Direct somatic embryogenesis from zygotic embryos of a timber-yielding leguminous tree, *Hardwickia binata* Roxb.

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We report here direct somatic embryogenesis from semi-mature zygotic embryos (60–75 days after anthesis) of a timber-yielding leguminous tree, *Hardwickia binata* Roxb., commonly known as Anjan. High frequency direct somatic embryogenesis was achieved on Murashige and Skoog's (MS) medium supplemented with 2.26 μM 2,4-dichlorophenoxyacetic acid (2,4-D). Somatic embryos proliferated rapidly in subsequent cultures. Various developmental stages were observed during the maturation of somatic embryos, including globular, heart-shaped, torpedo and cotyledonary stages. Maturation of somatic embryos was achieved by transferring isolated somatic embryos to MS medium containing 0.26 μM α-naphthaleneacetic acid (NAA) and 2.22 μM 6-benzylaminopurine (BAP) or 1.89 μM abscisic acid (ABA). In long-term cultures, secondary somatic embryos developed from isolated torpedo-shaped and cotyledonary embryos. Bipolarity of the somatic embryos was confirmed by histological studies.

HARDWICKIA BINATA Roxb. [commonly known as Anjan, family Leguminosae (Caesalpinieae)] is a multi-purpose deciduous, nearly evergreen tree of great economic value. The tree grows in the dry savannah forests of Deccan Peninsula, central India and certain parts of northern India. According to Troup[1], the wood of this tree is among the hardest and most dense of the trees growing in India. It is used for bridge and house construction and ornamental works, as the wood is very durable. The leaves are used for fodder and manure. The bark yields a strong fibre largely employed in making ropes, which is a source of income for the tribal population. The propagation of Anjan through seeds is unreliable due to poor germination and death of young seedlings under natural environmental conditions[2]. Clonal propagation of superior trees of good form, with a clear cylindrical bole and resistance to diseases, is of utmost importance. Somatic embryogenesis offers an alternative and efficient means for plant multiplication[3,4]. In recent years, somatic embryos are being used for developing synthetic seeds, shortening the breeding cycle and transformation studies. Somatic embryogenesis has been achieved in a number of dicotyledonous and monocotyledonous angiosperms. However, somatic embryogenesis has been reported for only a few woody species[5,6]. Woody plants, including leguminous trees appear to be recalcitrant to *in vitro* culture and plant regeneration[7,8]. Leguminous tree species are less studied and only a few are amenable to *in vitro* culturing[9].

In some leguminous tree species, somatic embryogenesis has been reported from callus cultures derived from hypocotyls in *Albizia richardiana*[10], from immature cotyledons in *Acacia catechu*[11] and *H. binata*[12], from endosperm in *Acacia nilotica*[13] and from semi-mature zygotic embryos in *Dalbergia sissoo*[14]. Direct somatic embryogenesis has been reported from immature embryos in *Dalbergia latifolia*[15]. We report here direct somatic embryogenesis from semi-mature zygotic embryos of *H. binata* Roxb. Direct somatic embryogenesis in this species will be useful in the improvement of this economically important tree species, as the technique requires fewer steps with concomitant reduction in labour, time and cost. Direct somatic embryogenesis is also preferred because it allows production of plants without somaclonal variation and in efficient cloning and genetic transformation[16].

Green pods of *H. binata* (60–75 days after anthesis) were collected from trees growing in the forest of Simrol Valley, located 20 km from Indore, Madhya Pradesh. The pods were washed under running tap water for 30 min, treated with tween-20 solution (10 drops/100 ml, v/v) for 15 min and washed again in running tap water. This was followed by Savlon antiseptic solution treatment (0.6 ml/100 ml, v/v) for 15 min and then washing with running tap water for 5 min. Further, sterilization treatments were done under a laminar-flow chamber. The pods were disinfected with 0.1% (w/v) freshly prepared aqueous mercuric chloride for 30 min, followed by washing with sterile distilled water. Finally, the pods were treated with 70% ethanol for 2 min and washed 4–5 times with sterile distilled water.

