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In vitro Plantlet Regeneration from Internode Explant of Native-olive (*Elaeocarpus robustus* roxb.)

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Abstract: Regeneration of multiple shoots via callus induction and organogenesis were achieved in native-olive (*Elaeocarpus robustus*). Callus induction and shoot buds regeneration were obtained from internode explants of *Elaeocarpus robustus* on their sufficient medium. The best organic callus was found on modified MS (MMS_1) medium supplemented with 0.5 mg L^{-1} BA+0.5 mg L^{-1} 2,4-D. Development of adventitious shoots occurred when the calli were subcultured on MMS_1 medium supplemented with BA and NAA. Maximum frequency (80%) of calli induced adventitious shoots with highest number of 14.05±1.56 shoots per callus were obtained when the medium was fortified with 1.0 mg L^{-1} BA+0.1 mg L^{-1} NAA. Plantlets developed roots when *in vitro* developed microcuttings were implanted on modified MS (MMS_2) medium with 0.2 mg L^{-1} of IBA. Within six weeks of transfer, 65% rooting was achieved on this medium. Rooted shoots (plantlets) were gradually acclimatized and successfully established under natural condition with about 50% survival rate.

Key words: *Elaeocarpus robustus*, internode explants, plantlet regeneration

INTRODUCTION

Adventitious shoots regeneration from callus is necessary for application of gene transfer technology and for screening plants for somaclonal variation. The latter purpose would be of particular interest in situations in which conventional breeding programmes have relatively little impact upon commercial production. *Elaeocarpus robustus* is an important woody minor fruit plants of Bangladesh. Although there is no well-known variety of this fruit, but it is cultivated in limited scale in Bangladesh. The native-olive tree is also said to be indigenous to the Chittagong and Sylhet forest of Bangladesh[1]. It is believed to have originated in Australia. A medium sized to big tree planted for its edible fruits and timber in many areas of the country[2].

Fruits of this plant are prescribed in diarrhoea and dysentery. Soup of the fruit are given for stimulating secretion from the test buds. It is rich in vitamin C. The green fruits are eaten fresh and also used in making soup, chutney, jelly and jam. Ethanolic extract of leaves are diuretic and cardiovascular stimulant and are also used in rheumatism. The native-olive tree is generally grown from seeds. It may also be propagated by vegetative means through stem cutting and layering. Clonal propagation of selected trees by air layering, approach grafting or budding onto seedlings rootstalks is possible, but the number of plants produced by this conventional method is relatively low[3,4]. The application of tissue culture methods for improvement and large-scale propagation of fruit trees have been well demonstrated[5,6]. This plants have received very little attention for its multiplication through *in vitro* culture. Regeneration of plantlets from shoot tip and nodal explants of mature native-olive trees has also been reported[7,8]. The earlier report described *in vitro* plantlets regeneration through axillary shoot proliferation. The present study describes organogenesis and plant regeneration from internode-derived callus in native-olive.

MATERIALS AND METHODS

Internodes of *Elaeocarpus robustus* were collected from field grown mature plants and washed thoroughly under running tap water, then treated with 1% Savlon for about 10 min. This followed by successive three washing with distilled water to make the material free from Savlon. Surface sterilization was carried out with 0.1% $HgCl_2$ for seven minutes followed by gentle shaking. The material was then washed with sterilized distilled water for five minutes giving four-five changes and then internode segments were divided into pieces as explants of size 1-1.5 cm. Each internode explant was then placed on MMS_1 medium (half strength of major salts and full

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strength of minor salts) supplemented with various concentrations of 2,4-D alone and combinations with BA/Kn for induction of callus.

Callus from these primary cultures was transferred to MMS_1 medium containing different concentrations and combinations of BA/Kn and NAA for shoot buds initiation. The calli were subcultured at monthly intervals on fresh medium of the same combination. Regenerated adventitious shoots were placed on MMS_2 medium (half strength of both major and minor salts) supplemented with various concentrations of auxins (NAA, IBA or IAA) singly for rooting.

The 1x6 inch culture tubes were used with one explant in each tube. For solidifying the medium, 8 g L^{-1} agar was used. The pH was adjusted to 5.7±0.1 prior to autoclaving and sterilized by autoclaving for 20 min at 1.1 kg cm^{-2} pressures at 121°C temperature. The cultures were maintained at 25±2°C under the cool white fluorescent lights for 16 h photoperiod with a photon flux density of about 70 μ mol m^{-2} s^{-1}.

