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SHORT COMMUNICATION

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Direct shoot organogenesis from leaf explants of *Embelia ribes* Burm. f.: a vulnerable medicinal plant

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Abstract An efficient system was developed for direct plant regeneration from in vitro-derived leaf explants of *Embelia ribes* Burm. f., a vulnerable medicinal woody climber of the Western Ghats of India. The in vitro procedure involved three steps that included induction of shoot initials from leaf tissue, regeneration and elongation of shoots from the shoot initials, and rooting of shoots. The induction of shoot initials was achieved on Murashige and Skoog (MS) solid medium supplemented with different concentrations of thidiazuron (TDZ). The best medium for shoot induction was MS with 0.272 μM TDZ. Numerous shoot primordia developed within 2–3 weeks on the leaf margin as well as on the midrib region, without any callus phase. In the second step, the shoot clumps separated from the leaf explant on transfer to MS basal medium, resulting in the differentiation of 90% of the shoot initials into well-developed shoots. The 2- to 3-cm-long shoots rooted on half-strength MS basal medium supplemented with 4.90 μM indole-3-butyric acid (IBA) and 3% (w/v) sucrose in the third stage. The rooted plants could be established in soil with 70% success. This protocol could be utilized for in vitro propagation and conservation of this important threatened medicinal plant.

Key words *Embelia ribes* · Shoot organogenesis · Medicinal woody climber · Vulnerable · Conservation

Introduction

Embelia ribes Burm. f. (Myrsinaceae), an important vulnerable medicinal plant, is a climbing shrub found in the semievergreen to evergreen forests of India, Sri Lanka, Malaysia, and China at altitudes above 400 m and up to 1200 m. The species is reported to be vulnerable in the Western Ghats of Tamil Nadu and Karnataka states of India, and at lower risk in Kerala state (Ravikumar and Ved 2000). Dried fruits of the plant are the source of an ayurvedic drug, *vidanga*. It is a highly esteemed medicinal plant having powerful anthelmintic, carminative, antibacterial, antibiotic, and hypoglycemic properties and is an ingredient in about 75 traditional ayurvedic drug formulations. The drug gained particular importance in view of the wide experimental and clinical trials on its contraceptive potential (Anon. 1966, 2002; Sivarajan and Balachandran 1994). The main active component, embelin, isolated from the berries has antifertility activity, which is reversible. The pharmacological and clinical investigations by various workers gave promising results about its antifertility activity without any side effects (Mitra 1995; Anon. 2002).

Propagation of the species is possible through seeds as well as vegetative means, although both methods are unreliable and difficult. The embryos are small when present and most of the seeds are abortive (Anon. 1990). Based on the preliminary studies conducted on seed and vegetative propagation, specific habitat conditions are required for its survival and growth. Regeneration is also very slow. All such factors, coupled with unsustainable and indiscriminate harvesting from the wild, have posed threats to this species. Thus, conventional propagation through seeds and vegetative cuttings is not sufficiently reliable or adequate to meet the demand for planting material. The development of an in vitro propagation method will be of great importance for the production of planting material to build up the resource base of this threatened species. This is the aim of the present study. No previous reports are available on in vitro propagation of this threatened medicinal plant.

Materials and methods

Establishment of in vitro shoot cultures

Mature fruit were collected from the wild populations of *Embelia ribes* in the Western Ghat forests of Kannur Dis-

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trict (1200 m a.s.l.), Kerala, India, during April 2003. Fruit were shade-dried and used immediately. They were soaked in water for 3 h and then treated with 0.1% (w/v) mercuric chloride solution and Tween 20 (2 drops in 100 ml) for 10 min. After rinsing four to five times with sterile distilled water, the surface-disinfected fruits were depulped and inoculated aseptically on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) containing 0.7% agar and 3% sucrose.

Shoot regeneration from leaf tissue

The in vitro raised seedlings served as explant source. Mature and semimature leaves along with a portion of the petiole were excised and were cultured on solid medium with their abaxial sides touching the medium. MS basal medium supplemented with 3% (w/v) sucrose and different concentrations of thidiazuron (TDZ), benzyl adenine (BA), and kinetin were tested for shoot regeneration experiments. The cultures were observed constantly for any response.

Shoot elongation and rooting of shoots

The clumps of shoots produced on the leaf tissue were excised from the parent culture and transferred to MS basal medium devoid of growth regulators for shoot elongation. After 4 weeks of culture, the elongated shoots (2–3 cm) were excised and kept for rooting. Full-strength and half-strength MS basal medium supplemented with indole-3-butyric acid (IBA) and 3% (w/v) sucrose were used.

Acclimatization

Rooted shoots were carefully taken out of the medium and washed thoroughly in running tap water to remove all traces of medium attached to the roots without damaging the roots. The plantlets were planted in 5-cm thermocol cups (Boss–CFC free; Utsav Polymers, Aurangabad, India) containing a mixture of sand and soil in the ratio 1:1. The cups were covered with polythene bags to maintain humidity and kept in a shade house where they were observed for further growth and establishment. After 20–25 days, the established plants were transplanted to polybags and then to pots containing garden soil and farmyard manure.

