases, rheumatism, tuberculosis and inflammatory conditions. There are also some evidences suggesting the anti-cancerous properties of ashwagandha in various cancers like breast, colon and prostate cancers[6] and some studies have also provided the mechanism of action of its active compound against cancer cells.[22] Other use of this plant is in improving immunity.[25] It is also beneficial as abortificient, amoebicidal, analgesic, antibacterial, antifertility and antispasmodic agent.[1] Roots of ashwagandha are also used in tranquilizing for senile frailty and preclusion of Alzheimer's disease.[3,16,19,23]

Traditional method for propagation of *Withania somnifera* is mainly carried out by seeds, but for propagation, the healthy and mature seeds are not always accessible because of poor feasibility of seed that exist only for a year.[15] Due to its high medicinal values, exploitation of this plant is being carried out on large scale for commercial purposes. Because of its overexploitation, it also has been now comprised in the list of threatened species by the IUCN.[7,21] Hence an efficient regeneration method for the development of this plant is required. Conventional propagation methods have proved to be insufficient to meet this challenge. Tissue culture technology can be utilized as an alternative method because of the advantage of this technology by producing good quality disease-free plantlets regardless of the external environment conditions.

Material and Methods

The young growing apical buds and nodes of *Withania somnifera* were used as explants gathered from FRI, Dehradun. The experiment was completed in PTC Lab of Biotechnology Department of Dolphin Institute of Biomedical and Natural Sciences, Manduwala, Dehradun.

Nodal part in the plant which was fresh and young was used as explant and washed. Initially these nodal segments were put under running water to wash off all the dirt particles from surface upto 15 minutes. After washing, segments were put in 1% tween-20 for 1 minute and washed with running water under tap for five times. Now different concentrations of $HgCl_2$ (0.1 and 0.2%) were allowed for surface sterilization

for different time duration under laminar hood and then washed with autoclaved water repeatedly two to three times. These nodal segments were again surface sterilized using 70% ethanol for 30 seconds and then finally washed with autoclaved water for 5 times to remove all the surfactants.

To absorb the surplus water, washed nodal segments were placed on the filter paper. The damaged, dead cells that had been exposed to chemicals were subsequently removed by cutting the nodal explants from both sides. Finally, the explants were arranged on the MS medium so that half of the nodal explants were in contact with the media.

Media Preparation: MS (Murashige and Skoog) media was used with 3% (w/v) sucrose. The medium was complemented with different growth regulators of different concentration. After adding all components, pH of the media was set to 5.8 before adding agar and then solidified with 0.8% (w/v) agar and finally autoclaved at 121ºC for 20 min.

The different nodal segments were cultivated on MS basal media complemented with different growth regulators (BAP, KN and 2,4-D) for shoot rejuvenation and callus initiation respectively. From this culture experiment, appropriate quantity and mixture of these growth promoters were identified in which the response was maximum. All inoculations were performed in the laminar airflow chamber. Scalpels and forceps were flame sterilized before and after each inoculation. The explants were cultured and incubated under white fluorescent light (2000 lux) for 16/8 hours light and dark conditions at 25 ± 2ºC for shoot initiation and proliferation. For callus initiation, explants were incubated in dark conditions at 25 ± 2°C.

Statistical analysis: After 3–4 weeks of culture, data for tests on callus induction, shoot initiation and shoot length were recorded. Three times each test was done in each experiment. The data was examined using the One-way ANOVA approach and presented as the mean SE of three replicated trials.

Results

Standardization of sterilization technique: During standardization of sterilization of explants with two different concentrations of $HgCl_2$ (0.2 and 0.1 % dipped for 3 and 5, 7 minutes), it was observed that highest viable culture was obtained with 0.1% $HgCl_2$ dipped for 7 minutes and explants remained green and healthy. However, increasing the time period also decreased the viability of explant. It was also observed that higher concentration of $HgCl_2$ also decreased the viability of explants with respect to time period. Thus, the finest results were found with 0.1% $HgCl_2$ dipped for 7 minutes (Table 1).