Seeds were isolated from the pods. Zygotic embryos were excised after removing the seed coat and aseptically cultured onto a semi-solid Murashige and Skoog (MS)[17] medium supplemented with various concentrations (0.45–13.57 μM) of 2,4-dichlorophenoxyacetic acid (2,4-D) or (0.53–16.11 μM) of α-naphthaleneacetic acid (NAA). The pH of the medium was adjusted to 5.75 with 0.1 N NaOH or 0.1 N HCl solution prior to adding 0.8% (w/v) agar-agar. The medium was steam-sterilized at 121°C at 1.06 kg/cm^2 for 15 min. For somatic embryo induction, cultures were maintained in the dark for 7 weeks, while for proliferation cultures were transferred to 16/8 h light/dark photoperiod at 25 ± 2°C. A light intensity of 40 μmol m^{-2} s^{-1} was provided by cool-white, fluorescent tubes (Philips, India). The cultures were transferred to fresh medium after an interval of 4 weeks. Explants with developing somatic embryos were cultured on proliferation medium (MS + 0.26 μM NAA + 2.22 μM BAP; MS + 0.53 μM NAA; or 1/2-MS + 0.53 μM NAA). The average number of embryos per explant was determined

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over a period of 40 weeks. For maturation and germination, somatic embryos were transferred to MS medium supplemented with growth regulators (MS + 0.26 μM NAA + 2.22 μM BAP or MS + 0.37–3.78 μM ABA) and also on MS medium without growth regulators. The cultures were maintained for a period of 4 years and many combinations of growth regulators were tried for the differentiation of plantlets from these somatic embryos and also from the fresh somatic embryos formed on the zygotic embryos in the initial cultures. In another experiment, somatic embryos were allowed to desiccate by transferring them to sterile empty petri plates for 7 and 15 days and also dehydrated for 15 and 30 days by transferring on MS medium containing 10% sucrose. Later, these were transferred to MS medium containing various growth regulators and also on MS medium without growth regulators (MSO).

For histological studies, the embryos were fixed in formaldehyde/glacialacetic acid/ethanol (FAA, 5 : 5 : 90, v/v/v) for 24 h, dehydrated through a graded tertiary butyl alcohol (TBA) series, each for 24 h and embedded in saturated paraffin wax. Embedded materials were sectioned at 5 μm thickness on a rotary microtome. Paraffin wax was removed by xylene prior to rehydration of the tissues in a graded ethanol series and staining the tissue with 1.0% (w/v) safranin. Tissues were briefly washed in water to remove excess stain and then dehydrated in a graded ethanol series. Photographs were taken with a binocular Nikon compound microscope with a Nikon camera, model F 601 (Japan).

Percentage response for direct somatic embryo formation and mean number of somatic embryos was determined after 9 weeks of culture. Experiments were repeated four times and each experiment consisted of 14 replicates. Various stages of somatic embryo development on different proliferation media were recorded over a period of 40 weeks. This experiment was repeated twice and each treatment consisted of 6 replicates. Standard errors of the means were calculated and a randomized complete block design (RCBD) was used for the analysis of variance. Fisher's least significant difference (LSD, $P < 0.05$) among mean values was calculated.

Among various 2,4-D concentrations tested, direct somatic embryogenesis with no intervening callus stage was observed from cultured semi-mature zygotic embryos on MS medium supplemented with 0.45–3.39 μM 2,4-D, when cultures were kept in the dark for 7 weeks. In contrast, higher concentrations of 2,4-D (4.52–13.57 μM) favoured root formation. Somatic embryogenesis occurred from zygotic embryos excised from 60–75-day-old semi-mature seeds. No response was obtained from the fully matured zygotic embryos excised from dry seeds. The maximum response for direct somatic embryo formation from the explant on MS medium containing 2.26 μM 2,4-D after 9 weeks of culture was 72.3% and the mean number of somatic embryos per responding explant was 18.3 (Table 1). Somatic embryos first developed on one part of the explant and later on all surfaces of the cultured zygotic embryos. Globular embryos first appeared as protuberances on the surface of the explant. Individual embryos enlarged into distinct bipolar structures and passed through each of the typical developmental stages (globular, heart, torpedo and cotyledonary). Sometimes, somatic embryos first appeared as heart or torpedo-shaped on the surface of zygotic embryos and finally matured into the cotyledonary stage (Figure 1 *a*, *b*). This indicated that sometimes early development of somatic embryos (globular to heart stage) occurred inside the explant and further development occurred after emergence of the embryos.

