Pak. J. Bot., 43(4): 1837-1842, 2011.

AN ATTEMPT TO CONSERVE *WITHANIA SOMNIFERA* (L.) DUNAL - A HIGHLY ESSENTIAL MEDICINAL PLANT, THROUGH *IN VITRO* CALLUS CULTURE

JYOTI RANJAN ROUT*, SANTI LATA SAHOO AND RITARANI DAS

Biochemistry and Molecular Biology Laboratory,
P.G. Department of Botany, Utkal University, Vani Vihar, Bhubaneswar-751004, Odisha, India
**Corresponding author: E-mail: routjr@gmail.com*

Abstract

A simple effective protocol was developed for conservation and plant propagation through callus cultures of *Withania somnifera* (Ashwagandha). Seed germination percentage reached a maximum value of 64.3% on ½ MS + GA_3 0.25 mg/l at third week of culture. Three different basal media compared for seed germination, MS was most effective. Out of 25 combinations of growth regulators evaluated, MS + 1.0 mg/l BA + 1.0 mg/l 2, 4-D found to be best for callus induction and proliferation regardless to explants. Among the four different explants tested, *In vivo* leaf explant was found most suitable for callus induction, proliferation and fresh weight gain. The highest callus induction frequency percentage 86.4% was recorded with *In vivo* leaf explant whereas, 43.4% in *In vitro* leaf explant at day 30 on MS augmented with 1.0 mg/l BA + 1.0 mg/l 2,4-D. Among different growth regulator combinations tested in augmentation with MS for shoot initiation and elongation, 2.0 mg/l BA + 1.0 mg/l NAA was the best eliciting a maximum of 82.3% shoot induction with highest shoot number 4.8 shoots/callus. The original callus was sub-cultured 2 times on fresh shoot multiplication medium after each harvest of the shoots. Of three different auxins tested for *In vitro* rooting, IBA was most effective compared to IPA and NAA. Half-strength MS medium containing IBA at an optimum concentration of 2.0 mg/l induced rooting in 83.1% of the *In vitro* derived shoots. The rooted plantlets were acclimated and eventually established in soil.

Introduction

Withania somnifera (L.) Dunal is an important medicinal plant that belongs to the family Solanaceae. It is popularly known as Ashwagandha or Winter Cherry (Andallu & Radhika, 2000). The plant is popularly known in India by different vernacular names like Punir (Hindi), Ashvaganda/Ashwagandha (Oriya & Bengli), Aksan (Punjab), Amukkira (Tamil), Tilli (Marathi) etc (Anonymous, 1988). It is described as an herbal tonic and health food in Vedas and considered as 'Indian Ginseng' in traditional Indian system of medicine (Singh *et al.,* 2001). It is a green shrub found throughout the drier parts of India, Baluchistan, Pakistan, Afghanistan, Sri Lanka, Congo, South Africa, Egypt, Morocco and Jordan. In India, it is widely grown in the provinces of Madhya Pradesh, Uttar Pradesh, plains of Punjab and northwestern parts of India like Gujarat and Rajasthan (Bhatia *et al.,* 1987). It is generally used as anti-inflammatory, anticancer, anti-stress and immune-modulator, adaptogenic, central nervous system, endocrine and cardiovascular activities, respectively (Bhattacharya *et al.,* 1997a; Rai *et al.,* 2003; Ahmad *et al.,* 2005; Mohanty *et al.,* 2004). *W. somnifera* is known to modulate the oxidative stress markers of the body. The root extract significantly reduced the lipid peroxidation (Dhuley, 1998) and increased the superoxide dismutase (SOD) and catalase (CAT) activity, thus possessing a free radical scavenging property (Panda & Kar, 1997). The active constituents of plant (Withaferin A, Sitoindosides VII–X) are reported to have an antioxidant activity which may contribute at least in part to the reported antistress, immune-modulatory, cognition facilitating, anti-inflammatory and anti-ageing properties (Bhattacharya *et al.,* 1997b).

Traditionally *W. somnifera* is propagated from seeds, but the mature and healthy seeds are not always available for germination. The viability period of seeds is very short and their germination is also poor (Vakeswaran & Krishnasamy, 2003). To meet the growing demand for pharmaceutical based industry, it is found necessary to multiply this species by adopting *in vitro* techniques. To our knowledge during the past years, successful *In vitro* plant regeneration has been achieved by using various explants for this medicinally important plant species (Sen & Sharma, 1991; Ray & Jha, 2002; Siddique *et al.,* 2004; Sivanesan & Murugesan, 2008).

