Journal of Applied Horticulture, 13(2): 150-153, 2011

Optimization of growth regulators and explant source for micropropagation and cost effective *ex vitro* rooting in 'Poshita' winter cherry (*Withania somnifera* L.)

A.A. Waman[1]*, B.N. Sathyanarayana[1], K. Umesha[1], Balakrishna Gowda[2], T.H. Ashok[3], A.M. Rajesh[1] and R.G. Guruprakash[1]

[1]*Plant Tissue Culture Laboratory, Department of Horticulture,* [2]*Department of Forestry and Environmental Sciences,* [3]*Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bengaluru-560 065, Karnataka, India. *E mail: ajit.hort595@yahoo.co.in*

Abstract

Withania somnifera (L.) Dunal., one of the 32 prioritized medicinal plants of India, is well known for its importance in the *Ayurveda* system of medicine. Attempt was made to establish an efficient plant regeneration protocol for a commercial cultivar 'Poshita' and to acclimatize the plantlets *ex vitro,* so as to reduce the cost. Results revealed that shoot induction was possible only after an intervening callus phase, irrespective of the concentration of growth regulators present in the nutrient pool. Nodal explant cultured on MS media supplemented with 1 mg L^{-1} BAP + 0.5 mg L^{-1} NAA showed superiority in callus induction capacity over epicotyls and leaves. Nodal segments when cultured on a media containing BAP alone could induce shoots in cent per cent explants. Highest number of shoots (5.8) was obtained in media containing 2 mg L^{-1} BAP. Number of adventitious buds was found to be maximum (13) with epicotyl explant and 1 mg L^{-1} BAP combination. Nodal explants cultured on high concentration of BAP (4 mg L^{-1}) showed highest incidence of malformed shoots (4.3). A total of 66.7 % plantlets could root and establish *ex vitro* even without auxin treatment and survival rate increased (87.5%) with increase in IBA concentration to 500 mg L^{-1}. The present protocol can be exploited on a commercial scale to obtain maximum benefits from the improved cultivar. Furthermore, *ex vitro* hardening can help to reduce the cost of production and thereby make the tissue culture industry more profitable.

Key words: Ashwagandha, Indian ginseng, *In vitro*, low cost options, medicinal plant, plant growth regulators, *Withania somnifera*

Introduction

Withania somnifera (L.) Dunal., commonly known as *Ashwagandha,* Indian ginseng or winter cherry, belongs to botanical family Solanaceae. The drug is officially used as a sedative in the Indian Pharmacopoeia. Tuberous roots are known to possess anti-tumour, anti-arthritic, anti-stress and anti-inflammatory properties (Malhotra *et al.,* 1961). Roots contain tropane alkaloids, several pyrazole alkaloids, withasomnine and steroidal lactones, withaferine-A and withanolides, and are being used as astringent, acrid, bitter, somniferous, stimulant, aphrodisiac, diuretic and tonic (Prajapati *et al.,* 2003). This species has a great scope due to its wide ranging pharmacological activities, but one of the major constraints in utilizing natural population is the existence of plant to plant variability. A large number of factors have been reported to influence the commercial exploitation of this crop, such as, seed propagation, limited seed viability (Rani and Grover, 1999), inherent variability due to sexual recombinations (Glotter *et al.,* 1973 and Kirson *et al.,* 1971), difficulties in fixing the variability generated by recombination and natural inability of the species to regenerate through vegetative propagation (Sen and Sharma, 1991).

The practical solution for the above mentioned difficulties lies in the use of improved cultivars and *in vitro* techniques for multiplication of this crop. Although *in vitro* studies have been made in ashwagandha (Deka *et al.,* 1999; Govindraju *et al.,* 2003; Kulkarni *et al.,* 1996; Manickam *et al.,* 2000; Rani *et al.,* 2003; Rani and Grover, 1999; Sivanesan and Murugesan, 2005; Sen and Sharma, 1991 and Telli *et al.,* 1999), there is a need to develop a protocol for rapid multiplication of a high yielding promising commercial cultivar 'Poshita', as different genotypes may respond in a different manner under *in vitro* condition. Secondly, a large number of commercial laboratories do not root micro-cuttings *in vitro*, because it is labour-intensive and expensive. This process has been estimated to account for approximately 35- 75% of the total cost of micropropagation (Debergh and Maene, 1981). Single step rooting of plantlets *ex vitro* and their acclimatization can bring down the production cost significantly. The present study describes an *in vitro* regeneration protocol and *ex vitro* rooting of *W. somnifera* cv. 'Poshita'.

