



## ***In vitro* propagation of *Withania somnifera* (L.) Dunal. from shoot apex explants**

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**Abstract:** *In vitro* rapid micropropagation of *Withania somnifera*; has been achieved using shoot apex explants, when cultured on MS medium with different auxins: Indole Butyric Acid (IBA) 0.5-4 mg/l, Indole Acetic Acid (IAA) 2 mg/l and Naphthalene Acetic Acid (NAA) 0.5-3 mg/l and cytokinin: Benzyl Amino Purine (BAP) 0.5-4 mg/l. NAA and BAP stimulated direct as well as callus mediated multiple shoot. IBA alone and in combination with IAA stimulated rhizogenesis, while IBA with NAA proliferated callus-mediated rhizogenesis. The rooted plantlets through sequential hardening process could be established in the field.

**Keywords:** Micropropagation, Shoot apex explant, Rhizogoneisis, *Withania somnifera*

### **INTRODUCTION**

*Withania somnifera* (L.) Dunal. is an erect, evergreen, perennial shrub. It is a potential medicinal plant that has been used medicinally in the treatment of tuberculosis, rheumatism, inflammatory conditions, cardiac diseases, and it is used as a general tonic, anti-stress drug and as an antitumor, antibiotic, anticonvulsant besides being an ingredient of several ayurvedic preparations (Mishra *et al.*, 2000; Kumar *et al.*, 2011 and Patel and Krishnamurthy, 2013). It is also useful as abortifacient, amoebicide, anodyne, bactericide, contraceptive, and spasmolytic (Asthana and Raina, 1989). With the increasing demand for the roots of *W. somnifera* in larger quantities, it is cultivated almost as a commercial crop in India. The production of *W. somnifera* roots through conventional methods of cultivation (seed) is less than the requirement due to numerous reasons viz. poor yield, takes long time, poor viability of seeds, susceptibility of the seeds and seedlings to fungal infections like seedling mortality and blight, leaf blight, seed rotting etc (Misra *et al.*, 1997). This medicinally significant plant species has been depleted from its natural habitat and is now included in the list of endangered species (Kanungo and Sahoo, 2011; Patel and Krishnamurthy 2013) by the International Union for Conservation of Nature and Natural Resources (Kavidra *et al.*, 2000; Supe *et al.*, 2006). The rapid multiplication of *W. somnifera* by tissue culture techniques can help to solve these problems

and the benefits are extensive in the agricultural world. The plant tissue culture is desirable in order to satisfy production demands, which has been developed for the mass propagation of medicinal plants (Thomas and Shankar, 2009), the conservation of particular and endangered species (Nagesh, 2008; Offord and Tyler, 2009; Kumar *et al.*, 2011). The present study was undertaken to establish an efficient protocol for rapid plant regeneration from shoot apex explants of *W. somnifera* (L.) Dunal.

### **MATERIALS AND METHODS**

The shoots of about 1.5-2 cm long were collected from apex of 2 years old genotype WA-20 of *W. somnifera* for standardization of regeneration protocol in Department of Agricultural Biotechnology and Molecular Biology at Rajendra Agricultural University Pusa, Bihar. Collected explants were first washed with running tap water (15 minutes) and then surface sterilized with Tween-20 (2 drop/100ml), 70% ethanol for 1-1 minutes and were rinsed in distilled water thrice. Then, they were taken to the laminar air flow chamber where treated with 0.1% HgCl<sub>2</sub> for 2 minutes and washed with sterile distilled water. It was then inoculated in the appropriate MS medium, which contained sucrose (3%), and pH (5.8). Cutting edge of the explants should make a direct contact with medium. The cultures were incubated at 24±2°C under 2000 lux light intensity provided by white fluorescent lamp for 16 hours photoperiod. The basal MS medium was used with derived supplementation of

phytohormones for callusing and induction of shoots and roots. The MS basal media were supplemented with different auxins: Indole Butyric Acid (IBA) 0.5-4 mg/l, Indole Acetic Acid (IAA) 2 mg/l and Naphthalene Acetic Acid (NAA) 0.5-3 mg/l and cytokinin: Benzyl Amino Purine (BAP) 0.5-4 mg/l. The rooted shoots were removed from the culture tube and washed with sterile water to remove traces of agar sticking on the roots and dipped in 0.2% (W/V) bavistin solution for 20 min. Shoots were transferred to plastic pots filled with sterile soil and kept in green house. Initially, the pots were covered with polybags. After 20 days, they were uncovered and shifted gradually from shade to sunlight.

