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ACKNOWEDGEMENTS. I acknowledge the support by Sri Jayachamarajendra College of Engineering, and also the referees whose observations have improved the quality of the paper.

Received 13 April 1998; revised accepted 14 September 1998

Induction of somatic embryogenesis and plant regeneration from leaf callus of *Terminalia arjuna* Bedd.

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A protocol for effective plant regeneration via somatic embryogenesis has been developed for *Terminalia arjuna* Bedd. Calluses were initiated from leaves of mature trees on Murashige and Skoog's medium (MS) supplemented with 5 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 0.01 mg l^{-1} kinetin, 3% sucrose and 0.8% agar. The calli showed differentiation of globular structures when transferred to the MS basal medium. Globular structures enlarged and produced secondary globular structures and/or somatic embryos. Continued production of globular structures, their differentiation into embryos and germination of embryos occurred on the MS medium with 3% sucrose and 0.8% agar. The plantlets were hardened and transferred to the soil. Such *in vitro* raised plants showed luxuriant growth in field condition.

TERMINALIA arjuna is one of the important Indian trees with multiple uses. Besides yielding timber, the tree is a primary host plant of the tropical tasar silkworm (*Antheraea mylitta*)[1,2]. The bark and different parts of the tree are medicinally important and also serve as a source of tannin[3,4]. The population of *Terminalia arjuna* show tremendous variation[5]. Owing to the poor knowledge on their breeding system, selection of improved progenies is difficult. There is an important need for identification of plus trees with higher nutritional value in leaves for silkworm and resistance to diseases and development of methods for their micropropagation.

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Somatic embryogenesis has the potential to speed up the propagation of forest trees[6,7]. The present paper describes induction of somatic embryogenesis in *Terminalia arjuna* from leaf callus and successful regeneration of plants.

Leaves (6–14 cm) of *Terminalia arjuna* were collected during April–May from a mature tree of the BHU campus. They were washed in running tap water for approximately one hour and were surface-sterilized in 1% v/v cetavlon solution with constant agitation for 25 min followed by washings in running tap water. They were re-sterilized in laminar flow hood with 70% ethanol for 30 s and with 0.05% $HgCl_2$ solution for 5 min. After washing in double distilled water 2 or 3 times, discs of 0.8 cm diameter were cut out from the leaves by means of a cork borer. These were cultured on MS medium supplemented with 3% sucrose, 0.8% agar and different concentrations (0.001 to 5 mg l^{-1}) and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (N^6-furfurylaminopurine) (Kn) or 6-Benzylaminopurine (BAP). When present in combinations, the concentration of 2,4-D was kept higher (1–5 mg l^{-1}) than that of BAP or Kn (0.001–0.1 mg l^{-1}).

The cultures were maintained at 25 ± 1°C with light intensity of 2000–3000 lux for 16 h photoperiod regulated by a timer.

The leaf discs showed proliferation into callus, which stopped after 3 weeks. Hence, the calli were transferred to the same initiation medium or to MS basal medium. After 2 or 3 subcultures on the MS basal medium, the calli were placed either on MS medium containing different concentrations of Kn, BAP, α-napthaleneacetic acid (NAA), indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) or were maintained on MS basal medium for studying the effect of plant growth regulators (PGRs) on their differentiation.

Globular structures appeared from the callus (obtained on MS + 5 mg l^{-1} 2,4-D + 0.01 mg l^{-1} Kn) after 8–10