Materials and methods

Plant material: Seeds of *W. somnifera* cv. Poshita were obtained from Central Institute of Medicinal and Aromatic Plants (CIMAP), Resource Centre, Bengaluru, India. Seeds were soaked in distilled water for 24 h, washed with 5% (v/v) Teepol detergent solution for 5 min, disinfected with 0.1% $HgCl_2$ for 12-13 min, rinsed for 3 times using sterile double distilled water, and placed in test tubes (150 x 25 mm) containing half strength MS medium (Murashige and Skoog, 1962) with 1 % sucrose without any growth regulators. Seeds were incubated for germination at 25 °C in the dark for a week and then under white fluorescent light (40

µmol m^{-2} s^{-1}, Philips, India) with 16: 8 h light: dark cycle. Sections of epicotyls (0.8-1.0 cm), leaves (0.8-1.0 cm^2) and nodes (0.5 cm) were prepared from *in vitro* grown seedlings of 30 days old. The seedlings at this age were 8-10 cm tall and on an average they had about 4-6 pairs of leaves. All the available epicotyls, leaves and nodes of each seedling were used to prepare explants.

Induction of multiple shoots, *ex vitro* rooting and acclimatization: Explants, after preparation, were inoculated on the full strength MS medium containing comparatively higher doses of 6-benzylaminopurine (BAP - 1, 2, 4 and 8 mg L^{-1}) in combination with lower doses of naphthalene acetic acid (NAA- 0, 0.5 and 1.0 mg L^{-1}) to get direct shoot induction and its subsequent proliferation. The explants were also inoculated on basal MS media which served as control. Cultures were maintained at 25 °C under white fluorescent light (40 µmol m^{-2} s^{-1}, Philips, India) with 16: 8 h light: dark cycle. Micro shoots (about 10 cm long) obtained from shoot induction experiments were kept for *ex vitro* root induction as well as hardening in a single step. Cuttings were dipped in indolebutyric acid (IBA) solution for 30 s and planted in small polypropylene containers (cups) containing agro peat- *Varshamruth* (Varsha Enterprises, Bengaluru) as a hardening medium. The plantlets were then kept in the tunnel house to maintain high humidity. Survival percentage was recorded at weekly interval.

Data analysis: Three explants were put in every bottle for direct regeneration studies and each bottle served as a replication. Five replications were maintained per treatment. For hardening experiment, eight plantlets were maintained in every treatment and each plantlet was taken as a replicate. Each experiment was repeated twice with similar results and the data presented are of one representative experiment. Statistical analysis was done according to the completely randomized design (simple and factorial analysis of variance).

Result and discussions

Irrespective of the explant and growth regulators used, callus was observed as an intermediate phase during the regeneration (Table 1). Among different explants used, nodal segment was found to be superior in induction of callus. In case of growth regulators, BAP in presence of NAA was far more superior to BAP alone. Nodal explant cultured on 1 mg L^{-1} BAP + 0.5 mg L^{-1} NAA, with a score of 6.33 was found to be best for callus induction.

Shoot induction response: Cent percent shoot induction was noticed from nodal segments when cultured on a media containing BAP alone (4 and 8 mg L^{-1}) (Table 2, Fig. 1A). Positive effects of BAP in shoot induction when used alone have already been reported in *Withania somnifera* L. (Rani and Grover, 1999 and Sivanesan and Murugesan, 2005). Leaf explants could induce shoots in 33.3 per cent explants on a medium with 8 mg L^{-1} BAP (Table 2, Fig. 1B). However, according to Sivanesan and Murugesan (2005), shoot induction from leaf explants was possible only at concentrations of BAP less than 1.5 mg L^{-1} and at higher concentrations only calli were produced. The reason for these contrasting findings may be attributed to the variation in genotypes and concentrations of endogenous hormones. In present study, an improved cultivar 'Poshita' was used, whereas, their report indicates the use of plants collected from wild. Epicotyls, on the other hand, could produce shoots only on the media supplemented with 1 and 2 mg L^{-1} BAP (Table 2, Fig. 1C). Kulkarni *et al.* (2000) observed that hypocotyls could produce shoots only on media containing 0.5 mg L^{-1} BAP, which shows that shoots obtained are completely dependent upon the interaction between explant and cytokinin.