## RESULTS AND DISCUSSION

The present study of *in vitro* propagation of *W. somnifera* includes multiple shoot formation from shoot tip explants, elongation of regenerated shoots and rooting and hardening of the elongated shoots.

**Callus and multiple shoot formation from shoot tip explants:** In this process axillary buds were forced to produce multiple shoots with higher concentration of cytokinin in comparison to auxin. Shoot tip explants of *W. somnifera* were inoculated on MS basal media with different concentrations (0.5, 1.0, 2.0, 3.0, 4.0) of BAP with (0.5, 1.0, 2.0, 3.0) of NAA for multiple shoot formation. Sprouting of shoot buds from the swollen basal part of the shoot apex explants was noticed after 10 days of incubation in presence of NAA and BAP. All the combinations of NAA with BAP developed multiple shoot buds with varying degree of callus at the base except MS medium 0.5mg/l BAP with 3.0 mg/l and 2.0 mg/l NAA having

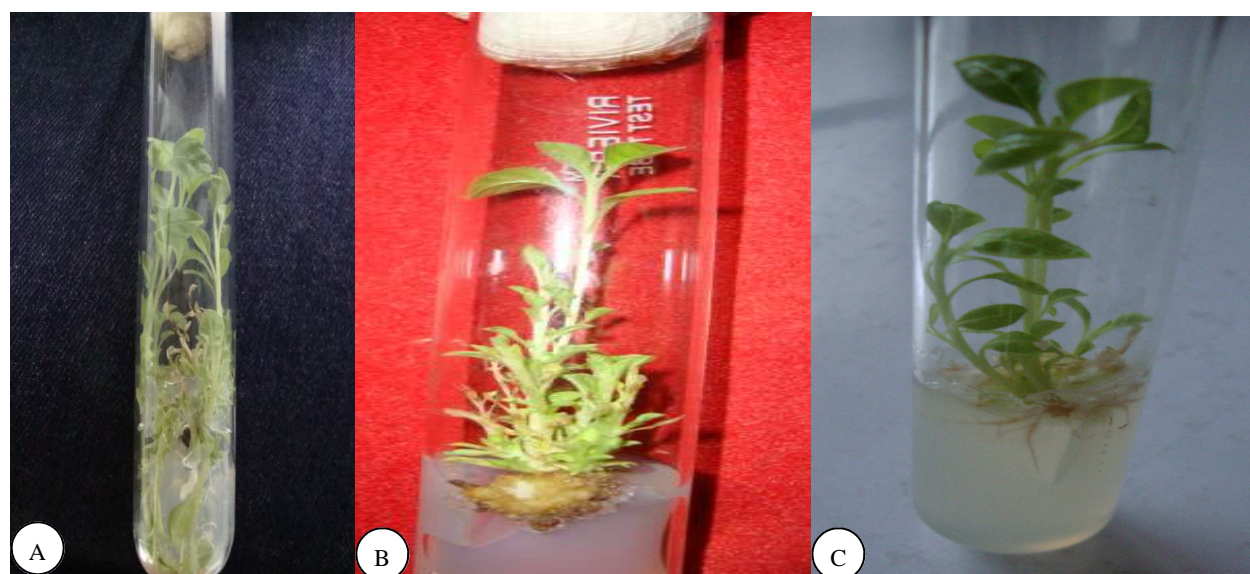
tendency to direct shoot regeneration (Plate 1a, b). The amount of callus formation and the number of shoot bud proliferation per explants gradually increased with the increasing concentrations of BAP along with a concomitant suppression of shoot elongation. Similar results were also reported by Ghosh and Banerjee (2003) in *Rauvolfia tetraphylla*. The best response of shoot differentiation was achieved at 4 mg/l BAP with 1mg/l NAA where about 7.56 shoot buds per explants as well as callus induction could be observed (Table 1). Ojha and Choudhary (2013) reported that stem explants of *Stevia rebaudiana* cultured on MS media having 0.5mg/L-1 BAP + 0.5 mg/L-1 NAA showed 65 % shoot proliferation while only 14% callusing found on higher conc. of 2.0 mg/L-1 BAP + 2.0 mg/L-1 NAA. Panwar *et al.* (2011) observed that the callus derived from leaves and stem explants cultured on MS medium containing BAP (2.0 mg l<sup>-1</sup>) with NAA (0.5 mg l<sup>-1</sup>) was most effective combination for multiple shoot induction and shoots per culture in *R. serpentina*. For multiple shoot induction BAP has played a key role. Atta-Alla and Van (1997) produced an average of 6.6 shoots of *Yucca alifolia* on MS medium with 4.4, 8.9 or 17.8µm BA+2.7µm NAA. The combination of NAA with BAP was effective in shoot bud production (Ghosh and Banerjee, 2003; Ojha and Choudhary, 2013). In the present study, the best callusing from the base of explants were recorded on 4 mg/l BAP and 1mg/l NAA.

