Pak. J. Bot., 42(6): 4087-4093, 2010.

RAPID *IN VITRO* MULTIPLICATION OF *ACACIA NILOTICA* SUBSP. *HEMISPHERICA*, A CRITICALLY ENDANGERED ENDEMIC TAXON

HAIDER ABBAS[1*], MUHAMMAD QAISER[2] AND BEENA NAQVI[3]

[1]Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, Karachi-75270, Pakistan,
[2]Federal Urdu University of Arts, Science and Technology, Karachi -75300, Pakistan,
[3]Plant Tissue Culture Laboratory, Pakistan Council for Scientific and Industrial Research (PCSIR) Laboratories Complex, Karachi-75280, Pakistan.
[*]Corresponding author: invitro.life@gmail.com

Abstract

Acacia nilotica Willd. ex Delile subsp. *hemispherica* is an endangered and endemic taxon reported from Southern Pakistan. Hence an urgent conservation strategy is required due to exposure of the taxon to habitat loss and its over-exploitation. A micropropagation system was developed for *Acacia nilotica* by comparing MS and B5 media effects on growth. Fresh seeds were collected from the wild, germinated *in vitro* and these seedlings were used as an explant source. The efficiency of B5 and MS medium was thoroughly examined by augmenting with various levels of BAP along with 0.5 mg/l NAA. MS media proved more appropriate than B5 medium and produced the highest number (4.23) of shoots with 43.2% shoot regeneration frequency in the presence of 2.0 mg/l BAP and 0.5 mg/l NAA. IAA (3.0 mg/l) produced maximum number (2.80) of roots along with the highest rooting frequency (95%).

Introduction

Acacia nilotica Willd. ex Delile subsp. *hemispherica* Ali & Faruqi (Fabaceae-Mimosoideae), is a large igloo shaped shrub with several stems arising from the base. It is an endemic taxon, confined on the dried stream beds in and around paradise point and adjoining areas near Karachi (Ali & Faruqi, 1969; Ali, 1973; Abbas, 2010). Habitat destruction and the continuous harvesting of native plants are the main factors affecting nearly all plant species (Malda *et al*., 1999; Beck & Dunlop, 2001) while, species rate of natural recruitment also has a direct influence on its extinction (Martin & Pardeep, 2003). Abbas (2010) has demonstrated that anthropogenic activities i.e., fuel wood cutting, grazing, poultry business and its extremely restricted geographic range (16.01 km^2) played a vital role in its habitat loss and low population.

According to Dewan (1992) the natural regeneration/recruitment of *Acacia nilotica* subsp. *indica* is quite low in India. During four years of field study (2005-2008), it was recorded that population of *Acacia nilotica* subsp. *hemispherica* is continuously declining with a threatening rate without having any natural recruitment. Hence, taxon was classified as Critically Endangered (CR), according to IUCN (2001) Red List Criteria and Categories (Abbas, 2010).

As the taxon is surviving in a hostile environment with a low population size, there is a dire need for a conservation strategy to avoid its extinction (Abbas, 2010). Although species conservation is achieved most effectively through the management of wild populations in its natural habitats (*in situ* conservation) (Maunder *et al*., 1998; Ramsay *et al*., 2000) but it is becoming impractical due to the disappearance of large wild areas (Englemann, 1991; Seeni & Decruse, 2007). Ex-situ conservation has gained the international recognition through its inclusion in the ninth article of Convention on Biological Diversity (CBD).

In vitro plant micropropagation or storage (conservation) reported as safer alternative for rare and endangered plant germplasm (De Langhe, 1984; Withers, 1989; Holobiuc *et al.*, 2009) and in case of species having poor and uncertain responses to conventional methods of propagation (Sarasan *et al*., 2006) it is the only feasible way to maintain a gene bank of plants for their future sustainable utilization (Martin & Pardeep, 2003).

There are no reports on the *in vitro* culture of *Acacia nilotica* subsp. *hemispherica*. The prime focus of the present investigation was to establish a rapid *in vitro* micropropagation/multiplication protocol for the taxon. The lack of published methods for *in vitro* culture of wild taxa and the limited amount of experimental plant material make the choice and development of initial culture medium for rare and threatened plants somewhat arbitrary (Krogstrup *et al.*, 2005).

