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In vitro high frequency plant regeneration of a tree legume *Tamarindus indica* (L.)

Pawan K. Jaiwal and Anju Gulati

Department of Bio-Sciences, M.D. University, Rohtak-124 001, India

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ABSTRACT

Optimal culture conditions for high frequency plant regeneration from excised cotyledons of Tamarindus indica were established. Maximum shoot bud differentiation (100%) occurred when the adaxial surface of the entire cotyledon (excised from 12-d old seedlings) was in contact with MS medium containing $5x10^{-6}$M BAP. On MS alone only roots were formed. Shoot or root formation was confined to nodal tissue at the top of the notch present on the adaxial surface at the proximal end of the cotyledon. Thirty-four to 95 shoots were regenerated in a 4 month period from individual cotyledons. Shoots were rooted on MS with $5.7x10^{-6}$ M IAA. IAA ($5.7x10^{-7}$M) alone induced complete plant formation. Regenerated plants were established in the soil with 70% success.

ABBREVIATIONS

BAP, 6-benzylaminopurine; KIN, kinetin; 2-iP, 6-Y-Y-dimethylallyl aminopurine; AdS, adenine sulphate; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA, 1-naphthalene acetic acid; MS, Murashige and Skoog (1962) medium.

INTRODUCTION

Micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass, and conservation of elite and rare germplasm (Bonga and Durzan, 1982; Bajaj, 1986). In general, woody taxa are difficult to regenerate in in vitro conditions. But, recently, some success has been achieved in a few leguminous tree species (see review by Dhawan, 1989). Tamarindus indica is a multipurpose leguminous tree used for timber, fire wood, medicine, food, confectionary, drink, paper and textile industries (Purseglove, 1968; Mascarenhas et al, 1987). Because this species is well adapted to pedoclimatic conditions of the semi-arid tropics and can grow well on poor soils, it has a good potential for afforestation of degraded areas. Traditional vegetative propagation methods have not been successful for multiplication of this species (Mascarenhas et al, 1987). Moreover, there are no reports of micropropagation of Tamarindus indica using tissue culture techniques except a preliminary note published on hypocotyl and shoot tip cultures of this species (Mascarenhas et al, 1987). Therefore, the present study was undertaken to define optimal culture conditions for high frequency plant regeneration from cotyledon explants of Tamarindus indica.

MATERIAL AND METHODS

Plant Material

Mature seeds of Tamarindus indica were collected from two selected trees growing in the Forest Department Nursery, Rohtak, India. To raise aseptic seedlings, uniform and healthy seeds were rinsed in 70% alcohol for 1 min and then sterilized with 0.1% (W/V) aqueous mercuric chloride solution for 20 min. After rinsing 4-5 times with sterile distilled water, the seeds were aseptically placed on filter paper bridges in culture tubes containing 20 ml of liquid MS basal medium. The seeds were germinated in 8-h dark and 16-h cool-white fluorescent light of 40 $\mu Em^{-2}s^{-1}$ at 25±2°C with 60% relative humidity. Entire cotyledons (1 cm x 1.5 cm) from 12-d-old seedlings were used as explants. Unless otherwise mentioned, the cotyledons were explanted with the proximal end embedded in the medium (Fig. 1).

Culture medium

In all experiments, MS basal medium was used, variously supplemented with BAP, KIN, 2-iP, AdS, IAA, NAA and activated charcoal (BDH, Analar grade). All media were adjusted to pH 5.8 using 0.1 N HCl or 0.1 N NaOH before autoclaving. The medium was gelled with 0.7% agar (Hi-media, Bombay). Routinely, 20 ml of molten medium was dispensed into culture tubes (150 mm x 25 mm) and plugged with non-absorbent cotton wrapped in one layer of cheese cloth. The culture tubes were then steam sterilized at 1.05 kg cm^{-2} for 20 min.

Culture conditions

All cultures were maintained under the same incubation conditions as for germination of seeds. For each treatment 24 cultures were raised and each experiment was conducted at least twice. Visual observations of the cultures were taken every week, and the effect of different

Offprint requests to: P. K. Jaiwal

treatments were quantified on the basis of the percentage of cultures showing response and the number of regenerants per culture.