Explants with developing somatic embryos at various developmental stages which were induced on MS medium supplemented with 2.26 μM 2,4-D, were further subcultured on fresh MS medium containing various growth regulators (MS + 0.26 μM NAA + 2.22 μM BAP; MS + 0.53 μM NAA; 1/2-MS + 0.53 μM NAA) for rapid proliferation. Of the various growth regulator combinations tested, 1/2-MS + 0.53 μM NAA appeared to be best for somatic embryo proliferation. Table 2 shows the mean number of somatic embryos per explant and various stages of somatic embryos on proliferation medium after 10, 15, 20, 25, 30, 35 and 40 weeks. After 40 weeks of culture on embryo proliferation medium (1/2-MS + 0.53 μM NAA), an average of 142.7 somatic embryos per explant was produced. It was observed that different media varied in their effectiveness for somatic embryo formation from the explant. On proliferation medium, a large number of somatic embryos of various developmental stages developed on the entire surface of the zygotic embryo explant (Figure 1 *c*, *d*).

Proliferation of somatic embryos occurred in two ways: (1) multiplication of somatic embryos from the explant through primary somatic embryogenesis; and (2) proliferation of secondary somatic embryos from already formed somatic embryos through repetitive embryogenesis.

Table 1. Response of zygotic embryo explants of *H. binata* for direct somatic embryo formation (data represent mean ± standard error of four experiments, each consisting of 14 explants)

Medium + growth regulator (μM)	Percentage response for direct somatic embryo formation from the explants (Mean ± SE)	Number of somatic embryos per explant (Mean ± SE)
MS + 2,4-D (0.45)	19.6 ± 3.0	4.0 ± 0.3
MS + 2,4-D (1.13)	39.2 ± 4.0	5.7 ± 0.4
MS + 2,4-D (2.26)	72.3 ± 3.8	18.3 ± 0.5
MS + 2,4-D (3.39)	19.6 ± 3.0	2.3 ± 0.2
MS + NAA (0.53)	–	–
MS + NAA (1.34)	–	–
MS + NAA (2.68)	–	–
MS + NAA (4.02)	–	–
Mean	37.7	7.6
LSD ($P < 0.05$)	12.3	1.4

(–), No response.