The requirement of the plant species is now met from natural population and pharmaceutical companies, leading to their gradual depletion. Hence, there is an urgent need for conservation and cultivation of this plant specie for future use. For the above, an efficient *In vitro* propagation method may play an important role in rapid multiplication and germplasm conservation of this rare, threatened or endangered aromatic medicinally important herb. The present investigation was made out to explore the possibilities for developing a reproducible protocol for induction and establishment of callus cultures of Ashwagandha followed by plant regeneration via *de novo* shoot organogenesis from *In vivo* explants. Because plants regenerated through callus culture are genetically different to the source material, free from pathogens and it is possible to produce a huge number of plantlets within a very short time period.

Materials and Methods

Seed collection: The fruits of *W. somnifera* used for this experiment were collected from Ramakrishna Mission Ashrama, Narendrapur, Kolkata. The seeds were removed from the berries, air dried and stored in paper bags at room temperature (25 ^{0}C). The various experiments were conducted 22 days after collection. After the collection of seeds, the viability test checked by tetrazolium method (Karam &Al-Salem, 2001).

Sterilization and culture conditions: Only mature and healthy seeds were washed thoroughly under running tap water for 30 min followed by treatment with an aqueous solution of 5% (cm^3 m^{-3}) teepol (Reckitt's Colman, Kolkata, India) for 10 min., and rinsed 5 times with double distilled water. The seeds were then surface disinfected with an aqueous solution of 0.1% (g dm^{-3}) $HgCl_2$ (Hi-Media, Mumbai, India) for 5 min and rinsed

five times with autoclaved double distilled water. The disinfected seeds were inoculated in 150 cm^3 Erlenmeyer flasks (Borosil, Bangalore, India) containing eight different planting media i.e., MS (Murashige & Skoog 1962), BM (Blayde's Medium 1966), B_5 medium (Gamborg *et al.,* 1968), ½ MS (half-strength MS), ¼ MS (one fourth MS), ½ MS + GA_3 0.1mg dm^{-3}, ½ MS + GA_3 0.25 mg/l (gibberellic acid) and ½ MS + GA_3 0.50 mg/l with 7.0 gm/l agar (Hi-media, Mumbai, India) for the evaluation of basal media for seed germination. The pH of the medium was adjusted to 5.8 ± 0.5 before autoclaving at 121°C and 104 kPa for 15 min. The seeds were allowed to germinate at 25 ± 1°C, 60 % relative humidity and 35 μmol m^{-2} s^{-1} photon flux density provided by cool white fluorescent tubes (Philips, Bangalore, India).

Callus induction and shoot regeneration: The young healthy leaves and internode segments of *Withania somnifera* were collected from garden grown plant and from *In vitro* seedlings. The *In vivo* explants were surface disinfected as described above for seeds. The explants were inoculated in 300 cm^3 screw-capped jars (Excel corporation, Alleppey, Kerala, India) containing MS supplemented with 0.5-3.0 mg/l BA alone or in combinations with 0.5-4.0 mg/l NAA/2,4-D (2,4-dichlorophenoxi acetic acid) for callus induction and proliferation. Whereas, for shoot regeneration MS was supplemented with 0.5-3.0 mg/l BA (N^6 benzylaminopurine)/Kin (kinaten) alone or in combination with 0.5-2.0 mg/l NAA. The pH of all the media was adjusted to 5.8 ± 0.5 before gelling with 0.8% agar. The original callus was sub-cultured two times on fresh shoot multiplication medium after each harvest of the shoots.

Induction of rooting and acclimatization: Well developed *In vitro* shoots (3.0-3.5 cm) with 3-4 fully expanded leaflets were excised and inoculated to 100 cm^3 Erlenmeyer flasks (Borosil, Bangalore, India) containing ½ MS medium gelled with 0.7% (g dm^{-3}) agar. The medium was augmented with 0.5 - 3.0 mg/l IBA (indole-3-butyric acid) or IPA (indole-3-propionic acid) or NAA (α-naphthaleneacetic acid). After 5-7 days of root initiation the rooted shoots were transferred to auxin-free ½ MS medium for further elongation of roots. All the cultures were maintained under similar conditions as described earlier for seed germination. Well developed rooted shoots were removed from the culture vessels, washed gently under running tap water and planted in pots containing a sterile mixture of sand, soil and cow-dung manure in the ratio of 1:1:1 (v/v). The plantlets were kept in the green house for acclimatization (2-3 weeks) before their subsequent transfer to the field. Humidity was maintained by sprinkling water regularly. Plants were gradually exposed to the normal conditions and finally transferred to the Botanical garden of Utkal University.

