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# Direct organogenesis in hypocotyl cultures of *Tamarindus indica*

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## Abstract

Direct differentiation of shoot buds from hypocotyl segments of 12-d-old seedlings of *Tamarindus indica* was obtained on Murashige and Skoog (MS) medium with or without growth regulators. The highest regeneration (66 %) and the maximum number of shoots (3 - 4) per explant were obtained from the explants on MS medium containing 6-benzylaminopurine ($5 \times 10^{-6}$ M). A maximum roots per shoot were produced on medium containing 3-indole butyric acid ($5 \times 10^{-6}$ M). The resulting plantlets were hardened and transferred to soil in pots where 75 % of them survived and resumed growth. Histological examination of explants suggests that the shoots were of *de novo* origin which would make this system suitable for transformation experiments.

*Additional key words*: plantlets, leguminous tree, tamarind.

## Introduction

Many tree species have been propagated *in vitro* from embryos, shoot tips, axillary buds and cotyledons (for review see Trigiano *et al.* 1993). However, organogenesis from non-meristematic explants has been achieved from only in a limited number of species, *e.g. Albizzia lebbeck* (Upadhayaya and Chandra 1983), *Dalbergia latifolia* (Nataraja and Sudha Devi 1985), *Sesbania grandifolia* (Shanker and Mohan Ram 1990), and *Acacia auriculiformis* (Rao and Prasad 1991). *Tamarindus indica* (tamarind) is used for timber, fire wood, as food, in medicine, paper and textile industries, *etc.* Tamarind can be also used for afforestation and reclamation of waste lands because of its low water requirements and ability to grow on nutrient deficient soils. Traditional vegetative propagation methods have not been successful for

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*Abbreviations*: BAP - 6-benzylaminopurine; KIN - kinetin; IAA - 3-indole acetic acid; IBA - 3-indole butyric acid; NAA - α-naphthalene acetic acid; NOA - β-napthoxy acetic acid.

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multiplication of this species (Mascarenhas *et al.* 1987), hence tissue culture methods have been employed. Tamarind plants have been regenerated from pre-existing meristem on explants like cotyledons, shoot tips and cotyledonary nodes (Mascarenhas *et al.* 1987, Jaiwal and Gulati 1991, 1992), however, there are no reports of plant regeneration from non-meristematic explants. In the present study, we describe direct and rapid regeneration of multiple shoot buds or shoots from hypocotyl explants of *T. indica* on simple culture medium. Histological studies are also presented to show origin and development of shoots.

## Materials and methods

**Explant preparation:** Seeds of *Tamarindus indica* L. collected from trees growing in Forest Department Nursery, Rohtak (India) were rinsed with 70 % alcohol for 1 min and then surface sterilized with 0.1 % (m/v) aqueous mercuric chloride solution for 20 min. The seeds were again rinsed 5 - 6 times with sterilized distilled water and then aseptically placed on filter paper bridge in borosil culture tubes (150 mm × 25 mm) containing 15 $cm^3$ of liquid Murashige and Skoog's (MS) basal medium with 3 % sucrose. The pH of medium was adjusted to 5.8 before autoclaving at 1.05 kg $cm^{-2}$. The seeds were germinated under 8 h dark and 16 h cool white fluorescent light (40 μmol $m^{-2}$ $s^{-1}$) at temperature 25 ± 2 °C. Hypocotyl segments (10 mm long) excised from 12-d-old seedlings were used as explants. In all experiments MS medium containing 3 % sucrose and 0.7 % agar, supplemented with various phytohormones was set to pH 5.8. Twenty $cm^3$ of gelled medium was dispensed into test tubes (150 mm × 25 mm) and plugged with non-absorbent cotton wrapped in cheese cloth, and slants were prepared after autoclaving at 1.05 kg $cm^{-2}$ for 20 min. All cultures were maintained under the same conditions as for seed germination. Visual observations of cultures were taken every week and the effect of different treatments were quantified on the basis of percent cultures showing response and the degree of response per culture. For each treatment 24 cultures were raised and each experiment was conducted twice. The data was subjected to analysis of variance and significant treatment differences were selected by Newman-Keul's multiple range test (Bruning and Kintz 1977).

**Regeneration of shoot buds and shoots:** For induction of shoots, hypocotyl explants were cultured on MS basal medium or supplemented with BAP or KIN either alone or in combinations. To test the optimal age of donor seedling for regeneration, hypocotyl explants were excised from 6-, 8-, 10-, 12- and 14-d-old seedlings and cultured on MS medium containing BAP ($5 \times 10^{-6}$ M ).

