Regeneration of plants via adventitious bud formation from mature zygotic embryo axis of tamarind (*Tamarindus indica* L.)

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A protocol for *in vitro* regeneration of plants via adventitious bud formation from mature embryo axis of tamarind (*Tamarindus indica* L.) was standardized. Explants consisting of longitudinal section of embryo axis with attached cotyledon were cultured in the Murashige and Skoog (MS) medium with various combinations and concentrations of α-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) and sucrose. Induction of adventitious shoot buds was achieved in the cut surface of the axis when cultured in a medium containing 2.69 µM NAA, 44.39 µM BAP and 4% sucrose. A medium consisting of 0.91 µM zeatin, 2.22 µM BAP, 0.41 µM calcium pantothenate and 0.40 µM biotin supported the differentiation of the buds to form elongated shoots. The shoots developed roots in a half strength MS medium with 2% sucrose following a 72 h treatment with auxin mixture in the dark. On transfer to soil 24 plants survived.

EXTENSIVE efforts to develop efficient regeneration systems for many legumes have resulted in a large array of *in vitro* protocols[1]. However, leguminous tree species are less studied and only a few are amenable to *in vitro* cultures[2]. The leguminous tree, tamarind (*Tamarindus indica* L.), is well adapted to semi-arid tropical regions and is a species of choice for sustainable development of wasteland. The pulp produced in the fruits of this tree is rich in tartaric acid and is extensively used in the production of drinks, jams and confectioneries. The seed contains 63% starch, 16% protein and 5.5% oil[3] and is a rich source of these natural products. Methods for manipulation of tamarind tissue *in vitro* have been restricted to shoot multiplication from meristematic buds and nodal explants of seedlings[4], or from cotyledonary nodal tissue of seedlings[5] and to a limited extent, shoot tips and nodal buds from mature trees[6]. However, the potential of micropropagation of tamarind has also been emphasized[4,7]. In this report we describe a method for *in vitro* regeneration of tamarind plantlet via adventitious bud formation from the cut surface of mature embryo axis explant.

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Tamarind seeds were collected from the mature pods of five trees growing locally. Seeds were washed with a detergent and surface sterilized using 0.1% (w/v) mercuric chloride ($HgCl_2$) for 15 min. Excess $HgCl_2$ was removed by repeated washing with sterile distilled water under aseptic conditions. Seeds were soaked in sterile distilled water for 4–5 h at room temperature and washed thoroughly before culturing in a germination medium consisting of half strength Murashige and Skoog (MS)[8] basal salts and vitamins with 2% sucrose (Qualigens, Glaxo India Ltd) and 5 g l^{-1} agar (Qualigens, Glaxo India Ltd). The pH of the medium was adjusted to 5.8 prior to addition of agar. Cultures were incubated in the dark at 25 ± 2°C. The cracked seed coat was removed on the fifth day and cotyledons were separated carefully to obtain two explants, each consisting of a longitudinal section of the embryo axis with an attached cotyledon (LSEC). Two types of explants from imbibed seeds were tested for morphogenetic activity, i.e. LSEC, and longitudinal section of the embryo axis without cotyledon. In spite of splitting the cotyledons carefully with controlled pressure, the embryo axis divided into two uneven longitudinal sections with the split being just below the plumule. As the split was oblique, often one explant had the plumule whereas the other was devoid of it. Explants were cultured with the cut surface of the embryo axis and the adaxial side of the cotyledon in direct contact with the medium in 85 × 15 mm petri dishes (Laxbro Manufacturing Company, Pune, India) with 20 ml MS medium supplemented with various concentrations of α-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) and sucrose singly and in combinations. The media were solidified with 0.5% agar. All growth regulators were procured from Sigma Chemicals, USA. Cultures were incubated for 4 weeks in light (16 h photoperiod, at 16 µE m^{-2} s^{-1}) at 25 ± 2°C. The number of explants showing caulogenic response along the cut surface of the axis were scored. The percentage response in different media was analysed statistically. After 4 weeks, LSEC with clusters of shoot buds was transferred to 250 ml Erlenmeyer flasks containing 50 ml liquid medium composed of MS with 0.91 µM zeatin, 2.22 µM BAP, 0.41 µM calcium pantothenate, 0.40 µM biotin and 2% sucrose for elongation. The cultures were incubated in light (16 h photoperiod, at 16 µE m^{-2} s^{-1}) at 25 ± 2°C on a gyratory shaker (80 rpm). After 6 weeks, the elongated shoots (2.5 cm and above) were isolated from the cluster and the main explant bearing the remaining small shoots and buds was transferred to a fresh medium of the same composition for elongation of a second batch of shoots. Rooting was induced in the elongated shoots using the method described earlier[4,6]. In brief, the method involves incubation of shoots for 72 h in a half strength MS liquid medium containing 2 ppm each of indole-3-acetic acid (IAA), indole-3-