Materials and Methods

Fresh seeds of *Acacia nilotica* subsp. *hemispherica*, were collected from the wild population and used as an initiating material for further study. Seeds were thoroughly washed for 20 minutes under running tap water, followed by quick dip in 95% ethanol for 20 seconds. Thereafter, surface sterilization was performed using 10% commercial bleach (Sodium Hypochlorite, NaOCl) solution containing 3-6 drops of Tween 20 in 200 ml solution for 15 minutes. Sterilized seeds were rinsed 3 times with autoclaved distilled water before inoculating on nutrient media. Different concentrations of growth regulators {i.e. 6-benzylaminopurine (BAP), Naphthalene Acetic Acid (NAA), Indole-3 Acetic Acid (IAA) and Indole Buteric Acid (IBA)} were incorporated in the media along with 3% sucrose. 0.6% Phytagel (P8169-Phytagel, Sigma-Aldrich, St. Louis. Mo. USA) was used as a gelling agent. For shoot induction/multiplication BAP was incorporated in MS (Mureshige & Skoog, 1962) and B5 (Gamborg *et al*., 1968) media at the level of 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 & 4.0 mg/l along with invariable dose of 0.5 mg/l NAA. IAA and IBA each was used at the level of 0.0, 1.0, 2.0 & 3.0 mg/l for root regeneration. The pH of the medium was adjusted to 5.8 prior to autoclave (121^{o}C for 15 minutes). The autoclaved media were properly stored in storage room till its use. Seeds were inoculated on specific media within laminar flow hood (Technical Scientific, Lahore, Pakistan) to avoid any contamination. Glass bottles with plastic caps were used for culture. After inoculating seeds on hormone free MS media, seeds were incubated at 25^{o}C under 16 hours of photoperiod. Light was provided using 40 watts, normal cool white florescent tubes (Philips-TL40W 54). Intensity of light ranged from 2000 to 3000 lux of energy. Sterile seedlings were used for the collection of explants (nodal segments) for establishing *In vitro* cultures. The nodal segments were cultured on different levels of growth hormones in MS or B5 media for the shoot induction/multiplication. Experiment was laid out according to completely randomised design (CRD) with 5 replicates per treatment. Established shoot cultures were sub-cultured after 4-6 months depending upon the growth and condition of media in the glass jars. Full strength MS media containing IAA or IBA, was used for root induction. The data was recorded on frequency (%) and number of shoots and roots in various combinations of media. The data was statistically analysed using Duncan's Multiple Range Test (DMR).

Results

i. Effect of BAP-NAA in B5 medium on shoot regeneration: Various levels of concentrations of BAP - NAA present in B5 medium had a significant effect on shoot regeneration frequency and number of shoots per explant in *Acacia nilotica* subsp.

hemispherica (Table 1). Results suggest that the means for frequency of regeneration and number of shoots per explant ranged 3.0 to 39.6% and 0.60 to 3.91, respectively. Maximum number of shoots per explant was recorded at 3.0 mg/l BAP - 0.5 mg/l NAA. Significantly low number of shoots (0.60) per explant was recorded in the absence of plant growth regulators.

ii. Effect of BAP-NAA in MS medium on shoot regeneration: BAP-NAA at different concentrations in MS medium had significant effect on shoot regeneration frequency and number of shoots per explant in *A. nilotica* subsp. *hemispherica*. The results (Table 2) showed that the means for frequency of regeneration and number of shoots per explant ranged 10.0 to 43.2% and 1 to 4.23, respectively. Maximum number of shoots (4.23) per explant was recorded on MS medium containing 2.0 mg/l BAP -0.5 mg/l NAA. However, statistically similar number of shoots per explant was recorded on MS medium containing 1 to 4 mg/l BAP -0.5 mg/l NAA. Single shoots per explant were recorded in the absence of plant growth regulators.

iii. Effect of IAA or IBA in MS medium on number of roots: Various concentrations of IAA or IBA in MS medium had significant effect on rooting frequency and number of roots per explant in *A. nilotica* subsp. *hemispherica*. The means for frequency of rooting and number of roots per explants are presented in Table 3. The results showed that the maximum rooting frequency of 95% was recorded on MS medium containing 3 mg/l IAA. By contrast, IBA was not promising and resulted in sharply reduced frequency of rooting on any concentration of IBA. Similarly, maximum number of 2.80 roots per explant was recorded on MS medium containing 3.0 mg/l IAA. In general, each increase in the concentration of auxins resulted in corresponding increase in the frequency and number of roots per explant, with maximum roots at 3.0 mg/l of both the auxins. The results showed no interaction between the type of auxin and their concentration. However, in numeric terms IAA was more appropriate compared to IBA. Negligible and weak roots were recorded in the absence of IAA or IBA or at 1 mg/l IAA or IBA in the rooting medium.