Table 1. Effect of BAP+NAA combinations (mg L^{-1}) on callus induction from epicotyls (E), nodal (N) and leaf (L) explants of *W. somnifera* L. cv. 'Poshita'

BAP + NAA (G)	Week 4				Week 6			
	Explants (Ex)				Explants (Ex)			
	E	N	L	Mean	E	N	L	Mean
1+0.0	1.30	3.10	0.70	1.70	2.30	3.30	1.30	2.30
1+0.5	4.13	6.07	2.93	4.38	4.87	6.33	4.07	5.09
1+1.0	2.10	4.33	2.93	3.12	2.20	5.00	3.73	3.64
2+0.0	1.50	4.60	1.07	2.39	2.40	4.80	1.20	2.80
2+0.5	3.40	4.90	2.67	3.66	3.60	5.10	3.41	4.04
2+1.0	3.07	4.00	2.07	3.05	3.60	4.53	2.86	3.66
4+0.0	0.80	4.00	3.07	2.62	1.07	5.00	3.73	3.27
4+0.5	3.81	4.27	3.00	3.69	4.60	4.47	4.20	4.42
4+1.0	3.40	5.27	3.80	4.16	3.87	6.13	5.07	5.02
8+0.0	1.60	3.53	3.40	2.84	1.93	4.93	4.67	3.84
8+0.5	3.87	5.47	3.93	4.42	4.87	6.27	5.07	5.40
8+1.0	3.53	5.07	3.80	4.13	3.93	5.87	5.20	5.00
Mean	2.71	4.55	2.78	3.35	3.27	5.14	3.71	4.04

	Week 4		Week 6	
LSD	*P*=0.05	*P*=0.01	*P*=0.05	*P*=0.01
Ex	0.28	0.37	0.33	0.43
G	0.55	0.73	0.65	0.86
Ex x G	0.96	1.26	1.13	1.49

Table 2. Shoot induction response (%), number of adventitious buds and leaves, and number of malformed shoots produced from various explants of *W. somnifera* L., cv. 'Poshita' on BAP and NAA supplemented media

Explants	Treatment (mg L^{-1})		Percentage response	Adventitious		Mean no. of malformed shoots
	BAP	NAA		Mean no. of buds	Mean no. of leaves	
After 4 weeks of culture						
Epicotyl	1	0.0	20.0	10.0	0.0	2.0
Node	1	0.0	50.0	2.2	7.0	0.0
Node	1	0.5	13.3	1.0	2.0	1.0
Node	1	1.0	6.7	2.0	4.0	0.0
Epicotyl	2	0.0	10.0	0.0	0.0	1.0
Node	2	0.0	80.0	3.7	9.3	3.0
Node	2	0.5	20.0	3.5	4.0	0.0
Node	4	0.0	100.0	4.0	7.6	4.0
Node	4	0.5	60.0	1.0	2.0	0.0
Node	4	1.0	20.0	3.3	4.0	3.0
Node	8	0.0	93.3	4.4	8.1	2.0
Leaf	8	0.0	33.3	3.5	6.6	0.0
After 6 weeks of culture						
Epicotyl	1	0.0	30.0	13.0	17.0	2.0
Node	1	0.0	60.0	2.7	4.3	0.0
Node	1	0.5	13.3	1.0	2.0	1.0
Node	1	1.0	6.7	3.0	0.0	0.0
Epicotyl	2	0.0	20.0	2.0	4.0	1.0
Node	2	0.0	90.0	4.6	8.2	3.5
Node	2	0.5	20.0	3.5	4.0	0.0
Node	4	0.0	100.0	4.2	8.5	4.3
Node	4	0.5	66.7	1.0	2.0	0.0
Node	4	1.0	20.0	3.7	6.0	3.0
Node	8	0.0	100.0	4.3	8.3	2.0
Leaf	8	0.0	33.3	4.0	0.8	0.0

