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EX SITU CONSERVATION *VIA* DIRECT *DE NOVO* ORGANOGENESIS FROM LEAF SEGMENTS OF *EMBELIA RIBES* BURM. F., A VULNERABLE MEDICINAL PLANT IN WESTERN GHATS

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Abstract: *De novo* regeneration of shoots was accomplished from the *in vitro* cultured leaf segments of *Embelia ribes* (Family Myrsinaceae), a vulnerable medicinal woody climber found in the Western Ghats of Indian peninsula. Leaf explants derived from *in vitro* raised shoots cultured on MS medium supplemented with 0.01-1.0 mgl^{-1} TDZ induced shoot initials from both basal and distal portion of the explants. MS medium supplemented with 0.1 mgl^{-1} TDZ induced a maximum of 11.14±0.63 shoot buds from leaf segments after 4 weeks of culture. Histological events leading to *de novo* organogenesis analyzed through free hand sectioning followed by staining in toluidine blue at periodical intervals of 7 days duration revealed the pattern of organogenesis *via* direct regeneration. The regenerated shoots were elongated in hormone-free medium and produced maximum 6.44±0.94 cm long, slender roots at 86.67% efficiency after 4 weeks of culture in ½MS medium supplemented with 0.5 mgl^{-1} NAA. Within 28 weeks, starting from a single leaf segment ~65,536 plantlets could be harvested. Plantlets thus obtained after a short hardening phase of 2-3 weeks followed by transfer to the ambient environment resulted in 78% establishment. The protocol demonstrated in the present investigation reveals that mass production of adventitious shoot buds from the meristemoids of parenchymal origin of leaf explants is a reliable source for *ex situ* conservation and consistent utilization of this plant genetic resource.

Key words: *De novo* shoot organogenesis, *Embelia ribes*, *Ex situ* conservation, TDZ

INTRODUCTION

Medicinal plants constitute a very important national resource and India has one of the richest plant based ethno-medicinal traditions in the world. More than 90% of the formulations under Ayurveda, Sidha, Unani, and Homoeopathy mainly contain plant-based raw materials. Overall, almost 2000 medicinal plants are used in the preparation of medicines, and 500 of them are most commonly used. For centuries, the forests have been the source of herbs and medicinal plants. In the last few decades, while the availability of medicinal plants collected from forests is becoming uncertain, demand for herbs and plants has been increasing due to concern interest in indigenous systems. Therefore, the forest resources are under double pressure and are not able to meet the demand of providing medicinal plants and herbs. A number of species have been rendered vulnerable to extinction due to lack of cultivation and also unscientific harvesting of these wild resources.

Embelia ribes Burm. F. (Myrsinaceae), a vulnerable woody climber in the Western Ghats has wide array of medicinal properties (Ravikumar and Ved, 2000). Dried fruits of this plant are considered as anthelmentic, antibacterial, astringent, carminative, stimulant, etc. It contains embelin, quercitol, an alkaloid christembin, a resinoid, tannins and minute quantities of volatile oil. It is one of the ingredients of cough syrup '*Koflet*'. They are used in the preparation of Ayurvedic antidiarrhoeal drug '*Diarex*' for infants and children. In fact, population of this species gradually decline due to increase in demand for various therapeutical applications (Anonymous, 2002). Conventional propagation through stem cuttings is time consuming and seed propagation seldom successful due to its abortive nature. Consequently there is an urgent need for the development of alternative methods for the *ex situ* conser-vation of this species. In this

context, micropropagation holds great promise for mass multiplication of plant species, particularly those are difficult to propagate, rare or endangered. Reports on efficient micropropagation protocols in *E. ribes* is meager (Raghu *et al*., 2006 ; Preetha *et al*., 2012). A protocol to maximize the regeneration of adventitious buds will be useful for rapid *in vitro* propagation of the species. Hence, the present study focused on the development of a regeneration system on direct *de novo* shoot organogenesis from leaf segments of *in vitro*-derived shoot cultures of mature plants, which will be a pre-requisite for *ex situ* conservation and consistent utilization of this plant genetic resource.

MATERIALS AND METHODS

Establishment of shoot cultures

Shoot cuttings were collected from more than 25 years old plant growing in the field gene bank of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI) during April-May season. Nodal segments were cut out, washed in a liquid detergent, Teepol (3 to 4 drops dissolved in 100 ml distilled water) for 15 min and subsequently washed in running tap water for 20 min. They were rinsed several times in distilled water and treated with 0.1% (w/v) mercuric chloride for 7 min followed by 4-5 rinses in sterile distilled water. Nodal segments (1-1.5 cm) excised aseptically were inoculated vertically in agar gelled MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mgl^{-1} Benzyl adenine (BA). The *in vitro* shoots thus differentiated from the nodal explants were transferred to MS medium supplemented with individual concentrations of BA or kinetin (0.1, 0.5, 1.0 and 2.0 mgl^{-1}) or a combination of 0.5, 1.0 and 2.0 mgl^{-1} BA and 0.1 mgl^{-1} α-naphthal-eneacetic acid (NAA) or Indole 3-acetic acid (IAA) to enhance the multiplication rates.