Culture conditions and data analysis

Uniform culture conditions were applied in all experiments. The pH of the medium was adjusted to 5.8 using 0.1 N NaOH, prior to autoclaving at 121°C for 20 min. The cultures were maintained at 25° ± 2°C under a 12-h photoperiod with a light intensity of 35–40 $\mu m^{-2} s^{-1}$ (Philips India, Mumbai, India).

All experiments were repeated at least three times with more than 12 replicates. Standard errors of means were calculated, and statistically significant mean differences were determined by the least significant difference (LSD) test.

Table 1. Effect of thidiazuron (TDZ) concentration on direct shoot organogenesis from in vitro-derived leaf explants cultured on MS medium

TDZ (μM)	Relative response (%)	Mean no. of shoots/explant
0.009	22.2 (± 0.6)	3.4 (± 0.6)a
0.023	31.6 (± 0.5)	4.6 (± 0.7)a
0.045	52.4 (± 0.4)	7.4 (± 0.7)b
0.091	54.3 (± 0.4)	10.2 (± 0.4)c
0.182	71.6 (± 0.3)	12.2 (± 0.5)d
0.272	83.6 (± 0.2)	16.3 (± 0.9)e
0.363	81.3 (± 0.2)	14.7 (± 0.9)de
0.454	62.8 (± 0.4)	9.6 (± 0.7)c

Each value was recorded after 28 days of incubation. Data are given as mean (± SE) of three independent experiments each with 12 replicates. Means followed by the same letters are not significantly different at $P = 0.05$ by the least significant difference test

Results and discussion

Shoot regeneration from leaf tissue

In the present investigation it was possible to obtain high frequency of shoot organogenesis directly from in vitro-derived leaf explants of *Embelia ribes* in TDZ-containing medium.

In the process of establishment of in vitro cultures, 20% of the seeds germinated after 45 days of incubation. The seedlings produced harvestable leaves within 30–40 days. Leaf explants derived from in vitro-raised seedlings exhibited differentiation of shoots in MS medium supplemented with different concentrations of TDZ ranging from 0.009 to 0.45 μM (Table 1). Semimature and expanded young leaves gave good response in all trials. Similar to the observations made in *Plumbago* species (Das and Rout 2002) and *Zizyphus jujuba* (Gu and Zhang 2005), shoot organogenesis in *E. ribes* is dependent on the maturity of explants.

The medium supplemented with BA or kinetin separately or the combination of the two did not give any response (data not shown). Within 2 weeks of incubation, direct shoot organogenesis was observed on the surface of leaf explants (Fig. 1a). Shoot initials developed in all parts of the lamina, although they were more concentrated in the midrib regions (Fig. 1b). At low concentrations of TDZ, more shoot initials developed at the petiole portion of the midrib and surrounding area (Fig. 1c).

The number of shoot initials increased with increasing concentration of TDZ. The highest rate of shoot bud regeneration took place in the medium containing 0.272 μM TDZ (Fig. 1d). Magioli et al. (1998) have reported the superiority of lower concentration of TDZ in *Solanum melongena* using similar explant. Such a response may perhaps be due to the increase in the levels of endogenous cytokinins by the effect of the growth regulator used. Hare and Van Staden (1994) reported that TDZ has a capacity to inhibit (at least partly)