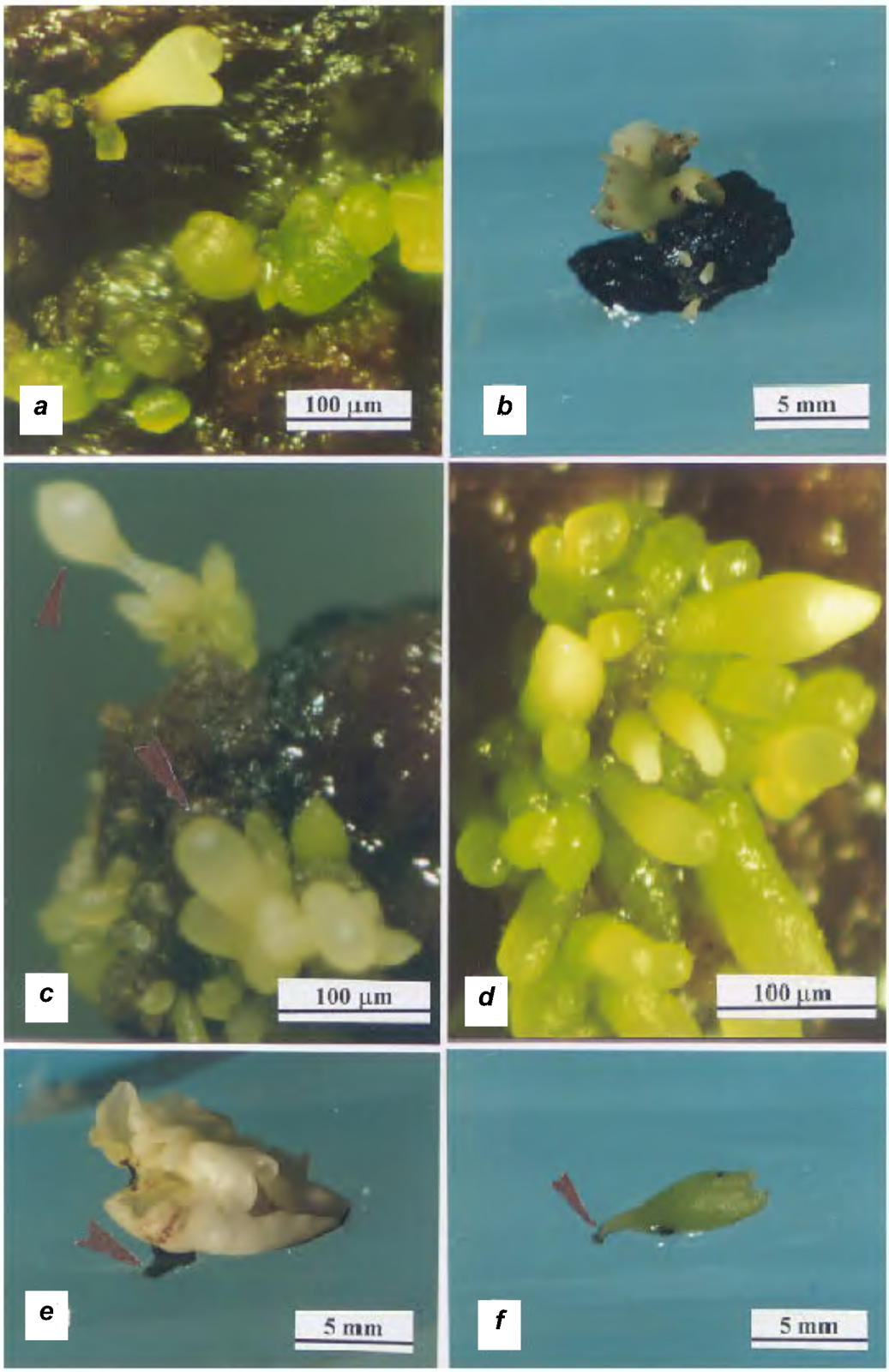

Figure 1. ***a*** and ***b***, Somatic embryo development from the zygotic embryo of *H. binata* on MS medium containing 2,4-D at 2.26 µM; ***c*** and ***d***, Different stages of somatic embryo development (arrows) on proliferation medium; ***e***, Somatic embryo showing fused cotyledons; ***f***, Somatic embryo showing plumule and radical zone.

Secondary somatic embryogenesis was achieved from torpedo and cotyledonary stage somatic embryos. Generally, 3–5 secondary embryos were developed, either from the base or tip of the somatic embryos.

Somatic embryos were maintained on MS medium supplemented with 1.11 µM BAP, 1/2-MS supplemented with 0.53 µM NAA + 1.11 µM BAP, and also on MSO, for a long term (over a period of 4 years). Secondary embryogenesis was observed in subsequent subcultures and globular embryos continued their development to the torpedo and cotyledonary stage. Addition of ABA in the medium promoted production of torpedo-stage embryos. To promote maturation and germination of somatic embryos, they were separated and cultured on MS medium containing various growth regulators and also on MSO. Of these media tested, MS + 0.26 µM NAA + 2.22 µM BAP and MS + 1.89 µM ABA proved to be best for somatic embryo maturation. Das *et al.*[12] reported somatic

Table 2. Mean number of *H. binata* somatic embryos on proliferation medium over a period of 40 weeks. The initiation medium was MS enriched with 2.26 µM 2,4-D; the explant was a zygotic embryo (data represent mean ± standard error of two experiments, each consisting of 6 replicates)