Table 1
Sterilization of explants with different concentration of $HgCl_2$ for different time duration

S.N.	Conc. of $HgCl_2$	Time duration	Observation
1	0.2%	3 min	Brown/Dead
2	0.1%	5 min	Green/ less contaminated
3	0.1%	7 min	Green/Healthy

Table 2
Analysis of data on Shoot Induction

Source of Variation	SS	df	MS	F	F crit	CD
Between Regulators	297.7693	4	74.44233	3462.434***	3.47805	0.1197
Within Regulators	0.215	10	0.0215			
Total	297.9843	14				

*** specifies significance at probability level p< 0.001

Table 3
Observation of shoot induction in explants on medium complemented with different type growth regulators (BAP and KN) with different concentrations

Regulators	Percentage of response (%)	Shoot induction Mean±SE	Significance	CD
BAP_1	12%	0.967 ± 0.088	*** (P<0.001)	0.1197
BAP_3	40%	3.967 ± 0.088		
$BAP_{1.5}+KN_1$	50%	5.033 ± 0.060		
BAP_2+KN_2	85%	12.00 ± 0.011		
BAP_4+KN_2	74.1%	12.00 ± 0.058		

Interpretation: The analysis revealed that the variation among the regulators was highly significant (at p< 0.001). The regulators BAP_2KN_2 and BAP_4KN_2 show maximum shoots induction. BP_1 shows the minimum shoot induction.

***In vitro* shoot induction and proliferation:** The action of different cytokinins on nodal explants for shoot induction was evaluated in this research work. Several shoots were directly induced when nodal segments cultured on MS media containing BAP (1.0-3.0mg/L) unaided and also in synergy with Kinetin. Nodal segments cultured on MS media lacking growth regulators failed to induce multiple shoots. Among all the concentrations of BAP (1.5 - 4.0 mg/L) + KN (1.0 - 2.0mg/L) used for shoot initiation, the MS media complemented with BAP and Kinetin treated explants achieved higher response (number of shoots per explant and average shoot length) than those treated with BAP alone.

After inoculation, explants were observed on daily basis for 2-3 weeks to check the growth and development of shoots by measuring their length. It was observed that shoots started generating after 1 week of inoculation. From this study, it was observed that BAP(2.0mg/L) + KN(2.0mg/L) was suggestively most active for encouraging shoot regeneration and proliferation (85 and 15) shooting (%) and number of shoots per explant respectively (Table 2) and BAP (4.0mg/L) + KN (2.0mg/L) promoted highest average length of shoot approx. 3.6cm (Table 3).

***In vitro* Callus initiation:** Nodes and leaf sections were cultivated on MS media complemented by varied concentrations of growth regulator 2,4-D (0.5- 2.0 mg/L) for callus production. Dissimilar response shown by different explants was due to the different concentration of 2-4-D. Nodal segments showed maximum light brown callusing of 75% from internodal segments cultivated on media added with 2.0 mg/L 2,4-D (Table 4). Callus initiation appeared after 20-30 days and callus was pale brown in color, compressed and crisp.