Rooting of shoots and transplantation

In vitro raised shoots measuring 3-4 cm were excised and cultured on 1/4 - strength MS, 1/2 - strength MS, MS, or MS containing IAA (5.7×10^{-7}, 5.7×10^{-6}M) or NAA, IAA, IBA (each at 5×10^{-6}M) or activated charcoal (0.2%). Plantlets with well-developed roots were removed from their culture vessel. After washing their roots in running tapwater, the plants were transferred to pots containing sterile vermiculite. Glass beakers were inverted over the plants to ensure high humidity during the first few days. After 15 d, the plants were transferred to soil in the nursery.

RESULTS AND DISCUSSION

Cotyledon explants enlarged considerably within 7 d on MS medium and after 10-15 d, 50% of the cultures directly produced 2-3 roots from the nodal tissue at the top of the notch present on the adaxial surface at the proximal end of explant. In older cultures, the roots were 10-15 cm in length with many laterals (Fig. 2).

Addition of different concentrations of BAP to MS medium suppressed root formation and induced shoot bud differentiation. Shoot buds appeared within 10-15 d after culture initiation and were restricted to the site from where the roots emerged on MS medium. Within 20-28 d these buds proliferated profusely on BAP containing medium. The multiple buds regenerated on BAP were transferred to MS medium containing GA_3 (5×10^{-6}M) (MGA) for elongation. Within 20 d, some of the buds developed into multiple shoots (2-6 cm in height) (Fig. 3), whereas the others remained stunted ($\leq$ 1.0 cm). The frequency of regeneration, the number of shoot buds, and the number of shoots per explant increased with increasing BAP concentration, reaching a maximum at 5×10^{-6}M and thereafter decreased with further increase in BAP (Table 1). The length of shoots showed an inverse relationship with concentration of BAP. BAP at 5×10^{-6}M gave 80% regeneration and produced 4-5 shoots (3-5 cm in length) and 30 shoot buds per explant. Well-developed shoots (more than 2 cm) were excised and transferred to rooting medium. The basal portions with unelongated shoot buds were excised from the cotyledons and subcultured on shoot proliferation medium, e.g. MS+BAP. On subculture the number of multiple shoot buds further increased, and an average of 34 shoots per explant were produced after elongation on MGA medium.

Table 1 : Regenerative response of cotyledons of Tamarindus indica on MS medium supplemented with different concentrations of BAP[a].
Culture period: 8 wks (4 wks for shoot multiplication + 4 wks for shoot elongation using MS+GA_3)

BAP conc. (M)	Total cultures regenerating (%)	% cultures with			Average number of			Average length of shoot(cm)/ explant.
		Shoots	Shoot buds	Roots	Shoots	Shoot buds	Roots	
0.0	50.0	0.0	0.0	50.0	0.0	0.0	2.5± 0.5	0.0
2.5×10^{-6}	54.5	54.5	9.10	0.0	3.0± 1.3	28.2 1.5	0.0	4.5± 0.4
5.0×10^{-6}	80.0	50.0	70.0	0.0	3.6± 0.7	30.0± 2.0	0.0	3.5± 0.2
10^{-5}	25.0	25.0	12.5	0.0	2.5± 1.5	10.0± 1.2	0.0	2.4± 0.2

Values are mean ± SE.
a Data based on 24 explants.

Effect of different cytokinins

The shoot multiplication response of BAP was compared with three other cytokinins: KIN, 2-iP and AdS at equimolar concentrations of 5×10^{-6}M (Table 2). The response of explants on AdS was largely the same as on basal MS medium. It induced only root differentiation directly from 58.3% of the explants. The other cytokinins induced shoot bud differentiation, but with different frequencies. BAP was the most effective cytokinin with regard to the number of cultures forming shoots (80%) and the number of shoots per explant (4-5). KIN was least effective in inducing shoot bud differentiation. This experiment demonstrated the requirement for a cytokinin, especially BAP, to induce shoot organogenesis from cotyledon explants of this species, similar to other leguminous tree species (Khatter and Mohan Ram, 1982, 1983; Kapoor and Gupta, 1986).

