Research in Biotechnology, 2(5): 34-39, 2011 **ISSN: 2229-791X**
www.researchinbiotechnology.com

Regular Article

In vitro plant regeneration from leaf explants of *Withania somnifera* (L) Dunal (Ashwaganda) - an important medicinal plant

Owk Aniel Kumar, Geddam Jyothirmayee, Sape Subba Tata*

Department of Botany, Andhra University, Visakhapatnam, India – 530 003
*Correspondence Email: s_tata_s@yahoo.co.in

***Withania somnifera* (L) Dunal (Ashwaganda) is a Solanaceous herb having numerous medicinal values widely used in traditional Ayurvedic drug preparations. An efficient protocol for *in vitro* plant regeneration via direct adventitious shoot proliferation from leaf explants of Ashwaganda is developed. MS medium containing 1.5mg/l BAP and 1.5mg/l IAA was found to be the best medium for maximum *in vitro* response i.e., 100(% shooting) and 68(shoots/explant). An improved *in vitro* shoot bud elongation and rooting was achieved on MS medium fortified with 0.15mg/l GA_3 and 5mg/l IBA respectively. Rooted plants were hardened and transplanted in earthen pots and were showed 80-90% survival during transplantation.**

Key words: Adventitious shoot, Ashwaganda, BAP, IAA, IBA, KN.

Medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries depend in part on plants for the production of pharmaceutical compounds (Chand *et al.*, 1997). WHO estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. The developed nations are also looking for eco-friendly treatment of various diseases through plant based source. In addition, many valuable herbal drugs have been discovered by knowing that particular plant was used by the ancient folk healers for the treatment of some kind of ailment (Ekka and Dixit, 2007). *Withania somnifera* (L) Dunal is a member of the family Solanaceae commonly known as Ashwaganda. Its root part rich in alkaloids (withanine) (Majumdar, 1955), which are valuable constitutes in traditional Ayurvedic drug preparations against many diseases viz., hiccup, female disorders, cough, rheumatism and dropsy (Kiritikar and Basu, 1975). Besides roots, the other parts of this plant also useful for the treatment of inflammatory conditions, tuberculosis and exhibits excellent anti-tumor and anti-bacterial activities (Devi and Sharada, 1992, Devi, 1996). Due to the indiscriminate collection of huge amount of this plant by local herbalists, Ayurvedic and Unany companies, this plant species is on the verge of extinction. Under such a situation it is important to develop techniques for rapid mass propagation of this species to meet up the commercial need and also for protecting the genetic erosion. *In vitro* micro propagation technology has sound and extensive potential for commercial rapid multiplication of plants because it is a quick method, allows round-the-year propagation of identical plants, and produces plants free from diseases. The conventional method of propagating this

species is through seeds, but seed viability is very poor and low germination limits its multiplication. Moreover, seed-derived progenies are not true to type, due to cross-pollination (Heywood, 1978). In *Withania,* several procedures were available for inducing *in vitro* response using leaf explants (Baburaj and Gunasekaran, 1995, Kulkarni *et al.,* 1996, Govindaraju *et al.,* 2003, Sivanesan and Murugesan, 2005, Shrivastava and Dubey, 2007, De Silva and Senarath, 2009, Valizadeh and Valizadeh, 2009, Dewir *et al.,* 2010, Ghimire *et al.,* 2010, Joshi and Padhya, 2010, Logesh *et al*., 2010, Sharma *et al.,* 2010, Singh *et al*., 2011). However, the rate of plant regeneration per explant is not sufficiently high to be practical application. Therefore, we report a rapid and efficient method for direct adventitious shoot bud proliferation from leaf explants of *Withania somnifera* (L) Dunal.

Materials and methods

Explant preparation

Seeds of *Withania somnifera* (L) Dunal genotype were obtained from Medicinal Garden of Botany Department, Andhra University, Visakhapatnam, India. The seeds were surface sterilized with 0.1% $HgCl_2$ and repeatedly washed in sterile distilled water. The seeds were then inoculated in glass containers with 50ml of half-strength MS medium (Murashige and Skoog, 1962) for germination. The leaf explants were derived from 30 days old seedlings grown *in vitro* transferring on to culture media.