Medium + hormone (µM)	Mean no. of somatic embryos/explant	Stages of somatic embryos			
		Globular	Heart-shaped	Torpedo-shaped	Cotyledonary
Time period: 10 weeks					
MS + NAA (0.26) + BAP (2.22)	20.0 ± 1.0	8.2 ± 0.5	4.8 ± 0.3	4.2 ± 0.3	2.8 ± 0.3
MS + NAA (0.53)	21.0 ± 0.8	9.2 ± 0.3	5.8 ± 0.1	4.3 ± 0.6	1.7 ± 0.2
1/2-MS + NAA (0.53)	23.4 ± 1.1	10.3 ± 0.8	6.8 ± 0.4	5.3 ± 0.4	1.0 ± 0.2
Mean	21.4	9.2	5.8	4.6	1.8
LSD ($P < 0.05$)	6.3	4.5	2.8	2.0	1.6
Time period: 15 weeks					
MS + NAA (0.26) + BAP (2.22)	23.5 ± 1.3	10.2 ± 0.5	5.3 ± 0.7	4.2 ± 0.4	3.8 ± 0.3
MS + NAA (0.53)	25.0 ± 1.1	11.5 ± 0.7	6.5 ± 0.2	5.0 ± 0.6	2.0 ± 0.2
1/2-MS + NAA (0.53)	28.2 ± 0.9	12.7 ± 0.9	7.5 ± 0.3	6.2 ± 0.5	1.8 ± 0.1
Mean	25.5	11.4	6.4	5.1	2.6
LSD ($P < 0.05$)	7.7	5.0	3.9	5.1	2.5
Time period: 20 weeks					
MS + NAA (0.26) + BAP (2.22)	26.3 ± 1.9	12.0 ± 1.0	5.8 ± 0.8	4.3 ± 0.6	4.2 ± 0.3
MS + NAA (0.53)	32.0 ± 1.2	15.5 ± 0.6	7.7 ± 0.3	5.8 ± 0.7	3.0 ± 0.2
1/2-MS + NAA (0.53)	37.0 ± 1.3	15.2 ± 0.7	10.0 ± 0.5	8.8 ± 0.7	3.0 ± 0.4
Mean	31.7	14.2	7.8	6.3	3.4
LSD ($P < 0.05$)	9.3	6.0	4.5	2.5	2.0
Time period: 25 weeks					
MS + NAA (0.26) + BAP (2.22)	38.4 ± 1.0	15.7 ± 0.7	9.8 ± 0.8	7.2 ± 0.5	5.7 ± 0.3
MS + NAA (0.53)	48.8 ± 1.1	21.2 ± 0.4	11.2 ± 0.8	9.7 ± 0.5	6.7 ± 0.3
1/2-MS + NAA (0.53)	57.2 ± 2.3	21.0 ± 1.3	13.5 ± 1.4	15.5 ± 1.0	7.2 ± 0.8
Mean	48.1	19.3	11.5	10.8	6.5
LSD ($P < 0.05$)	10.7	7.7	4.5	3.4	3.5
Time period: 30 weeks					
MS + NAA (0.26) + BAP (2.22)	47.6 ± 1.1	19.3 ± 1.6	11.0 ± 0.6	8.8 ± 0.7	8.5 ± 0.5
MS + NAA (0.53)	69.6 ± 1.0	26.2 ± 0.3	19.8 ± 0.1	13.8 ± 1.1	9.8 ± 0.6
1/2-MS + NAA (0.53)	90.7 ± 2.3	29.3 ± 1.5	25.7 ± 2.0	25.5 ± 1.6	10.2 ± 1.4
Mean	69.3	24.9	18.8	16.0	9.5
LSD ($P < 0.05$)	12.3	6.6	6.1	4.6	3.8
Time period: 35 weeks					
MS + NAA (0.26) + BAP (2.22)	61.9 ± 1.2	23.5 ± 1.6	13.2 ± 1.0	12.0 ± 1.4	13.2 ± 1.3
MS + NAA (0.53)	95.7 ± 1.6	32.7 ± 0.8	28.5 ± 0.8	19.5 ± 1.9	15.0 ± 0.2
1/2-MS + NAA (0.53)	120.3 ± 3.2	43.7 ± 1.6	32.2 ± 1.0	31.2 ± 1.9	13.2 ± 1.5
Mean	92.6	33.3	24.6	20.9	13.8
LSD ($P < 0.05$)	12.3	5.3	6.5	5.3	6.5
Time period: 40 weeks					
MS + NAA (0.26) + BAP (2.22)	77.3 ± 1.0	30.3 ± 1.4	15.0 ± 1.1	15.8 ± 1.7	16.2 ± 1.3
MS + NAA (0.53)	115.6 ± 1.5	41.5 ± 0.6	33.8 ± 0.4	23.0 ± 0.8	17.3 ± 0.3
1/2-MS + NAA (0.53)	142.7 ± 2.3	56.0 ± 2.0	37.2 ± 0.9	34.2 ± 2.3	15.3 ± 0.8
Mean	111.9	42.6	28.7	24.3	16.3
LSD ($P < 0.05$)	10.7	7.6	8.2	8.0	6.0