RESULTS AND DISCUSSION

Callus initiation was observed from cut end of the explants within four weeks of incubation. By the sixth weeks, almost every explant turned into a mass of callus when internode segments were cultured on MMS_1 medium with various levels of 2,4-D (0.1-1.0 mg L^{-1}) alone and combination with BA/Kn (0.5-2.0 mg L^{-1}) were shown in Table 1. The highest frequency of callus induction (75%) was observed on MMS_1 medium containing 0.5 mg L^{-1} BA+0.5 mg L^{-1} 2,4-D. The texture of the callus was compact and greenish in colour (Fig. 1A). No shoot formation was observed when the calli were maintained in the same media for a longer period.

The calli were transferred on MMS_1 medium containing BA/Kn (0.5-2.0 mg L^{-1}) and NAA (0.1-0.5 mg L^{-1}) for shoot bud differentiation. The callus produced bud primordial and these buds developed into normal shoots within four weeks of subculture (Fig. 1B). Highest percentage (80) of callus produced shoots and maximum number 14.05±1.56 of shoots per culture were obtained in medium containing 1.0 mg L^{-1} BA with 0.1 mg L^{-1} NAA (Fig. 1C and D) from each culture. The adventitious shoot regeneration to different combinations of BA/Kn with NAA is shown in Table 2.

In this experiment, it was found that 2,4-D along or in combination with BA or Kn could induce callus but for better proliferation auxin (NAA) and cytokinin (BA, Kn) were required. Rao and Chopra[9], Pandey and Gonopathy[10] also used 2,4-D and along with BA for

Table 1: Effects of different concentrations of 2,4-D and combinations with BA/Kn on callus induction from internode explants of *Elaeocarpus robustus*. Data (x̄±S.E) were recorded after 6 weeks of culture

Growth regulators (mg L^{-1})	Percentage of cultures callusing	Callus colour	Intensity of callus growth
2,4-D			
0.1	30	W	+
0.2	35	Crw	++
0.5	45	Gr	+++
1.0	30	Crw	++
BA+2,4-D			
0.5+0.1	40	Cr	++
0.5+0.2	60	Gr	++
0.5+0.5	75	Gr	+++
1.0+0.1	35	Crw	++
1.0+0.2	55	Cr	++
1.0+0.5	65	Gr	+++
2.0+0.1	40	Cr	++
2.0+0.2	35	Crw	++
2.0+0.5	30	W	++
Kn+2,4-D			
0.5+0.1	30	Crw	+
0.5+0.2	50	Cr	++
0.5+0.5	65	Gr	+++
1.0+0.1	25	W	++
1.0+0.2	40	Crw	++
1.0+0.5	55	Gr	+++
2.0+0.1	35	Cr	+
2.0+0.2	25	Crw	++
2.0+0.5	20	W	+

Callus growth rating value = (+) poor, (++) moderate and (+++) profuse callus formation; Gr= Greenish, Cr=Creamy, Crw= Creamy white and W= White colour

induction of callus with 100% frequency. It was observed that 1.0 mg L^{-1} BA in combination with 0.1 mg L^{-1} NAA was the best medium supplementation for proliferating the adventitious shoots from internode explants. Similar observations were made on internode segments culture of Citrus[11,12] and *Adhatoda vasica*[13]. Comparatively higher levels of the cytokinin (1.0-2.0 mg L^{-1}) with a lower level of the auxin (0.1-0.5 mg L^{-1}) was essential for the differentiation of adventitious shoots. Similar observation were made on seedling leaf segments culture of carambola[14] and stem callus culture of jackfruit[15]. Superior effect of BA-NAA combination on adventitious bud proliferation from leaf explant has also been reported by Islam *et al*.[16] for *Aegle marmelos*. Organogenesis and plants formation in presence of BA with NAA was reported from cotyledon explants[17,18].

Subculture of initially produced internode-derived callus on MMS_1 with 1.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA yielded a higher number of adventitious shoots production and the number of shoots increased gradually from subculture to subculture. At the initial stage of culture four-six shoot buds sprouted from a culture on suitable medium within four weeks and a cluster of ten-twelve shoots was produced after one subculture. This cluster of shoots was divided into pieces and each piece was subcultured individually on the same medium that

