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In Vitro Shoot Regeneration from Embryonic Axis of a Multipurpose Vulnerable Leguminous Tree, *Saraca indica* L.

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ABSTRACT

Saraca indica L. syn. *Saraca asoca* (Roxb.) De Wilde (Caesalpinaceae) is a commercially important evergreen medicinal tree. The annual demand for its bark is 10,724 tonnes and is growing at 15% per annum in India. Commercial exploitation for its bark has led to it becoming vulnerable, resulting in poor regeneration in its natural habitat. Tissue culture offers a viable alternative for production of a large number of plantlets within a short period of time. The present paper describes the formation of shoots through embryonic axis under the influence of two cytokinins, viz., 6-benzyladenine (BA) and zeatin (Zn). Embryonic axes were excised from one-month old immature seeds and a 1-2 mm cut was introduced at the radicle end. The embryonic axis was inoculated onto B_5 medium supplemented with BA (0 and 2.5 µM) and Zn (0, 5, 10 and 20 µM) to study the effect on direct or indirect organogenesis. BA had a significant effect on the number of shoots formed and callus formation (%). After one month B_5 medium supplemented with 2.5 µM BA regenerated both shoots and callus from embryonic axes. The dose of Zn and its interaction with BA did not significantly affect callus formation, shoot formation or the number of shoots. These shoots were multiplied further on 2.5 µM BA supplemented medium.

Keywords: callus formation, Caesalpinaceae, cytokinins, embryonic axis, micropropagation
Abbreviations: BA, -benzyladenine; **B_5**, Gamborg's medium, **Zn**, zeatin

INTRODUCTION

Leguminous forest trees are of special interest because of their economic and ecological importance. Further, tree legumes are well known for their utility to mankind, but relatively less success has been achieved in their propagation using stem or root cuttings (Rajadurai *et al*. 1989). Thus, over the years an increasing interest has been shown in the application of *in vitro* techniques for their rapid propagation as evinced by reports on tissue culture of *Acacia koa* (Skolmen and Mapes 1976), *Sesbenia grandiflora* (Khattar and Mohan Ram 1983), *Albizia richardiana* (Tomar and Gupta 1988), *Prosopis cineraria* (Shekhawat *et al*. 1993), *Dalbergia sissoo* (Chauhan *et al.* 1996), *Bauhinia vahlii* (Upreti and Dhar 1996), *Acacia catechu* (Kaur and Kant 2000; Sahni and Gupta 2002), *Acacia sinuata* (Vengadesan 2003), *Pterocarpus marsupium* (Chand and Singh 2004; Tiwari *et al*. 2004; Anis *et al*. 2005; Husain *et al*. 2007, 2008) *Pterocarpus santalinus* (Prakash *et al*. 2006), *Robinia ambigua* (Guo *et al*. 2006) and *Cassia siamea* (Parveen *et al*. 2010). Extensive efforts to develop efficient regeneration systems for many legumes have resulted in a large array of *in vitro* protocols (Rout 2005; Veltcheva *et al*. 2005; Agrawal and Sardar 2006; Faisal *et al*. 2006; Shahzad *et al*. 2007; Siddique and Anis 2007; Aasim *et al*. 2008). However, forest trees in general and Leguminosae in particular, have proved to be recalcitrant for mass propagation by tissue culture (Anis *et al*. 2005; Veltcheva *et al*. 2005; Agrawal and Sardar 2006). *Saraca indica* L. syn. *Saraca asoca* (Roxb.) De Wilde (Caesalpinaceae, common name Sita Ashok) is a low-branched, evergreen tree, reaching a height of 6-9 m or more with a dense crown of horizontally spreading branches distributed in India, Bhutan, Nepal, Myanmar, Malaysia and Sri Lanka. It is often planted as an ornamental or avenue tree due to its religious significance. This scared tree has a wide spectrum of medicinal properties. The bark is employed in Indian systems of medicine to cure internal uterine haemorrhages, colic piles, ulcers, dyspepsia and to beautify the complexion. The flowers are considered useful in acute dysentery and diabetes and as an excellent uterine tonic (Anon 1972, Kirtikar and Basu 1981; Karki and Williams 1999). The fruits are chewed by tribal people as a substitute for areca nuts (Troup 1983). The annual demand for its bark is 10,724 tonnes and is growing at 15% per annum in India. The demand is largely met from the wild, leading to its depletion in its natural habitats. Thus, IUCN has categorized it as globally vulnerable (IUCN 2004). The seeds retain viability for only about 2 months and so should be sown as soon as possible after collection. Vegetative propagation methods for this species have, however, not been reported for production of large quantity of its clonal planting stock. The above information about *S. indica* clearly advocates its multiplication through different modes of *in vitro* regeneration. Thus, in the present study effect of two cytokinins, viz., BA (6-benzyladenine) and zeatin (Zn) on formation of shoots along embryonic axis of immature seeds was investigated. To the best of our knowledge, this is the first report of *in vitro* shoot formation in *S. indica*.