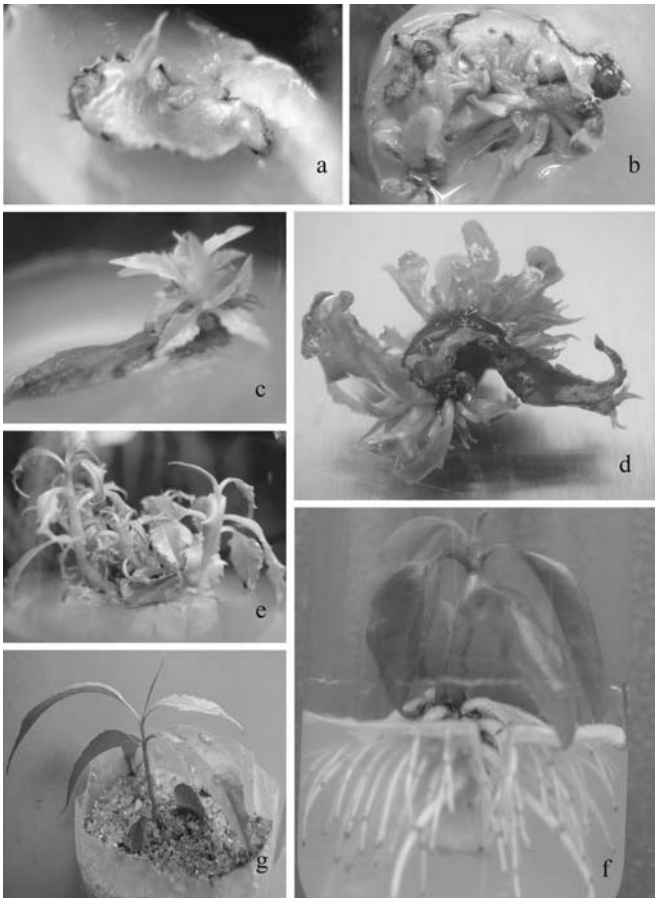

Fig. 1a–g. Direct shoot organogenesis from leaf explants of *Embelia ribes*. **a** Initiation of shoot primordia on leaf surface within 2 weeks, **b**, **c** shoot organogenesis concentrated on the midrib (**b**) and petiolar (**c**) regions, **d** shoot bud regeneration from all over the leaf surface in Murashige and Skoog (MS) medium with 0.272 μM thidiazuron (TDZ), **e** elongation of shoots in MS basal medium, **f** rooting of in vitro shoots in MS medium with 4.90 μM indole-3-butyric acid, **g** acclimatized plantlet

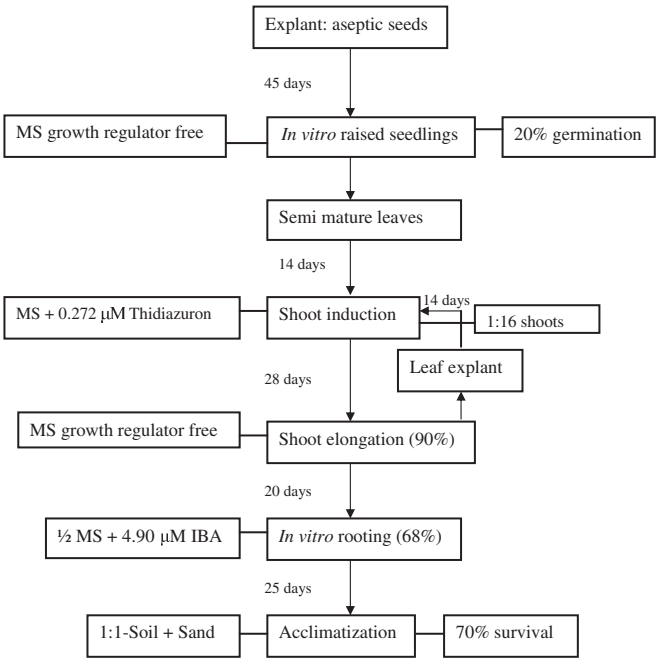

Fig. 2. Schematic representation for the micropropagation protocol of *Embelia ribes* using leaf explants. *IBA*, indole-3-butyric acid

the action of cytokinin oxidase, which in turn may increase the levels of endogenous cytokinins. However, higher concentrations caused reduction in shoot length and callusing. Multiple shoot initials induced by TDZ failed to elongate. Similar results were observed in other species (Preece and Imel 1991; Dalal and Rai 2004). Cytokinins commonly stimulate shoot proliferation and inhibit their elongation. Therefore, inhibition of shoot elongation by TDZ may be consistent with its high cytokinin activity (Huetteman and Preece 1993).

Shoot elongation and rooting of shoots

The length of the shoots obtained in the regeneration medium containing TDZ was not suitable for persistent rooting. Therefore, after 28 days, the clumps of shoot initials (0.5–1.0 cm) were excised from the parent culture and transferred to growth regulator-free MS basal medium for elongation. The shoot buds elongated sufficiently in this medium within 20 days (Fig. 1e). These differentiated cultures were further used as the source of leaf explant for shoot induction in subsequent cycles. Thus, the three-step plant regeneration protocol was completed within 132 days initially and the subsequent cycles were completed within 87 days (Fig. 2).

Shoot tips measuring about 1.5–2 cm in length with two or three leaves were used for rooting. The auxin IBA, which often induces rooting in tissue culture, was employed in the present study. Results differed depending upon the concentration of IBA as well as the strength of the basal medium. However, rooting percentage was lower in full-strength MS medium than in half-strength medium. Among the media tried (data not shown) half-strength MS medium supplemented with 4.90 μM IBA and 3% sucrose was the best. Profuse root formation was observed after 1 month of incubation (Fig. 1f).

Acclimatization

Rooted plantlets were successfully transferred to thermocol cups containing sand and soil (1:1 v/v mixture) and new growths were observed after 3 weeks (Fig. 1g). These plants exhibited a 70% survival rate 1 month after transfer.

In conclusion, an efficient protocol was developed for micropropagation of *E. ribes*, a vulnerable medicinal plant of Western Ghats of India. Our results show that micropropagation of this plant has a three-step protocol and TDZ is required for adventitious shoot regeneration from the leaf explants. Similar results have also been reported earlier in *Alstromeria* species by Lin et al. (1997). This protocol provides a successful and rapid technique that can be used for the propagation and ex situ conservation of this

important species. Application of this protocol could help in minimizing the pressure on wild populations and contribute to the conservation of the valuable flora of the Western Ghats. In addition to this, the present system could permit genetic transformation studies in this threatened medicinal liana in the near future.

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