Statistical analysis: For callus induction and proliferation experiment each treatment consisted of 05 culture vials and with 03 explants/flask whereas, for shoot regeneration experiment each treatment consisted of 05 culture vessels and the experimental unit was 04 callus/vessel. In the rooting experiment, each treatment consisted of 05 flasks and 02 shoot/experimental unit. The above three experiments was repeated thrice at an interval of 7 days. Visual observations of cultures showing callus induction and fresh weight gain (Initial weight of explant – Final weight of callus at days 30), shoot differentiation and the number of shoots/explant, shoot length, root number and root length were recorded at day 30. Data were analyzed using analysis of variance (ANOVA) for a completely randomized design (CRD). Duncan's New Multiple Range Test (DMRT) (Gomez & Gomez, 1984) was used to separate the means for significant effect.

Results and Discussion

The freshly collected seeds did not germinate satisfactorily in the soil condition (data not shown). Of the eight different planting media evaluated for seed germination ½ MS + 0.25 mg/l GA_3 showed the highest 64.3 % (Fig. 1 A), the lowest 11.3 % on B_5 and no response on BM basal medium was observed (Table 1). Among three basal media tested as planting media, MS was found to be the best as compare to B_5 and BM. Further seed germination percentage was found to be better on ½ MS compared to MS. According to Vakeswaran & Krishnasamy (2003), seed viability of *W. somnifera* was as low as 5% at the end of one year. The maximum percentage of seed germination (46%) obtained by Kambizi *et al.,* 2006 is still very low, considering the fact that tetrazolium chloride test indicated 78.8% seed viability. This discrepancy explains why there is low natural regeneration with consequent low plant populations of *W. somnifera* in the wild.

Table 1. Evaluation of germination percentage on different basal / planting media of *W. somnifera*.

Planting media	Germination days after inoculation	% of germination
BM	No response	No response
B_5	30	11.3^{f}
MS	28	38.1^{d}
½ MS	25	61.7^{b}
¼ MS	26	34.1^{de}
½ MS + GA_3 0.10 mg/l	22	55.9^{c}
½ MS + GA_3 0.25 mg/l	20	64.3^{a}
½ MS + GA_3 0.50 mg/l	20	61.3^{b}

Data pooled from 3 separate experiments each with 04 flasks containing 20 seeds per flask. Mean values within the column with same superscript are not significantly different (*p<0.05*; Duncan's New Multiple Range Test).