All the cultures were incubated in a culture room maintained at 25±2°C under 12 hr photoperiod and illumination at 50-60 $\mu Em^{-2}S^{-1}$ provided by fluorescent tubes (Philips India Ltd., Mumbai). Observations on multiple shoot initiation were made periodically and data were recorded 4 weeks onwards.

Direct de novo shoot organogenesis from leaf discs

Leaf discs (0.5 cm^2) from *in vitro*-derived shoots cut into basal and distal portions were transferred to MS medium supplemented with 0.01, 0.05, 0.1, 0.3, 0.5 and 1.0 mgl^{-1} thidiazuron (TDZ) for inducing *de novo* organogenesis. The cultures were incubated under optimum conditions as described earlier. Shoot initials along with leaf discs cultured in TDZ supplemented MS media were removed at seven days interval for histological analysis through fresh free hand sections followed by toluidine blue staining.

Shoot elongation, rooting and field transfer

The shoot clumps obtained *via* direct organogenesis were subcultured in ½ MS medium for 4 weeks to promote their elongation. To induce rooting, the elongated shoots (> 4 cm) were transferred to ½ MS medium supplemented with 0.1, 0.2, 0.5, 1.0 mgL^{-1} NAA/ IAA/ Indole 3-butyric acid (IBA). After 4-6 weeks, the plantlets were taken out from the flasks, washed thoroughly in running tap water followed by treating with 0.1% Dithane M45 for 5 min and transplanted in plastic cups filled with a mixture of garden soil and pure river sand mixture (1:1). They were kept for hardening inside the mist chamber under high humidity. After 2 weeks of hardening, the plantlets were transferred to the poly bags filled with pure river sand, top soil and cattle manure in the ratio 3:1:1 for nursery establishment.

Statistical analysis

Each treatment consisted of 10 replicates and was repeated thrice. The data on the number of shoots and shoot length recorded after 30 days of culture were subjected to analysis of variance (ANOVA) and the means compared by Duncan's multiple range test at $p \leq 0.05$ using the SPSS/ PC + version 10 (SPSS Inc., Chicago, USA, 1999).

RESULTS AND DISCUSSION

Establishment of *de novo* shoots

In the present investigation, a protocol for direct *de novo* shoot organogenesis was standardized from leaf segments of *in vitro* shoots which were obtained from nodal segments cultured in MS

medium supplemented with 0.5 mgl^{-1} BA and 0.1 mgl^{-1} IAA (Fig. 1a). Single node cuttings of micro shoots transferred to MS medium supplemented with 0.1 to 2.0 mgl^{-1} BA/ kinetin or BA (0.1 to 2.0 mgl^{-1}) in combination with 0.1 mgl^{-1} IAA/ NAA showed shoot multiplication. The supplementation of BA either alone or in combination with IAA is advantageous to get good multiplication rates (Preetha *et al*., 2012). Leaf segments dissected out from such *in vitro* shoots cultured in MS medium supplemented with 0.01-1.0 mgl^{-1} TDZ evoked the production of *de novo* shoots after 4 weeks.

The effect of three cytokinins viz. BA, kinetin and TDZ on direct *de novo* shoot induction from leaf explants was analyzed in the study. Medium supplemented with BA or kinetin did not induce direct regeneration of shoots. In *E. ribes*, MS medium supplemented with 0.1 mgl^{-1} TDZ induced a maximum of 11.14±0.63 shoot buds from leaf segments (Fig. 1b). Frequency of multiple shoot induction was greater in the basal part of the leaf segments than from the distal part (Fig. 2). The potentiality of TDZ in aiding rapid plant regeneration through organogenesis in a number of species has been reported earlier by Murch *et al*. (2000).

Histological studies of the leaf segments at periodic intervals confirmed that the shoot buds were developed from the meristemoid areas in the midrib and it is another option for high frequency direct organogenesis from leaf explants of *E. ribes*. L.S. of the *in vitro* leaf segment prior to organogenesis revealed the presence of collateral vascular bundles limited by parenchymatic bundle along the mesophyll.