butyric acid (IBA), indole-3-propionic acid (IPA) and NAA, prior to culturing them for 4 weeks in a medium devoid of growth regulators. Rooted plantlets were washed thoroughly under running tap water to remove the medium adhering to the root system prior to transfer of these plantlets to 10 × 5 cm polybags containing 3 parts soil to 1 part sand. The plantlets were maintained in light at 25 ± 2°C in the hardening room in trays covered with glass sheets to minimize loss of moisture. After 20 days, the covers were removed and the plants were maintained for 10 days prior to transfer to pots in the greenhouse.

The caulogenic buds developed on LSEC were subjected to histological studies. Pieces of explants (ranging 5–7 mm in length) were fixed in FAA (formalin:glacial acetic acid:alcohol::5:5:90 by volume) for 48 h at room temperature. Tissues were dehydrated and embedded in paraffin according to Sharma and Sharma[9]. Serial sections, 8–10 µm, were cut using a Reichert–Jung 2050 rotary microtome. Sections double stained with haematoxylin–eosin and mounted with DPX (Loba Chemie) were studied to confirm the origin of the shoot buds.

Tamarind seeds cultured in the germination medium swelled in 4 days. Multiple cracks which appeared in the seed coat (Figure 1 *a*) facilitated its removal. The presence of seed coat resulted in browning of the medium and inhibited germination in culture. Out of the two explants tested, only LSEC (Figure 1 *b*) responded in culture. Orientation of the explant plays an important role in regeneration[5,10]. In the present study, shoot buds appeared on the expanded cut surface in 3 weeks while it was in contact with the medium. In contrast, explants which lost contact with the medium failed to respond. The section of embryo axis devoid of cotyledon also failed to respond. These observations confirmed the importance of attachment of the cotyledon with the embryo axis and the orientation of the explant, to obtain a