MATERIALS AND METHODS

Culture establishment

Green pods were collected from a single tree, 90 days after appearance of first flowers on the tree (**Fig. 1A**). The pods were thoroughly washed under running tap water (**Fig. 1B**). Thereafter, they were continuously agitated in 2% solution of Cetrimide (ICI Ltd., Patiala, India) for 30 min. After thorough rinsing three-four times in distilled water to remove all traces of soap, they were soaked in 0.2% aqueous solution of fungicide Bavistin® (BASF India Ltd., Mumbai, India) and 0.5% aqueous solution of antibiotic Ambistryn-S® (Piramal Healthcare Ltd., Mumbai, India) for 30 min each. Surface sterilization of pods was carried out under aseptic conditions with 0.6% aqueous $HgCl_2$ solution for 15 min.

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Fig. 1 (**A**) Flowering tree of *Saraca indica* L.; (**B**) green pods; (**C**) seeds inside the pods, (**D, E**) Shoot formation from embryonic axis on B_5 medium supplemented with 2.5 µM BA.

Then the pods were rinsed thoroughly in sterile distilled water three times. The pods were opened with the help of sterile forceps and scalpel and the white immature seeds (around one month after seed formation started) were taken out (**Fig. 1C**). The seeds were split longitudinally and embryonic axis was excised. A 1-2 mm cut was given at the radicle end and the embryos were inoculated individually on B_5 medium (Gamborg *et al*. 1968) supplemented with 6-benzyl adenine (BA, 0 and 2.5 µM) and zeatin (Zn, 0, 5, 10 and 20 µM) to study the effect on direct or indirect organogenesis. Observations on callus formation (%) shoot formation (%) and the number of shoots formed was recorded after 4 weeks.

Culture conditions

The MS medium supplemented with 3% (w/v) sucrose (DSM, Dhampure, India), 0.8% agar (Qualigens Pvt. Ltd., Mumbai, India) and 0.01% (w/v) *myo*-inositol was used in all experiments. The pH of the medium was adjusted to 5.8, prior to autoclaving for 15 min at 15 psi. Each explant was cultured in a 25 mm × 150 mm culture tube containing 15 ml of sterilized semisolid medium for culture establishment. The cultures were incubated at a temperature of 25 ± 2°C under 16-h daily illumination (approx. 45 µ mol m^{-2} s^{-1}) with white fluorescent light in the culture room.

Statistical analysis

The experiment comprised of three replicates with ten samples per treatment. The data was analysed with SX statistical package according to a completely randomized design using two-way analysis of variance. The interactions of the treatments were studied in factorial combinations. Data expressed in percentage was transformed using the arc sine transformations (Gomez and Gomez 1984). The significance of the data was ascertained by the F-test and Least Significant Difference (LSD) values at p=0.05.

