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In vitro Rapid Regeneration from Cotyledon Explant of Native-olive (*Elaeocarpus robustus* Roxb.)

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Abstract: In the present study an efficient tissue culture method for high rate of shoot regeneration was developed for *Elaeocarpus robustus*. Cotyledonary explants with and without petiolar end were cultured in ½ MS medium supplemented with auxin and cytokinin. Explant with the petiolar/proximal end showed a better response than those without it in respect of shoot initiation. Half strength of MS medium supplemented with 1.0 mg l^{-1} BA+0.5 mg l^{-1} 2,4-D was found to be best for producing organogenic callus. The best shoot proliferation was observed when cotyledon-derived callus was subcultured in medium fortified with a combination of 1.0 mg l^{-1} BA+0.5 mg l^{-1} Kn+0.1 mg l^{-1} NAA. *In vitro* elongated shoots were rooted with 80% success by treating them ½ in MS medium supplemented with 0.2 mg l^{-1} IBA and incubated under 30°C in dark for initial one week. Rooted shoots (plantlets) were successfully established into soil and the survival rate of the plantlets was about 55%.

Key words: Regeneration, *in vitro*, cotyledon explants, *Elaeocarpus robustus*

INTRODUCTION

The native-olive (*Elaeocarpus robustus* Roxb.) is a moderably big tree planted for its edible fruits and timber in many areas of the country[1]. The plant is believed to have originated in Australia, however, it is well grown in Bangladesh. It is the most popular fruit among the few little known fruits of Bangladesh. It is cultivated in all districts of Bangladesh and also occurs wild in the evergreen forest of Chittagong and Sylhet[2]. Fruits are rich in vitamin C and are prescribed in diarrhoea and dysentery. Soup of the fruit is also given for stimulating secretion from the test buds. The green fruits are eaten fresh and also used in making soup, chutney, jelly and jam.

Nature of organogenic response in the cultured plant parts mostly determines the aim and objective of a *in vitro* plant propagation system[3,4]. Plants originated through axillary shoot proliferation method are more stable and very rarely show any genetic variation within the clone[5,6]. However, usable variation does occur in micropropagated plants originated through adventitious shoot proliferation methods, particularly when regeneration of shoots associated with intermediate callusing stage[7,8].

The native-olive tree is generally grown from seeds. The application of tissue culture methods for improvement and large-scale propagation of fruit trees have been well demonstrated[9,10]. Despite being very important as fruit and timber tree the native-olive plant has received very little attention for its multiplication through *in vitro* culture. However, some aspects of *in vitro* plant regeneration from shoot tip and nodal explants of mature native-olive trees have been reported earlier[11,12]. The present paper describes organogenesis and plant regeneration from cotyledon-derived callus cultures of native-olive.

MATERIALS AND METHODS

Seeds of *Elaeocarpus robustus* were collected from the mature fruits and cotyledons were excised from these seeds. Excised cotyledons were washed in distilled water and then surface sterilized with 0.1% $HgCl_2$ solution for 5 min. followed by thorough washing with sterilized distilled water. Two types of explants namely, cotyledon with petiolar / proximal end and without petiolar end / with distal end were cultured in ½ MS medium supplemented with different combinations of BA, Kn and 2,4-D for callus induction. After six weeks, calli were subcultured in conical flasks containing different combinations of cytokinin and auxin supplemented media for shoot induction. Multiple shoots developed in the medium containing 1.0 mg l^{-1} BA+0.5 mg l^{-1} Kn+0.1 mg l^{-1} NAA. Elongated shoots were excised, made into microcuttings (2-3 cm) and transferred to ½ MS medium with 0.1-1.0 mgl^{-1} of either IBA, NAA or IAA for rooting. All media