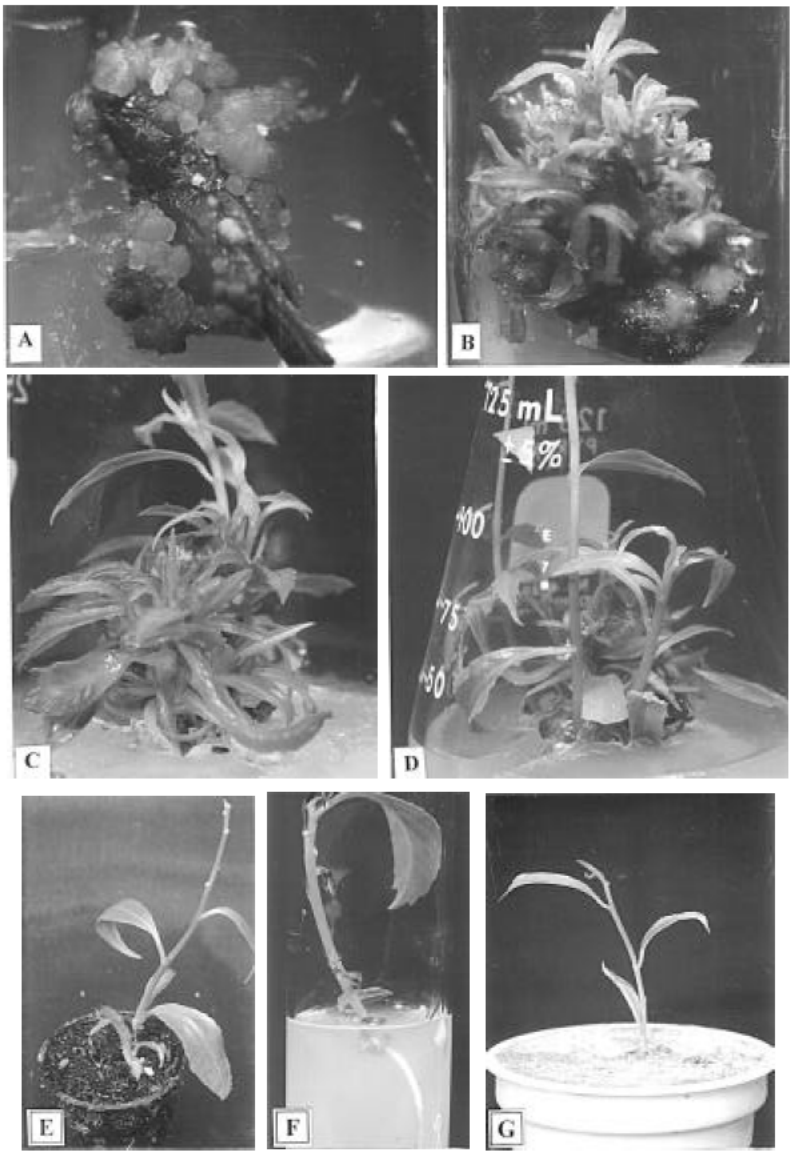

Fig.1: A-G: Callus induction, organogenesis and development of plantlet in *Elaeocarpus robustus*

A. Induction of callus on internode explants on MMS_1 supplemented with 0.5 mg L^{-1} BA+0.5 mg L^{-1} 2,4-D after six weeks in culture.
B. Shoot bud formation from callus on MMS_1 containing 1.0 mg L^{-1} BA+0.1 mg L^{-1} NAA after seven weeks in subculture.
C. Development and elongation of shoot bud primordia after six weeks in subculture.
D. Development and elongation of shoots on MMS_1 supplemented with 1.0 mg L^{-1} BA+0.1 mg L^{-1} NAA after ten weeks in culture.
E. Development of roots from the base of excised shoot on MMS_2 fortified with 0.2 mg L^{-1} IBA after six weeks in culture.
F. Regenerated plantlet on the coco-peat after four weeks of transfer under *ex vitro* condition.
G Acclimatization of plantlets on the soil after eight weeks of transfer under outside condition

Table 2: Effects of different concentrations and combinations of NAA, BA and Kn on organogenesis and plantlet regeneration from internode-derived callus of *Elaeocarpus robustus*. Data (x̄±S.E) were recorded after 8 weeks of culture

