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# MULTIPLE SHOOT FORMATION FROM SOMATIC EMBRYOS OF *HEVEA BRASILIENSIS*

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Multiple shoot formation from germinating somatic embryos of *Hevea brasiliensis* has been achieved. Among the different cytokinins tested, high levels of benzyladenine (BA) and a comparatively lower concentration of thidiazuron (TDZ) proved to be effective in bringing about multiple shoot formation. However, further elongation and rooting of the multiple shoots induced in the presence of BA was better than that of TDZ. Wounding helped in inducing multiple shoots from the somatic embryos. An average of 3.45 micro-shoots per explant could be induced by this system and each micro-shoot developed into a full plantlet.

Key words : *Hevea brasiliensis*, Micropropagation, Multiple shoots, Somatic embryogenesis.

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## INTRODUCTION

Plant regeneration by somatic embryo-genesis of *Hevea brasiliensis* has been achieved in several laboratories using different explant sources (Asokan *et al.*, 1992a, b; Carron *et al.*, 1995; Chen, 1984; Jayasree *et al.*, 1999). However, success rate in the conversion of somatic embryos into full plantlets still remains low. The low percentage of germination and regeneration of the somatic embryos is one of the factors limiting the application of somatic embryogenesis as a mass-scale propagation system. In somatic embryogenesis, usually a single shoot develops to form a single plantlet from one somatic embryo. Multiple shoot induction from germinating somatic embryos could be an alternative method of regeneration for systems with low embryo induction as well as plant regeneration frequency. Multiple shoot production from cotyledonary node explants of zygotic embryos has been described for several species like pea (Jackson and Hobbs, 1990), litchi (Das *et al.*, 1999), pigeon pea (Shivaprakash *et al.*, 1994) and cotton (Agrawal *et al.*, 1997). Multiple shoot induction from somatic embryos is only rarely reported, mainly in legumes (Wright *et al.*, 1991; Distabanjong and Geneve, 1997). Multiple shoot formation from somatic embryos of tree crops has not, so far, been reported. This article reports, for the first time, induction of multiple shoots from mature cotyledonary somatic embryos of *H. brasiliensis*.

## MATERIALS AND METHODS

### Plant material

Immature inflorescences from mature trees of *Hevea brasiliensis*, clone RRII 105, were washed in running tap water for 10 min and sterilized with $HgCl_2$ (0.1%) containing two drops of Tween 20 for 3 min and rinsed five times with sterile distilled water. These sterile explants were placed in basal MS (Murashige and Skoog, 1962) medium supplemented with B5 vitamins, 4.5 µM 2,4-dichloro phenoxy acetic acid (2,4-D), 2.7 µM naphthalene acetic acid (NAA) and 2.3 µM kinetin (KIN) and maintained in the dark at 25°C for callus induction. The compact, nodular calli formed were transferred to embryo induction medium which consisted of MS basal medium with B5 vitamins, sucrose (7%), 4.4 µM benzyl adenine (BA), 2.9 µM giberellic acid ($GA_3$) and 1.0 µM NAA. Embryogenic calli emerged after about three months in culture and pro-embryo clusters appeared subsequently. These embryo clusters were matured in hormone-free MS medium with sucrose (10%). Within one to two months in this medium, mature embryos of torpedo and cotyledonary stages started appearing along with a number of abnormal embryos characterised with swollen or fused cotyledons. Normal cotyledonary embryos were selected from such embryo masses for multiple shoot induction.

### Effect of media composition

Various levels of four cytokinins BA (4.4 - 44.0 µM), KIN (4.6 - 46.0 µM), 2-isopentenyl adenine (2ip) (4.9 - 49.0 µM) and thidiazuron (TDZ) (0.5 - 5.0 µM) were incorporated into two basal media, *viz.* MS and woody plant medium (WPM) of Lloyd and McCown (1981), supplemented with 5 per cent coconut milk, 0.2 per cent activated charcoal and solidified with 0.2% phytagel. The pH of the medium was adjusted to 5.6 before autoclaving at 121°C for 15 min.