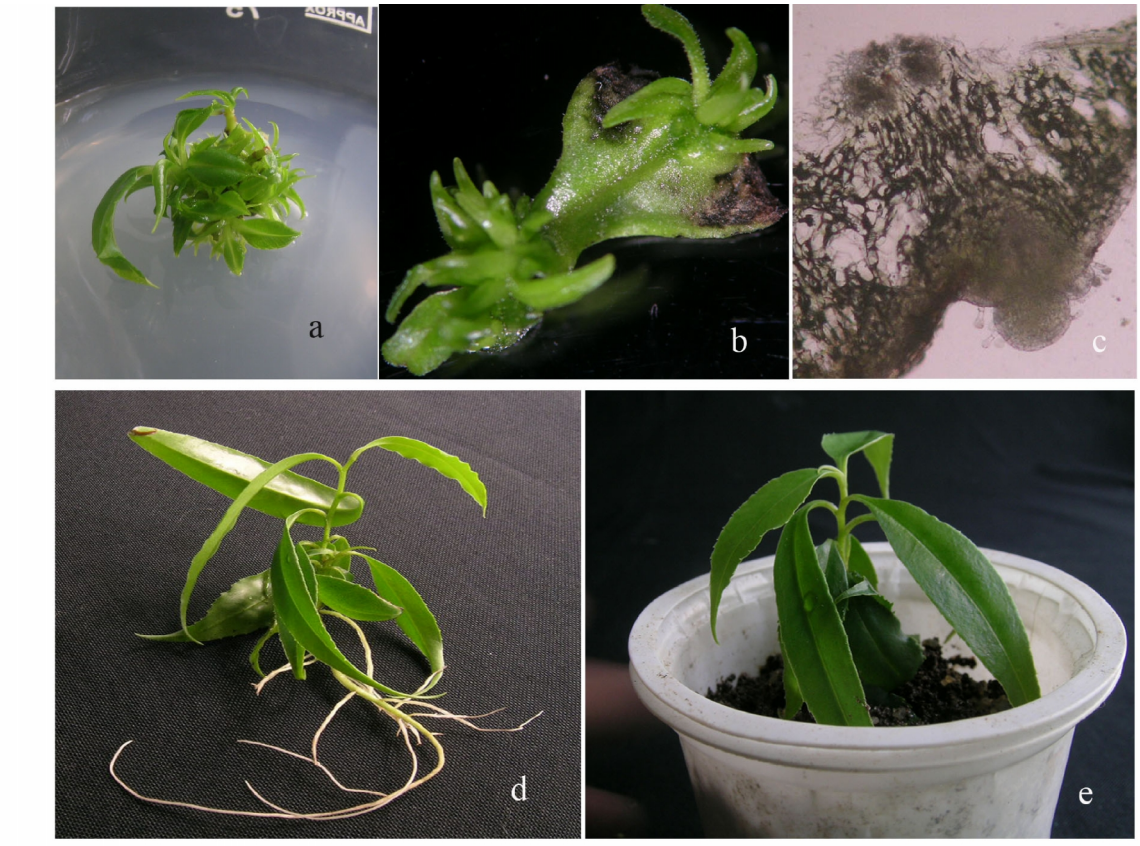

Figure. 1a-d.-*De novo* organogeness from leaf segments of *Embelia ribes*.
(a) Multiple shoots produced in 0.1 mg/l IAA and 0.5 mg/l BA; **(b)** Direct regeneration of shoots from leaf segments in 0.1 mg/l TDZ after 21 days of cultur; (c) Microscopic view of *de novo* organogenesis from leaf segments; (d) Rooting of elongated shoots in ½ MS medium augmented with 0.5 mgl/1 NAA; (e) Plantlets transferred to potting mix after 4 weeks of hardening.

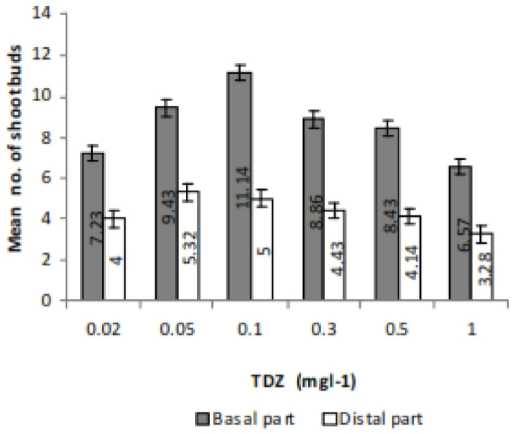

Fig. 2. Effect of TDZ on direct *de novo* shoot organogenesis from leaf segments of *E. ribes*