Discussion and Conclusion

In vitro techniques are the useful means for the production of plantlets from young and mature trees with a lower risk of genetic instability (Rao and Lee, 1986). Rout *et al.* (2008) observed the highest number of shoots per explant (4.21) from nodal cuttings of seedlings of *Acacia chuandra,* cultured on MS medium containing 1.5 mg/l of BAP - 0.05 mg/l IAA. Mathur & Chandra (1983) also observed development of multiple shoots from nodal explants of *Acacia nilotica* on auxin (0.5-1.0 mg/l IAA) containing media. While, Arya & Shekhawat (1987), Coleman & Ernst (1990) and Dewan *et al*. (1992) reported cytokinins as an obligatory part of the media for shoot differentiation. Their results are in agreement with our studies, where nodal segments obtained from two week old seedlings induced maximum number of 4.23 and 4.12 shoots per explant on MS medium, containing 2.0 mg/l BAP and 0.5 mg/l NAA and 2.5 mg/l BAP and 0.5 mg/l NAA, respectively. Our results are also in conformation with Rout *et al*. (2008), who obtained 4.21 shoots per explant using 1.5 mg/l BAP - 0.05 mg/l IAA. However, the results are not in agreement with Kaur *et al*. (1998), who recorded 8-10 shoot per explant (*Acacia catechu* Willd) from nodal explants on MS medium containing 4.0 mg/l of BAP - 0.5 mg/l NAA.

Table 1. Effect of BAP-NAA in B5 medium on frequency and number of shoots per explant from nodal cuttings of seedlings of *Acacia nilotica* subsp. *hemispherica*.

BAP concentrations (mg/l)	NAA concentrations (mg/l)	Frequency of shoot regeneration (%)	Mean number of shoots per explant
0	0	3.0c	0.60c
0.5	0.5	22.0b	2.20b
1.0	0.5	37.6a	3.61a
1.5	0.5	33.0a	3.11ab
2.0	0.5	35.0a	3.50a
2.5	0.5	37.6a	3.75a
3.0	0.5	39.6a	3.91a
3.5	0.5	32.6a	3.25a
4.0	0.5	32.0a	3.41a

Values within column followed by similar letters are non-significantly different in accordance with Duncan's Multiple Range Test

Table 2. Effect of BAP-NAA in MS medium on frequency and number of shoots per explant of *Acacia nilotica* subsp. *Hemispherica*.

BAP concentrations (mg/l)	NAA concentrations (mg/l)	Frequency of shoot regeneration (%)	Mean number of shoots per explant
0	0	10.0c	1.0c
0.5	0.5	22.0c	2.20bc
1.0	0.5	32.4ab	3.20ab
1.5	0.5	33.6ab	3.26ab
2.0	0.5	43.2a	4,23a
2.5	0.5	41.2a	4.12a
3.0	0.5	39.6a	3.95a
3.5	0.5	39.0a	3.91a
4.0	0.5	41.6a	4.18a

Values within column followed by similar letters are non-significantly different in accordance with Duncan's Multiple Range Test

Table 3. Effect of IAA and IBA in MS medium on *in vitro* rooting of *Acacia nilotica* subsp. *Hemispherica*.

Concentration mg/L	Frequency (%) of root regeneration		Number of roots per explants	
	IAA	IBA	IAA	IBA
0	10.0d	5.0c	0.40b	0.20b
1.0	20.0c	5.00c	0.60b	0.00b
2.0	60.0b	10.0b	1.20b	1.00a
3.0	95.0a	25.0a	2.80a	1.20a

Values within column followed by similar letters are non-significantly different in accordance with Duncan's Multiple Range Test

While, using B5 media for shoot regeneration, maximum number of shoots (3.91) were recorded, when nodal explants were taken from two weeks old seedlings and inoculated at 3.0 mg/l BAP - 0.5 mg/l NAA. However, a previous study by Dewan *et al*. (1992), showed higher number of shoots (6.3) per nodal explant on B5 medium supplemented with 1.5 mg/l BAP. The results of this study correspond with those of Dewan *et al*. (1992) and Gupta & Agrawal (1992) for *A. nilotica;* however, Mittal *et al*. (1995) and Gupta & Agrawal (1992) recorded multiple shoots from nodal segments on B5 medium containing different concentrations of BA in *A. auriculiformis*.