Table 3. Shoot induction on different combinations of growth regulators from *de novo* and pre existing meristems of different explants of *W. somnifera* L. cv. 'Poshita'

Explants	Treatment (mg L^{-1})		*De novo* meristem			Pre-existing meristems		
	BAP	NAA	No. of shoots	Mean shoot length (cm)	No. of leaves	No. of shoots	Mean shoot length (cm)	No. of leaves
After 4 weeks of culture								
Epicotyl	1	0.0	3.0	1.2	4.7	0.0	0.0	0.0
Node	1	0.0	2.0	0.9	4.0	3.0	1.6	5.6
Node	1	0.5	1.5	0.7	2.9	0.0	0.0	0.0
Node	1	1.0	2.0	1.2	5.0	0.0	0.0	0.0
Epicotyl	2	0.0	2.0	0.8	6.5	0.0	0.0	0.0
Node	2	0.0	4.0	1.5	5.2	3.0	1.1	6.3
Node	2	0.5	0.0	0.0	0.0	1.0	1.2	3.0
Node	4	0.0	2.7	1.2	5.7	1.8	1.1	5.3
Node	4	0.5	2.0	0.9	6.3	1.0	0.8	4.0
Node	4	1.0	2.0	0.8	4.0	0.0	0.0	0.0
Node	8	0.0	2.8	1.2	4.9	2.1	1.3	6.2
Leaf	8	0.0	2.0	1.4	2.5	0.0	0.0	0.0
After 6 weeks of culture								
Epicotyl	1	0.0	3.0	1.2	5.2	0.0	0.0	0.0
Node	1	0.0	2.5	0.8	3.0	2.7	1.7	8.4
Node	1	0.5	1.5	0.8	4.0	0.0	0.0	0.0
Node	1	1.0	2.0	1.2	0.0	0.0	0.0	0.0
Epicotyl	2	0.0	2.0	0.9	7.0	0.0	0.0	0.0
Node	2	0.0	4.0	1.3	6.1	1.8	0.7	4.1
Node	2	0.5	0.0	0.0	0.0	1.0	1.2	3.0
Node	4	0.0	2.7	1.5	6.4	2.2	1.0	4.6
Node	4	0.5	2.0	0.9	5.8	1.0	0.8	4.0
Node	4	1.0	2.0	0.8	4.0	0.0	0.0	0.0
Node	8	0.0	2.7	1.3	5.3	2.4	1.3	6.2
Leaf	8	0.0	3.0	1.4	6.3	0.0	0.0	0.0

***De novo* meristem production:** Nodal explants when cultured on media supplemented with 2 mg L^{-1} BAP recorded maximum number of shoots (4) both after 4 and 6 weeks of culture, whereas, epicotyls cultured on the same concentration of BAP resulted in production of maximum number of leaves (6.5 and 7.0 at 4th and 6th week) (Table 3, Fig. 2A). These results are in accordance with the earlier findings in ashwagandha (Rani and Grover, 1999), *Solanum pseudo capsicum* (Baburaj and Gunasekaran, 1994) and *Solanum khasianum* (Bhalsing and Maheshwari, 1997). Highest number of adventitious buds, 10 and 13 (after 4 and 6 weeks), was obtained from epicotyls when cultured on a medium containing 1 mg L^{-1} BAP (Fig. 2B).

Shoots from pre-existing meristems: After 4 weeks of culture, number of shoots was found to be maximum (3) with node + 1 and 2 mg L^{-1} BAP combinations (Table 3). Maximum shoot length of 1.6 cm was obtained with 1 mg L^{-1} BAP. At 6th week, nodal segment on 1 mg L^{-1} was found to be superior in all the parameters. These results are in accordance with the earlier findings by Kulkarni *et al.* (2000) in ashwagandha.