Micropropagation

The basal nutrient medium containing MS salts and vitamins was used with IAA (indole -3- acetic acid), BAP (6-benzyl aminopurine) and KN(kinetin). In the first experiment, the effects of BAP and KN were examined individually at the concentrations of 0.5-3.0mg/l and in the second experiment, IAA at the concentration of 1.5 and 2.0mg/l was combined with BAP and KN. Subculture at every two weeks to the same medium the number of shoot buds were recorded after five weeks of culture, then the shoot buds were elongated on MS media with various levels of GA_3 (0.05-0.25mg/l) after one week of culture. To test their rooting capacity, the *in vitro* elongated shoots were excised and transferred on to MS media fortified with various concentrations of IBA (1.0-8.0mg/l). The rooting i.e., frequency of rooting (%), root length (cm) and number of roots per shoot were noted after two weeks of culture.

In vitro conditions

All media were supplemented with 3% sucrose and 0.8% agar, the pH of the media was adjusted to 5.8 with 1N NaOH or 1N HCl prior to autoclaving. The cultures were maintained at 25 ± 2^0C air temperatures in a culture room with a 16 hour photoperiod under an illumination of 20 m mol $m^{-2}s^{-1}$ photosynthetic photon flux density, provided by cool-white fluorescent light.

Acclimatization

Plants with roots were transferred during two weeks, after washing of the agar with distilled water and to pots with a mixture of soilrite (1:1). Potted plantlets were covered with transparent polythene membrane to ensure high humidity and watered every three days with half-strength MS salts solution for two weeks in order to acclimatize plants to field conditions. After two weeks the acclimatized plants were transferred to pots containing normal garden soil and maintained in greenhouse under natural day length conditions.

Statistical analysis

Experiments were set up in Randomized Block Design (RBD) and each experiment was replicated thrice. Observations recorded on the percentage of response, number of shoots per explant, number of roots per shoot and root length. Mean and standard errors were carried out for each treatment.

Results and Discussion

Leaf explants of *Withania somnifera* (L) Dunal when cultured on MS basal medium

supplemented with various levels of 0.5-3.0mg/l BAP and KN alone or in combination with 1.5-2mg/l IAA showed direct shoot bud differentiation (Table 1).

Table 1: Effect of culture medium on *in vitro* multiple shoot induction from leaf explants of *Withania somnifera* (L) Dunal.

Plant growth regulators (mg/l)		Shooting (%)	Shoot No. / explant
BAP	**IAA**		
0.0	0.0	00.0±0.00	00.0±0.00
0.5	0.0	72.0±0.08	12.0±0.06
1.0	0.0	80.0±0.20	21.0±0.08
1.5	0.0	98.0±0.00	30.0±0.04
2.0	0.0	90.0±0.10	23.0±0.14
2.5	0.0	88.0±0.16	16.0±0.09
3.0	0.0	80.0±0.06	9.0±0.10
0.5	1.5	86.0±0.14	36.0±0.12
1.0	1.5	90.0±0.22	44.0±0.08
1.5	1.5	100.0±0.00	68.0±0.04
2.0	1.5	84.0±0.23	53.0±0.07
2.5	1.5	70.0±0.14	44.0±0.11
3.0	1.5	62.0±0.27	35.0±0.21
0.5	2.0	68.0±0.19	10.0±0.12
1.0	2.0	75.0±0.08	19.0±0.09
1.5	2.0	87.0±0.24	30.0±0.05
2.0	2.0	81.0±0.26	26.0±0.16
2.5	2.0	70.8±0.13	13.0±0.12
3.0	2.0	64.0±0.20	6.0±0.08
KN	**IAA**		
0.0	0.0	00.0±0.00	00.0±0.00
0.5	0.0	60.0±0.06	4.8±0.11
1.0	0.0	68.0±0.14	6.0±0.06
1.5	0.0	72.5±0.11	10.8±0.16
2.0	0.0	82.0±0.19	14.0±0.08
2.5	0.0	75.0±0.24	11.2±0.13
3.0	0.0	63.0±0.12	7.3±0.22
0.5	1.5	69.0±0.08	29.0±0.16
1.0	1.5	74.5±0.20	36.0±0.08
1.5	1.5	79.0±0.16	41.8±0.13
2.0	1.5	94.0±0.00	54.0±0.20
2.5	1.5	82.0±0.10	30.0±0.16
3.0	1.5	66.0±0.22	21.0±0.11
0.5	2.0	63.0±0.15	10.6±0.04
1.0	2.0	70.8±0.19	16.9±0.10
1.5	2.0	78.5±0.08	22.0±0.08
2.0	2.0	85.0±0.24	26.3±0.14
2.5	2.0	74.0±0.04	14.0±0.08
3.0	2.0	59.0±0.16	9.2±0.16