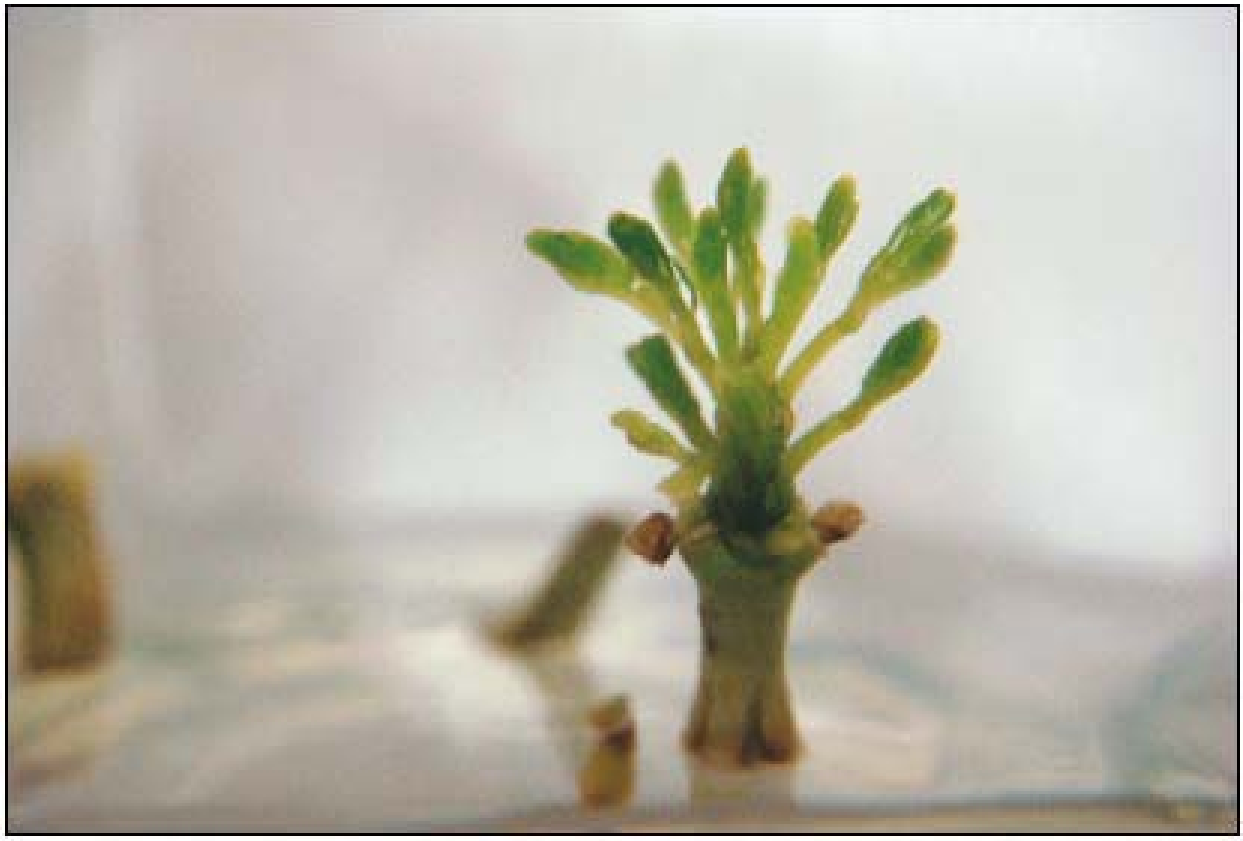

Fig. 1. Multiple shoot regeneration from nodal explant of *Acacia nilotica* subsp. *hemispherica* after 16 weeks of culture.

Fig. 2. Rooting of *Acacia nilotica* subsp. *hemispherica* on MS medium containing 30.0 mg/l IAA.