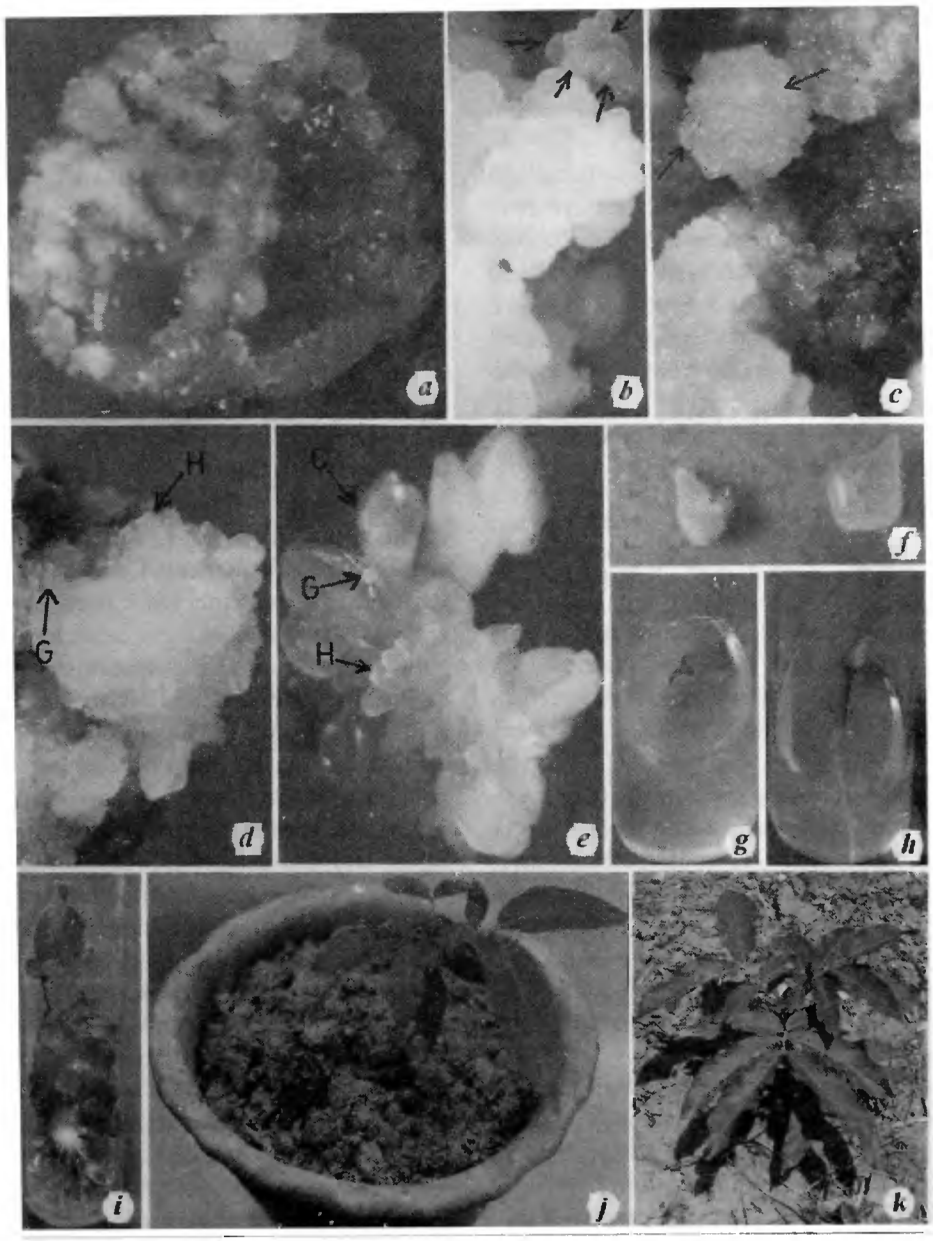

Figure 1. *a*, Callus formation from leaf disc on MS + 5 mg l^{-1} 2,4-D + 0.01 mg l^{-1} Kn (× 10); *b*, Globular structure (shown with arrows) formation from the margin of friable callus on a MS basal medium after 8–12 weeks of callus initiation (× 10); *c*, Globular structure with several protuberances (shown with arrows) on its surface (× 10); *d*, Differentiation of somatic embryos from approximately 4-week-old globular structure (× 10); *e*, Different stages of development of fused embryo (× 10); *f*, Early stages of cotyledonary embryos (× 10); *g*, Two-week-old cotyledonary embryo placed for germination on a MS basal medium (× 0.958); *h*, Root formation and elongation of the hypocotyl region (× 0.958); *i*, Plantlet with well-developed leaves, stem and root (× 0.708); *j*, Three-month-old plant growing under laboratory conditions in an earthen pot containing garden soil and farmyard manure mixture (× 0.527); *k*, One-year-old *in vitro* raised plant growing in the garden (× 0.129). G, Globular embryo; H, Heart-stage embryo; C, Cotyledonary embryo.

weeks of culture initiation and the effect of different concentrations of sucrose (1–5%) was studied on the formation of globular structures. Isolated globular structures were placed for 4 weeks on MS medium with gibberellic acid (GA_3) or abscisic acid (ABA) (0.01–5 mg l^{-1}) or MS medium with varying concentrations of sucrose (1–5%) to ascertain whether these treatments would promote their morphogenesis. For germination, 2 week-old cotyledonary embryos were transferred either to MS medium with GA_3 or ABA or to MS medium with different concentrations of sucrose (1–5%) and observed after 4 weeks.