However, the explants cultured on MS medium without growth regulators failed to induce shoot proliferation. BAP alone or combined with IAA was found more effective than KN or its IAA combined forms. The maximum *in vitro* response i.e.,

shooting (%) (100 and 94.0±0.12) and shoot number per explant (68.0±0.04 and 54.0±0.20) were obtained on MS medium supplemented with BAP(1.5mg/l) + IAA (1.5mg/l) and KN(2mg/l) + IAA(1.5mg/l) respectively (Fig.1a&b). The results obtained were strongly supported by earlier reports in *Withania* leaf explants *in vitro* response (Kulkarni *et al.* 1996, Govindaraju *et al.,* 1999, Shrivastava and Dubey, 2007, Dewir *et al.,* 2010, Ghimire *et al.,* 2010, Logesh *et al.,* 2010) while, Joshi and Padhya (2010), Sivanesan and Murugesan (2005) who found that KN alone or KN+BAP ranks as the best shoot inductor on leaf explants of *Withania*. These results differ from what has been reported by Valizadeh and Valizadeh (2009), De Silva and Senarath (2009) who found that leaf explants failed to respond shoot induction. The result indicated that the culture medium i.e., MS+BAP(1.5mg/l) + IAA(1.5mg/l) was better than any other media in the present investigation. This may due to the synergetic effect of IAA with BAP on the enhancement of shoot multiplication (Sudha and Seeni, 1994). The shoots(2-3cm) were excised from the adventitious shoot clusters and were elongated on MS media with various levels of GA_3(0.05-0.25mg/l), maximum elongation of shoot length(cm) 15±0.20 was obtained on MS+GA_3 (0.15mg/l) (Table 2 and Fig. 1c).