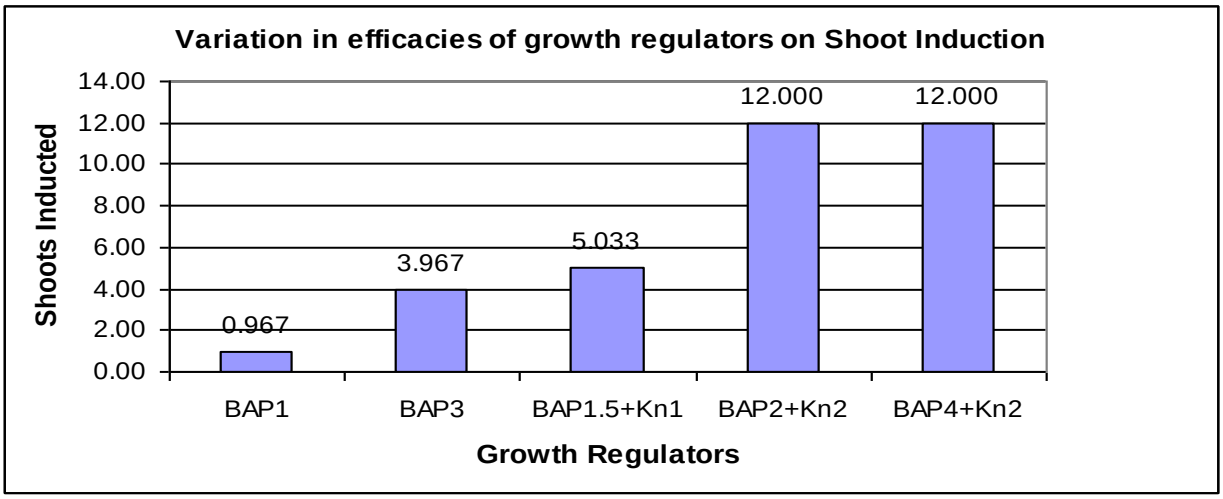

Figure 1: Graphical illustration of the effect of MS medium with growth regulators on shoot induction

Table 4
Analysis of data on Shoot Lengths

Source of Variation	SS	Df	MS	F	F crit	CD
Between Regulators	17.71829	4	4.429573	728.548***	3.47805	0.0637
Within Regulators	0.0608	10	0.00608			
Total	17.77909	14				

*** specifies significance at probability level p< 0.001

Table 5
Average shoot lengths of explants on MS medium with different concentration of plant growth regulators

Regulators	Percentage of length induction	Shoot length induction Mean±SE	Significance	CD
MSB_1	5.8%	0.467 ± 0.09	*** (P<0.001)	0.0637
MSB_3	13%	1.300 ± 0.01		
$MSB_{1.5}K_1$	15.6%	1.600 ± 0.01		
MSB_2K_2	18.3%	2.583 ± 0.04		
MSB_4K_2	22.3%	3.613 ± 0.02		

Interpretation: The analysis revealed that the variation among the regulators was highly significant (at p< 0.001). The regulator MSB_4KN_2 shows maximum shoots length closely followed by (MSB_2KN_2) whereas MSB_1 shows the minimum shoot lengths.

