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In vitro Plant Regeneration from Cotyledon-derived Callus of Three Varieties Pummelo (*Citrus grandis* [L.] Osb.)

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Abstract: Cotyledon explants from ***in vitro*** grown seedlings were cultured on half-strength of MS medium with different growth regulators for ***in vitro*** indirect regeneration of shoots. Optimum callus formation, when cotyledon explants were cultured on MS containing 1.0 mg l^{-1} BAP with 5.0 mg l^{-1} NAA. After that the calli were used for shoot regeneration by transferring them half-strength MS medium supplemented with only cytokinin. Maximum percentage of shoot regeneration was obtained on half strength MS medium in the presence of 1.0 mg l^{-1} BAP from callus in three varieties [Var.-1 (pink colour), Var.-2 (white colour) and Var.-3 (red colour)] of pummelo. For rooting, shoot cuttings were cultured on half strength MS salts with 0.1-1.0 mg l^{-1} NAA, IBA or IAA. The best and healthy rooting was observed on 0.1 mg l^{-1} NAA. The plantlets of three varieties were successfully established on soil. About 95% of plantlets survived under ***ex vitro*** condition.

Key words: ***In vitro***, pummelo, ***Citrus grandis***, cotyledon, regeneration

Introduction

In Bangladesh pummelo (***Citrus grandis*** [L.] Osb.) is one of the commonly grown fruit trees and it is the largest producer among the ***Citrus*** crops grown in the country. Chaturevedy ***et al.*** (1974a, b); first attempt to propagated pummelo tree by using tissue culture techniques and they observed somatic embryogenesis and some biochemical aspects of shoot organogenesis from callus. Song ***et al.*** (1991) were described embryogenic callus induction of ***Citrus grandis*** was cultured on Murashige and Tucker medium with various concentrations and combinations of growth regulators. Latter, ***in vitro*** protocols for regenerating complete plants using axillary and adventitious shoot proliferation from seedling explants of pummelo (Amin and Akhtar, 1993; Baruah ***et al.***, 1995; Paudyal and Haq, 2000). More recently, Begum ***et al.*** (2001) were also obtained ***in vitro*** clonal propagation from field grown mature plants of pummelo.

Micropropagation and tissue culture of sweet orange was achieved by Duran ***et al.*** (1992). Porntip (1993) was described in tissue culture of lime. ***In vitro*** micropropagation of seedless 'Cohen' citrange was described by Grosser ***et al.*** (1994). Bowman (1995) has reported micropropagation of citrus rootstocks. Cotyledon tissue culture and shoot buds on roots in ***Citrus*** were studied by Teo ***et al.*** (1988). Subsequently, Carimi ***et al.*** (1999) have also reported somatic embryogenesis and plant regeneration from pistil thin cell layers of ***Citrus***. The present research investigation with an objective to develop the best possible protocol for ***in vitro*** indirect regenerated shoots of cotyledon segments from three varieties of pummelo and establishment of microplants under field condition.

Materials and Methods

Healthy seeds were collected from pummelo fruits of the field grown plants. Then the seeds were sterilized with 20% solution of a commercial bleach "Clorox" (5% available chlorine in Sodium hypochlorite) for 15 minutes and then washed three - four times with autoclaved distilled water inside the laminar air-flow chamber. The sterilized seeds were inoculated on MMS_1 (half strength of major salt but full strength of minor salt) medium for ***in vitro*** germination. The cotyledon explants (1-1.5 cm) were collected from these ***in vitro*** germinated seedlings and inoculated on half-strength MS medium supplemented with different concentrations of BAP (0.5-2.0 mg l^{-1}) in combinations of NAA (0.1-1.0 mg l^{-1}). For adventitious root induction half -strength of MS medium was assayed with 0.1-1.0 mg l^{-1} NAA, IBA or IAA. The pH of the medium was adjusted to 5.7±0.1 and solidified by (6-7 gm l^{-1}) agar and dispensed into glass tubes. The medium was autoclaved at 121 E C and at 1.1 kg / cm^2 pressure for 20 minutes. After inoculation all the cultures were grown under a photoperiod of 16 hrs light from white flourescent tubes and at a temperature of 26±1 EC. Data on number of shoots per culture and mean shoot length were recorded after 10 weeks of culture. Besides, data on days required to rooting, percentage of cutting rooted, number of root per cutting and average length of root were recorded. The rooted plantlets were then transplanted into 10 cm diameter pots containing a mixture of garden soil, sand and compost (2:1:2).