RESULTS AND DISCUSSION

Effect of BA and Zn on callus and shoot formation on embryonic axis

1. Callus formation

Brown callus was obtained 10-15 days after inoculation, on the surface of embryonic axis of immature seeds of *S. indica*. BA individually had statistically significant effect on formation of callus on embryonic axis (**Table 1**). Callus formation (64%) was obtained on B_5 medium supplemented with 2.5 µM BA, which was an enhancement of 94.8% over the control. Zn individually did not have any significant effect on callus formation and callus obtained on all four treatments of Zn was statistically on par. Similarly, the interactions between BA and Zn had statistically non-significant effect on callus formation. Significant effect of BA on production of callus on embryonic axes has been reported in other tree species like *Juglans regia* (Heile-Sudholt *et al*. 1986) and *Pinus pinaster* (Rancillac 1991). In contrast to our results, callus on embryonic axis was obtained by using kinetin in lentil, *Lens culinaris* Medik (Nafees *et al*. 2009).

2. Shoot formation

Shoot initiation could be seen as small green bud like structures after 15 days. Different doses of BA, Zn and interactions between BA and Zn did not have significant effect on shoot formation (**Table 2**).

3. Number of shoots

The number of shoots formed on the embryonic axis was significantly influenced by BA. On B_5 medium supplemented with 2.5 µM BA, 0.74 shoots were produced, registering an increase of 85% over the control (**Fig. 1D, 1E**). Similarly, Vieitez and Vieitez (1980) produced axillary shoot multiplication from embryonic axes on 1 mg/l BA in *Castanea sativa*. Multiple shoots were regenerated from immature embryos of *Populus deltoides* cultured on MS medium supplemented with 0.5 mg/l BA, 150 mg/l PVP and 250 mg/l glutamine (Kouider *et al.* 1984). Similar effects of BA on shoot formation has been reported by other workers in *Pinus ponderosa* (Ellis and Bilderback 1989); *Picea engel-*

Table 1 Effect of BA, zeatin and their interactions on callus formation (%) in embryonic axis of *Saraca indicia* L.

BA (µM)	Zeatin (µM)				Mean
	0	5	10	20	
0	25.00	50.00	22.00	44.30	35.33
	(25.02)	(45.00)	(23.39)	(41.69)	(33.77)
2.5	63.70	66.70	83.30	41.70	63.83
	(53.11)	(55.00)	(74.96)	(35.02)	(54.52)
Mean	44.33	58.33	52.67	43.00	
	(39.06)	(50.00)	(49.18)	(38.35)	
		LSD 5%			
BA (B)		21.68			
Zeatin (Z)		N.S.			
B x Z		N.S.			

The values in parenthesis are arc sine transformed.
LSD: Least significant Difference, N.S.: Not significant.

Table 2 Effect of BA, zeatin and their interactions on shoot formation (%) in embryonic axis of *Saraca indicia* L.

BA (µM)	Zeatin (µM)				Mean
	0	5	10	20	
0	50.00	33.33	19.33	36.00	34.67
	(45.00)	(35.00)	(21.71)	(36.69)	(34.60)
2.5	55.33	41.67	58.33	41.67	49.25
	(48.11)	(40.00)	(50.00)	(40.00)	(44.53)
Mean	52.67	37.50	38.83	38.83	
	(46.55)	(37.50)	(35.85)	(38.34)	
		LSD 5%			
BA (B)		N.S.			
Zeatin (Z)		N.S.			
B x Z		N.S.			

The values in parenthesis are arc sine transformed.
LSD: Least significant Difference, N.S.: Not significant.

Table 3 Effect of BA, zeatin and their interactions on number of shoots formed in embryonic axis of *Saraca indicia* L.

BA (µM)	Zeatin (µM)				Mean
	0	5	10	20	
0	0.50	0.33	0.19	0.58	0.40
2.5	0.80	0.98	0.75	0.42	0.74
Mean	0.65	0.65	0.47	0.50	
		LSD 5%			
BA (B)		0.30			
Zeatin (Z)		N.S.			
B x Z		N.S.			