NAA	BA	Kn	Morphogenic response	% of cultures showing response	Mean no. of total shoots per culture
0.1	0.5	0.0	Slow callus growth with poor shoot primordia	40	6.62±0.27
0.5	0.5	0.0	Rapid callus growth with poor shoot primordia	25	5.15±0.12
0.1	1.0	0.0	Slow callus growth with considerable shoot primordia	80	14.05±0.56
0.5	1.0	0.0	Slow callus growth with reasonable shoot primordia	60	9.25±0.15
0.1	2.0	0.0	Slow callus growth with reasonable shoot primordia	50	8.00±0.26
0.5	2.0	0.0	Slow callus growth with poor shoot primordia	45	7.86±0.18
0.1	0.0	0.5	Slow callus growth with poor shoot primordia	25	4.45±0.29
0.5	0.0	0.5	Rapid callus growth with poor shoot primordia	20	4.15±0.13
0.1	0.0	1.0	Slow callus growth with considerable shoot primordia	60	8.45±0.24
0.5	0.0	1.0	Slow callus growth with reasonable shoot primordia	50	7.24±0.16
0.1	0.0	2.0	Slow callus growth with reasonable shoot primordia	45	6.00±0.18
0.5	0.0	2.0	Slow callus growth with poor shoot primordia	35	4.86±0.27

Table 3: Effects of different concentrations of auxins on adventitious root formation from the *in vitro* grown microcutting cultured on MMS_2 medium. There were 15-20 microcuttings in each treatment. Data (x̄±S.E.) were recorded after 6 weeks of culture

Different concentration auxin (mg L^{-1})	% of micro-cutting rooted	Number of root per microcutting	Average length of the root (cm)
IBA			
0.1	35	1.35±0.12	2.20±0.21
0.2	65	2.25±0.21	3.00±0.46
0.5	50	1.65±0.15	2.25±0.18
1.0	-	-	-
NAA			
0.1	30	1.25±0.21	1.80±0.24
0.2	55	1.85±0.23	2.45±0.14
0.5	45	1.65±0.18	1.90±0.23
1.0	-	-	-
IAA			
0.1	20	1.00±0.23	1.55±0.18
0.2	40	1.45±0.26	2.30±0.16
0.5	30	1.25±0.21	1.85±0.13
1.0	-	-	-

(-) No response

produced a bunch of 20-30 shoots totally within 4 weeks of further subculture. Results of this study indicate that large scale propagation of native-olive is feasible by tissue culture methods and 100s of plants can be regenerated from 1 cm internode explant within forth subculture. However, after forth subculture the shoot multiplication rate of the subcultured material was tended to be constant. Amin and Jaiswal[19], Norton and Norton[20] have also reported variation in multiplication rate and shoot number with gradual increase of subculture period.

Microcuttings (1-1.5 cm) prepared from *in vitro* proliferated shoots were cultured on MMS_2 medium with 0.1-1.0 mg L^{-1} of either IBA, NAA or IAA for adventitious rooting in 1x6 inch tubes. The effects of these three auxins on percentage of root formation, number of roots per shoot and length of the longest root were recorded after six weeks of culture. The rooting response to different auxins is shown in Table 3. Percentage of root induction and number of roots per shoots were markedly influenced by the concentration and nature of the auxin. Among the three types of auxins used, IBA was found to be the best for root induction. 65% shoots produced roots when they were cultured in the MMS_2 medium with 0.2 mg L^{-1} IBA (Fig. 1E). Root formation on media containing IAA was not as good as that in IBA and NAA. Maximum number of 55 and 40% microcuttings produced roots on medium supplemented with 0.2 mg L^{-1} NAA and 0.2 mg L^{-1} IAA. The highest number of roots per shoot (2.25±0.21) with their maximum length (3.00±0.46 cm) was found on MMS_2 medium containing 0.2 mg L^{-1} IBA.

Higher concentration (1.0 mg L^{-1}) of IBA, NAA or IAA could not form any root but produced callus at the cut bases of the shoots. Being stable nature IBA has been the preferred auxin for adventitious root initiation in many species[8,21,22]. Hustchinson[23] found IBA as superior auxin to IAA or NAA for *in vitro* rooting of apple shoots, while Amin and Jaiswal[19] observed its superiority for rooting of guava and jackfruit micro-cuttings. After sufficient development of roots, the established regenerated plants were transferred into the soil. The propagation of *in vitro* plantlets were not transferred readily to an open soil environment. Before transplantation, the regenerated plantlets were out from *in vitro* condition washed thoroughly and then planted into pots containing coco-peat (Fig. 1F). Humidity was maintained by covering the pot with polythene bags for five-seven days. Within 20-25 days the plantlets were established on garden soil (Fig. 1G). All the transplanted plants were morphologically similar to mother plant. Through the present investigation, techniques for *in vitro* establishment of culture, callus induction, regeneration and multiplication of shoots and rooting of the shoots have been standardized. Information from different steps of the present study would be used for initiating further experiment on tissue culture of *Elaeocarpus robustus*.

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