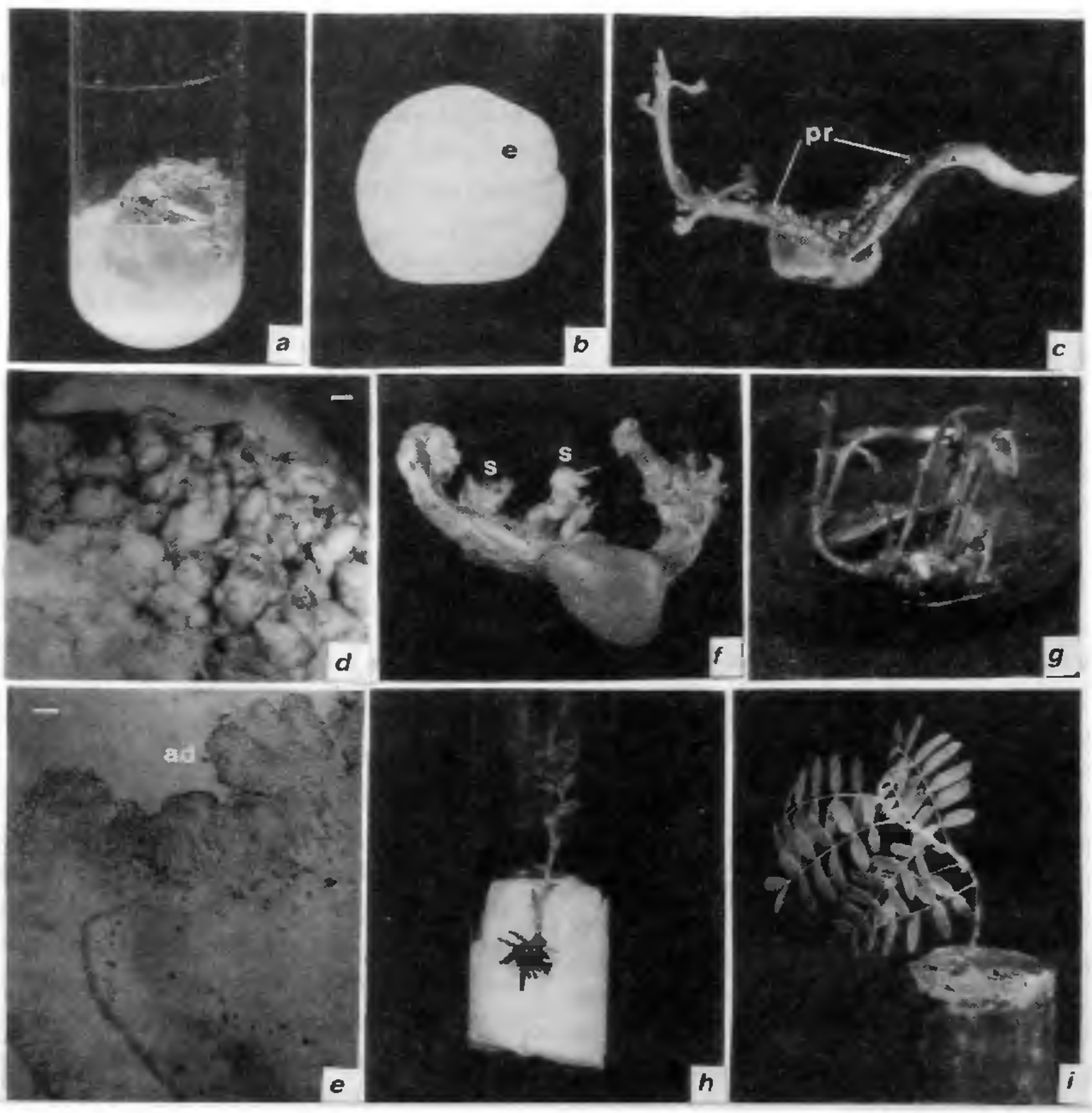

Figure 1. Plant regeneration via organogenesis in *Tamarindus indica* L. from longitudinal section of embryo axis with attached cotyledon; ***a***, Swollen seed with cracks on seed coat; ***b***, Longitudinal section of split embryo axis (e) explant with attached cotyledon (LSEC); ***c***, Shoot bud protuberances (pr) on the elongated cut surface of LSEC explant; ***d***, Magnified view of shoot buds (bar = 500 µm); ***e***, Light micrograph showing the formation of adventitious (ad) buds from subepidermal region of the explant (bar = 100 µm); ***f***, Shoot bud(s) differentiated from the protuberances; ***g***, Elongated shoots developed from shoot buds in liquid medium; ***h***, Rooting of elongated shoot; ***i***, Plant in soil.