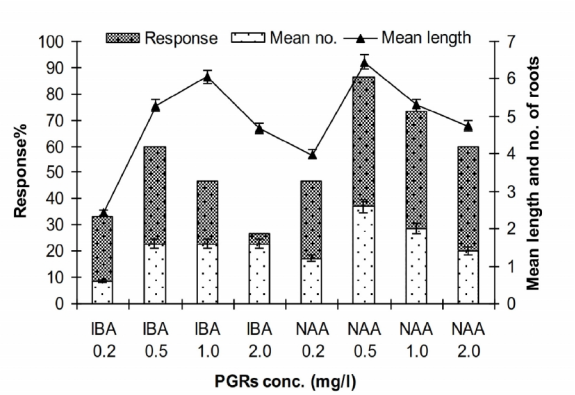

Fig. 3. Root induction from *in vitro* shoots of *E. ribes in ½ MS medium supplimented with different concentration of IBA/NAA*

The vascular bundles in the midrib portion of lamina were surrounded by the fundamental parenchyma. Initial histological alteration in the leaf explant and first divisions in the fundamental parenchyma were noticed after seven days. In the leaf blade, the abaxial epidermal cells increased in size and the hypodermal cells divided in several planes. On the 14th day, periclinal divisions in the mesophyll close to the vasculature were observed. Further divisions led to the formation of meristematic areas consisting of small cells with dense cytoplasm and prominent nucleus similar to those described as meristemoids by Saravitz *et al*. (1993) indicating early stages of shoot bud differentiation. On subsequent divisions, they give rise to small protrusions of tissue (Fig. 1c). All the leaf blade meristemoids were from the

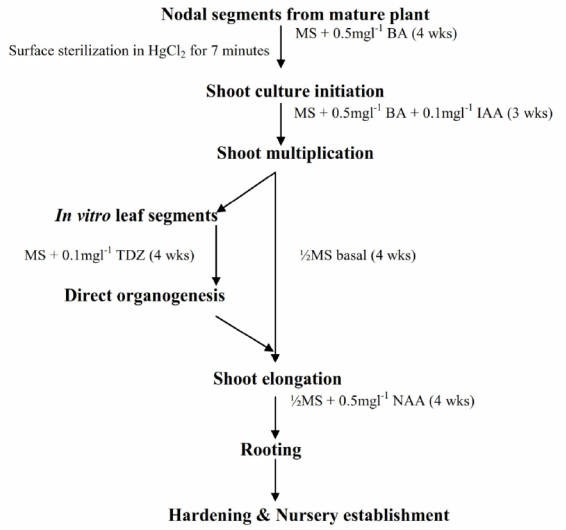

Fig. 4. Schematic representation for mass multiplication protocol of *Embelia ribes via de novo* organogenesis from leaf explants

abaxial epidermal cells. As the development proceeded, on the 21st day of culture, disorganized vascular system in the mid rib was observed due to increased activity of bundle sheath parenchyma providing evidence of the shoot bud development and its vascular connection with the explant tissue. Presence of several shoot buds were observed after 28 days confirming that they were originated from the meristematic centres (meristemoids) from the mid rib region of the leaf tissue.

Shoot elongation, rooting and field transfer

The *de novo* shoots obtained from leaf segments were having thick condensed internodes posturing difficulty for further multiplication and rooting phase. Such stunted shoots when subcultured to ½MS hormone-free medium permitted their elongation and produced elongated shoots with broad leaves. Similar observations were reported in *Azadirachta*, where transfer of shoot cultures in hormone-free or low PGR supplemented media at periodic intervals was recommended for shoot elongation of underdeveloped shoots (Eeswara *et al*., 1998).

The elongated shoots upon transfer to root induction medium in presence of IBA brought in thick and stout roots while rooting did not occur in medium augmented with various

concentrations of IAA (Fig. 3). The regenerated shoots produced maximum 6.44±0.94 cm long, slender roots at 86.67% efficiency after 4 weeks of culture in ½ MS medium supplemented with 0.5 mgl^{-1} NAA (Fig. 1d). The competence of NAA for root induction of *in vitro*-raised shoots of other woody species is well documented (Deora and Shekhawat, 1995).

Thus within a very short duration of 28 weeks, starting from a single leaf segment ~65,536 rooted plantlets could be harvested. Plantlets thus obtained have undergone a short hardening phase of 2-3 weeks followed by transfer to the ambient environment which resulted in 78% establishment (Fig. 1e). Thus the reliable rapid multiplication protocol demonstrated here through *de novo* shoot organogenesis in *E. ribes* (Fig. 4) is the first report which can be offered as another option for large scale production, *ex situ* conservation and sustainable utilization of this vulnerable woody medicinal climber.

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