**Rooting and transplantation:** Well developed shoots were excised and cultured on full or half strength MS medium supplemented with $5 \times 10^{-6}$ M IAA, IBA, NAA and NOA and 0.2 % activated charcoal either singly or in various combinations. The shoots with well developed roots were washed with distilled water to remove the agar and the plantlets were transferred to pots containing sterilized soil:sand:vermiculite

(2:1:1). Each plant was covered with polythene bag to ensure high humidity for first 15 d and then transferred to field.

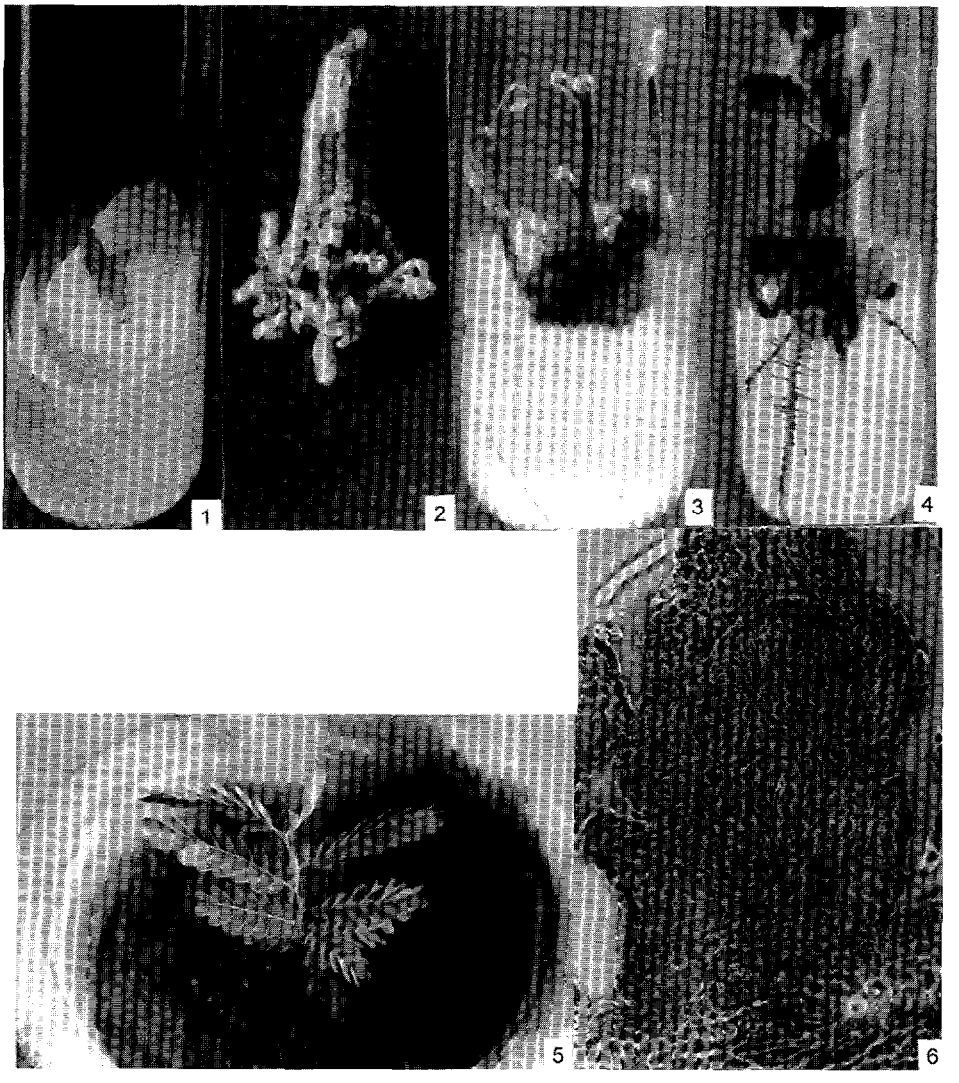
**Histological studies:** To study the ontogeny of adventitious shoot differentiation in cultures, hypocotyl explants after different days of culture on MS or MS + BAP ($5 \times 10^{-6}$ M) were fixed in FAA (95 % ethanol + glacial acetic acid + 40 % formaldehyde + water, 10:1:2:7 by volume ), serially dehydrated with ethanol and *t*-butanol and finally embedded in paraffin. Serial longitudinal and transverse sections of 10 μm in thickness were cut using rotary microtome. The resulting paraffin ribbons were mounted on glass slides and passed through a series of deparaffinizing solution. The sections were stained with safranin and haemotoxylin and were examined under an *Olympus* microscope.