The young plantlets were transferred to agar-solidified MS medium or to MS liquid medium supplemented with 3% sucrose for further growth. The roots of plantlets either from solid or liquid medium were repeatedly

washed with running tap water and transferred to acid and cetavlon washed sterilized sand. The sand was irrigated with the liquid MS medium without sucrose. For each experiment, 25 plantlets were transferred to sand in conical flasks capped with cotton plugs.

Hardened plantlets were kept in unautoclaved soil and sand mixture (1:1) in plastic pots. The latter were covered with polythene bags and kept under white fluorescent light. After one month, plantlets were transferred to earthen pots with garden soil and farmyard manure mixture (1:1). The exposure to sunlight was increased gradually and plantlets were finally transferred to the garden. Data were analysed statistically[8].

The following formula was used to analyse data regarding callus formation from leaf discs.

$$\% \text{ Explant response} = \frac{\text{Explant showing response}}{\text{Total number of explants}} \times 100$$

% Callus formation per explant: Whole surface area of the leaf disc was assumed to be 100% and observations were taken by calculating the percentage of the surface area of the leaf disc showing callus. Here values were only approximations.

Leaf discs cultured on media containing PGRs enlarged and curled within a week. Subsequently discs showed initiation of callus after one week from all parts, i.e. cut margin, midrib, veinal or intraveinal region and from both adaxial and abaxial surfaces. The discs cultured on the MS basal medium failed to respond and dried up within a week.

The callusing response from leaf discs varied depending on the concentrations and combinations of PGRs. 100% explants showed initiation of callus when the MS medium was supplemented with 3 or 4 mg l^{-1} 2,4-D. However, the percentage of callus formation per explant increased when the medium containued combinations of 2,4-D with 0.01 mg l^{-1} Kn. Maximum % callus formation per explant (58.57%) occurred on MS + 5 mg l^{-1} 2,4-D + 0.01 mg l^{-1} Kn (Figure 1 *a*).

The calli were either friable or compact and their growth ceased after three weeks on the initiation medium. Hence calli were transferred to a fresh initiation medium or MS basal medium. On transfer to PGR containing media, the calli became compact and degenerated after 5–9 weeks. Calli transferred to the MS basal medium from different initiation media also degenerated after 3–4 weeks of transfer with the exception of calli initiated on 4 or 5 mg l^{-1} 2,4-D + 0.01 mg l^{-1} Kn. The calli produced by 4 or 5 mg l^{-1} 2,4-D with 0.01 mg l^{-1} Kn became proliferative and friable on the MS basal medium.

Proliferative calli, which were initiated on media containing 2,4-D (4 and 5 mg l^{-1}) and Kn (0.01 mg l^{-1}) were subcultured on the MS basal medium two or three times. Such calli showed some proliferation when transferred to MS medium containing Kn, BAP, NAA, IAA or IBA, but did not show any morphogenetic response. Calli initiated on medium + 5 mg l^{-1} 2,4-D + 0.01 mg l^{-1} Kn when maintained on MS basal medium showed formation of white, shiny globular structures. The number of globular structures formed was maximum (24.6 ± 3.9) (± S.E.) on the MS medium containing 2% sucrose.

Globular structures turned green, enlarged and showed protuberances on their surfaces (Figure 1 *c*) which developed into embryos and/or secondary globular structures (Figure 1 *d*). These secondary globular structures again gave rise to embryos and/or tertiary globular structures. Globular structures and globular embryos were similar in appearance in the early stages. But cells of globular structures were enucleated with large vacuoles, stained lightly and measured 71.4 ± 4.7 µm (± S.E.) and cells of globular embryos were smaller (26.7 ± 5.6 µm), nucleated and darkly stained. The occurrence of fused embryos was common (Figure 1 *e*) and embryo development was asynchronous. The average number of secondary globular structures and embryos arising from one globular structure was 17.8 ± 5.04 (Figure 2) on MS + 3% sucrose. ABA at different concentrations caused inhibition of conversion of globular structures to somatic embryos. Curiously GA_3 at 0.5 mg l^{-1} stimulated production of 10.43 ± 0.57 embryos and secondary globular structures per globular structure. GA_3 completely inhibited the processes at other concentrations.

The fully-developed embryos had two free cotyledons or fused cotyledons (Figure 1 *e*, *f*). Germination started on the MS basal medium with sucrose in the two-week-old embryos by the formation of a root followed by the shoot. 100% germination of normal cotyledonary embryos occurred on the MS medium supplemented with 3% sucrose. Addition of GA_3 lowered the percentage of germination of embryos. However, fused cotyledonary embryos showed maximum germination (66.7%) on 3 mg l^{-1} GA_3 containing medium. ABA at all concentrations inhibited the germination of both normal and fused embryos. Growth of young plantlets was better on the MS solid medium than in liquid medium.