LSD: Least significant Difference, N.S.: Not significant.

manii (Harry and Thorpe 1991); *Fagus slyvatica* (Vieitiz *et al.* 1993); *Prunus mume* (Ning *et al*. 2007). In many tree species belonging to family- Leguminosae, multiple shoot buds formation was also observed from the embryonic axis on medium incorporated with BA, viz., *Prosopis tamarugo* (Nandwani and Ramawat 1992), *Tamarindus indica* (Mehta *et al.* 2000), *Sophora toromiro* (Jordan *et al*. 2001). In common bean (*Phaseolus vulgaris* L.) also many workers have achieved shoot formation via embryonic axis using BA (Arellano *et al*. 2009; Gatica Arias *et al*. 2010; Kwapata *et al*. 2010). Thus, the results achieved with *S. indica* are in conformity with earlier reports that have shown BA to be the most suitable cytokinin for inducing shoot regeneration in legumes.

Zn doses and interactions between BA and Zn did not have significant effect on number of shoots (**Table 3**). The decotylated embryos or embryonic axes are really dependent on the nutrient elements of the medium. In many cases, exogenously supplied hormones are not required for embryo culture. For some species the medium may be supplemented with hormones at a very low level, in order to better reproduce the conditions of the *in ovulo* environment (Monnier 1995). Thus, in our study only low concentration of BA significantly affected number of shoots whereas the high doses of Zn did not have any statistically significant influence. These shoots are being further multiplied on B_5 medium supplemented with 2.5 µM BA.

CONCLUSION

This study has thrown light on the utility of embryonic axis of immature seeds as a means of shoot formation in a commercially important vulnerable medicinal tree *Saraca indica*, which has not been explored for micropropagation studies earlier.

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REFERENCES

Aasim M, Khawar KM, Özcan S (2008) *In vitro* micropropagation from shoot meristems of Turkish cowpea (*Vigna unguiculata* L.) cv. Akkiz. *Bangladesh Journal of Botany* **37**, 149-154

Agrawal V, Sardar PR (2006) *In vitro* propagation of *Cassia angustifolia* through leaflet and cotyledon derived calli. *Biologia Plantarum* **50**, 118-122

Anis M, Husain MK, Shahzad A (2005) *In vitro* plantlet regeneration of *Pterocarpus marsupium* Roxb., an endangered leguminous tree. *Current Science* **88**, 861-863

Anon (1972) *The Wealth of India, Raw Materials* (Vol IX), Rh-So, CSIR, New Delhi, pp 232-234

Arellano J, Fuentes SI, Castillo-España P, Hernández G (2008) Regeneration of different cultivars of common bean (*Phaseolus vulgaris* L.) via indirect organogenesis. *Plant Cell, Tissue and Organ Culture* **96**, 11-18

Chand S, Singh AK (2004) *In vitro* shoot regeneration from cotyledonary node explants of a multipurpose leguminous tree, *Pterocarpus marsupium* Roxb. *In Vitro Cellular and Developmental Biology – Plant* **40**, 464-466

Chauhan VA, Josekutty PC, Jasraj YT, Prathapasenan G (1996) Rapid *in vitro* multiplication of *Dalbergia sissoo* Roxb. *Journal of Plant Biochemistry and Biotechnology* **5**, 117-118

Ellis D, Bilderback D (1989) Temporal competence of embryonic *Pinus ponderosa* cotyledons to form multiple buds *in vitro*. *American Journal of Botany* **76**, 348-355

Faisal M, Siddique I, Anis M (2006) *In vitro* rapid regeneration of plantlets from nodal explants of *Mucuna pruriens* – a valuable medicinal plant. *Annals of Applied Biology* **148**, 1-6

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soyabean root cells. *Experimental Cell Research* **50**, 151-158

Gatica Arias AM, Valverde JM, Fonseca PR, Melara MV (2010) *In vitro* plant regeneration system for common bean (*Phaseolus vulgaris*): Effect of N^6-benzylaminopurine and adenine sulphate. *Electronic Journal of Biotechnology* **13**, 1