### Effect of wounding

In a separate experiment, a longitudinal cut was made in between the cotyledons so as to injure the apical meristem. These wounded explants were compared with the intact embryos in order to assess the effect of wounding on multiple shoot induction. All the cultures were incubated at 25°C under cool, white, fluorescent light at 30 µE per $m^2$ per s with a 16 h photoperiod. Each treatment consisted of 20 explants and each experiment was repeated thrice. Observations were recorded after two months in culture.

### Elongation of micro-shoots

Explants which responded with formation of multiple shoots were transferred to WPM, fortified with 1.45µM $GA_3$ for shoot elongation and maintained under the same culture conditions for about one month.

### Rooting of micro-shoots

Root initiation of the elongated micro-shoots was attempted by separating individual micro-shoots and culturing them in WPM devoid of growth regulators. The rooted plants were transferred to polybags for acclimatization and later established in the field.

### Histology

Samples were fixed in formalin-acetic acid-alcohol mixture (Johansen, 1940). Microtome sections of embryos embedded in paraffin wax (Tissue Prep 2, 55-57°C) were cut (8-10 µm) and stained with toluidine blue for general histology. Observations and photomicrographs were taken using a Leica Diaplan microscope.

## RESULTS AND DISCUSSION

It was observed that the basal media components as well as the type and concentration of cytokinins influenced multiple shoot induction from the somatic embryos of *H. brasiliensis*. Among the two basal media tried, WPM was found to stimulate multiple shoot formation whereas MS was ineffective. The growth regulators, BA and TDZ, gave rise to multiple shoots from the cultured embryos while KIN and 2ip failed to do so. The percentage of explants with multiple shoot initiation (Fig. 1) as well as the mean number of shoots per responding explant in the presence of BA or TDZ are presented in Table 1. Among the different treatments, maximum explant response was observed with BA at 22.0 µM and TDZ at 2.0 µM. TDZ at 1.0 µM was statistically on par with 2.0 µM TDZ. Moreover, mean number of shoots at 2.0 µM TDZ was significantly higher than that obtained in the presence of BA at all concentrations.

