Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

ANSInet
an open access publisher
http://ansinet.com

Asian Journal of Plant Sciences 7 (6): 551-556, 2008
ISSN 1682-3974

An Efficient Regeneration from Nodal Explants of *Withania somnifera* Dunal

I. Sivanesan and K. Murugesan
Centre for Advanced Studies in Botany, University of Madras, Guindy Campus,
Chennai-600 025, Tamil Nadu, India

Abstract: An efficient protocol for *in vitro* propagation of *Withania somnifera* is described by axillary shoot multiplication. Nodal explants of *W. somnifera* differentiated shoot buds on MS, SH and B_5 media containing different concentrations and combinations of auxin and cytokinin. Among the media tested, MS medium was found to be the most favorable for induction of multiple shoots than B_5 and SH media. Maximum number of multiple shoots was obtained on MS medium containing BAP+IAA each at 1.5 mg L^{-1}. The microshoots were separated from the multiple shoots, transferred to MS medium supplemented with 0.3 mg L^{-1} GA_3 favoured maximum elongation of shoots. Regenerated shoots produced highest number of roots when transferred to half strength MS medium supplemented with 0.8% (w/v) agar, 3% (w/v) sucrose and 2.0 mg L^{-1} IBA. The regenerated plantlets were hardened in the greenhouse and successfully transferred in soil with 87% survival rate.

Key words: Axillary shoot multiplication, B_5 medium, direct regeneration, medicinal plant, Schenk and Hilderbrandt

INTRODUCTION

Withania somnifera Dunal. is an erect, evergreen, perennial shrub. *W. somnifera* grows in open and undisturbed lands in Australia, Eastern Asia and Africa. It is also found throughout the drier parts of India in waste places and on bunds, in Deccan, Mysore, Coimbatore and E. Carnatic (Gamble, 1918). This plant is reputed to have adaptogenic, tonic, analgesic, antipyretic, anti-inflammatory and abortifacient properties. Its roots were prescribed for hiccup, female disorders, cough, rheumatism and dropsy (Kiritikar and Basu, 1975). The leaves contain withanolides like withaferin A that exhibit anti-bacterial and anti-tumor properties (Devi and Sharada, 1992; Devi, 1996). *W. somnifera* an important medicinal shrub is being exploited on a large scale on a commercial basis for its medicinal value. Because of over exploitation, this plant is becoming a member in the endangered plant species (Antonisamy and Mamickam, 1999). Micropropagation is an useful tool for conservation and multiplication of several medicinal plants (Sivanesan, 2007b; Sivanesan and Jeong, 2007a, b). There have been few reports to date on micropropagation in this plant species using various explants (Kulkarni *et al*., 1999; Rani and Grover, 1999; Kulkarni *et al*., 2000; Mamickam *et al*., 2000; Sivanesan and Murugesan, 2005; Sivanesan, 2007a). However, only few reports are available axillary shoot multiplication through nodal explants. Furthermore, they used *in vitro* grown shoots or seedlings as explants source not field grown mature plants. The majority of plants used for medicines are harvested from the wild. Hence, there is an urgent need for conservation and cultivation of this plant species for future use. Multiplication of elite clone has potential impact on pharmaceutical industries, thus, we investigate an efficient *in vitro* propagation method using mature field grown plants. In this study, it is reported that an *in vitro* method for rapid regeneration using nodal explants of *W. somnifera*.

MATERIALS AND METHODS

The plants were collected from in and around Shevaroy hills, Salem District, Tamil Nadu, India during the month of Jan 1999. The plants were grown in a net house at the Madras University, Botany Field Research Laboratory, Chennai, India and used for experimental studies. Explants were excised from the field grown plants and were washed thoroughly in running tap water for 30 min then in Teepol for 10 min and washed several times with distilled water. Explants immersed in 70% (v/v) ethanol for 1 min followed by mercuric chloride solution 0.2% (w/v) for 5 min and washed 4 times with sterile distilled water. The explants were blotted in sterile filter