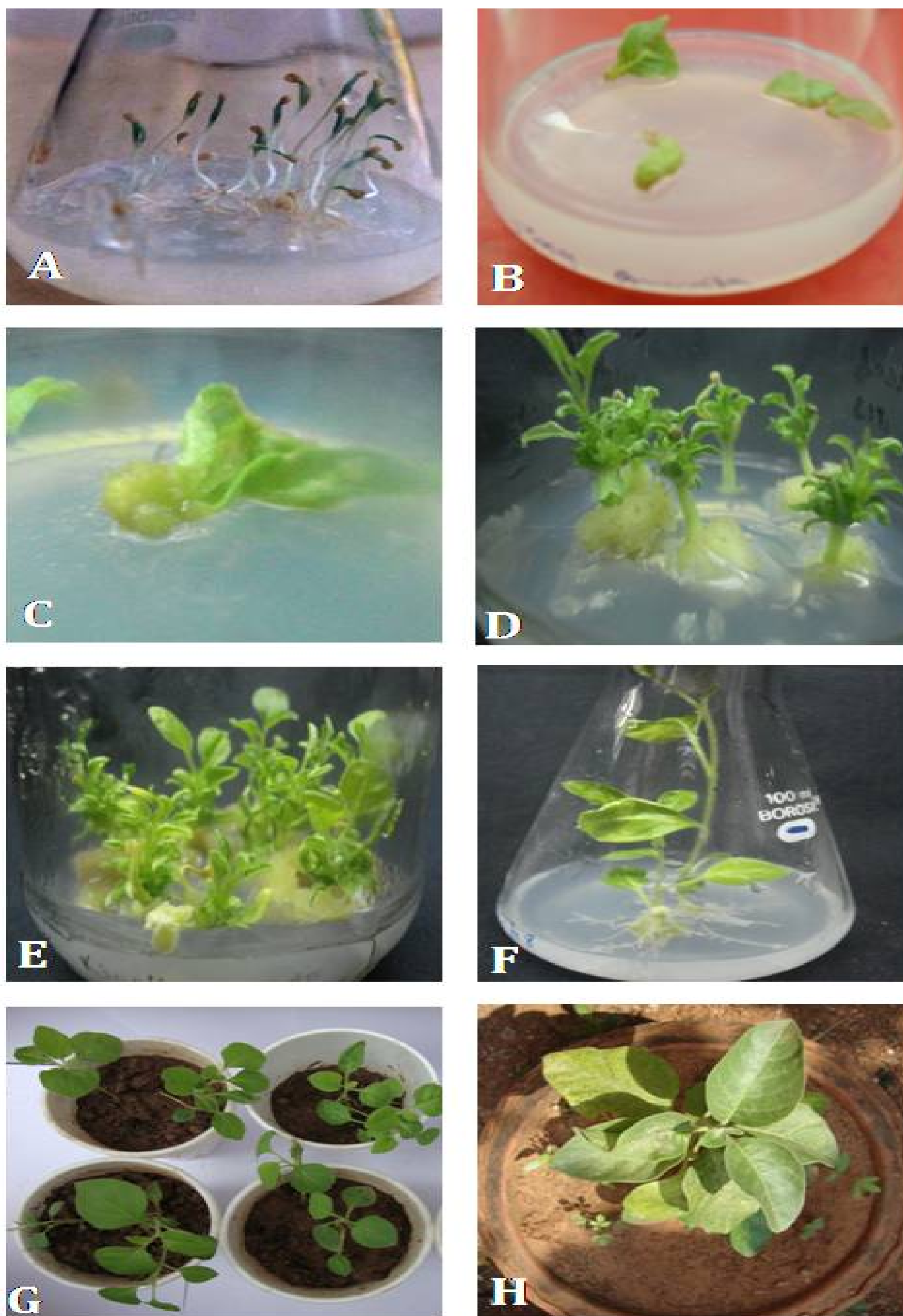

Fig. 1. a) *In vitro* seed germination of *Withania somnifera* L. in ½ MS + 0.25mg/l GA_3. b) Callus induction from *In vitro* leaf explants on MS + 1.0mg/l BA + 1.0mg/l 2,4-D at 30 d. c) Callus induction from *In vivo* leaf explants on MS + 1.0mg/l BA + 1.0mg/l 2,4-D at 30 d. d) Shoot initiation from *In vitro* leaf derived callus 2.0mg/l BA + 1.0mg/l NAA. e) Shoot initiation from *In vivo* leaf derived callus 2.0mg/l BA + 1.0mg/l NAA. f) A rooted shoot on ½ MS + 2.0mg/l IBA after 10 d. of culture. g) An acclimated plant in sterile mixture of sand, soil and cow-dung manure (1:1:1). h) A acclimated plant in garden soil.

From this study, it is evident that germination of seeds of this plant in the wild would be largely dependent on light and temperature conditions. These conditions and hormonal additives (gibberellins) should be taken into consideration in the economic cultivation of this valuable plant. The basal media devoid of GA_3 showed poor response and inclusion of GA_3 increased the germination percentage. On increasing the concentration of GA_3 the germination percentage was reduced. GA_3 has been found to induce and enhance the germination in *W. somnifera*, which is in consonance with earlier findings in *Silybum marianum* and *Lipidium sativum* (Saba, 1998). GA_3 is known to obviate the requirement of seeds for various environmental clues as it promotes germination and counteracts the inhibitory effect of ABA (Bewley & Black, 1994). Gibberellins appear to play a role in two different stages of germination. One occurs at the initial enzyme induction in their transcription from the chromosomes. The second is in the activation of reserve food mobilizing system (Hartman *et al.,* 1990).