***Ex vitro* rooting and acclimatization:** Data pertaining to establishment of plantlets after IBA treatments is presented in the Table 4. A total of 66.7 per cent plantlets could survive even without auxin treatment. But the survival rate increased with increase in IBA concentration upto 500 mg L^{-1} (87.5%) and remained stable at 1000 mg L^{-1}. Micropropagated plants showed good growth and uniformity *ex vitro* and exhibited normal development. The probable reason for good response

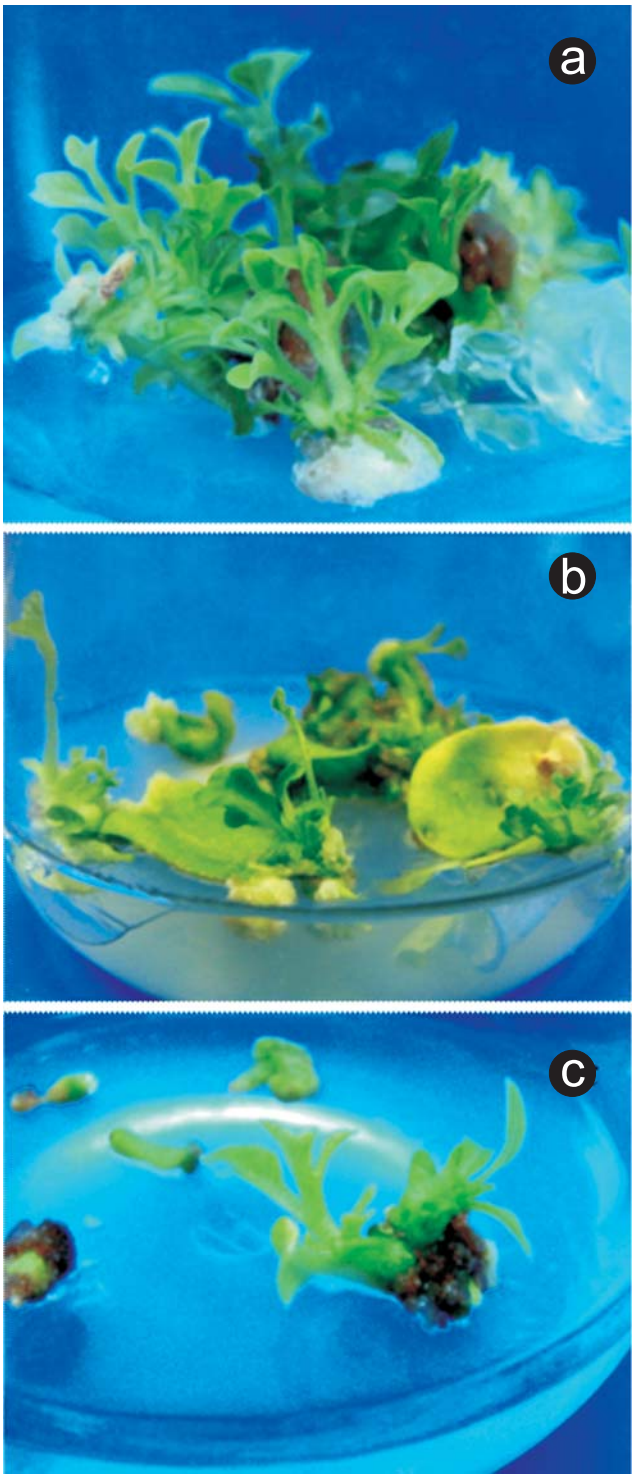

Fig. 1. Direct shoot regeneration from various explants of *W. somnifera* cv. 'Poshita'; a. Shoot regeneration from nodal explants on medium containing 2 mg L^{-1} BAP, b. Shoot regeneration from leaf explants on medium containing 8 mg L^{-1} BAP, c. Shoot regeneration from epicotyl explants on medium containing 1 mg L^{-1} BAP.