Corresponding Author: Iyyakkannu Sivanesan, Department of Horticulture, Division of Applied Life Science (BK21), Graduate School, Gyeongsang National University, Jinju, Korea 660-701
Tel: +82-55-751-5489 Fax: +82-55-757-7542

paper to remove excess water and then remove the cut ends. The nodal segments were cultured on MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972) and B_5 (Gamborg *et al.*, 1968) media supplemented with different concentrations and combinations of plant growth regulators (auxins and cytokinins). The pH of the medium was adjusted to 5.7 using 0.1 N NaOH or 0.1 N HCl prior to autoclave at 121°C for 15 min. Cultures were incubated at 25±2°C under 16 h photoperiod with 45 μmol m^{-2} sec^{-1} irradiance provided by cool white fluorescent light (PHILIPS 40 W tubes). The microshoots were transfer into shoot elongation medium containing different concentrations of GA_3. Individual shoots, which were grown about 3-4 cm long transferred to half strength MS medium containing IAA, IBA or NAA for rooting. The MS medium was supplemented with 2.0 mg L^{-1} IBA and glucose, maltose, fructose, lactose or sucrose at 3% (w/v), to study the effect of carbon source on *in vitro* rooting. The rooted plantlets were transferred into plastic cups containing soil: sand: vermiculite (1:1:1). The plantlets were maintained under the same controlled environmental conditions for 3 weeks and watered once in 2 days with half strength MS basal salts, subsequently they were transferred to polythene cover and kept in greenhouse after four weeks the plantlets transfer to the field. Data were statistically analyzed by analysis of variance (ANOVA) followed by Duncan multiple range test at 5% probability level. Data analysis was performed using SAS computer package (SAS Institute Inc., NC, USA).

RESULTS

Effect of cytokinins on shoot induction: BAP was supplemented into various media at concentration ranging from 0.1-5.0 mg L^{-1}. Each nodal segment cultured on MS medium without plant growth regulators was differentiated into a single shoot within a period of 2 weeks (Fig. 1a). When BAP was supplemented in MS medium at concentration ranging from 0.1-3.0 mg L^{-1} promote multiple shoot formation (Fig. 1b). However, at 4.0 and 5.0 mg L^{-1} there was no regeneration from the nodal explants. A maximum of 17 shoots were induced from the nodal explants at 1.0 mg L^{-1} BAP and the percentage of shoot induction was 84. In SH medium the maximum shoot regeneration giving 9 shoots at 2.0 mg L^{-1} BAP (Table 1). Where as in the case of B_5 medium 7 shoots produced at the same concentration and percentage of shoot regeneration was 70% which was comparatively less than those produced by the MS medium. MS medium containing 1.0 mg L^{-1} kin induced highest number of shoots 7. At higher concentrations (4.0 and 5.0 mg L^{-1}) there was no regeneration. Nodal explants produced an average of 5 shoots when the SH medium was supplemented with 1.0 mg L^{-1} kin. However, the maximum number of shoots when compared to others was 8 in B_5 medium with 1.5 mg L^{-1} kin and the percentage of shoot induction was 68% (Table 1).

Effect of combination of BAP and auxins on shoot induction: BAP and IAA each at 1.5 mg L^{-1} optimum for shoot multiplication. The number of shoots obtained was 22 and the percentage of shoot induction was 89. 0.1-0.5 mg L^{-1} (Fig. 2a, b), poor shoot induction was observed which gradually increased from 1.0-2.0 mg L^{-1} and there was a decrease at 3.0-5.0 mg L^{-1} BAP. A mean of 12 shoots were induced from the nodal explants at a concentrations of 1.5 mg L^{-1} each of BAP and NAA and the percentage of shoot induction was 78 (Table 2). At lower concentrations only a few shoots were produced.