No callus was formed when the explants were cultured on growth regulator free MS basal medium. Out of 55 combinations of growth regulators tested, MS + 1.0 mg/l BA + 1.0 mg/l 2,4-D found to be most suitable for callus induction and proliferation regardless to explants. Among the four different explants tested, *In vivo* leaf explant was found most suitable for callus induction, proliferation and fresh weight gain. The highest callus induction frequency percentage 86.4 % was recorded with *In vivo* leaf explants (Fig. 1C) whereas, 43.3% in *In vitro* leaf explant (Fig. 1B) at day 30 on MS augmented with 1.0 mg/l BA + 1.0 mg/l 2,4-D (Table 2). *In vivo* leaf explant showed maximum 81.6% organogenic callus, whereas, nodal explant showed low percentage in *Centella asiatica* (Mohapatra *et al.,* 2008). According to Siddique *et al.,* 2004 in *W. somnifera* 85% of organogenic callus was observed in nodal segments in MS medium containing 1.0 mg/l BAP and 2.0 mg/l Kin whereas, direct regeneration from *In vitro* leaf explants was observed by Kulkarni *et al.,* 1996. Similar type of response was observed in different medicinal plants as *Centella asiatica* (Mohaptra *et al.,* 2008) and *Vitex trifolia* (Hiregoudar *et al.,* 2006).

Compact green calli derived from *In vivo* leaf explant were cultured on the MS and MS augmented with cyokinin for shoot initiation. It was observed that MS without any cytokinin did not show any sign of shoot induction. Highest percentage of shoot regeneration (82.3 %) with 4.8 shoots per callus of shoot length 4.3cm was achieved on MS supplemented with 2.0 mg/l BA + 1.0 mg/l NAA (Table 3, Fig 1 D & E). The original callus was sub-cultured 2 times on fresh shoot multiplication medium after each harvest of the shoots. Shoot regeneration was reported to have occurred in leaf explants, but this was accompanied by callus formation (Malabadi & Nataraja, 2001), whereas direct shoot regeneration obtained in a combination of BA and NAA in *Lathyrus sativus* (Barik *et al.,* 2005). A cytokinin supplement to MS was essential to induce shoot proliferation. A combination of cytokinin and auxin improves the percentage of shoot regeneration as well as the shoot number and shoot length. Similar type of response was observed in medicinal plants like *W. somnifera* (Kulkarni *et al.,* 1996; Saritha & Naidu, 2007), *Abutilon indicum* (Rout *et al.,* 2009), *Bryophyllum pinnatum, Bryophyllum daigremontianum* (Naz *et al.,* 2009).

Table 2. Effect of growth regulators on the callus induction, proliferation and fresh weight gain of *Withania somnifera* at day 30.

MS + Growth regulators ($mg\ dm^{-3}$)	Callus induction frequency (%)				Callus fresh weight gain (g)			
	In vitro explants		*In vivo* explants		*In vitro* explants		*In vivo* explants	
	Leaf	Internode	Leaf	Internode	Leaf	Internode	Leaf	Internode
Control	----	----	----	----	----	----	----	----
BA								
0.5	08.1	06.0	09.1^{i}	07.3^{h}	0.31	0.27	1.21^{f}	1.22^{d}
1.0	24.4	17.1	28.0^{g}	25.3^{fg}	0.95	0.85	1.71^{de}	1.63^{cd}
1.5	22.1	11.3	22.5^{h}	20.1^{g}	0.81	0.83	1.38^{f}	1.21^{d}
2.0	---	---	---	---	---	---	---	---
3.0	---	---	---	---	---	---	---	---
BA + 2, 4-D								
1.0 + 0.5	17.3	15.3	67.3^{de}	47.3^{ef}	1.30	1.52	2.10^{d}	2.00^{cd}
1.0 + 1.0	43.4	38.3	86.4^{a}	78.3^{a}	2.50	2.31	5.13^{a}	4.21^{a}
1.0 + 2.0	38.1	33.1	83.3^{b}	68.9^{b}	2.10	2.05	3.81^{b}	2.97^{bc}
1.0 + 3.0	34.3	28.4	73.1^{c}	57.9^{c}	1.91	1.81	3.70^{b}	2.13^{c}
1.0 + 4.0	34.3	28.4	68.1^{d}	57.9^{c}	1.93	1.83	3.21^{bc}	2.11^{c}
BA + NAA								
1.0 + 0.5	15.1	11.1	53.3^{f}	41.1^{f}	1.11	1.18	2.31^{d}	1.91^{cd}
1.0 + 1.0	39.4	37.4	71.1^{c}	68.3^{b}	1.93	2.05	3.92^{b}	3.24^{b}
1.0 + 2.0	32.1	31.3	63.4^{e}	53.1^{d}	1.84	1.86	1.88^{de}	2.81^{bc}
1.0 + 3.0	28.9	30.1	51.3^{f}	48.9^{e}	1.73	1.57	1.57^{de}	1.42^{d}
1.0 + 4.0	28.1	30.1	51.3^{f}	48.9^{e}	1.77	1.53	1.32^{f}	1.22^{d}