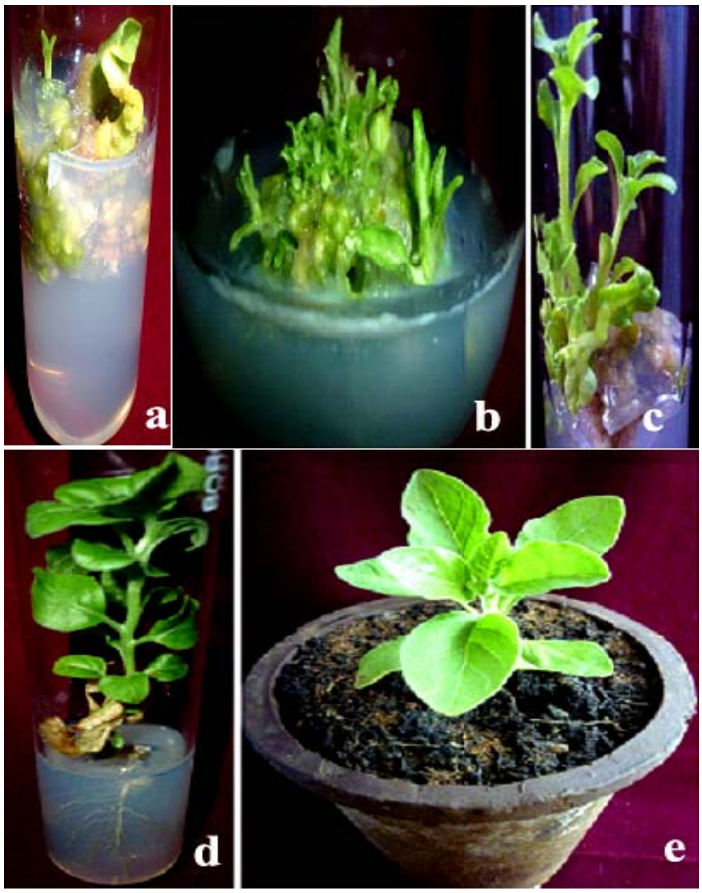

Figure 1(a-e): *In vitro* regeneration of *Withania somnifera* (L) Dunal using leaf explants; a: initiation of multiple shoots at the cut ends of leaf on MS+BAP(1.5mg/l)+IAA(1.5mg/l); b: proliferation of multiple shoots on MS+BAP(1.5mg/l)+IAA(1.5mg/l); c: elongation of shoot buds on MS+GA_3(0.15mg/l); d: rooting of elongated shoot on MS+IBA(5mg/l); e: Established plant in earthen pot.

In consonance to the present study, Sivanesan and Murugesan (2008) reported that elongation of shoots on GA_3 in *Withania*. The elongated shoots were excised and implanted on MS media supplemented with different concentrations of IBA(1-8mg/l), which induced *in vitro* rooting at the basal ends of the shoots after two weeks of culture (Table 3 and Fig. 1d). The optimum rooting efficiency (%) (64.0±0.22) as well as the best root number per shoot (12.8±0.16) and root length(cm) (11.0±0.05) were achieved on MS medium fortified with IBA(5.0mg/l). Similar observations were reported in *Withania* by Valizadeh and Valizadeh (2009), Joshi and Padhya (2010), Logesh et al. (2010), Sharma *et al.,* (2010). Our results show that 68 shoots per explant can be considered as very good regeneration in comparison to 10 to 16 shoots reported by Kulkarni et al. (1996), Joshi and Padhya (2010), Logesh *et al.,* (2010), Sharma *et al.,* (2010). The regenerated plants showed 80-90% survival during hardening and acclimatization and there were no observable differences between the parent plants and *in vitro* regenerated plants (Fig. 1e).

Table 2: Effect of GA_3 on *in vitro* shoot elongation

Plant growth regulator(mg/l)	Shoot length(cm)
GA_3	
0.00	0.0±0.00
0.05	8.0±0.08
0.10	11.4±0.12
0.15	15.0±0.20
0.20	12.4±0.24
0.25	10.2±0.18

Conclusions

Hence, an efficient and reproducible protocol was developed for *in vitro* direct multiplication of Ashwaganda from leaf explants which would be helpful in future for clonal multiplication of Ashwaganda germplasm to fulfill the needs of various biotechnological and pharmaceutical industries.

Table 3: Effect of IBA on *in vitro* rooting

Plant growth regulator (mg/l)	Rooting (%)	Root No. /shoot	Root length (cm)
IBA			
0.0	00.0±0.00	0.0±0.00	0.0±0.00
1.0	12.0±0.20	2.0±0.06	1.8±0.05
2.0	14.0±0.08	2.2±0.02	3.6±0.04
3.0	19.0±0.10	3.6±0.11	4.2±0.03
4.0	25.0±0.19	6.4±0.08	5.8±0.03
5.0	64.0±0.22	12.8±0.16	11.0±0.05
6.0	40.0±0.09	8.0±0.04	8.2±0.09
7.0	29.0±0.16	6.8±0.15	6.3±0.06
8.0	16.0±0.24	5.6±0.05	5.0±0.10

Acknowledgements

One of the authors (O. Aniel Kumar) is grateful to UGC-SAP, Department of Botany, Andhra University, Visakhapatnam for providing financial assistance.

References

Baburaj, S., Gunasekaran, K. 1995. *In vitro* differentiation of shoots from leaf callus cultures of *Withania somnifera* (L) Dunal. *J. Indian Bot. Soc.* **74:** 323-324.

Chand, S., Sahrawat, A.K., Prakash, D.V.S.S.R. 1997. *In vitro* culture of *Pimpinella anisum* L (anise). *J Plant Biochem. Biotech.* **6:** 1-5.