Shoot elongation: GA_3 was supplemented into MS medium at concentrations ranging from 0.01-2.0 mg L^{-1} to test its effect on shoot length. Maximum shoot length of 12 cm was obtained at 0.3 mg L^{-1} GA_3. About 9.42 cm of shoot length was obtained at 0.5 mg L^{-1} and there was poor effect at higher concentrations (1.0, 1.5 and

Table 1: Effect of cytokinins on multiple shoot formation from nodal explants of *W. somnifera*

	No. of shoots per explant						Shoot induction (%)					
	BAP			KIN			BAP			KIN		
Conc. (mg L^{-1})	MS	SH	B_5	MS	SH	B_5	MS	SH	B_5	MS	SH	B_5
0.1	4.2i	-	1.3l	1.4l	2.1k	-	31.0n	-	46.0j	43.9k	26.6o	-
0.3	7.0f	2.0	1.8	4.6	2.8	-	40.5l	28.0	53.9h	60.8g	34.0m	-
0.5	9.2e	4.2i	3.0j	5.2h	3.5	2.0k	47.8j	34.0m	58.0h	71.4d	43.0k	48.0j
1.0	17.2a	4.7i	4.0i	7.0f	5.0h	3.0j	84.8b	36.0m	60.2g	81.7b	45.2k	56.0h
1.5	14.0b	5.4h	5.3h	4.6i	4.0i	8.2ef	87.6a	49.2i	61.4g	78.9bc	39.6l	68.0e
2.0	12.2c	9.0e	7.0f	3.4j	2.0k	5.7g	72.0d	58.0h	70.0d	75.6d	33.9m	66.0f
3.0	10.2d	6.2g	3.0j	2.8k	-	4.0i	60.7g	50.0i	68.0e	68.0e	-	59.0gh
4.0	8.5e	-	-	-	-	-	57.1h	-	-	-	-	-
5.0	6.0g	-	-	-	-	-	51.4i	-	-	-	-	-

Means having same letter in a column were not significantly different by Duncan's comparison test $p<0.05$ level

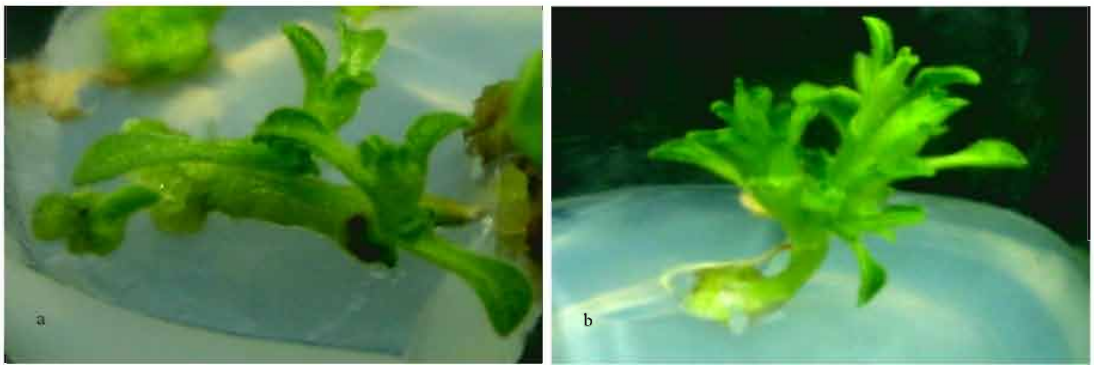

Fig. 1: Effect of cytokinins on multiple shoot induction from nodal explants of *W. somnifera* (2 weeks old), (a) axillary shoot initiated from nodal explants cultured on MS basal medium with out plant growth regulators, (b) multiple shoot initiated from nodal explants cultured on MS basal medium supplemented with 1.0 mg L^{-1} BAP