Gomez KA, Gomez AA (1984) *Statistical Procedures for Agricultural Research* (2nd Edn), John Willey and Sons, NY, 680 pp

Guo W-L, Li Y-D, Gong L, Li F-X, Dong Y-S, Liu B (2006) Efficient micropropagation of *Robinia ambigua* var. *idahoensis* (Idaho locust) and detection of genomic variation by ISSR markers. *Plant Cell, Tissue and Organ Culture* **84**, 343-351

Harry IS, Thorpe TA (1991) Engelmann Spruce (*Picea engelmanii*). In: Bajaj YPS (Ed) *Biotechnology in Agriculture and Forestry Trees III* (Vol 16), Springer-Verlag, Berlin, pp 408-422

Heile-Sudholt C, Huetteman CA, Preece JE, van Sambeek JW, Gaffney GR (1986) *In vitro* embryonic axis and seedling shoot tip culture of *Juglans nigra* L. *Plant Cell, Tissue and Organ Culture* **6**, 189-197

Husain MK, Anis M, Shahzad A (2007) *In vitro* propagation of Indian Kino (*Pterocarpus marsupium* Roxb.) using thidiazuron. *In Vitro Cellular and Developmental Biology – Plant* **43**, 59-64

Husain MK, Anis M, Shahzad A (2008) *In vitro* propagation of a multipurpose leguminous tree (*Pterocarpus marsupium* Roxb.) using nodal explants. *Acta Physiologiae Plantarum* **30**, 353-359

IUCN (2004) IUCN Red List of Threatened Species. www.iucnredlist.org

Jordan M, Larrain M, Tapia A, Roveraro C (2001) *In vitro* regeneration of *Sophora toromiro* from seedling explants. *Plant Cell, Tissue and Organ Culture* **66**, 89-95

Karki M, Williams JT (1999) Priority species of medicinal plants in South Asia. International Development Research Centre, New Delhi, India, 22 pp

Kaur K, Kant U (2000) Clonal propagation of *Acacia catechu* Willd. by shoot tip culture. *Plant Growth Regulation* **31**, 143-145

Khattar S, Mohan Ram HY (1983) Organogenesis and plantlet formation *in vitro* in *Sesbania grandiflora*. *Indian Journal of Experimental Biology* **21**, 251-253

Kirtikar KR, Basu BD (1981) *Indian Medicinal Plants* (Vol II), B/s Bishen Singh Mahendra Pal Singh, Dehra Dun, India, pp 883-884

Kouider M, Skirvin RM, Saladin KP, Dawson JO, Jokela JJ (1984) A

method to culture immature embryos of *Populous deltoides in vitro*. *Canadian Journal of Forest Research* **14**, 956-958

Kwapata K, Sabzikar R, Sticklen MB, Kelly JD (2010) *In vitro* regeneration and morphogenesis studies in common bean. *Plant Cell, Tissue and Organ Culture* **100**, 97-105

Mehta UJ, Krishnamurthy KV, Hazra S (2000) Regeneration of plants via adventitious bud formation from mature zygotic embryo axis of tamarind (*Tamarindus indica* L.). *Current Science* **78**, 1231-1234

Monnier M (1995) Culture of zygotic embryos. In: Thorpe TA (Ed) *In Vitro Embryogenesis in Plants*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 117-153

Nafees A. (2009) Comparative study of meristem and callus regenerated lentil (*Lens culinaris* Medik) plants. *Electronic Journal of Environmental Agricultural and Food Chemistry* **8**, 76-80

Nandwani D, Ramawat KG (1992) High frequency plantlets regeneration from seedling explants of *Prosopis tamarugo*. *Plant Cell, Tissue and Organ Culture* **29**, 173-178

Ning GG, Bai SP, Bao MZ, Liu L (2007) Factors affecting plantlet regeneration from *in vitro* cultured immature embryos and cotyledons of *Prunus mume* "Xue mei". *In Vitro Cellular and Developmental Biology – Plant* **43**, 225-230