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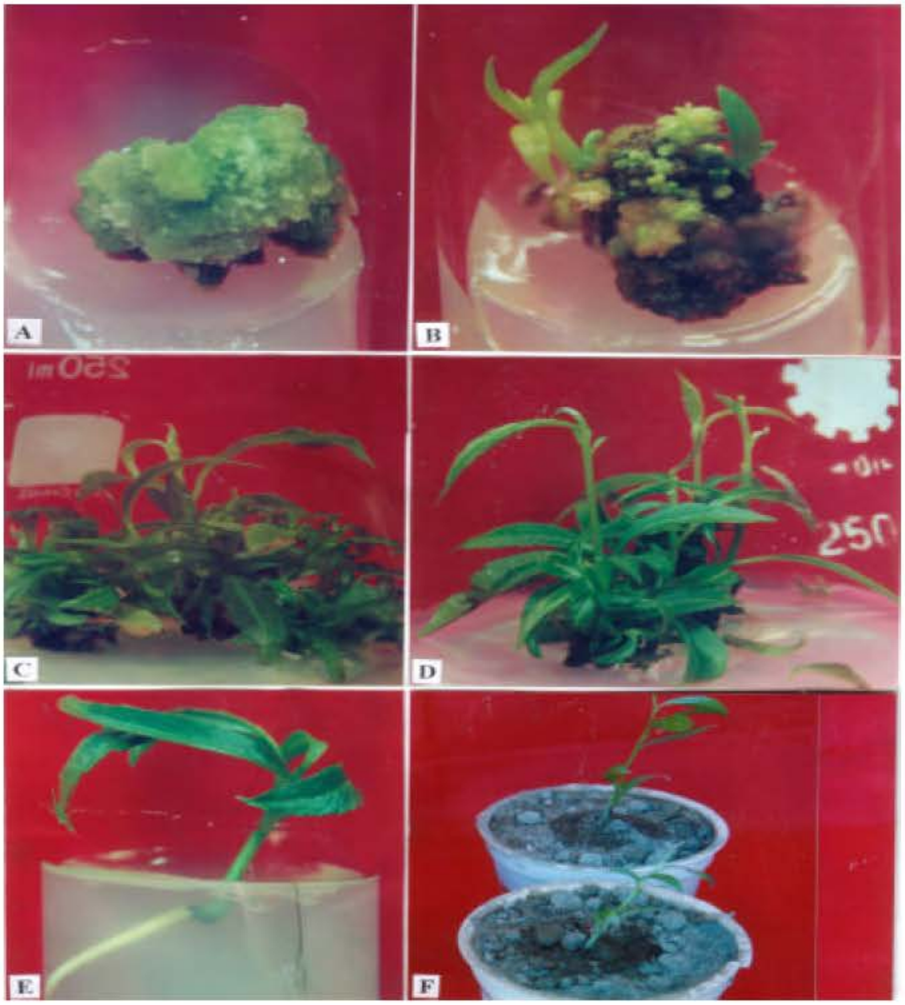

Fig. 1 A-F: Callus induction and organogenesis in *Elaeocarpus robustus*.

A. Induction of callus on cytoledon explants on ½ MS supplemented with 1.0 mg l^{-1} BA+0.5 mg l^{-1} 2,4-D after six weeks in culture
B. Shoot bud formation from callus on ½ MS containing 1.0 mg l^{-1} BA+0.5 mg l^{-1} Kn+0.1 mg l^{-1} NAA after eight weeks in culture
C. Development of shoots from shoot bud primordia after ten weeks in culture
D. Development of shoots on ½ MS supplemented with 1.0 mg l^{-1} BA+0.5 mg l^{-1} Kn+0.1 mg l^{-1} NAA after twelve weeks in culture
E. Development of roots from the base of excised shoot on ½ MS fortified with 0.2 mg l^{-1} IBA after five weeks in culture
F. Regenerated plantlet on the soil after four weeks of transfer under *in vitro* condition

were gelled with 0.7% agar (BDH) and supplemented with 30 mg l^{-1} sucrose. The pH of the medium was adjusted to 5.7±0.1 prior to autoclaving at 121°C under 1.1 kg cm^{-2} pressure for 20 min. The cultures were maintained in a growth room under 16 h photoperiod at 26±1°C.