embryogenesis from callus cultures of *H. binata*. However, complete plantlet formation was not achieved. With callus cultures, there have been numerous reports of cytological irregularities leading to the regenerated plants being mutant or polyploid. Lester and Berbee[18] reported within-clone variation in plants derived from callus cultures in *Populus* spp. Regeneration via embryogenesis is better for obtaining genetically uniform plants than through organogenic differentiation[19]. In this paper, we report direct somatic embryogenesis (without callus phase) from semi-mature zygotic embryos of *H. binata*. Zygotic embryogenic tissue is more amenable to somatic embryogenesis, because it contains the pre-embryogenic determined cells.

In our experiments, mature embryos formed one or more cotyledons, although some of these had unequally-sized cotyledons and others had fused cotyledons (Figure 1 *e*). The cotyledonary embryo population was very heterogeneous, differing in size and shape. Mature somatic embryos observed in the present study were classified into six morphological classes, i.e. monocotyledonary, dicotyledonary, cup-shaped, young zygotic embryo, trumpet and cauliflower. Germination of somatic embryos was evident with the emergence of cotyledons, a plumule and a radicular zone (Figure 1 *f*). However, they did not form a shoot or root, although several combinations of growth regulators were tried over a 4-year period. Desiccation of somatic embryos (for 7 and 15 days) and dehydration with sucrose (for 15 and 30 days) were also carried out, but were found ineffective for germination of somatic embryos.

Direct somatic embryogenesis in cultures proceeds from cells which are already determined for embryogenic development. This requires growth regulators and favourable conditions to allow these pre-embryogenic determined cells to undergo cell division and expression of embryogenesis[16]. Somatic embryogenesis is generally believed to be triggered by an auxin and for many plants, 2,4-D has been widely regarded to be effective for somatic embryogenesis. At higher concentration of auxin, probably the population of embryogenic cells drops due to their disruption and elongation and the embryogenic potential of the culture is lost[20]. Using two kinds of auxins (2,4-D and NAA) in our experiments, embryogenesis in *H. binata* occurred from the explants only in 2,4-D (0.45–3.39 µM)-enriched medium. Histological studies confirmed the formation of various stages of somatic embryos (Figure 2 *a*). Longitudinal sections of torpedo-shaped embryos showed an apical tip with two cotyledons and the posterior end showed a root pole. Examination of serial longitudinal sections of torpedo-shaped embryos showed a bilateral symmetry with cotyledon formation (Figure 2 *b*). Secondary somatic embryogenesis was also confirmed by histological studies. Secondary somatic embryogenesis has been reported in some tree species such as *Eucalyptus citriodora*[21], *Picea abies*[22], *Hevea brasiliensis*[23], *Olea europaea*[24] and *Acer palmatum*[25].