## Results and discussion

**Regeneration of shoot buds and shoots:** Hypocotyl explants enlarged considerably within seven days of culture on MS basal medium (Fig. 1) and directly produced single shoot (8 - 10 mm in length) with small amounts of callus at the proximal end of the explants in 10 % of the cultures after 60 d. Direct regeneration of shoots from hypocotyl explants on basal medium have also been reported in *Sesbania bispinosa* (Kapoor and Gupta 1986) and in *Dalbergia sissoo* (Suman 1992). Addition of $5 \times 10^{-6}$ M BAP induced direct differentiation of multiple shoot buds (3 - 4) in 66 % of the explants along with the formation of yellowish brown callus. The shoot buds started to form within 40 - 45 d from the proximal end of the explants. But occasionally they differentiated at both the ends or all over the surface of the explants

Table 1. Effect of different concentrations of BAP, KIN either alone or in combinations on regeneration of hypocotyl explants of *Tamarindus indica* after 8 weeks of culture. Means of 24 replications ± SE. Means followed by the same letter are not significantly different according to Newman-Keul's multiple range test ($P = 0.05$).

| BAP [M] | KIN [M] | Regenerating shoots [%] | callus [%] | Number of shoots [explant$^{-1}$] | Length of shoot [mm explant$^{-1}$] |
|---|---|---|---|---|---|
| 0 | 0 | 10 | 20 | 1.0 ± 0.0 | 8 |
| $5 \times 10^{-7}$ | 0 | 0 | 70 | 0 | 0 |
| $5 \times 10^{-6}$ | 0 | 66 | 100 | 3.1 ± 0.3 b | 20 |
| $5 \times 10^{-5}$ | 0 | 0 | 0 | 0 | 0 |
| 0 | $5 \times 10^{-7}$ | 8 | 60 | 1.0 ± 0.0 a | 10 |
| 0 | $5 \times 10^{-6}$ | 0 | 80 | 0 | 0 |
| 0 | $5 \times 10^{-5}$ | 0 | 90 | 0 | 0 |
| $5 \times 10^{-5}$ | $5 \times 10^{-7}$ | 0 | 9 | 0 | 0 |
| $5 \times 10^{-5}$ | $5 \times 10^{-5}$ | 9 | 9 | 1.0 ± 0.0 a | 5 |
| $5 \times 10^{-7}$ | $5 \times 10^{-5}$ | 0 | 16 | 0 | 0 |
| $5 \times 10^{-7}$ | $5 \times 10^{-7}$ | 25 | 58 | 2.7 ± 0.2 b | 8 |




Figs. 1 - 5. Plantlet formation from hypocotyl explants of *Tamarindus indica*: 1 - Hypocotyl explant at the time of culture; 2 and 3 - differentiation of multiple shoots from morphological upper end (2) and all over the surface of explant (3) on MS + BAP($5 \times 10^{-6}$ M) after 8 weeks of culture; 4 - root formation at the base of regenerated shoot; 5 - a regenerated plant growing in pot containing normal garden soil, photographed two months after transplantation.
Fig. 6. Cross-section of hypocotyl explant showing a young developing shoot bud surrounded by leaf primordia.

(Figs. 2 and 3). These buds subsequently developed into multiple shoots which attained a length of 33.5 mm after 60 d. BAP at lower concentration $5 \times 10^{-7}$ M induced only callusing in 70 % of the explants but the shoot buds did not differentiate from these callused explants even on prolonged incubation on $5 \times 10^{-7}$ M BAP containing medium. On the contrary, BAP at higher concentration ($5 \times 10^{-5}$ M) failed to elicit any morphogenic response (Table 1). On the other hand, KIN only at lower concentration $5 \times 10^{-7}$ M induced differentiation of single shoot in 8.3 % of the cultures. Higher concentration of KIN ($5 \times 10^{-5}$, $5 \times 10^{-6}$ M) completely inhibited shoot bud production but yield relatively large amount of callus. BAP was found to be more effective for shoot multiplication than KIN. This is in accordance with previous reports on other tree legumes (Khatter and Mohan Ram 1982, Kapoor and Gupta 1986, Tomar and Gupta 1988, Rai and Chandra 1988). BAP ($5 \times 10^{-7}$ M) in combination with KIN ($5 \times 10^{-7}$ M) enhanced the number of shoot buds (Table 1). Similar increase in shoot buds with BAP + KIN was reported in *Dalbergia latifolia* (Rai and Chandra 1988, Swamy *et al.* 1992).

**Age of explant:** The age of explant affects the rate of shoot regeneration in tree legumes. The explants excised from 12-d-old seedlings were found to be most responsive for shoot regeneration as they produced the maximum number of shoot buds (3 - 4) in 66 % of the cultures (Table 2). Similar results were obtained in *Sesbania grandiflora* (Shanker and Mohan Ram 1990).