RESULTS AND DISCUSSION

Callus induction: In order to obtain callus cotyledon explants were cultured in ½ MS medium supplemented with a cytokinin (1.0 mg l^{-1} BA) and an auxin (0.5 mg l^{-1}

Table 1: Effects of different concentrations of 2,4-D in combinations with BA/Kn on callus induction from cotyledon of *Elaeocarpus robustus*. Data ($\bar{x}$±S.E) were recorded after 6 weeks of culture

Growth regulators (mg l^{-1})	No. of cultures callusing (%)	Intensity of callus growth
2,4-D		
0.1	25	+
0.2	35	++
0.5	40	++
1.0	50	+++
2.0	35	++
BA+2,4-D		
0.5+0.1	40	+
0.5+0.2	60	++
0.5+0.5	65	++
1.0+0.1	30	++
1.0+0.2	65	++
1.0+0.5	87	+++
2.0+0.1	20	+
2.0+0.2	40	+
2.0+0.5	45	++
Kn+2,4-D		
0.5+0.1	20	+
0.5+0.2	55	++
0.5+0.5	65	++
1.0+0.1	25	++
1.0+0.2	55	++
1.0+0.5	70	+++
2.0+0.1	20	+
2.0+0.2	35	++
2.0+0.5	40	++

Callus growth rating value = (+) poor, (++) moderate and (+++) profuse callus formation

2,4-D) combination (Table 1). Within six weeks of culture callus formation was observed from the cut margins of the explants (Fig. 1A). Of the two types of explants, maximum of 87% explants responded when cotyledons with attached petiolar ends were used as explants. Compared to this figure, only 50% explants responded when cotyledons without petiolar end were used as explants (data not shown in the table). However, the morphogenic callus was compact and light green in colour, which showed the formation of a number of adventitious shoot buds within seven-eight weeks of subculture.

Shoot development: The callus developed into shoot buds (Fig. 1B) when they were subcultured in the medium supplemented with either BA and Kn alone or in combination with NAA and IBA. However, the presence of an auxin in the shoot development medium accelerated the regeneration process and of the two auxins, NAA was found to be more effective (Fig. 1C-D). The highest number of shoots per explant (7.45±0.18) was achieved in the medium with 1.0 mg l^{-1} BA+0.5 mg l^{-1} Kn+0.1 mg l^{-1} NAA (Table 2) using cotyledon explant with proximal end. Compared to this, the number of shoots per culture was only 4.34±0.13 in the same media when cotyledon explant with distal end was used (data not shown in the table).

Table 2: Effects of the cytokinins (BA, Kn) and the auxins (NAA, IBA) at different concentrations and combinations in ½ MS medium on callus formation and shoot regeneration from the callus tissue of *Elaeocarpus robustus*. Data were taken after 9 weeks of culture