Data pooled from 3 separate experiments each with 05 flasks containing 03 explants per flask. Mean values within the column with same superscript are not significantly different ($p<0.05$; Duncan's New Multiple Range Test).
--: No response, Fresh weight gain: Initial weight of explant - Final weight at day 30.

Table 3. Shoot regeneration from calli derived from leaf explant following 25 days culture on MS medium supplemented with different growth regulators.

MS + Growth regulators (mg dm^{-3})	% of shoot regeneration	Shoots/ callus	Shoot length (cm)
Control	--	--	--
BA			
1.0	38.3^{e}	2.5^{de}	1.1^{g}
1.5	51.6^{cd}	2.6^{de}	2.9^{d}
2.0	58.3^{b}	3.9^{b}	3.8^{b}
3.0	29.1^{fg}	3.3^{c}	3.1^{cd}
Kin			
1.0	--	--	--
1.5	27.3^{h}	2.1^{f}	2.3^{de}
2.0	31.1^{f}	3.3^{c}	2.8^{d}
3.0	20.5^{i}	2.9^{d}	2.1^{e}
BA + NAA			
2.0 + 0.5	54.7^{c}	3.1^{c}	3.4^{c}
2.0 + 1.0	82.3^{a}	4.8^{a}	4.3^{a}
2.0 + 2.0	51.3^{cd}	3.9^{b}	3.2^{d}
Kin + NAA			
2.0 + 0.5	48.1^{d}	3.1^{c}	2.1^{e}
2.0 + 1.0	51.1^{cd}	3.5^{bc}	2.5^{de}
2.0 + 2.0	45.3^{de}	2.5^{de}	2.0^{f}

Data pooled from 3 separate experiments each with 05 flasks containing 04 calli per flask. Mean values within the column with same superscript are not significantly different (*p<0.05*; Duncan's New Multiple Range Test).
-- : No response,

Half-strength MS medium without any auxin supplement failed to induce roots in the excised shoots even after 30 days of culture. Addition of an auxin to the medium was essential to induce rooting in the excised shoots. Of the three different auxins tested, best result was recorded with IBA (Table 4). Half-strength MS medium containing IBA at an optimum concentration of 2.0 mg/l induced rooting in 83.1 % of the shoots in 8-10 day and from each shoot 5.2 roots developed (Fig. 1F). IBA has been reported to have a stimulatory effect on root induction in many medicinal plant species including *W. somnifera* (Siddique *et al.,* 2004), *Centella asiatica* (Mohapatra *et al.,* 2008) and Ginger (Sultana *et al.,* 2009). Upon transfer to a half-strength MS medium free of auxins primary roots as well as shoots elongated. With higher concentration of IBA 3.0 mg/l rooting did not occur, instead there was callus formation at the base. Our results are corroborated by an earlier report on *Clitoria ternatea* (Lakshmanan & Dhanlakshmi, 1990), which demonstrated that IBA 0.5 mg/l was most effective in resulting root formation in isolated *In vitro* shoots (3-5 roots/shoot).