Parveen S, Shahzad A, Saema S (2010) *In vitro* plant regeneration system for *Cassia siamea* Lam., a leguminous tree of economic importance. *Agroforestry Systems* **80**, 109-116

Prakash E, Khan SW, Shaik P, Rao S, Thoguru JV, Meru ES (2006) Micropropagation of red sanders (*Pterocarpus santalinus* L.) using mature nodal explants. *Journal of Forest Research* **11**, 329-335

Rajadurai D, Rao AN, Loh CS (1989) *In vitro* studies in two leguminous species. In: Rao AN, Yusoff A Mohd (Eds) *Proceedings of Seminar on Tissue Culture of Forest Species*, FRIM and IDRC, Kuala Lumpur, pp 104-128

Rancillac M (1991) Maritime pine (*Pinus pinaster* Sol.). In: Bajaj YPS (Ed) *Biotechnology in Agriculture and Forestry Trees III* (Vol 16), Springer-Verlag, Berlin, Heidelberg, pp 318-338

Rout G (2005) Micropropagation of *Clitoria ternatea* Linn. (*Fabaceae*) – An important medicinal plant. *In Vitro Cellular and Developmental Biology – Plant* **41**, 516-519

Sahni R, Gupta SC (2002) *In vitro* plantlet regeneration from seedling nodal explants of *Acacia catechu*. *Indian Journal of Experimental Biology* **40**, 1382-1386

Shahzad A, Faisal M, Anis M (2007) Micropropagation through excised root culture of *Clitoria ternatea* and comparison between *in vitro*-regenerated plants and seedlings. *Annals of Applied Biology* **150**, 341-349

Shekhawat NS, Rathore TS, Singh RP, Deora NS, Rao SR (1993) Factors affecting *in vitro* cloning of *Prosopis cineraria. Plant Growth Regulation* **12**, 273-280

Siddique I, Anis M (2007) *In vitro* shoot multiplication and plantlet regeneration from nodal explants of *Cassia angustifolia* (Vahl.): a medicinal plant. *Acta Physiologiae Plantarum* **29**, 233-238

Skolmen RG, Mapes MO (1976) *Acacia kao* Gray plantlets from somatic callus tissue. *Journal of Heredity* **67**, 114-115

Tiwari S, Shah P, Singh K (2004) *In vitro* propagation of *Pterocarpus marsupium* Roxb.: An endangered medicinal tree. *Indian Journal of Biotechnology* **3**, 422-425

Tomar UK, Gupta SC (1988) Somatic embryogenesis and organogenesis in callus cultures of a tree legume – *Albizia richardiana* King. *Plant Cell Reports* **7**, 70-73

Troup RS (1983 reprint) *The Silviculture of Indian Trees: Leguminosae (Caesalpinieae) to Verbenaceae* (Vol IV), The Controller of Publications, Government of India, Delhi, pp 230-231

Upreti J, Dhar U (1996) Micropropagation of *Bauhinia vahlii* Wight & Arnott – a leguminous liana. *Plant Cell Reports* **16**, 250-254

Vieitez AM, Vieitez VE (1980) Plantlet formation from embryonic tissue of chestnut grown *in vitro*. *Physiologia Plantaram* **50**, 127-130

Vieitez AM, Ferro EM, Ballester A (1993) Micropropagation of *Fagus sylvatica* L. *In Vitro Cellular and Developmental Biology – Plant* **29**, 183-188

Vengadesan G, Ganapathi A, Pream Anand R, Selvaraj N (2003) *In vitro* propagation of *Acacia sinuata* (Lour.) Merr. from nodal segments of a 10-year-old tree. *In Vitro Cellular and Developmental Biology – Plant* **39**, 409-414

Veltcheva M, Svetleva D, Petkova SP, Perl A (2005) *In vitro* regeneration and genetic transformation of common bean (*Phaseolus vulgaris* L.) – Problems and progress. *Scientia Horticulturae* **107**, 2-10