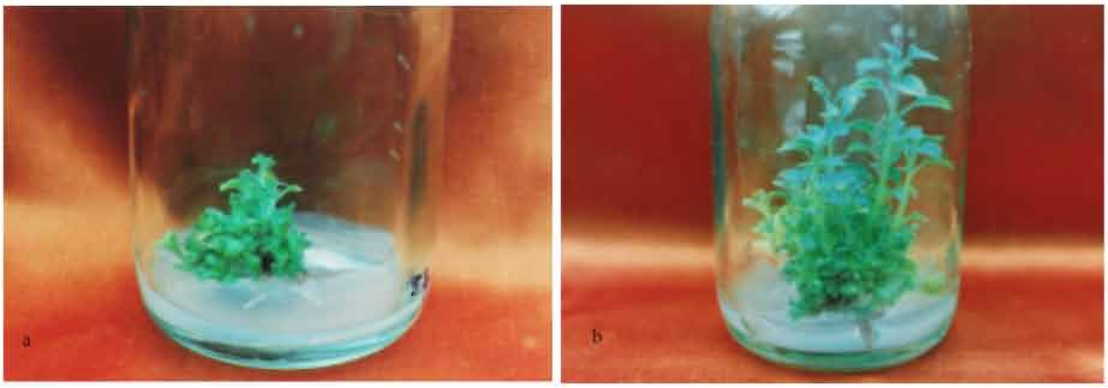

Fig. 2: Multiple shoot induction from nodal explants cultured on MS medium fortified with BAP and IAA each at 1.5 mg L^{-1} (Different stages of multiplication), (a) 2 weeks old and (b) 5 weeks old

Table 2: Effect of different concentration and combination of BAP and auxins on multiple shoot regeneration from nodal explants of *W. somnifera*

Conc. (mg L^{-1})		No. of shoots per explant		Shoot induction (%)	
BAP	Auxins	BAP+IAA	BAP+NAA	BAP+IAA	BAP+NAA
0.1	0.1	3.0j	3.0j	60.0g	52.0hi
0.3	0.3	5.4gh	4.4i	63.2f	56.1hg
0.5	0.5	9.0e	8.5ef	70.0f	69.1ef
1.0	1.0	17.0c	10.0e	83.0c	71.2e
1.5	1.5	22.0a	13.6d	89.0b	78.4d
2.0	2.0	20.4b	9.0e	90.6a	72.8de
3.0	1.0	14.3d	6.8g	76.4d	64.0f
4.0	1.0	8.3f	5.0h	53.7h	50.5i
5.0	1.0	6.2g	2.0k	48.0j	44.0k

Means having same letter in a column were not significantly different by Duncan's comparison test $p<0.05$ level

2.0 mg L^{-1}), the shoot length measured 4.8, 5.0 and 4.0 cm, respectively (Table 3, Fig. 3).

Rooting and acclimatization: Regenerated shoots were subjected to root induction using MS half strength medium supplemented with IAA, IBA and NAA. Sixteen roots were produced when I BA was supplemented into the medium at 2.0 mg L^{-1} and 100% root induction was observed at this concentration (Table 4). The corresponding root length was 10.5 cm. The carbon

Table 3: Effect of GA_3 on shoot elongation

Conc. (mg L^{-1})	Shoot length (cm)
0.01	2.3f
0.1	6.1cd
0.3	12.0a
0.5	9.4b
0.75	7.0c
1.0	4.8d
1.5	5.0d
2.0	4.0e

Means having same letter in a column were not significantly different by Duncan's comparison test $p<0.05$ level

Table 4. Effect of auxins on root induction

	No of roots per shoot			Root length (cm)			Root induction (%)		
Conc (mg L^{-1})	IAA	IBA	NAA	IAA	IBA	NAA	IAA	IBA	NAA
0 1	2 3g	3 0f	3 0f	3 1f	2 6g	1 6h	36 0k	42 0j	76 0f
0 5	4 0f	5 0e	4 0f	3 5f	2 7g	1 8h	53 2h	48 0i	80 3e
1 0	7 0d	5 7e	4 5ef	6 0c	4 1e	2 1g	57 2g	94 2bc	83 0d
1 5	8 3cd	10 3bc	6 6de	5 7c	4 9d	2 3g	84 4d	96 0b	81 0e
2 0	11 0b	16 0a	-	4 0e	10 5a	-	97 0b	100 0a	-
3 0	10 0bc	9 0c	-	4 3de	8 1b	-	95 0bc	91 0c	-

Means having same letter in a column were not significantly different by Duncan's comparison test p<0 05 level

Table 5: Effect of different carbon source on root induction in *W somnifera*

Carbon source (3%)	No of roots	Root length	Root induction (%)
Glucose	9 1b	4 7d	68 0c
Maltose	5 2d	4 2d	57 0e
Lactose	6 0c	5 0c	79 0b
Fructose	6 7c	6 5b	65 0d
Sucrose	16 0a	10 5a	100 0a