Table 4. Influence of different auxins on rooting of the *In vitro* derived shoots of *Withania somnifera.*

½ MS + Auxins	% Of rooting	Roots/ shoot	Root length (cm)
Control	--	--	--
IBA			
0.5	65.7^{c}	3.2^{e}	4.3^{bc}
1.0	78.3^{b}	3.9^{c}	4.9^{b}
2.0	83.1^{a}	5.2^{a}	6.2^{a}
3.0	callus	callus	callus
IPA			
0.5	41.5^{f}	3.8^{cd}	3.6^{e}
1.0	62.1^{cd}	4.3^{b}	3.8^{d}
2.0	47.7^{e}	3.1^{e}	3.7^{e}
3.0	callus	callus	callus
NAA			
0.5	--	--	--
1.0	38.3^{fg}	2.0^{f}	3.2^{f}
2.0	31.1^{h}	1.3^{fg}	3.7^{e}
3.0	callus	callus	callus

Data pooled from 3 separate experiments each with 05 flasks containing 02 shoots per flask. Mean values within the column with same superscript are not significantly different (*p<0.05*; Duncan's New Multiple Range Test).
-- : No response

Plantlets with 4-6 fully expanded leaflets and well-developed roots were successfully acclimated in the plant growth chamber and eventually established in soil. The percentage of survival of the plantlets after transfer to sterile mixture of sand, soil and cow-dung manure in the ratio of 1:1:1 (v/v) was 75% (Fig. 1G) and about 81% of the plants transferred to garden soil survived (Fig. 1H).

The protocol reported herein for *In vitro* plant regeneration of *Withania somnifera* via callus cultures will considerably facilitate large-scale propagation and conservation of this multipurpose medicinally important plant specie as well as enable production of somaclones.

Acknowledgements

The financial support of the SAP-DRS Programme of UGC, New Delhi is gratefully acknowledged. The authors are also grateful to the Ramakrishna Mission Ashrama, Narendrapur, Kolkata for providing the seeds of Ashwagandha.

References

Ahmad, M., S. Saleem, A.S. Ahmad, M.A. Ansari, S. Yousuf and M.N. Hoda. 2005. Neuroprotective effects of *Withania somnifera* on 6-hydroxydopamine induced Parkinsonism in rats. *Hum. & Exp. Toxicol.,* 24: 137–147.

Andallu, B. and B. Radhika, 2000. Hypoglycemic diuretic and hypocholesterolemic effect of winter cherry (*Withania somnifera* Dunal) root. *Ind. J. of Exp. Bio.,* 38: 607-609.

Anonymous. 1988. The Wealth of India: *A Dictionary of Indian Raw Materials and Industrial Products. Publication and Information Directorate*, CSIR, New Delhi, India. Vol. 2.

Barik, D.P., U. Mohapatra and P.K. Chand. 2005. High frequency *in vitro* regeneration of *Lathyrus sativus* L. *Biologia Plantarum,* 49 (4): 637-639.

Bewley, J.D. and M. Black, 1994. *Seeds: Physiology of Developmental and Germination* . New York: Plenum Press.

Bhatia, P., S.I.S. Rattan, J. Cavallius and B.F.C. Clark. 1987. *Withania somnifera* (Ashwagandha) a so-called rejuvenator inhibits growth and macromolecular synthesis of human cells. *Med. Sci. Res.,* 15: 515-516.

Bhattacharya, S.K., K.S. Satyan and A. Chakrabarti. 1997a. Effect of Trasina, an Ayurvedic herbal formulation, on pancreatic islet superoxide dismutase activity in hyperglycaemic rats. *Ind. J. of Exp. Bio.,* 35: 297-299.

Bhattacharya, S.K., K.S. Satyan and S. Ghosal. 1997b. Antioxidant activity of glycowithanolides from *Withania somnifera*. *Ind. J. of Exp. Bio.,* 35: 236-239.

Blaydes, D.F. 1966. Interaction of kinetin and various inhibitors in the growth of soybean tissue. *Physiology Plant*, 19: 748-753.

Dhuley, J.N. 1998. Effect of Ashwagandha on lipid peroxidation in stress-induced animals. *Journal of Ethnopharmacology*, 60: 173-178.

Gamborg, O.L., R.A. Miller and K. Ojima. 1968. Plant tissue culture methods. *Experimental Cell Research*, 50:151-158.

Gomez, K.A. and A.A. Gomez, 1984. *Statistical Procedure for Agricultural Research*. John Wiley & Sons, New York.

Hartman, H.T., D.E. Kester and D.E. Davies. 1990. *Plant Propagation, principles and practices*. 5th Edn., Prentice Hall Inc., Englewood, Cliffs, NJ., USA.

Hiregoudar, L.V., H.N. Murthy, J.G. Bhat, A. Nayeem, B.P. Hema, E.J. Hahn and K.Y. Paek. 2006. Rapid clonal propagation of *Vitex trifolia*. *Biologia Plantarum,* 50: 291-294.

