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Micropropagation of *Elaeocarpus robustus* Roxb.

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Abstract Multiple shoots were obtained from shoot tips and nodal explants of 20-year-old trees of *Elaeocarpus robustus* on Murashige and Skoog's medium supplemented with 0.5 mg l^{-1} each of BA and Kn. Explants taken from in vitro-proliferated shoots subsequently produced multiple shoots when cultured on the same basal medium containing 0.5 mg l^{-1} each of BA and Kn. Repeated subculture resulted in rapid shoot multiplication at an average rate of 10 new shoots per subculture. The addition of CM (10%) and CH (100 mg l^{-1}) to the medium enhanced the number of shoots up to 20 per subculture and increased the length of shoots. In vitro-raised shoots were rooted on half-strength MS medium containing 1.0 mg l^{-1} IBA and 0.5 mg l^{-1} IAA. Following transplantation in the field 85% of the plantlets survived and grew uniformly.

Key words *Elaeocarpus robustus* · Fruit plant · Micropropagation

Abbreviations *NAA* α-Naphthalenacetic acid · *IBA* indole-3-butyric acid · *IPA* indole-3-propionic acid · *IAA* indole-3-acetic acid · *Kn* kinetin · *BA* 6-benzylaminopurine · *CN* coconut milk · *CH* casein hydrolysate

Introduction

Vegetative or clonal propagation provides several advantages for the improvement of perennial fruit crops. Clonal multiplication of superior phenotypes and valuable breeding stocks can be used for establishing tree improvement and seed production orchards (Dunstan et al. 1992). Micropropagation methods are specifically applicable to species for which clonal propagation is required. Thus, it is necessary to develop the essential micropropagation technology and then proceed to scale up to meet the needs of a particular operation (Gamborg and Phillips 1995).

Elaeocarpus robustus Roxb. (Family, Elaeocarpaceae) is an evergreen species commonly found in tropical countries and native to Bangladesh (Das 1987). The fleshy sour fruits provide vitamin C and are eaten raw or cooked and pickled. The tree has a fine-textured, moderately hard and strong wood which is suitable for making furniture and musical instruments. Due to cross pollination, propagation through seeds dose not conserve true-to-type fruit in this plant, and clonal propagation through conventional methods like cuttings or grafting have not been successful. The objective of the study reported here was to establish an efficient and reproducible method for the micropropagation of *Elaeocarpus robustus* from mature trees.

Materials and methods

Plant materials and culture media

Small stem twigs were collected in the spring from coppiced branches of 20-year-old trees of *E. robustus* growing in a field of the Jahangirnagar University Campus Dhaka. Expanded leaves were first removed, and stems were washed in an agitated solution of liquid detergent for 15 min and later washed in running tap water for 1 h. Surface sterilization was carried out with 0.1% mercuric chloride (BDH) for 5 min. Stems were thoroughly rinsed with sterile distilled water and cut into pieces (1.0–1.5 cm long) before being implanted into the culture medium.

The basal medium consisted of the mineral salts and organic nutrients of the MS medium (Murashige and Skoog 1962), 3% sucrose and 0.8% Difco agar. Depending on the experiment, the basal medium was variously supplemented with factorial combinations of different growth regulators such as BA, Kn, IPA, IBA, NAA, IAA at different concentrations (0.2–2.5 mg l^{-1}). The effects of CM and CH on shoot growth and development were also determined. All of the supplements were added to the molten agar, and the pH of the medium was adjusted to 5.8, before autoclaving at 1.06 kg cm^{-1} and 121 °C for 15 min in culture tubes (150×25 mm) or 100-ml conical

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flasks (Corning brand). The cultures were maintained at 24°±2°C under a 16-h photoperiod with a light intensity of 60–70 μE m^{-2} s^{-1} from Phillips cool-white fluorescent tubes. There were 20 explants per treatment and all experiments were repeated thrice.