Devi, P.U., Sharada, A.C. 1992. *In vivo* growth inhibitory effect of *Withania somnifera* (Ashwaganda) on a transplantable mouse tumor, Sarcoma 180. *Indian J. Exp. Biol.* **30:** 169-172.

Devi, P.U. 1996. *Withania somnifera* Dunal(Ashwaganda): Potential plant source of a promising drug for cancer chemotherapy and radio sensitization. *Indian J. Exp. Biol.* **34:** 927-932.

De Silva, M.A.N., Senarath, W.T.P.S.K. 2009. *In vitro* mass propagation and greenhouse establishment of *Withania somnifera* (L.) Dunal (Solanaceae) and comparison of growth and chemical compounds of tissue cultured and seed raised plants. *J. Natn. Sci. Foundation Sri Lanka*. **37**(4): 249-255.

Dewir, Y.H., Chakrabarty, D., Lee, S.H., Hahn, E.J., Paek, K.Y. 2010. Indirect regeneration of *Withania somnifera* and comparative analysis of withanolides in *in vitro* and greenhouse grown plants. *Biologia Plantarum*. **54(2):** 357-360.

Ekka, R.N., Dixit, V. 2007. Ethno-pharmacognostical studies of medicinal plants of Jashpur district, Chattisgarh. *Int. J. Green Phar.* **1:** 2-4.

Ghimire, B.K, Seong, E.S., Kim, E.H., Lamsal, K., Yu, C.Y., Chung, I.M. 2010. Direct shoot organogenesis from petiole and leaf discs of *Withania somnifera* (L.) Dunal. *African Journal of Biotechnology.* **9(44):** 7453-7461.

Govindaraju, B., Rao, S.R., Venugopal, R.B., Kiran, S.G., Kaviraj, C.P., Rao, S. 2003. High frequency plant regeneration in Ashwaganda (*Withania somnifera* (L.) Dunal): An important medicinal plant. *Plant Cell Biotechnol. Mol. Biol.* **4(1):** 49-56.

Heywood, V.H. 1978. Flowering Plants of the World, Oxford Univ. Press, UK. pp.239–240.

Joshi, A.G., Padhya, M.A. 2010. Shoot regeneration from leaf explants of *Withania somnifera* (L.) Dunal. *Notulae Scientia Biologicae*. **2(1):** 63-65.

Kiritikar, K.R., and Basu, B.D. 1975. Indian Medicinal Plants. Bishen Singh Mahendra Pal Singh, Dehra Dun. **3:** 1774-1777.

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

Logesh, P., Settu, A., Thangavel, K., Ganapathi, A. 2010. Direct *in vitro* regeneration of *Withania somnifera* (L.) Dunal. through leaf disc Culture. *International Journal of Biological Technology*. **1(3):** 1-4.

Majumdar, D.N. 1955. *Withania somnifera* Dunal. Part II: alkaloidal constituents and their chemical characterization. *Ind. J. Pharm.* **17:** 158-161.

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

Sharma, M.M., Ali, D.J., Batra, A. 2010. Plant regeneration through *in vitro* somatic embryogenesis in Ashwaganda (*Withania somnifera* (L.) Dunal). *Researcher.* **2(3):** 1-6.

Shrivastava, S., Dubey, P.K. 2007. *In vitro* callus induction and shoot regeneration in *Withania somnifera* Dunal. *International Journal of Biotechnology and Biochemistry*. **24:** 10-19.

Singh, S., Tanwer, B.S., Khan, M. 2011. Callus induction and *in vivo* and *in vitro* comparative study of primary metabolites of *Withania Somnifera. Advances in Applied Science Research.* **2(3):** 47-52**.**

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

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

Sudha, C.C., Seeni, S. 1994. *In vitro* propagation and field establishment of *Adhatoda beddomei* CB Clarke: a rare medicinal plant. *Plant Cell Reporter*. **13:** 203-207.

Valizadeh, J., Valizadeh, M. 2009. *In vitro* callus induction and plant regeneration from *Withania coagulans*: A valuable medicinal Plant. *Pakistan Journal of Biological Sciences.* **12(21):** 1415-1419.