with *Varshamruth* may be attributed to the optimum conditions such as good water retention, richness in nutrients, aeration and good drainage provided by the substrate. These favourable conditions may also have resulted in better vascular connections between root and shoot. This increase in survival may be due to a more developed and efficient rooting system as observed in Rhododendron (Almeida *et al.,* 2005). On contrary, Grout and Aston (1977) observed abnormal transition zone between root and shoots when microshoots rooted *in vitro*. The vascular

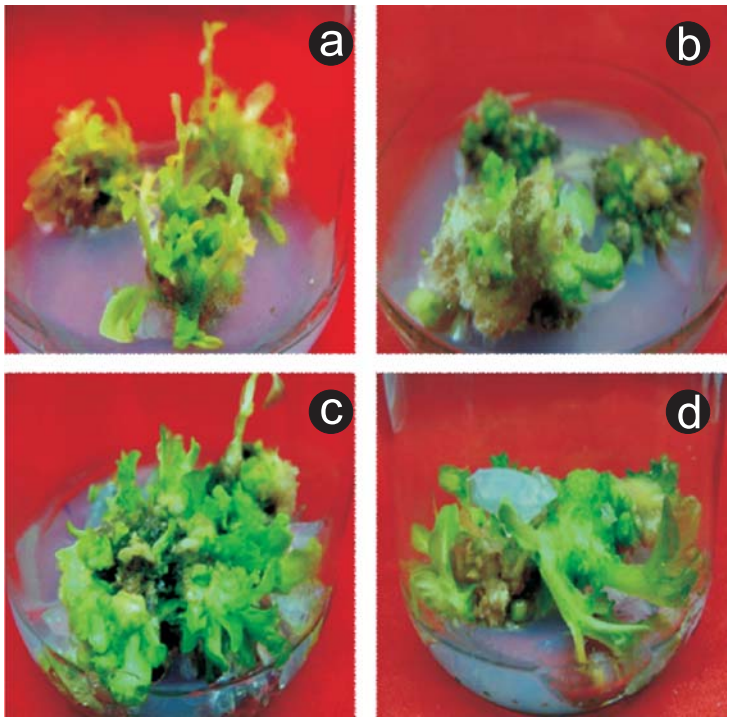

Fig. 2. Different kind of responses obtained during direct regeneration of *W. somnifera* cv. 'Poshita' on media containing various combinations of BAP and NAA; a. *De novo* shoots, b. Adventitious buds, c. Adventitious leaves, d. Malformed shoots

Table 4. Effect of different concentrations of IBA on *ex vitro* rooting of micro-cuttings of *W. somnifera* L. cv. Poshita

Treatments (mg L^{-1})	Cumulative percentage of plant death				Plant survival (%)
	Week 1	Week 2	Week 3	Week 4	
Control (no auxin)	0	22.2	22.2	33.3	66.7
IBA 250	0	12.5	12.5	25.0	75.0
IBA 500	0	12.5	12.5	12.5	87.5
IBA 1000	0	12.5	12.5	12.5	87.5

connection was poorly formed and narrow when observed at the time of plantlets removed from culture. This restricted water uptake from the root into the shoot. Rani *et al.* (2003) established rooted plantlets of ashwagandha on simple soil media with 60% survival rate. In contrast, the present one step rooting and acclimatization technique with 87.5% survival not only save the time and labour but is cost effective too.

The study revealed that different genotypes respond differently to the treatments under *in vitro* conditions and thus a specific protocol is needed to be developed for different varieties. The present protocol can be exploited on a commercial scale to obtain maximum benefits from the improved variety 'Poshita'. Furthermore, the concept of *ex vitro* hardening can help to reduce the cost of production and thereby make the tissue culture industry more profitable.

Acknowledgements

The authors are grateful to CIMAP, RC, Bengaluru for the supply of seeds. Research Fellowship awarded by Indian Council for Agricultural Research, New Delhi to the senior author is acknowledged.

References

Almeida, R., S. Gonc`Alves and A. Romano, 2005. *In vitro* micropropagation of endangered *Rhododendron ponticum* L. subsp. *baeticum* (Boissier & Reuter) Handel-Mazzetti. *Biodiver. Conserv.,* 14: 1059-1069.