Acclimatization and establishment of plants in soil

Four-to six-week-old regenerants with well-developed roots were removed from the culture tubes and washed free of agar. They were then dipped in 0.2% cupravit (Cu-oxychloride) (Bayer) fungicide for 2 min and subsequently transplanted into plastic trays containing sterilized alluvial soil and river sand (1:1). The tray containing plantlets was covered with a transparent polyethylene lid to maintain high humidity, and the microcuttings were misted thrice each day. Following 3 weeks at 24°±2°C under a 16-h photoperiod the lid was removed and the plantlets were transplanted singly to earthen pots containing sterile sand, soild and humus (1:2:1) at ambient room temperature (28°±2°C) with indirect sunlight. After 2 months they were planted out.

Results and discussion

Initiation of shoot cultures

The surfcae-sterilization procedure for explants described in the Materials and methods yielded 90% aseptic cultures. Media containing Kn alone did not form multiple shoots (data not presented). BA alone induced multiple shoots but at a low frequency (Table 1). On the combinations of 0.5–1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn, 76–85% of the cultures showed an induction of shoots within 3 weeks with 4–5 shoots per culture (Table 1). The only auxin-cytokinin combinations that promoted shoot induction were BA-NAA combinations (Table 1); other auxins tested failed to promote shoot formation (data not shown).

Table 1 Effect of growth regulators in MS basal medium on shoot induction and number of shoots per culture established from apical and nodal bud explants of *E. robustus*. Data taken after 28 days of culture

Growth regulators (mg l^{-1})	Percentage of cultures with induced shoots (%)[a]	Number of shoots per explant[a]
BA		
0.5	15.7±2.6	1.8±0.6
1.0	17.9±1.6	1.7±0.7
1.5	32.1±4.3	2.0±0.8
2.0	26.2±2.1	2.1±0.6
2.5	20.6±7.2	1.8±0.4
BA + NAA		
1.0+0.2	41.3±4.6	1.7±0.6
1.5+0.5	49.6±3.4	1.9±0.7
1.5+1.0	38.7±4.2	2.1±0.6
2.0+1.0	37.8±3.2	2.1±0.8
BA + Kn		
0.2+0.2	51.6±5.9	2.5±0.6
0.5+0.5	85.3±5.3	4.6±0.9
1.0+0.5	76.4±6.2	4.2±0.8
1.0+1.0	71.4±3.5	3.8±0.8
1.5+1.0	59.5±4.1	3.1±0.5

[a] The values represent the mean (±SE) of three independent experiments. Twenty explants were used for each experiment

Table 2 Effects of coconut milk (CM) and casein hydrolysate (CH) on the number of shoots regenerated when shoots were cultured in MS medium with constant concentrations (0.5 mg l^{-1} each) of BA and Kn

CH (mg l^{-1})	CM (% v/v)	Number of shoots[a] ±SE
0	0	10.2±1.6
	5	11.6±1.3
	10	12.2±1.7
	15	12.8±1.9
	20	9.3±1.5
50	0	12.6±1.7
	5	12.8±1.3
	10	15.6±1.9
	15	13.5±1.8
	20	10.6±2.1
100	0	12.9±1.6
	5	18.5±2.2
	10	20.2±1.7
	15	15.7±2.3
	20	11.8±1.5
150	0	7.5±0.9
	5	10.3±1.2
	10	15.2±1.8
	15	12.1±1.6
	20	8.3±1.4

[a] The values represent the mean (±SE) of three independent experiments. Ten explants were used for each experiment

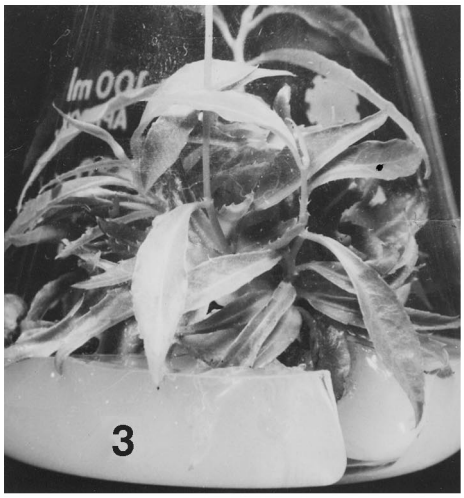
Shoot multiplication

At the time of subculturing, newly formed shoots were separated and transplanted to fresh medium of the same composition (0.5 mg l^{-1} each of BA and Kn). The shoots continued to proliferate through several subcultures with an average of 10 new shoots per transfer (Figs. 1, 2). In an attempt to enhance shoot proliferation, we added CH (50–200 mg l^{-1}) and CM (5–20% v/v) to the medium.