Table 2 : Effect of different cytokinins at equimolar concentrations of 5×10^{-6}M on T. indica shoot multiplication from cotyledon explants[a].
Basal medium : MS
Culture period : 8 wks.

Media	Total cultures regenerating(%)	% Cultures with			Average number of			Average length of shoot(cm)/ explant
		Shoots	Shoot buds	Roots	Shoots	Shoot buds	Roots	
MS	50.0	0.0	0.0	50.0	0.0	0.0	2.5± 0.5	0.0
MS+BAP	80.0	50.0	70.0	0.0	3.6 ± 0.7	30.0 ± 2.0	0.0	3.5± 0.2
MS+KIN	9.09	9.09	0.0	0.0	3.0± 0.2	0.0	0.0	3.0± 0.3
MS+AdS	58.3	0.0	0.0	58.3	0.0	0.0	1.5± 0.05	0.0
MS+2iP	41.6	8.3	41.6	0.0	1.0± 0.0	11.6± 3.0	0.0	3.5± 0.2

Values are mean ± SE.
a Data based on 24 explants.

Effect of IAA concentrations

Cotyledons cultured on media supplemented with different concentrations of IAA (5.7×10^{-8} to 5.7×10^{-6}M) directly produced 2 to 3 roots (5 to 10 cm long) with many small laterals at the proximal end. A linear relationship between the percentage of explants forming roots and the concen-

Table 3 : Effect of different concentrations of IAA alone and in combination with BAP on differentiation in cotyledon cultures of *T. indica*[a]. Culture period : 8 weeks; Basal medium : MS

Growth regulator (M)		Total cultures regenerating(%)	% cultures with				Average number of		Average length of shoot (cm)/explant
BAP	IAA		Shoots	Shoot buds	Roots	Plantlets	Shoots	Shoot buds	
0	0	50.0	0.0	0.0	50.0	0.0	0.0	0.0	0.0
0	$5.7x10^{-8}$	27.2	0.0	0.0	27.2	0.0	0.0	0.0	0.0
0	$5.7x10^{-7}$	63.9	9.09	0.0	36.3	18.2	1.0± 0.0	0.0	3.6± 0.9
0	$5.7x10^{-6}$	83.3	0.0	0.0	83.3	0.0	0.0	0.0	0.0
$5.0x10^{-6}$	$5.7x10^{-8}$	40.0	40.0	30.0	0.0	0.0	15.0± 1.2	55.0± 2.8	3.5± 0.8
$5.0x10^{-6}$	$5.7x10^{-7}$	20.2	0.0	20.0	0.0	0.0	12.0± 0.8	20.0± 1.8	3.0± 0.7
$5.0x10^{-6}$	$5.7x10^{-6}$	11.1	11.1	11.1	0.0	0.0	10.0± 0.7	15.0± 1.2	2.8± 0.5

Values are mean ± SE.
a Data based on 24 explants.

centration of IAA was observed. However, in about 20% of the explants, IAA ($5.7x10^{-7}$M) also induced single shoots having 2-3 nodes (Table 3). These shoots subsequently developed roots at the base, resulting in complete plantlets (4-5 cm in height) (Fig. 4).

Interaction of BAP and IAA

The regeneration response of explants decreased significantly when BAP ($5x10^{-6}$M) was supplemented with different concentrations ($5.7x10^{-8}$ to $5.7x10^{-6}$M) of IAA. The number of shoots per explant, however, increased three-fold compared to BAP alone (Table 1,3). A synergistic effect of auxin and cytokinin on shoot multiplication was also reported in *Albizzia lebbeck* (Upadhyaya and Chandra, 1983).