Table 2. Effect of age of explant on shoot regeneration from hypocotyl explants of *Tamarindus indica* after 8 weeks of culture. Means of 24 replications ± SE. Means followed by the same letter are not significantly different according to Newman-Keul's multiple range test ($P = 0.05$).

| Age of explant [d] | Regenerating cultures [%] | Number of shoots [explant$^{-1}$] | Length of shoots [mm explant$^{-1}$] |
|---|---|---|---|
| 6 | 27 | 2.0 ± 1.0 a | 7.0 ± 0.3 |
| 8 | 30 | 2.3 ± 0.9 a | 7.0 ± 0.3 |
| 10 | 43 | 2.7 ± 0.3 ac | 15.0 ± 0.8 |
| 12 | 66 | 3.2 ± 0.2 bc | 20.0 ± 0.4 |
| 14 | 28 | 2.5 ± 0.5 a | 10.0 ± 0.5 |

**Rooting of excised shoots:** Leafy shoots regenerated from hypocotyl explants were excised and transferred to rooting medium to obtain complete plantlets. For this, half or full strength MS basal medium supplemented with single or various combinations of auxins like IAA, IBA, NOA, NAA and activated charcoal (0.2 %) were used (Table 3). Out of the various auxins used, IBA at $5 \times 10^{-6}$ M was found to be optimal for rooting (Fig. 4). No root induction was observed with NOA and NAA (each at $5 \times 10^{-6}$ M). However, in *Prosopis cineraria*, NOA was the most effective auxin for inducing rooting (Kackar *et al.* 1991, 1992). IAA ($5 \times 10^{-6}$ M) in combination with activated charcoal (0.2 %) directly (without callus) induced adventitious roots at the cut ends of the shoots. Excised shoots treated with IAA ($5 \times 10^{-6}$ M) in liquid MS

medium also induced no callusing at the cut ends. Root regeneration decreased in half strength of MS basal medium supplemented either with IBA ($5 \times 10^{-6}$ M) or IAA ($5 \times 10^{-6}$ M). This was in contrast to Swamy *et al.* (1992) who reported the half strength MS basal medium supplemented with IBA ($5 \times 10^{-5}$ M ) as optimal for root induction in *Dalbergia latifolia.*

Table 3. Effect of different auxins added to MS basal medium either alone or in combination on *in vitro* rooting of isolated shoots excised from hypocotyl explants of *Tamarindus indica* after 6 weeks. Means of 24 replications ± SE. Means followed by the same letter are not significantly different according to Newman-Keul's multiple range test ($P = 0.05$).

| Auxin | Concentration [M] | Rooting [%] | Roots number [shoot$^{-1}$] | Root length [mm shoot$^{-1}$] |
|---|---|---|---|---|
| IBA | $5 \times 10^{-6}$ | 87 | 3.1 ± 0.4 a | 20.1 ± 0.3 |
| | $5 \times 10^{-6}$ | 62 | 4.6 ± 0.8 b | 10.0 ± 0.6 |
| IAA | $5 \times 10^{-6}$ | 58 | 2.8 ± 0.5 ad | 13.1 ± 0.1 |
| | $5 \times 10^{-6}$ | 50 | 2.6 ± 0.3 ad | 18.1 ± 0.6 |
| NOA | $5 \times 10^{-6}$ | 0 | 0 | 0 |
| NAA | $5 \times 10^{-6}$ | 0 | 0 | 0 |
| IAA + IBA | $5 \times 10^{-6}$ each | 60 | 11.1 ± 4.2 c | 16.0 ± 0.5 |
| IAA + charcoal | $5 \times 10^{-6}$ + 0.2 % | 50 | 2.0 ± 0.6 d | 14.2 ± 0.1 |

**Transplantation:** Auxins in general produced small amount of callus at the cut end of the shoots and subsequently roots arise from callus. This shows that vasculature of roots are not directly connected with the vasculature of shoots. Therefore, on transfer to soil these shoots showed low percentage of survival. However, when 0.2 % activated charcoal was added to MS + IAA ($5 \times 10^{-6}$ M), root formation occurred directly from cut end of the shoot without any callusing. Hence, these plantlets showed higher survival upon transplantation to soil (Fig. 5).

**Histology of hypocotyl explant:** Histological studies revealed *de novo* formation of shoots. The mitotic activity was localised in the epidermal and subepidermal cell layers of cultured explants. The formation of meristemoids occurred within four weeks in the parenchymatous type of cells produced by proliferating explants. These meristemoids developed into buds (Fig. 6) which emerge out on the surface and ultimately developed into adventitious shoots within six weeks.

The present study demonstrates that the hypocotyl, a non-meristematic explant directly produced multiple shoots *de novo* on a simple culture medium. Such regeneration systems are also suitable for transformation experiments.

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