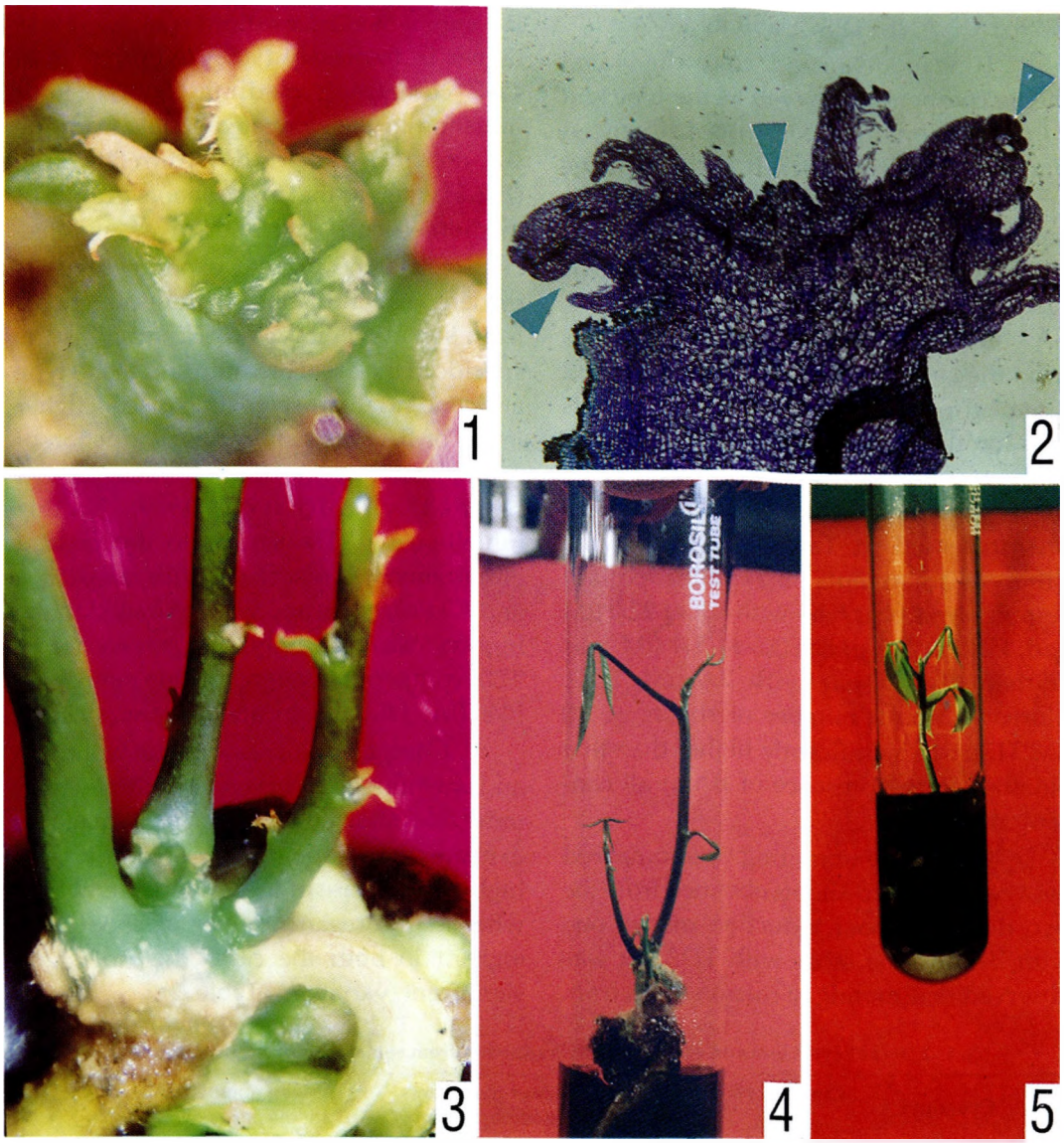
Percentage of rooting was compared between the micro-shoots obtained from the two different shoot induction media, one with BA and the other with TDZ. It was observed that rooting of micro-shoots from BA supplemented medium was rapid and higher in frequency (90%) than that from TDZ containing medium (50%). Root initiation in the elongated shoots from the BA medium occurred within 20 days (Fig. 5) whereas that in the shoots from TDZ medium occurred at about two months.

From the present experiments it is evident that induction of multiple shoots from somatic embryos of *H. brasiliensis* is specific to the basal medium and to the type and concentration of cytokinins used. Higher levels of BA was required for induction of multiple shoots in *Hevea*, similar to that reported for litchi (Das *et al.*, 1999). Rooting inhibition by TDZ similar to the present observation was reported in *Vitis rotundifolia* and *Rhododendron* and was attributed to the high cytokinin activity and persistence of TDZ compared to amino purine cytokinins like BA (Huetteman and Preece, 1993).

Comparison of responses of wounded and unwounded embryos for multiple shoot induction showed that the percentage of explants that responded was significantly higher in the case of the wounded embryos whereas the mean number of shoots per explant in both the cases did not show significant variation (Table 2).