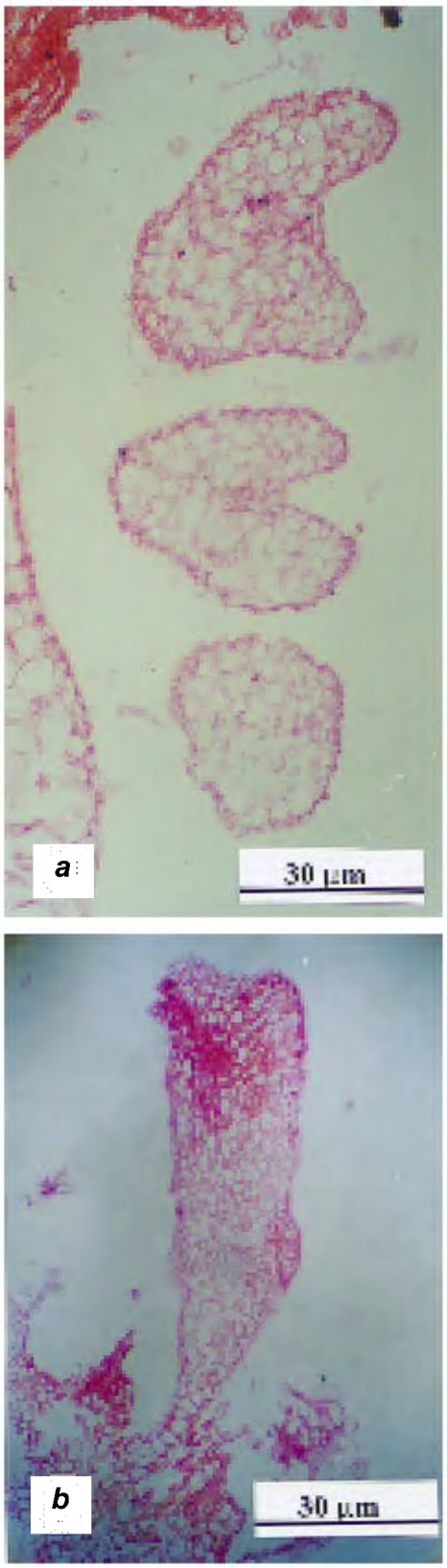

Figure 2. ***a***, Longitudinal section showing various developmental stages of somatic embryos; ***b***, Longitudinal section of a torpedo-shaped embryo showing cotyledon formation.

H. binata is a biologically significant system because it retains embryogenic potential even after 4 years of repeated subcultures. In this tree species, we obtained direct somatic embryogenesis without an intervening callus phase. Somatic embryos developed in clusters and multiplied rapidly. Several generations of somatic embryos have been initiated with no decrease in embryogenic potential. Bipolarity of the somatic embryos was confirmed by histological studies. The results showed that in *H. binata*, it is possible to obtain direct somatic embryogenesis from semi-mature zygotic embryos. Such studies are potentially useful in obtaining artificial seeds and experiments on genetic transformation.

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A new species of frog in the genus *Nyctibatrachus* (Anura: Ranidae) from Western Ghats, India

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A new species of *Nyctibatrachus* is described from the Western Ghats part of Kudremukh National Park, Karnataka, South India. This torrent species is differentiated from all known species in the genus by its very large size (SVL: snout vent length range 52–84 mm), stout body, rough and highly wrinkled dorsum, presence of two prominent folds in the tympanic region, prominent vomerine teeth located horizontally on highly-elevated strong ridges situated far behind the choanae. Hindlimb is short, tibio-tarsal articulation reaching the tympanic region, tips of fingers and toes dilated into prominent discs with circum-marginal grooves. Prominent and highly developed tubercles equal to the size of their respective terminal phalangial discs are present. The new species is compared with closely related congeneric species (*N. humayuni*, *N. major* and *N. vasanthi*). It markedly differs in colouration, size of tubercles, length of the forelimb, hindlimb, foot and tibia (calculated as a per cent of respective SVL) with a high degree of squared Euclidean dissimilarity.

HILLY terrain and forests of the Western Ghats of India are an abode for various amphibian species. This region harbours as many as 123 species[1–3]. Kudremukh National Park is located in the central Western Ghats (13°10′–13°26′N and 75°05′–75°10′E) and comprises highly complex vegetation mosaic of tropical evergreen forest, shola-grassland and mixed semi-evergreen forest, richly nourished by several hill streams and torrents. Biotic

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