Means having same letter in a column were not significantly different by Duncan's comparison test p<0 05 level

Fig. 3: Elongation of microshoots cultured on MS medium supplemented with 0.3 mg L^{-1} GA_3

Fig. 4: Root induction of excised regenerated shoots cultured on half strength MS medium supplemented with 2.0 mg L^{-1} IBA after 30 days

Fig. 5: Acclimatized plants were ready for field transfer

source, each at 3% concentration (Fructose, glucose, lactose, maltose and sucrose) was tested for root induction. Among the carbon source sucrose gave 100% root induction (Table 5, Fig. 4). The rooted plantlets successfully transferred to the field and the percentage of survival was 87% (Fig. 5).

DISCUSSION

Withania somnifera is used in several indigenous drug preparations. Because of overexploitation, this plant had entered in the endangered plants' list. Tissue culture is an important technique wherein numerous plants (clones) could be produced from a single explant. Therefore, this plant was micro propagated using nodal explants, which would lead to a large-scale multiplication of this plant. Kulkarni *et al.* (2000) stated that nodal explants formed multiple shoots both from pre-existing and *de novo* buds on MS medium containing

0.1-0.5 mg L^{-1} BAP. Similar results were obtained in the present study. Manickam *et al.* (2000) reported that the greatest number of shoots 8 was obtained from the nodal explants when MS medium was supplemented with 1.0 mg L^{-1} BAP. In this present study we obtained an average of 17 shoots at the same concentration. Handique and Bora (1999) also observed the same in *Houttuynia cordata* and in *Pistacio vera* (Onay, 2000). In this study, three different media (MS, SH and B_5) was used to test the efficacy of shoot induction. Among the three media MS was found to be the best medium. The need of MS salts for shoot multiplication shows the moderate salt requirement for the growth of *W. somnifera*. The MS medium was supplemented with different combinations and concentrations of auxins and cytokinin to promote shoot multiplication BAP and IAA together in the medium each at 1.5 mg L^{-1} produced maximum shoots. It was similar to the findings of Salvi *et al.* (2001) in Neem where, the highest number of shoots was produced in combination of BAP and IAA. Similar results were also observed by others Tivarekar and Eapen (2001) in Mung bean; Singh and Sehgal (1999) in *Ocimum sanctum*. Synergistic effect of auxins in combination with BAP on the enhancement of shoot multiplication was observed in *Dubrisia myoporoides* (Kukreja and Mathur, 1985) and *Velariana wallichi* (Mathur *et al.*, 1988). Shoot buds induced on the optimum medium failed to elongate rapidly. Therefore, microshoots were transferred to MS medium supplemented with different concentrations of GA_3. GA_3 when tried at 0.3 mg L^{-1} increased the shoot length by upto 12 cm in *W. somnifera*. This was supported by the observations of Amutha *et al.* (2003) in *Vigna radiata*. Complete root induction was obtained in *W. somnifera*, cultured in half strength MS medium that contained 2 mg L^{-1} IBA, which was similar to the results observed by Manickam *et al.* (2000), Rani and Grover (1999), Sivanesan and Murugesan (2005) and Sivanesan (2007a). Root induction was tried using half strength MS medium supplemented with different carbon sources. Among them, sucrose proved to be the best carbon source. This was similar to the findings of Marin and Marin (1998) in *Prunus* sp. The root growth in glucose or fructose containing medium was remarkably slower than that was in sucrose amended medium (Inomata *et al.*, 1993). A successful tissue culture method of propagation must result in re-establishment in soil of a high frequency of the tissue culture derived plants. In this study, regenerated plantlets of *W. somnifera* were transferred to the field with 87% of survival rate. Rani and Grover (1999) obtained 83% of survival rate in the same plant. Manickam *et al.* (2000) observed only 84% while Kulkarni *et al.* (2000) observed 100% of survival rate in *W. somnifera*. Micropropagation through tissue culture enables the large scale production of the plants. In this study, an efficient protocol was developed for regeneration of *W. somnifera* by using nodal explants.