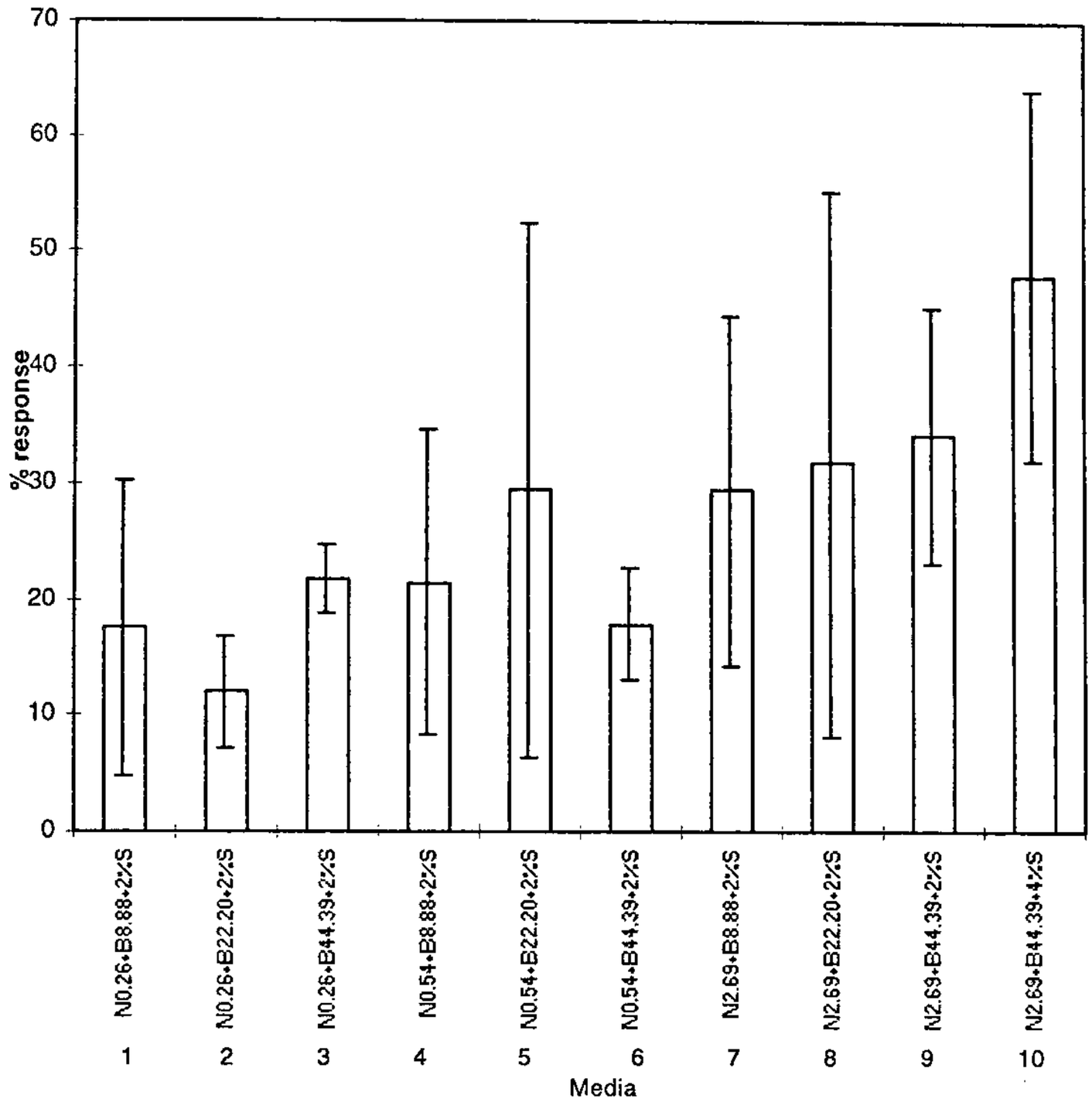

Figure 2. Frequency of caulogenic response in mature embryo axis of tamarind in varying combinations of NAA and BAP. The increased response in the last bar indicates enhancement of caulogenesis due to additional sucrose.

caulogenic response. The cotyledon of the LSEC explants turned green within 4 days of culture in the induction medium. The split axis showed elongation and broadening within 6 days. The buds appeared on the cut surface of the axis (Figure 1 *c*) in contact with the medium. Both explants with or without plumule responded in culture.

Direct organogenesis via adventitious caulogenesis has been noted earlier in other tree legumes such as *Albizzia lebbeck*[11], *Sesbania bispinosa*[12], *Albizzia* spp.[13], *A. falcataria*[14], *S. grandiflora*[15], *A. procera*[16] and *A. julibrissin*[17]. The only report on adventitions caulogenesis in tamarind[18] describes shoot formation mostly from the proximal end of hypocotyl explants of 12-day-old seedling in presence of BAP.

In the present study, BAP at concentrations of 2.22 to 66.60 µM singly failed to induce adventitious buds at the cut surface of the embryo axis which was devoid of meristem. Differentiation of root meristem was restricted in the presence of BAP. Roots proliferated from the existing root meristem of the original explants cultured in lower concentrations (0.54 to 5.37 µM) of NAA. In higher concentrations (26.85 to 80.55 µM), rooting was associated with profuse callusing.