Full strength MS medium containing different concentrations of IBA, IAA or NAA was found appropriate for rooting in *Acacia mangium* (Galiana *et al*., 1991; Darus, 1991; Bhaskar & Subhash, 1995; Toda *et al*., 1995; Nanda *et al*., 2004); *Acacia auriculiformis* (Das *et al*., 1993; Toda *et al*., 1995); *Acacia koa* (Skolmen & Mapes, 1976); *Acacia saligna* (Barakat & El-Lakany, 1992); *Acacia mearnsii* (Huang *et al*., 1994); *Acacia sinuata* (Vengadesan *et al*., 2002); *Acacia chundra* (Rout *et al*., 2008) and *Acacia catechu* (Kaur *et al*., 1998). These studies corroborate the findings of previous works where, *in vitro* grown 1-4 cm long shoots of *Acacia* sp. were rooted on MS medium containing different concentrations of IBA or IAA. In the current investigation, greater number (2.80) of roots was recorded in the presence of 3.0 mg/l IAA, while IBA at the same concentration did not able to produce more then 1.20 roots per shoot explant.

Thus it is concluded that MS medium is appropriate for *in vitro* culture and rooting of *A. nilotica* subsp. *hemispherica*. Hence, *Acacia nilotica* subsp. *hemispherica* can successfully be multiplied or conserved *in vitro* by using MS medium augmented with a combination of BAP (2.5 mg/l) - KIN (0.5 mg/l) - NAA (0.5 mg/l) for acquiring the highest level of shoot multiplication, while 3.0 mg/l IAA in the same medium found precise for root regeneration.

References

Abbas, H. 2010. `*Ex-situ* conservation of some threatened endemic and rare plants of southern Pakistan through tissue culture`. Ph.D. Thesis, University of Karachi, Pakistan.

Ali, S.I. 1973. Mimosaceae. In: *Flora of Pakistan*. (Eds.): E. Nasir & S.I. Ali. No. 36. Department of Botany University of Karachi and National Herbarium Islamabad.

Ali, S.I. and S. A. Faruqi. 1969. A taxonomic study of *Acacia nilotica* complex in West Pakistan. *Pakistan Journal of Botany* 1: 1-8.

Arya, H.C. and N.S. Shekhawat 1987. In: *Proceedings of the Symposium on Plant Cell and Tissue Culture of Economically Important Plants.* (Ed.):G.M. Reddy. Hyderabad, India; 160-163.

Barakat, M.N. and M.H. El-Lakany. 1992. Clonal propagation *Acacia saligna* by shoot tip culture. *Euphytica*, 59: 103-107.

Beck, S.L. and F.A. Dunlop. 2001. Micropropagation of the *Acacia* species: a review. *In Vitro Cellular & Developmental Biology Plant*, 37: 531-8

Bhaskar, P. and K. Subhash. 1995. Micropropagation of *Acacia mangium* Willd through nodal bud culture. *Indian Journal of Experimental Biology*, 34: 590-591.

Coleman, G.D. and S.G. Ernst. 1990. Axiliary shoot proliferation and growth of *Populus deltoides* shoot culture. *Plant Cell Report*, 9: 165-167.

Darus, H.A. 1991. Multiplication of *Acacia mangium* by stem cuttings and tissue culture techniques. *Advanced Tropical Acacia Research Proceedings Series*, 35: 32-35.

Das, P.K., V. Chakravarti and S. Maity. 1993. Plantlets formation in tissue culture from cotyledon of *Acacia auriculiformis* A. Cunn. ex Benth. *Indian Journal of Forestry*, 16: 189-192.

De Langhe, E. A. L. 1984. The role of *in vitro* techniques in germplasm conservation. In: *Crop Genetic Resources: Conservation and Evaluation*: (Eds.): J.H.W. Holden & J.T. Williams 131-137. London: Allen & Unwin.

Dewan, A., K. Nanda and S.C. Gupta. 1992. *In vitro* micro-propagation of *Acacia nilotica* subsp. *indica* Brenan via cotyledonary nodes. *Plant Cell Report*, 12: 18-21.

Englemann, F. 1991. *In vitro* conservation of tropical plant germplasm: a review. *Euphytica*, 57: 227-243.

Galiana, A.A., A. Tibok and E. Duhoux. 1991. *In vitro* propagation of the nitrogen fixing, tree legume *Acacia mangium* Willd. *Plant Soil*, 135: 151-159.

Gamborg, O.L., R.A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, 50: 151-158.

Gupta, S.C. and V.P. Agrawal. 1992. Micropropagation of woody taxa and plant productivity. In: *Role of Biotechnology in Agriculture*. *International Science Publisher*, (Eds.): B.N. Prasad, G.P.S. Ghimire, V.P. Agrawal. New York; 37-52.

Holobiuc, I., R. Blându and V. Cristea. 2009. Research concerning *in vitro* conservation of the rare plant species *Dianthus nardiformis* Janka, *The Journal of Biotechnology & Biotechnological Equipment*, 23(2): 221-224.