Size of explant

Each cotyledon was sliced into two equal parts either longitudinally or transversely, and each part was cultured on MS+BAP ($5x10^{-6}$M). The longitudinal halves and distal halves of transversely sliced cotyledons did not show any regeneration response (Table 4). However, the distal halves showed small papillar growths on the cut surface. The proximal halves of transversely sliced cotyledons showed 100% regeneration and produced 2-3 shoots/explant. The regeneration rate of segments (1 cm^3) from the proximal ends of cotyledons decreased and the number of shoots per explant increased by 2-fold compared to proximal halves. This experiment showed that shoot formation is restricted to the cotyledonary nodal tissue in this species.

Orientation of explant

Entire cotyledons were planted on MS+ BAP ($5x10^{-6}$M) medium in five different orientations and their shoot forming response was compared after 28 d (Table 5). The shoot forming response decreased drastically when (i) the distal end of the cotyledon was embedded in the medium; (ii) the abaxial surface of cotyledon was in contact with medium and (iii) the cotyledon was embedded longitudinally at a right angle to the surface of the medium. However, when the adaxial surface

Table 4 : Effect of explant size on regeneration in cotyledon cultures of *T. indica*[a].
Culture medium : MS + BAP ($5x10^{-6}$M)
Culture period : 8 wks.

Cotyledon size	Total cultures regenerating(%)	% cultures with		Average number of		Average length of shoot (cm)/explant
		Shoots	Shoot buds	Shoots	Shoot buds	
Complete cotyledon	80.0	50.0	70.0	3.6± 0.7	30.0± 2.0	3.5± 0.2
Longitudinal half	0.0	0.0	0.0	0.0	0.0	0.0
Transverse proximal half	100.0	55.5	100.0	2.0± 0.6	15.0± 1.8	3.0± 0.2
Transverse distal half	0.0	0.0	0.0	0.0	0.0	0.0
Cotyledon segment (1 cm^3) from proximal end	77.7	77.7	77.7	4.6± 0.9	15.0± 1.5	3.5± 0.2

Values are mean ± SE; a Data based on 24 explants.

of the explant was placed on the medium, the regeneration frequency and the number of shoots per explant increased greatly (100%, 95 shoots per explant in 16 weeks) in comparison to when the proximal end of the cotyledon was embedded in the medium (80%, 34 shoots per explant in 16 weeks). The size and orientation of the cultured explant have important roles in in vitro plant regeneration in legumes (Wright et al, 1987; Gulati and Jaiwal, 1990). In this study, placement of the adaxial explant surface in direct contact with the medium was not essential but did enhance the morphogenetic response.

Table 5 : Effect of orientation of cotyledon explants on regeneration of *T. indica*[a].
Culture medium : MS + BAP ($5x10^{-6}$M)
Culture period : 8 wks.

Orientation of explant	Total cultures regenerating(%)	% culture with		Average number of		Average length of shoot(cm)/ explant
		Shoots	Shoot buds	Shoots	Shoot buds	
Proximal end embedded in medium	80.0	50.0	70.0	3.6± 0.7	30.0[b] ± 2.0	3.5± 0.2
Distal end embedded in medium	16.6	16.6	0.0	2.0± 0.0	0.0	2.5± 0.2
Adaxial surface of explant in contact with medium	100.0	80.0	100.0	30.0± 2.5	65.0[b] ± 3.5	5.0± 0.2
Abaxial surface of explant in contact with medium	28.5	28.5	0.0	2.5± 0.7	0.0	3.0± 0.2
Explant embedded longitudinally at right angle to the medium	14.3	14.3	14.3	3.0± 0.2	15.0± 1.2	3.0± 0.2

Values are mean ± SE.
a Data based on 24 explants.
b On further subculture (8 wks), these buds elongated into shoots.

Rooting of shoots and transplantation

MS + IAA ($5.7x10^{-6}$M) induced a small amount of callus at the cut end of shoots, from which 4-5 roots (5-6 cm in length) with laterals emerged within 20 d in 100% of the cultures (Fig. 5). This treatment not only induced roots but also promoted shoot growth (length as well as the number of axillary branches). One hundred plantlets were transferred to pots (Fig.6) and later established in soil in the nursery where 70% of them survived and resumed growth.