Growth regulators (mg l^{-1})	% of shoot formation	Intensity of callus growth	No. of total shoots per culture	Average length of shoots per culture
BA				
0.5	45	+	3.25±0.26	2.41±0.16
1.0	55	+	3.56±0.15	2.53±0.08
2.0	40	+	3.10±0.18	2.35±0.22
Kn				
0.5	40	+	2.85±0.25	2.22±0.14
1.0	45	+	3.28±0.16	2.35±0.18
2.0	30	+	2.40±0.20	2.00±0.21
BA+NAA				
1.0+0.1	50	+	4.55±0.32	3.21±0.26
1.0+0.2	65	++	4.95±0.18	3.26±0.18
1.0+0.5	70	+++	5.20±0.26	3.54±0.24
2.0+0.1	40	+	4.85±0.24	3.15±0.20
2.0+0.2	45	++	4.20±0.16	2.65±0.18
2.0+0.5	55	+++	4.10±0.12	2.45±0.26
BA+IBA				
1.0+0.1	35	+	3.90±0.31	2.85±0.19
1.0+0.2	45	++	4.10±0.25	3.15±0.16
1.0+0.5	60	++	4.68±0.23	3.25±0.12
2.0+0.1	30	++	4.00±0.21	3.10±0.15
2.0+0.2	35	++	3.75±0.10	2.65±0.23
2.0+0.5	45	++	3.28±0.24	2.40±0.25
Kn+NAA				
1.0+0.1	40	+	3.55±0.43	2.80±0.45
1.0+0.2	50	++	3.84±0.25	2.90±0.32
1.0+0.5	65	+++	4.26±0.20	3.00±0.16
2.0+0.1	25	++	3.68±0.21	2.85±0.25
2.0+0.2	35	++	3.12±0.17	2.60±0.31
2.0+0.5	45	++	2.80±0.23	2.35±0.21
Kn+IBA				
1.0+0.1	25	+	3.15±0.24	2.60±0.26
1.0+0.2	40	+	3.54±0.13	2.80±0.31
1.0+0.5	50	++	3.85±0.16	2.86±0.26
2.0+0.1	20	++	3.50±0.22	2.72±0.22
2.0+0.2	30	++	2.92±0.25	2.58±0.25
2.0+0.5	40	++	2.56±0.13	2.45±0.12
BA+Kn+NAA				
0.5+0.5+0.1	60	+	5.35±0.26	3.10±0.35
0.5+0.5+0.2	50	++	4.85±0.24	2.80±0.26
1.0+0.5+0.1	85	++	7.45±0.18	3.55±0.23
1.0+0.5+0.2	70	++	6.00±0.22	3.25±0.13
2.0+0.5+0.1	40	+	3.84±0.31	2.65±0.26
2.0+0.5+0.2	35	+	3.24±0.16	2.25±0.10
BA+Kn+IBA				
0.5+0.5+0.1	50	+	4.16±0.17	2.60±0.36
0.5+0.5+0.2	40	++	4.00±0.21	2.55±0.16
1.0+0.5+0.1	75	++	5.25±0.25	3.20±0.19
1.0+0.5+0.2	60	++	4.50±0.20	2.85±0.20
2.0+0.5+0.1	35	+	3.64±0.15	2.50±0.30
2.0+0.5+0.2	25	+	3.10±0.23	2.10±0.16

Callus growth rating value = (+) poor, (++) moderate and (+++) profuse callus formation

BA and Kn alone at 1.0 mg l^{-1} induced shoot development in more than 55 and 45% cultures, respectively. In higher concentrations (2.0 mg l^{-1}) of BA or Kn in combination of higher levels (0.5 mg l^{-1}) of NAA or IBA, only few shoots proliferated that inhibiting the elongation of other shoots. Shoot production and plantlet formation decreased further when cytokinin and auxin

Table 3: Effects of different concentrations of auxins on adventitious root formation from the *in vitro* grown microcuttings cultured on ½ MS medium. There were 12-15 micro-cuttings in each treatment. Data (x̄± S.E) were recorded after 6 weeks of culture

Types of auxin	Different concentration of auxin (mg l^{-1})	% of micro-cutting rooted	Number of root per micro-cutting	Average length of the root (cm)
IBA	0.1	35	1.30±0.16	2.42±0.18
	0.2	60	1.85±0.20	3.25±0.26
	0.5	45	1.55±0.18	2.65±0.15
	1.0	-	-	-
NAA	0.1	35	1.30±0.22	2.45±0.21
	0.2	50	1.60±0.25	3.00±0.15
	0.5	45	1.45±0.16	2.58±0.24
	1.0	-	-	-
IAA	0.1	25	1.15±0.22	2.00±0.15
	0.2	40	1.50±0.20	2.80±0.18
	0.5	30	1.35±0.18	2.45±0.13
	1.0	-	-	-

(-) indicates no response

Table 4: Effects of dark treatment and temperature during initial one week of rooting of micro-cuttings on media contains 0.2 mg l^{-1} of IBA. Each treatment consisted of 15-20 microcuttings and data (x̄±SE) were recorded after 5 weeks of total culture

Culture condition	% of micro -cutting rooted	No. of root per micro-cutting	Average length of the root (cm)	Callus formation at the cutting base
Temperature 30°C plus dark treatment 1 week	80	2.25±0.18	4.55±0.25	+
Temperature 25°C plus dark treatment 1 week	60	1.80±0.14	4.00±0.20	+
Temperature 25°C plus culture under 16 h photoperiod for 6 weeks	50	1.52±0.12	3.25±0.21	++

(+) poor, (++) considerable callus formation

levels were increased in the proliferation medium in later subcultures.