**Elongation of elongated shoots:** Of the different tired concentration of NAA and BAP, the best result of shoot elongation and direct shoot regeneration were observed at 0.5mg/l BAP with 3.0mg/l and 2.0mg/l NAA (Plate 1a). The shoots attained a length of 4.22 cm

**Table 1.** *In vitro* response from shoot apex explants of *W. somnifera* cultured on different compositions of (BAP, NAA) MS media.

Phytohormone in mg/l		Response (%)	No. of shoots (Mean±SD)	Average length shoot in cm (Mean±SD)	Induction of callus*	Nature of callus
BAP	NAA					
0.5	0.5	73	3.28±0.41	3.34±0.35	+	S,F,W
0.5	1.0	53	4.44±0.27	3.64±0.38	+	S,F,WB
0.5	2.0	57	3.36±0.41	4.12±0.28	-	-
0.5	3.0	55	3.26±0.62	4.22±0.41	-	-
1.0	1.0	65	6.18±0.36	3.22±0.78	+	S,F,WB
1.0	0.5	70	6.22±0.31	2.58±0.20	++	S,F,WB
2.0	0.5	68	6.50±0.64	2.60±0.32	+++	F,B
3.0	0.5	74	6.68±0.51	2.76±0.42	+++	F,WB
4.0	0.5	75	6.80±0.50	2.98±0.59	++++	S,F,WB
4.0	1.0	85	7.56±0.59	2.28±0.26	+++++	F,W

\*Visual observation; number of + sign indicates relative amount of callus; - = no response; W= white; B=brown; F=friable; SF=semifriable.



**Plate 1.** *In vitro* responses from shoot apex in genotype WA-20 of *W. somnifera* (a) Initiation of multiple shoots (b) Callus mediated shoot differentiation (c) Rooting of regenerated shoots .

**Table 2.** *In vitro* response from shoot apex explants of *W. somnifera* cultured on different compositions of MS media.