CONCLUSIONS

Regeneration of plants directly from the explant has been used for in vitro propagation of herbaceous and woody plant species (Dhawan and Bhojwani, 1987). The use of cotyledon explants for in vitro plant regeneration has several advantages. A large number of cotyledons can be obtained by growing seeds under sterile conditions for a short period of time and at any time throughout the year. Microbial contamination of such explants is rarely a serious problem. Moreover, cotyledons have been shown to possess high morphogenetic potential (Fazekas et al, 1986). Cotyledon systems have been used recently for high frequency Agrobacterium - mediated gene transfer in Sesbania rostrata (Vlachova, 1987), Arabidopsis thaliana (Patton and Meinke, 1988) and Brassica napus (Moloney et al, 1989). Shoots obtained from cotyledon explants have also provided a spectrum of somaclonal variants (Gulati and Jaiwal, 1990).

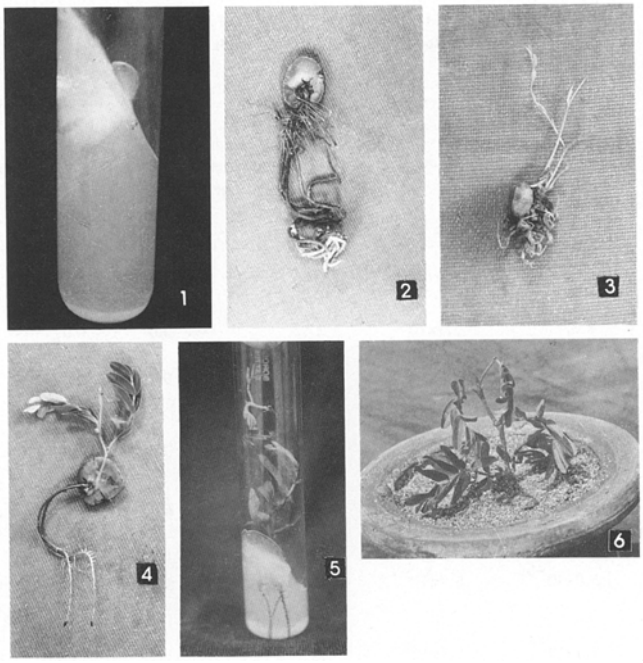

Fig.1, Cotyledon explant of *T. indica* with proximal end embedded in the medium at the time of culture; Fig. 2, Cotyledon explant of *T. indica* showing direct root formation on MS medium, 28 d after culture; Fig.3, Multiple shoots of *T. indica* arising directly from the cotyledon explant on MS containing BAP ($5x10^{-6}$M), 28 d after culture; Fig.4, A well developed *T. indica* plantlet from a cotyledon explant on MS containing IAA ($5.7x10^{-7}$M); Fig. 5, Isolated *T. indica* shoot on MS + IAA ($5.7x10^{-8}$M) showing root formation; Fig. 6, Plantlet of *T. indica* after transferred to a small pot containing sterile vermiculite.

Shoot bud differentiation from cotyledon derived callus has been reported in few tree legumes i.e. Sesbania sesban (Khattar and Mohan Ram, 1982), S. grandiflora (Khattar and Mohan Ram, 1983), S. bispinosa (Kapoor and Gupta, 1986), Dalbergia lanceolaria (Anand and Bir, 1984), and Albizzia lebbeck (Upadhyaya and Chandra, 1983; Varghese and Kaur, 1988). In contrast to these reports, in the present study shoot buds were differentiated directly from the explant. Shoot organogenesis from the cotyledon was determined by the presence of a cytokinin in the medium. Shoot multiplication was enhanced by the additional application of an auxin. Previous researchers have shown that cotyledon segments of *T. indica* produced only callus on BAP and KIN supplemented MS medium (Mascarenhas

et al, 1987). However, the present results showed that when the adaxial surface of the entire cotyledon excised from 12-d-old seedlings was in contact with BAP ($5x10^{-6}$) medium, as many as 95 shoots were produced from 100% of the cultures in 16 weeks. Thus cotyledon nodal tissues form adventitious shoots and roots with high frequencies under fairly simple culture conditions.

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