REFERENCES

Amutha, S., A. Ganapathi and M. Muruganantham, 2003. *In vitro* organogenesis and plant formation in *Vigna radiata* (L.). Wilczek. Plant Cell Tissue Org. Cult., 72: 203-207.

Devi, P.U., A.C. Sharada, F.E. Solomon and M.S. Kamath, 1992. *In vivo* growth inhibitory effect of *Withania somnifera* (Ashwagandha) on a transplantable mouse tumour, Sarcoma 180. Indian J. Exp. Biol., 30: 169-172.

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

Gamble, J.S., 1918. Flora of the Presidency of Madras 2. Botanical Survey of India, Calcutta, India.

Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirement of suspension cultures of soybean root cells. Exp. Cell Res., 50: 151-158.

Handique, P.J. and P. Bora, 1999. *In vitro* regeneration of a medicinal plant *Houttuynia cordata* Thunb. from nodal explants. Curr. Sci., 76: 1245-1247.

Inomata, S., M. Yokoyama, Y. Gozu, T. Shimizu and M. Yanagi, 1993. Growth pattern and ginsenoside production of Agrobacterium-transformed *Panax ginseng* roots. Plant Cell Rep., 12: 681-686.

Kiritikar, K.R. and B.D. Basu, 1975. Indian Medicinal Plants 3. 1st Edn., Bishen Singh Mahendra Pal Sing, Dehra Dun .

Kukreja, A.K. and A.K. Mathur, 1985. Tissue culture studies in *Duboisia myoporoides*; 1. Plant regeneration and clonal propagation by stem node cultures. Planta Med., 51: 93-96.

Kulkarni, A.A., S.R. Thangane and K.V. Krishnamoorthy, 1999. *Withania somnifera* (L.) Dunal. In: Biotechnological Applications of Plant Tissue and Cell Culture. Ravishankar, G.A. (Ed.). Oxford, IBH, India, pp: 156-159.

Kulkarni, A.A., S.R. Thangane and K.V. Krishnamoorthy, 2000. Direct shoot regeneration from node, internode, hypocotyl and embryo explants of *W. somnifera*. Plant Cell Tissue Org. Cult., 62: 203-209.

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

Marin, M.L. and J.A. Marin, 1998. Excised rootstock roots cultured *in vitro*. Plant Cell Rep., 18: 350-355.

Mathur, J., P.S. Ahuja, A. Mathur, A.K. Kukreja and N.C. Shah, 1988. *In vitro* propagation of *Valeriana wallichii*. Planta Med., 54: 82-83.

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

Onay, A., 2000. Micropropagation of Pistachio from mature trees. Plant Cell Tissue Org. Cult., 60: 159-163.

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

Salvi, N.D., H. Singh, S. Tivarekar and S. Eapen, 2001. Plant regeneration from different explants of neem. Plant Cell Tissue Org. Cult., 65: 159-162.

Schenk, R.U. and A.C. Hildebrandt, 1972. Medium and techniques for induction and growth of monocotyledons and dicotyledonous plant cell cultures. Can. J. Bot., 50: 199-204.

Singh, N.K. and C.B. Sehgal, 1999. Micropropagation of Holy Basil (*Ocimum sanctum* Linn.) from young inflorescences of mature plants. Plant Growth Regul., 29: 161-166.

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

Sivanesan, I., 2007a. Direct regeneration from apical bud explants of *Withania somnifera* Dunal. Indian J. Biotechnol., 16: 125-127.

Sivanesan, I., 2007b. Shoot regeneration and somaclonal variation from leaf callus cultures of *Plumbago zeylanica* Linn. Asian J. Plant Sci., 6: 83-86.

Sivanesan, I. and B.R. Jeong, 2007a. Micropropagation and *in vitro* flowering in *Pentanema indicum* Ling. Plant Biotechnol., 24: 527-532.

Sivanesan, I. and B.R. Jeong, 2007b. Direct shoot regeneration from nodal explants of *Sida cordifolia* Linn. *In vitro* Cell Dev. Biol. Plant, 43: 436-441.

Tivarekar, S. and S. Eapen, 2001. High frequency plant regeneration from immature cotyledons of mungbean. Plant Cell Tissue Organ Cult., 66: 227-230.