Phytohormone in mg/l			Response (%)	No.of root/shoots (Mean±SD)	Average length root in cm (Mean±SD)	Induction of callus	Nature of callus
IBA	IAA	NAA					
0.5			89	10.06±0.53	5.07±0.47	-	-
1.0			78	6.66±1.08	4.49±1.08	-	-
1.5			78	6.23±0.33	3.72±0.20	-	-
2.0			63	5.12±0.65	3.40±0.68	-	-
3.0			39	2.25±0.39	1.08±0.10	-	-
2.0	2.0		56	1.64±0.40	1.50±0.26	-	-
4.0	2.0		48	1.75±0.14	1.76±0.23	-	-
4.0		2.0	68	1.69±0.12	2.00±0.28	+++	F,W
2.0		2.0	75	2.34±0.20	1.32±0.13	+++	S,F,WG

\*Visual observation; number of + sign indicates relative amount of callus; - = no response; W= white; B=brown; F=friable; SF=semifriable.

within 25 days of inoculation (Table 1). From this study, it is inferred that 0.5mg/l BAP with 3.0mg/l NAA was the best for the elongation of regenerated shoots from the shoot tip explants. Increasing the concentration of BAP augment the multiplication of shoot buds markedly, but did not affect the elongation of shoots. However, Ghosh and Banerjee (2003) reported that increasing concentration of NAA did not affect the multiplication of shoot bud, but suppressed the elongation of shoots when shoot apex of *Rauvolfia tetraphylla* cultured on MS media supplemented with different concentrations of NAA and BAP. Adhikari and Pant (2013) observed the best growth of callus in the MS medium supplemented with 0.5 mgL<sup>-1</sup>BAP +

1.5 mgL<sup>-1</sup> NAA after 8 weeks in stem disc of *W. somnifera*. In contrast to present study, BAP and IBA were used to induce elongation of multiple shoots in *Vigna mungo* (Agnihotri et al., 2001) and *Momordica charantia* (Agrawal and Kamal, 2004).

**Rooting and hardening of regenerated shoots:** In the present study, auxins were successfully used to produce roots from regenerated shoots. The elongated shoots were excised from the shoot clumps and inoculated on MS medium containing different concentrations IBA (0.5,1.0,1.5,2.0,3.0) individually and in combinations with IAA and NAA with concentrations (2.0,4.0).The root proliferation was observed after 14-18 days of inoculation. Of these

different concentrations of auxins tried, the maximum percentage (89%) of rooting as well as number of root per culture (10.06) with average elongated length of root (5.07) was observed at 0.5mg/l IBA (Plate 1c). The roots were long, pale white, robust with root hairs. Pawar *et al.* (2001) observed rhizogenesis with a frequency of 90 ±5 on MS media containing IAA at 4µM and NAA at 8 µM whereas Govindaraju *et al.* (2003), also observed the root induction of the differentiated shoots on the half strength MS media (both liquid and solid) with IBA (0.5-1.0mg/l) alone and in combination with IAA (0.5mg/l) in *W. somnifera*. IBA alone was found more suitable for root induction than NAA and IAA (1.64-2.34no.of root/shoot). Similar results were observed in which maximum shoots rooted when cultured on MS medium containing optimal concentration of IBA (Manickam *et al.*, 2000; Rani *et al.*, 2003; Kumar *et al.*, 2011 and Udayakumar *et al.*, 2013). This suggested that the addition of IBA to rooting medium improved rooting efficiency of shoots. IBA used alone in low concentration showed positive results of rooting while in combination with NAA and IAA at high concentration did not show positive results (1-4 roots/shoot) of rooting. Patel and Krishnamurthy (2013) observed that the regenerated shoots of *W. somnifera* cultured on MS media with 0.4mg/l NAA+0.4mg/l IBA and produced maximum rooting (80-90%) with 4-5 roots/shoot.

After 30 days of inoculation on rooting medium, the rooted plantlets were removed from the culture tube and washed with distilled water. These *in vitro* derived plantlets were transferred to plastic pots containing mixture of sand and FYM in 1:1 ratio for 18 days for hardening. A maximum of 68% of plantlets were hardened successfully. It was concluded from this study that plant regeneration from shoot apex of *W. somnifera* through callus phase offered a great potential in genetic transformation of this important species. The protocol can be exploited for *in vitro* generating new genetic variability and production of bioactive constituents from the callus.

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