Root formation: The prepared microcuttings were induced to root formation *in vitro* by culturing them on half strength of MS medium with 0.1-1.0 mg l^{-1} either of IBA, NAA or IAA. Among the three types of auxin, IBA was found to be most effective at different concentrations tested for producing roots at the cut margins of the microcuttings. Among different auxin concentrations, 0.2 mg l^{-1} IBA was proved to be the best concentrations of auxins for proper rooting; in which 60% microcuttings rooted within six weeks of culture (Table 3). Rooting frequency should be increased to eighty percent when the microcuttings were cultured on ½ MS medium containing 0.2 mg l^{-1} IBA and incubated under 30°C in dark for initial one week (Fig. 1E). In this treatment, the highest number of roots per microcutting was 2.25±0.18 and their maximum length was 4.55±0.25 cm (Table 4).

The *in vitro* regenerated plantlets acclimated better under *ex vitro* condition when they were transferred on specially made plastic trays containing coco-peat as potting mix and taking special care not to damage the roots. In the first week of transplantation, plantlets were moistened uniformly at periodic intervals and kept covered in polythene tent for providing the condition of high humidity and sufficient light. The polythene cover was removed periodically and progressively whenever leaves appeared water soaked. Subsequently the regenerated plantlets were transferred to larger earthen pots filled with organic manured soil when new growth started on coco-peat after two-three weeks of transfer (Fig. 1F). Through this process of acclimatization, regenerated plantlets were established under *ex vitro* condition with a survival percentage 55 of transplants.

Regeneration of complete plantlets *in vitro* from the native-olive explants was found to be difficult. Nevertheless, results of the present investigation are in agreement with those of many other studies. Adventitious shoot regeneration was also observed on cotyledon segments cultured of *Theobroma cacao*[13] and in leaf-derived callus cultures of *Aegle marmelos*[14]. Of the two types of explant used, shoot proliferation from cotyledon explant with attached proximal end was greater than that of cotyledons without petiolar half, indicating a polarity in the regeneration potential of cotyledon explants. Similar observations were found in apple[15] and in *Echeveria elegans*[16]. Shoot regeneration from the cotyledon explant has been achieved in a wide range of plant species using cytokinin with auxin in combination[15,17].

Among the three types of auxin used, IBA was found to be the best for root induction. The findings are in agreement with those observed in other plant species such as pomegranate[18] *Ficus religiosa*[19], carambola[20]. Complete darkness for initial one week and comparatively higher temperature (30°C) during root initiation phase was found to promote rooting frequency in *E. robustus* microcuttings. The findings are in agreement with those observed in jackfruit[21] and carambola[20]. Apple microcutting also showed increased rooting in response to dark treatment[22]. Percentage of cuttings rooted and number of roots per cuttings were improved by raising the ambient temperatures of the culture from 25 to 30°C similar increased rooting frequency at 30°C than at 25°C has also been observed in guava[23] and apple[24,25].

About 55% of the regenerated plantlets could tolerate and survive under *ex vitro* environment or field conditions. A number of plantlets was lost due to damping off and necrosis during acclimatization in *ex vitro* condition. Loss of regenerates due to such symptoms was also observed in *Eucalyptus tereticornis* [26]. Due to the delicate nature of *in vitro* regenerated plantlets, special arrangement such as controlled green house conditions, use of soil free potting mix like perlite, vermiculite, peat plugs and application of fungicides are needed for easy and successful acclimatization of plantlets[27].

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