Plantlets grown on semi-solid MS medium showed 75.13 ± 3.88% survival on sterilized sand. For survival and establishment in the sand and soil mixture, the plantlets required hardening in sand for a minimum period of 4 weeks. During hardening, covering of plantlet with a polythene bag or beaker for at least 3 weeks was essential. Twenty five such hardened plants transferred to pots and two to field condition are showing luxuriant growth.

In *T. arjuna*, a potentially embryogenic callus was produced from all parts of the leaf disc. The calli formed from different regions were similar in nature and in their responses. In some plants, nature and response of calli are reported to be dependent on the site of their initiation[9,10].

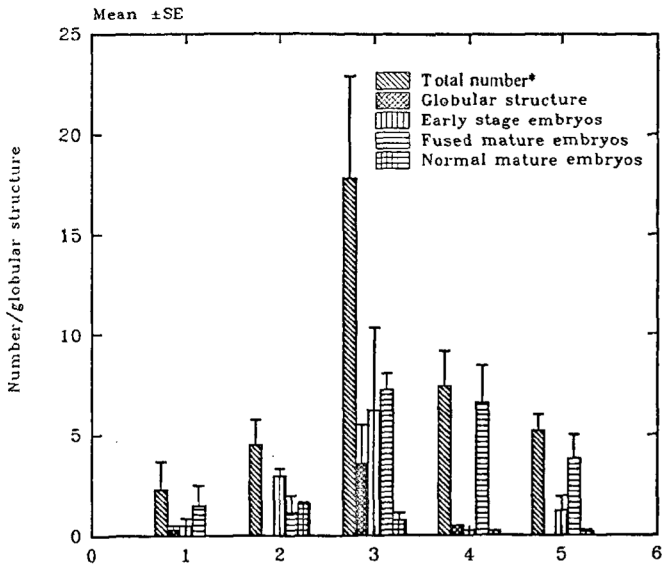

Figure 2. Effect of concentration of sucrose on differentiation from globular structure. *Total number indicates sum of number of embryos and globular structures. ANOVA values: For 1% sucrose: non-significant; 2% sucrose: significant at P 0.01 level; 3% sucrose: significant at P 0.05 level; 4% sucrose: significant at P 0.01 level; 5% sucrose: significant at P 0.05 level.

In the present work, calli differentiated white, shiny globular structures which gave rise to either secondary globular structures or embryos. A recycling of process through differentiation of secondary or tertiary globular structures was also observed. Somatic embryo formation from globular structures as described here is observed only in a few plants[11–14].

Globular structure formation from calli was restricted only to the leaf calli initiated on 5 mg l^{-1} 2,4-D + 0.01 mg l^{-1} Kn medium. Ruffoni and Massabo[15] have also observed a relationship between the number of embryos formed and concentration of 2,4-D in the induction media in *Lisianthus russellianus*. The requirement of cytokinin in addition to auxin observed in *T. arjuna* has also been reported in *Sapindus trifoliatus*[16] and *Euphoria longan*[17].

In the present study proliferation of embryogenic calli and embryo formation through differentiation of globular structures occurred even when PGRs were withdrawn from the medium. According to Zimmerman[18], removal of auxin results in the inactivation of a number of genes so that the embryogenesis program can proceed further.

Observations on germination of somatic embryos in the present study showed maximum germination of normal cotyledonary embryos on the MS basal medium, whereas maximum germination of fused cotyledonary embryos occurred on MS + GA_3. Somatic embryos of *Elymus giganteus*[19] also germinated when transferred onto a semi-solid MS medium without PGRs. But in several plants, addition of growth regulators in the medium was essential for germination of somatic embryos[9,20].

In the present study, the plantlets growing on a solidified MS medium survived better on sterilized sand irrigated with MS liquid medium without sucrose. Purohit *et al.*[21] have also observed that gradual shifting of the plants from a medium to culture bottles with low nutrients without saccharose allowed stress compelling plants to become partially autotrophic.

About thousand plants of *T. arjuna* can be produced within 6 months from the calli of one single leaf disc. This is a more effective means of propagation when compared to other available propagation methods of the tree. Besides this, the regenerative potential of leaf disc cells into embryos can be a useful tool for its genetic engineering studies.

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ACKNOWLEDGEMENT. N.K. thanks UGC, Delhi for junior and senior research fellowships.

Received 1 June 1998; revised accepted 8 September 1998