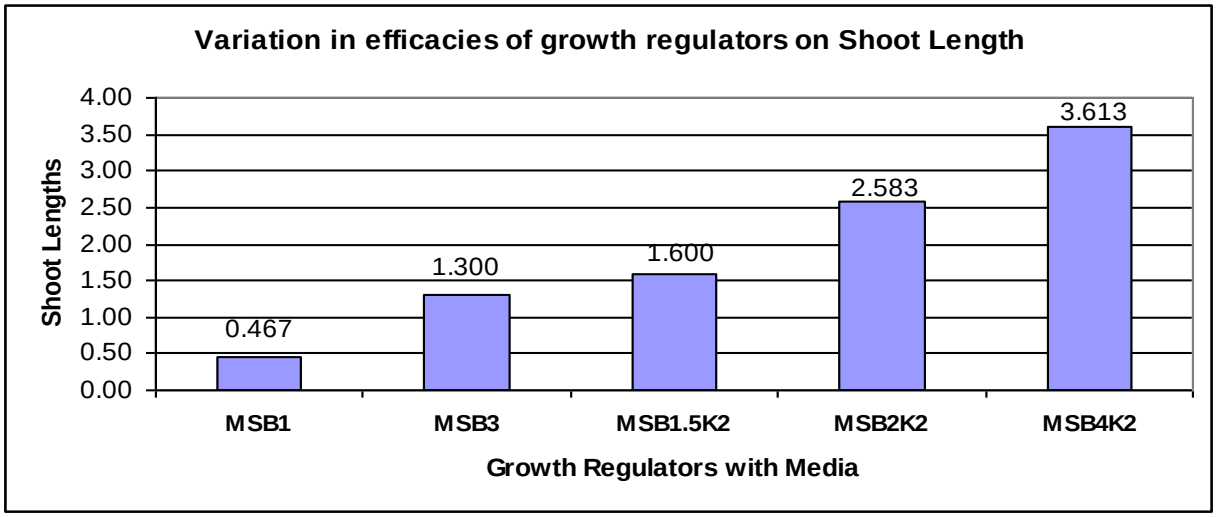

Figure 2: Graphical illustration of variation in efficacies of various growth regulators in MS media on shoot length induction

Table 6
Analysis of data on Callus Induction

Source of Variation	SS	Df	MS	F	F crit	CD
Between Regulators	47.95167	2	23.97583	5634.007***	5.143253	0.0533
Within Regulators	0.025533	6	0.004256			
Total	47.9772	8				

*** specifies significance at probability level p< 0.001

Table 7
Observation of the callus induction in explants on medium added with different growth regulators with different concentrations

Regulators	Percentage of response	Callus induction Mean±SE	Significance	CD
2,4-D0.5	8.3%	0.533 ± 0.038	*** (p<0.001)	0.0533
2,4-D1.5	33.3%	2.017 ± 0.044		
2,4-D2.0	75.2%	6.000 ± 0.029		

Interpretation: The analysis revealed that the variation among the regulators was highly significant (at p< 0.001). The regulator 2,4-D (2.0) shows maximum callus initiation whereas 2, 4- D (0.5) shows minimum callus initiation.

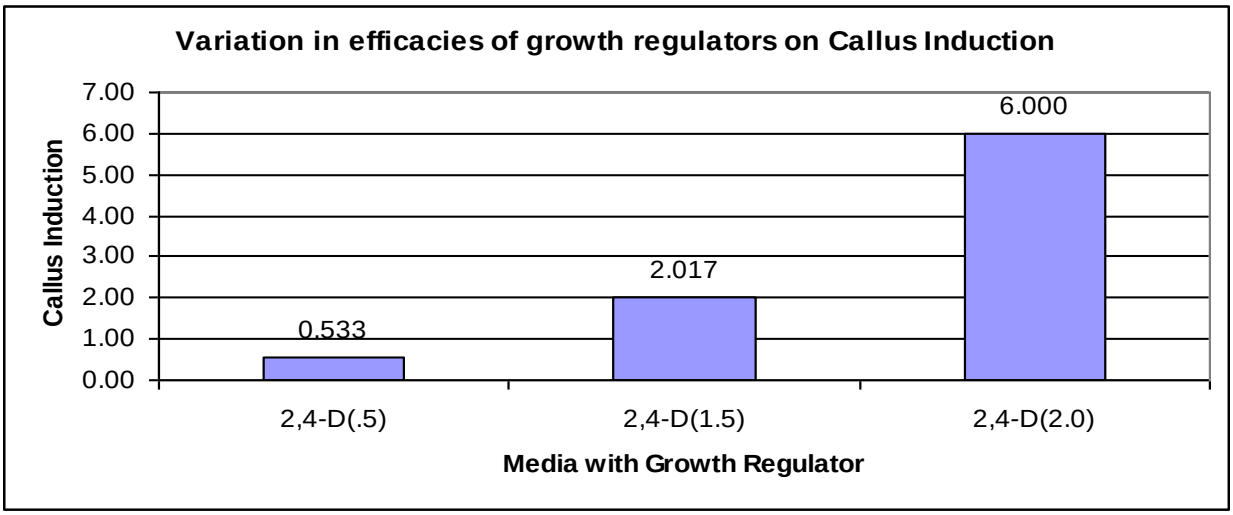

Figure 3: Graphical illustration of the effect of MS media with different regulators on callus initiation