The addition of 100 mg l^{-1} CH to the medium increased the number of new shoots per transfer to 20 (Fig. 3, Table 2). Thus, the optimal medium routinely used for multiplication of a large number (20) of shoots with the proper length was MS basal medium with 0.5 mg l^{-1} BA + 0.5 mg l^{-1} Kn + 10% CM + 100 mg l^{-1} CH. Minocha (1987) reported that the addition of casein hydrolysate at 500 mg l^{-1} to the medium for the culture of shoot tips of paper bark birch resulted in significant increases in growth. There are other reports on the effects of the addition of complex organic substances on the growth in culture of woody species (Gamborg et al. 1976; Thorpe 1992).

Rooting

Without auxin treatment, shoots obtained from the multiplication culture failed to root. Well-developed shoots were



Figs. 1, 2 Multiple shoot formation from the shoot-tip explant (**Fig. 1**) and nodal explant (**Fig. 2**) of *E. robustus* on MS medium with 0.5 mg l^{-1} BA+0.5 mg l^{-1} Kn

Fig. 3 Elongation and multiplication of shoots cultured in MS medium supplemented with 0.5 mg l^{-1} each of BA and Kn+10% CM+100 mg l^{-1} CH

Fig. 4 Root formation on a regenerated shoot using half-strength MS with 1.0 mg l^{-1} IBA+0.5 mg l^{-1} IAA

excised from the culture flask and implanted individually on root induction medium containing half-strength MS basal medium with different concentrations and combinations of IBA, NAA and IAA. IBA at 1.0 mg l^{-1}+0.5 mg l^{-1} IAA was found to be the best combination of auxins, providing 100% rooting in 23 days (Table 3, Fig. 4). The first roots began to emerge after 12 days. In contrast, only NAA produced rooted shoots from *Eucalyptus citriodora* (Gupta et al. 1981), while IBA+NAA was effective for *Artocarpus heterophyllus* (Roy et al. 1993). Three auxins in combination, IBA, IAA and IPA, were essential for inducing roots in *Tectona grandis* (Gupta et al. 1980).

Table 3 Effects of auxins in half-strength MS medium with 3% sucrose on root formation from regenerated shoots of *E. robustus*. Data were recorded after 28 days for culture

Growth regulators (mg l^{-1})			Shoots rooted (%) [a]
IBA	0.5		40.2 ± 5.3
IBA	1.0		51.3 ± 3.2
IBA	1.5		41.8 ± 7.2
NAA	0.5		0
NAA	1.0		0
NAA	1.5		0
IAA	0.5		29.6 ± 7.2
IAA	1.0		22.2 ± 3.6
IAA	1.5		26.4 ± 8.2
IBA	0.5+IAA	0.5	86.2 ± 8.6
IBA	1.0+IAA	0.5	100.0 ± 0
IBA	1.0+NAA	1.0	76.6 ± 7.3
IBA	0.5+NAA	0.5	46.8 ± 8.3
IBA	1.0+NAA	0.5	58.7 ± 5.6
IBA	1.0+NAA	1.0	56.5 ± 7.4
IBA	1.5+NAA	1.0	48.7 ± 5.8

[a] The values represent the mean (± SE) of three independent experiments. Twenty explants were used for each experiment

Acclimatization and establishment of plantlets in soil

After 4–6 weeks in rooting medium, the rooted shoots were transferred to plastic trays. About 92% of the transplanted plants survived. At the time of acclimation the shoots elongated, and the leaves expanded and turned deep green. The plant also grew more vigorously when transplanted to the potting mix. After 2 months the plants were planted out into the field, where 85% of the plants survived. To date they are still growing with full vigor and uniformity.

The results of this investigation clearly show that apical and nodal buds of coppiced shoots from selected mature trees of *Elaeocarpus robustus* are capable of producing multiple shoots in vitro, which can then be rooted to form complete plantlets. Moreover, the multiplication potential continued for an extended time, and the survival of regenerants in the fild was satisfactory. The technique described here provides a promising method for propagation on a commercial scale as well as for the conservation of superior genetic strains.

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