Kambizi, L., P.O. Adebola and A.J. Afolayan, 2006. Effects of temperature, pre-chilling and light on seed germination of *Withania somnifera* ; a high value medicinal plant.- *South African Journal of Botany,* 72: 11-14.

Karam, N.S. and M.M. Al-Salem, 2001. Breaking dormancy in *Arbutus andrachne* L. seeds by stratification and gibberellic acid. *Seed science and technology,* 29: 51-56.

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

Lakshmanan, K.K. and S. Dhanalakshmi, 1990. Callus, organogenesis and plantlet formation in tissue cultures of *Clitoria ternatea*. *Annal Botany* , 66: 451-455.

Malabadi, R. B. and K. Nataraja, 2001. Shoot regeneration in leaf explants of *Clitoria ternatea* L. cultured *in vitro*. *Phytomorphology*, 51: 169-171.

Mohanty, I., D.S. Arya, A. Dinda, K.K. Talwar, S. Joshi and S.K. Gupta. 2004. Mechanisms of cardioprotective effect of *Withania somnifera* in experimentally induced myocardial infarction. *Basic and Clinical Pharmacology and Toxicology*, 94: 184-90.

Mohapatra, H., D.P. Barik and S.P. Rath. 2008. *In vitro* regeneration of medicinal plant *Centella asiatica. Biologia Plantarum,* 52 (2): 339-342.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiology Plant,* 15: 473-497.

Naz, S., S. Javad, S. Ilyas and A. Ali. 2009. An efficient protocol for rapid multiplication of *Bryophyllum pinnatum* and *Bryophyllum daigremontianum. Pak. J. Bot*., 41(5): 2347-2355.

Panda, S. and A. Kar, 1997. Evidence for free radical scavenging activity of Ashwagandha root powder in mice. *Indian Journal of Physiology and Pharmacology,* 41: 424-426.

Rai, D., G. Bhatia, T. Sen and G. Palit. 2003. Anti-stress effects of Ginkgo biloba and Panax ginseng a comparative study. *Journal of Pharmacological Sciences*, 93: 458-64.

Ray, S. and S. Jha. 2002. Regeneration of *Withania somnifera* plants. *Journal of Tropical Medicinal Plants,* 3 (1): 89-95.

Rout, J.R., M. Mishra, R. Das and SL. Sahoo. 2009. *In vitro* Micropropagation of *Abutilon indicum* L. through Leaf Explants. *Plant Tissue Cult. & Biotech.*, 19 (2): 177-184.

Saba. 1998. *In vitro studies on micropropagation and secondary metabolite of Lepidium sativum L. and Silybum marianum*. Ph.D thesis, Hamdard University, New Delhi.

Saritha, K.V. and C.V. Naidu. 2007. *In vitro* flowering of *Withania somnifera* Dunal.—An important antitumor medicinal plant. *Plant Science,* 172: 847–851.

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

Siddique, N.A., M.A. Bari, S. Shahnewaz, M.H. Rahman, M.S.I. Khan M.S. Islam. 2004. Plant regeneration of *Withania somnifera* (L.) Dunal (Ashwagandha) from nodal segments derived callus an endangered medicinal plant in Bangladesh. *Journal of Biological Science*, 4(2): 219-223.

Singh, B., A.K. Saxena, K.K. Chandan, D.K. Gupta, K.K. Bhutani and K.K. Anand. 2001. Adaptogenic activity of a novel withanolide-free aqueous fraction from the roots of *Withania somnifera* Dunal. *Phytotherapy Research,* 15: 311-318.

Sivanesan, I. and K. Murugesan, 2008. An efficient regeneration from nodal explnts of *Withania somnifera* Dunal. *Asian Journal of Plant Sciences*,7(6): 551-556.

Sultana, A., L. Hassan, S.D. Ahmad, A.H. Shah, F. Batool, M.A. Islam, R. Rahman and S. Moonmoon. 2009. *In vitro* regeneration of ginger using leaf, shoot tip and root explants. *Pak. J. Bot*., 41(4): 1667-1676.

Vakeswaran, V. and V. Krishnasamy, 2003. Improvement in storability o f Ashwagandha (*Withania somnifera* Dunal). seeds through pre-storage treatments by triggering their physiological and biochemical properties. *Seed Technology,* 25: 203.

(Received for publication 20 January 2010)