Figure 4: Bud Breaking

Figure 5: Shoot Elongation

Discussion

The results presented that a very simple and effective clonal propagation can be positively achieved for *W. somnifera* through *in vitro* tissue culture technique. The sterilization procedure adopted resulted in 80-90% aseptic and responsive cultures. The sterilization of explants was carried out at concentration of $HgCl_2$ for different time and was allowed to culture on basal MS medium. The explants were treated with 0.2% and 0.1% for 3 minute and 7 minutes respectively showed survivability. Similar finding was also related with the work done by Autade et al[1].

Likewise, Mir et al[9], Bhoyar et al[2], Misra et al[10], Fatima and Anis[4] studied effects of different concentrations of surface disinfectants ($HgCl_2$) along with variation in treatment time duration.

Figure 6: Shoot Proliferation

In the current study, BAP and KN were used equally together for shoot proliferation and growth in the initial stages and showed good shooting response (85%) when used in concentration with BAP (2.0mg/L) and KN (2.0mg/L) and better results were obtained. This is in accordance with the reports of Kumar et al[7] who also achieved high shoot multiplication rate in MS medium comprising BAP along with KN. Comparable results were also testified by Darwesh et al[3]. Stimulating effects of BAP on formation of multiple shoots has been testified earlier in various therapeutic plants.[13,20] MS media with nodal explants supplemented

with BAP for shoot development were also reported by Autade et al.[1]

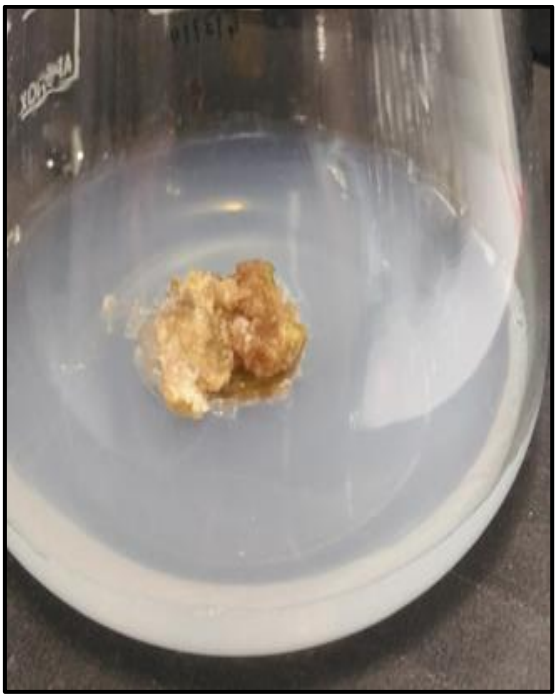

Figure 7: Induction of Callus

2,4-D (2.0mg/L) was optimized superior for the callus initiation. This is in accordance with Valizadeh and Valizadeh[23] who reported the highest callus growth in MS media complemented with 2,4-D and KN. Similar findings were also conveyed by Bhoyar et al.[2] Nagpal et al[11] also reported best callus induction in medium containing 2,4-D. The results were in accordance with Rani et al.[13] *In vitro* tissue culture technique is an important technique that is used for rapid production of diseases free plants in order to preserve the genome of rare, endangered and therapeutic plants.

Conclusion

Withania somnifera, a valuable therapeutic plant is being exploited on a large scale for its medicinal values on a commercial level. *In vitro* technology can be used as an alternative for large scale propagation of this medicinally important plant. From our research, we can conclude that the phytoregulators (BAP+KN) are the best suited combination for shoot initiation and propagation of *W. somnifera*.

As we have seen, plant growth regulators in MS medium induce shoot bud growth, because of which, the plant grows more effectively. These results show that it is possible to propagate *W. somnifera* endangered plant species through *in vitro* clonal propagation in order to overcome the threat of the extinction.

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