Baburaj, S. and K. Gunasekaran, 1994. Regeneration of plants from leaf callus cultures of *Solanum pseudo capsicum* L. *Indian J. Expt. Biol*., 32: 141-143.

Bhalsingh, S.R. and V.L. Maheshwari, 1997. *In vitro* culture and regeneration of *Solanum khasianum* and extraction of solasodine. *Plant Cell Rpt.,* 16: 39-40.

Debergh, P.C. and L.J. Maene, 1981. A scheme for commercial propagation of ornamental plants by tissue culture. *Sci. Hort*., 14: 335-345.

Deka, A.C., M.C. Kalita and A. Baruah, 1999. *In vitro* micropropagation of a potent herbal medicinal plant – *Withania somnifera*. *Environ. Ecol.,* 17: 594-596.

Glotter, E., I. Kirson, A. Abraham and D. Lavie, 1973. Constituents of *Withania somnifera Dunal.* XIII- The withanolides of an Indian chemotype- The withanolides of chemotype III. *Tetrahedron,* 29: 1353-1364.

Govindraju, B., S.K. Rao, R.B. Venugopal, S.G. Kiran, C.P. Kaviraj, R. Srinath and S. Rao, 2003. High frequency plant regeneration in ashwagandha *(Withania somnifera):* an important medicinal plant. *Plant Cell Biotechnol. Mol. Biol*., 4: 49-56.

Grout, B.W.W. and M.J. Aston, 1977. Transplanting of cauliflower plants regenerated from meristem culture I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. *Hort. Res.,* 17: 1-7.

Kirson, I., E. Glotter, D. Lavie and A. Abraham, 1971. Constituents of *Withania somnifera* Dunal. XII. The withanolides of an Indian chemotype. *J. Chem. Soc*., 11: 2032-2044.

Kulkarni, A.A., S.R. Thengane and K.V. Krishnamurthy, 1996. Direct *in vitro* regeneration of leaf explants of *Withania somnifera. Plant Sci.,* 119: 163-168.

Kulkarni, A.A., S.R. Thengane and K.V. Krishnamurthy, 2000. Direct shoot regeneration from node, internode, hypocotyls and embryo explants of *Withania somnifera. Plant Cell Tissue Org. Cult.*, 62: 203-209.

Malhotra, C.L., P.K. Das, N.S. Dhalla and K. Prasad, 1961. Studies on *Withania ashwagandha* Kaul.- Part-3: The effect of total alkaloids on the cardiovascular system and respiration. *Indian J. Med. Res*., 49: 448-460.

Manickam, V.S., M.R. Elango and R. Antonisamy, 2000. Regeneration of Indian ginseng plantlets from stem callus. *Plant Cell Tissue Org. Cult.,* 62: 181-185.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-479.

Prajapati, N.D., S.S. Purohit, A.K. Sharma and T. Kumar, 2003. *A Handbook of Medicinal Plants: A Complete Source Book.* Agribios, Jodhpur, India.

Rani, G. and I.S. Grover, 1999. *In vitro* callus induction and regeneration studies in *Withania somnifera. Plant Cell Tissue Org. Cult*., 57: 23-27.

Rani, G., G.S. Virk and A. Nagpal, 2003. Callus induction and plantlet regeneration in *Withania somnifera* (L.). *In vitro Cell Dev. Biol.– Plant*., 39: 468-474.

Sen, J. and A.K. Sharma, 1991. Micropropagation of *Withania somnifera* from germinating seeds and shoot tips. *Plant Cell Tissue Org. Cult.*, 26: 71-73.

Sivanesan, I. and K. Murugesan, 2005. *In vitro* adventitious shoot formation from leaf explants of *Withania somnifera* (L.). *Plant Cell Biotechnol. Mol. Biol*., 6: 163-166.

Telli, N.P., N.H. Patil, H.M. Pathak, S.R. Bhalsingh and V.L. Maheshwari., 1999. *Withania somnifera* (Ashwagandha) regeneration through meristem culture. *J. Plant Biochem. Biotechnol.,* 8: 109-111.

Received: December, 2010; Revised: August, 2011; Accepted: September, 2011