Various combinations of NAA (0.26–2.69 µM) and BAP (8.88–44.39 µM) were tested for induction of caulogenic response. The response was noted in all the explants in the form of protuberances (Figure 1 *c* and *d*) on the cut surface of the embryo axis. The frequency of response, determined in percentage is expressed in the bar diagram (Figure 2). It is apparent that the magnitude of response varied among the first 9 combinations tested, but the differences within these combinations were not statistically significant. The medium containing 2.69 µM NAA, 44.39 µM BAP and 2% sucrose (medium 9) was more effective and induced the response in 34% of the cultures. Increase of sucrose concentration in this medium from 2 to 4% (medium 10) accentuated the frequency of meristematic response dramatically to 48% in the LSEC explants. The statistical analysis of the data generated from the 10 media combinations (Figure 2) was carried out using ANOVA (Table 1). It shows that the caulogenic response in medium 10 is significantly higher compared to the initial 9 combinations which had 2% sucrose. Further increase in the concentration of sucrose to 6% induced browning of media which was detrimental for the growth of the shoots.

Table 1. ANOVA

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F	F-table
Treatment	9	3079.650	342.1834	3.232	~2.1
Error	45	4764.179	105.8706		
Total	54	7843.829			

Significant at 5%.

Histological studies confirmed that the small protuberances on the cut surface of the elongated axis (Figure 1 *d*) were adventitious buds originating from the subepidermal region of the explant (Figure 1 *e*). These could be distinguished as shoot buds within 20 days of culture (Figure 1 *f*). Exposure of cultures to the initiation medium for more than 4 weeks resulted in dedifferentiation of shoot buds which led to callus formation. This could be circumvented by transferring explants with shoot buds to an elongation medium containing 0.91 µM zeatin in addition to 2.22 µM BAP, 0.41 µM calcium pantothenate and 0.40 µM biotin. Shoot elongation was asynchronous and 1–12 shoots elongated from each explant within 6 weeks (Figure 1 *g*). The length of the shoots ranged from 1 to 5 cm. Shoot formation reduced (3–4 per culture) in the second passage and the remaining shoot buds dedifferentiated into callus. For clonal propagation of tamarind, a combination of kinetin (0.93 µM), BAP (2.22 µM), calcium pantothenate (0.41 µM) and biotin (0.40 µM) was used for induction and elongation of shoots from the existing meristem[4,6]. However, in the present study substitution of kinetin with 0.91 µM zeatin was more effective in promoting differentiation and elongation of shoot buds by retarding the dedifferentiation of buds often observed in the presence of kinetin.

Clonal propagation of tamarind from the existing meristems of seedlings was reported earlier from this laboratory[4,6]. The protocol used for rooting of meristem derived microshoots was followed for rooting of the shoots developed from the adventitious buds in the present study. Root initiation was observed in about 10–15 days (Figure 1 *h*). Further incubation for 15–20 days resulted in a healthy root system. In all, 170 shoots were kept for rooting, out of which 108 shoots rooted. All these plants were transferred to soil in polybags. At this stage a majority of the plantlets had unopened leaves. It was observed that the shoots of the plants with unopened leaves deteriorated rapidly and dried from the tips. On the contrary, plants with fully or partially opened leaves stabilized in soil within 30 days. Twenty-four plants could be recovered by this method (Figure 1 *i*) and they survived on transfer to pots in the greenhouse.

In contrast to earlier reports in tamarind on proliferation of existing meristems[4–6], the present study deals with proliferation of shoots via adventitious bud formation in non-meristematic regions of the mature zygotic embryo axis. This system could be used to understand the initial events of adventitious bud formation in tree legumes.

The large number of adventitious buds along the surface of the LSEC explant of tamarind indicates that the totipotent cells are probably distributed all along the explant which expresses on wounding in the presence of growth regulators. Ideally, bud formation associated with the wounding site is often used for *Agrobacterium*-mediated transformation to generate genetically-modified plants with desired characteristics.

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