Table 1. **Effect of cytokinins on multiple shoot induction from somatic embryos of *H. brasiliensis***

| Cytokinin | Concentration (µM) | Percentage of explants forming multiple shoots | Mean number of shoots per explant |
|---|---|---|---|
| BA | 4.40 | 00.00 | 0.00 |
| | 13.20 | 00.00 | 0.00 |
| | 22.00 | 56.67 | 3.13 |
| | 44.00 | 45.00 | 2.84 |
| TDZ | 01.00 | 55.00 | 3.18 |
| | 02.00 | 61.67 | 3.45 |
| | 05.00 | 35.00 | 3.00 |
| | 10.00 | 26.67 | 2.43 |
| CD (0.05) | | 13.34 | 0.30 |




Figs.1-5. Development of multiple shoots in somatic embryos of *H. brasiliensis*.. 1. Multiple shoots developing from a single somatic embryo; 2. Longitudinal section of a somatic embryo showing the origin of multiple shoots; 3. Elongated micro-shoots; 4. A plantlet showing multiple shoots with leaflets; 5. A micro-shoot separated and rooted in hormone-free medium

Distabanjong and Geneve (1997) reported that, in eastern red-bud, wounding increased the number of shoots formed per explant and did not increase the frequency of explants that formed shoots.

Micro-shoots obtained in the presence of cytokinins did not elongate in the same medium but elongated on transfer to the medium containing $GA_3$ (Fig.3).

Table 2. **Effect of wounding on multiple shoot induction**

| Treatment | Explant response (%) | | | Mean No. of shoots per explant | | |
|---|---|---|---|---|---|---|
| | Unwounded | Wounded | Mean | Unwounded | Wounded | Mean |
| T1 | 54.33 | 70.00 | 62.16 | 3.07 | 3.05 | 3.06 |
| T2 | 47.33 | 63.66 | 55.50 | 2.40 | 2.96 | 2.68 |
| T3 | 53.33 | 67.33 | 60.33 | 2.99 | 3.06 | 3.03 |
| T4 | 46.33 | 69.33 | 57.83 | 3.15 | 3.26 | 3.21 |
| Mean | 50.33 | 67.58 | | 2.91 | 3.09 | |
| CD (cytrokinins) | | | NS | | | 0.31 |
| CD (wounding) | | | 3.63 | | | NS |
| CD (cytokinin x wounding) | | | NS | | | NS |

T1 = 22.0 µM BA; T2 = 44.0 µM BA; T3 = 1.0 µM TDZ;l T4 = 2.0 µM TDZ

Requirement of another medium, rather than the induction medium, for shoot elongation was reported for several species like cotton apple, pear, populus and rhododendron (Huetteman and Preece, 1993) where multiple shoots were induced from cotyledonary nodes (Agrawal *et al.*, 1997). In the present study, it was also observed that the multiple shoots were not uniform and did not appear simultaneously. The micro-shoots which developed first, elongated faster on transfer to $GA_3$ containing medium compared to those developed later (Fig. 4). However, when the longer micro-shoots were separated for rooting, the smaller ones started to grow faster.

Histological observation of the samples with multiple shoots confirmed the origin of the multiple shoots (Fig. 2). Three distinct shoot meristems were noticed in a single embryo.

Asynchronous development of the embryos was observed in *H. brasiliensis*. As reported in several other species like eastern redbud (Distabanjong and Geneve, 1997), white spruce (Kong and Yeung, 1994) and *Abies nordmanniana* (Norgaard, 1997), during somatic embryo-genesis of *H. brasiliensis*, a large number of malformed or abnormal embryos appeared most of which did not undergo further normal development into plantlets. Full plantlets could be obtained mostly from the normal embryos, as a result of which the total number of plantlets obtained through somatic embryogenesis remained much lower as compared to the number of somatic embryos induced. Induction of multiple shoots and subsequent rooting of the micro-shoots is an alternative to compensate for this low plant regeneration frequency. The present experiments have shown that 3 to 4 shoots per explant could be induced each of which could give rise to a full plantlet. In addition, the micro-shoots obtained from the somatic embryos could be induced to form additional shoots from axillary buds so as to boost up shoot multiplication from somatic embryos (Distabanjong and Geneve, 1997).

The micro-shoots developed from the somatic embryos are highly juvenile in nature and resemble seedlings. These can be utilized for micropropagation through micro cuttings and axillary bud proliferation. Moreover, this system could be made use of in genetic engineering experiments where plant regeneration frequency from transgenic embryos is far too low.

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