Huang, F.H., J.M. Al-Khayri and E.E. Gbur. 1994. Micropropagation of *Acacia mearnsii*. *In Vitro Cellular Developmental Biology–Plant*, 30: 70-74.

Kaur, K., B. Verma and U. Kant 1998. Plants obtained from the Kahir tree (*Acacia catechu* Willd) using mature nodal segments. *Plant Cell Report*, 17: 427-429.

Krogstrup, P., J.I. Find, D.J. Gurskov and M.M.H. Kristensen. 2005. Micropropagation of Socotran fig, *Dorstenia gigas* Scrweinf. Ex. Balf. F. – a threatened species, endemic to the island of Socotra, Yemen. *In Vitro Cellular & Developmental Biology – Plant*, 41: 81-86.

Malda. G., H. Suzán and R. Backhaus. 1999. *In vitro* culture as a potential method for the conservation of endangered plants possessing crassulacean acid metabolism. *Scientia Horticulturae*, 81: 71-87.

Martin, K.P. and A.K. Pradeep. 2003. Simple strategy for the in vitro conservation of Ipsea malabarica an endemic and endangered orchid of the Western Ghat of Keral, India. *Plant Cell, Tissue and Organ Culture*, 74: 197-200.

Mathur, I. and N. Chandra. 1983. Induced regeneration in stem explants of *Acacia nilotica*. *Current Science*, 52: 882-883.

Maunder, M., S. Higgens and A. Culham. 1998. Neither common nor garden; the garden as refuge for threatened plant species. *Curtis Botanical Magazine*, 15: 124-132.

Mittal, A., Agarwal, R. and S.C. Gupta. 1989. *In vitro* development of plantlets from axillary buds of *Acacia auriculiformis* – a leguminous tree. *Plant Cell Tissue and Organ Culture*, 19: 65-70.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15: 473-497.

Nanda, R.M., P. Das and G.R. Rout. 2004. *In vitro* clonal propagation of *Acacia mangium* Willd. and its evaluation of genetic stability through RAPD marker. *Annals of Forest Science* 61: 381-386.

Ramsay, M.M., A.D. Jackson and R.A. Porley. 2000. A pilot study for exsitu conservation of UK bryophytes. In: (Eds.): BGCI, EuroGard 2000 – II European Botanic Gardens Congress. Canary Islands, Las Palmas de Gran Canaria, Spain; 52-57.

Rao, A.N. and S.K. Lee. 1986. An overview of the *in vitro* propagation of woody plants and plantation crops. In: *Plant tissue culture and its agricultural application*. (Eds.): L.A. Withers, and P.G. Aldeson. University Press Cambridge, UK; 123-138.

Rout, G.R., Senapati and S.K.S. Aparajeta. 2008. Micropropagation of *Acacia chundra* (Roxb.) DC. *Horticultural Science*, 35(1): 22-26.

Sarasan, V., R. Cripps, M.M. Ramsay, C. Atherton, M. McMichen, G. Prendergast and J.K. Rowntree. 2006. Conservation *in vitro* of threatened plants - progress in the past decade. *In Vitro Cellular & Developmental Biology – Plant*, 42: 206-214.

Seeni, S. and S.W. Decruse. 2007. *In vitro* multiplication and restoration of selected rare, endangered and threatened plants of India. In: *Proceedings of 3rd Global Botanic Garden Congress* (3GBGC), Wuhan, China.

Skolmen, R.G. and O.M. Mapes. 1976. *Acacia koa* gray plantlets from somatic callus tissue. *Journal of Heredity*, 67: 114-115.

Toda, T., M. Tajima and Brini. 1995. Tissue culture of *Acacia mangium* and *Acacia auriculiformis* and *Acacia* hybrid. *Bulletin of National Tree Breeding Center*, 13: 81-94.

Vengadesan, G., A. Ganapathi, R.P. Anand and V.R. Anbazhagan. 2002. *In vitro* propagation of *Acacia sinuata* (Lour.) Merr. via cotyledonary nodes. *Agroforestory Systems*, 55: 9-15.

Withers, L. A. 1989. *In vitro* conservation and germplasm utilization. In: (Eds.): A.D.H. Brown, D.R. Marshall, O.H. Frankel & J.T. Williams. The Use of Plant Genetic Resources: 309-334. Cambridge: Cambridge University Press.

(Received for publication 17 December 2009)