Results and Discussion

Establishment of callus cultures

In the present study, the cultured explants of cotyledon segments responded to callus formation and subsequently to shoot regeneration under the influence of growth regulators. The cotyledon explants from three varieties of pummelo were collected from 5-6 weeks old aseptically grown seedlings and cultured on MS medium supplemented with different concentrations and combinations of cytokinin and auxin. Among different concentrations and combinations of the growth regulator in MS medium 1.0 mg l^{-1} BAP + 5.0 mg l^{-1} NAA was found to be best for callusing (Fig. $1A_1$, A_2 and A_3). Besides this combination, other combinations also showed slight callusing as indicated in Table 1. For callusing, the cotyledon explants were cultured on MS medium supplemented with low concentration of cytokinin and high concentration of auxin. Among various combinations of BAP and NAA the cultured explants showed best callus on the medium containing 1.0 mg l^{-1} BAP with 5.0 mg l^{-1} NAA. This combination showed maximum frequency of 86.6% explants for Var.-1, 73.3% explants for Var.-2 and 53.3% explants for Var.-3 for callus production. After establishing of the callus, the next step was to induce shoot formation.

Induction of indirect shoot regeneration

The callus were used for shoot regeneration by transferring them half-strength MS medium supplemented with only cytokinin (BAP and Kn). The maximum number of shoot per culture was found to be 6.5±0.61 for Var.-1, 5.5±0.21 for Var.-2 and 5.1±0.14 for Var.-3, while the maximum number of usable shoots per culture was observed to be 5.5±0.01 for Var.-1, 4.2±0.16 for Var.-2

Table 1: Effects of different concentrations and combinations of cytokinin with auxin for induction of callus from the cotyledon explant of three varieties pummelo. There were 15 explants each treatment and data ($\bar{x}$±S.E) were collected after 10 weeks

Growth regulators	% of explants showing callus formation			Intensity of callus growth		
(mg l^{-1})	Var.-1	Var.-2	Var.-3	Var.-1	Var.-2	Var.-3
BAP+NAA						
0.5+4.0	53.3	20.0	13.3	+	+	+
+5.0	60.0	26.6	20.0	+++	+++	+++
+6.0	40.0	6.6	6.6	++	++	++
1.0+4.0	66.6	66.6	33.3	++	++	++
+5.0	86.6	73.3	53.3	+++	+++	+++
+6.0	33.3	26.6	20.0	++	++	++
2.0+4.0	46.6	33.3	33.3	+	+	+
+5.0	53.3	46.6	40.0	++	++	++
+6.0	33.3	26.6	20.0	-	-	-
BAP+IBA						
0.5+4.0	46.6	13.3	13.3	+	+	+
+5.0	53.3	20.0	20.0	++	++	++
+6.0	33.3	26.6	20.0	-	-	-
1.0+4.0	53.3	40.0	33.3	+	+	+
+5.0	66.6	53.3	40.0	++	++	++
+6.0	53.3	26.6	20.0	+	+	+
2.0+4.0	33.3	26.6	33.3	+	+	+
+5.0	40.0	33.3	40.0	++	++	++
+6.0	33.3	20.0	20.0	-	-	-
BAP+IAA						
0.5+4.0	40.0	13.3	13.3	+	+	+
+5.0	40.0	20.0	13.3	+	+	+
+6.0	20.0	26.6	20.0	-	-	-
1.0+4.0	40.0	33.0	26.6	+	+	+
+5.0	53.3	40.0	33.3	+	+	+
+6.0	20.0	13.3	13.3	+	+	+
2.0+4.0	13.0	13.3	13.3	+	+	+
+5.0	20.0	13.3	13.3	+	+	+
+6.0	6.6	6.6	6.6	-	-	-

(-) indicate no response; (+) slight callusing; (++) considerable callusing; (+++) intensive callusing

and 3.8±0.08 for Var.-3, average length of shoot was found to be 3.1±0.16 cm for Var.-1, 2.8±0.11 cm for Var.-2 and 2.5±0.08 cm for Var.-3 at half-strength MS containing 1.0 mg l^{-1} BAP (Fig. 1 B_1, B_2 and B_3). On the other hand, it was also observed that when the callus was transferred to

Table 2: Effects of different concentrations of cytokinin on regeneration of adventitious shoot from cotyledon derived callus of three varieties pummelo

Growth regulators (mg l^{-1})	No. of total shoots/culture	No. of usable shoots/culture	Average length of shoots (cm)
Var.-1			
BAP			
0.5	2.5±0.05	2.0±0.02	2.3±0.24
1.0	6.5±0.61	5.5±0.01	3.8±0.16
2.0	3.8±0.24	2.5±041	2.1±0.61
Kn			
0.5	2.0±0.32	1.6±0.03	1.5±0.65
1.0	4.5±0.12	3.1±0.21	2.8±0.67
2.0	2.5±0.15	2.1±0.14	2.3±0.12
Var.-2			
BAP			
0.5	2.1±0.21	1.6±0.21	2.3±0.21
1.0	5.3±0.21	4.2±0.16	3.5±0.11
2.0	3.5±0.11	2.1±0.15	2.0± 0.46
Kn			
0.5	1.5±0.38	1.2±0.21	1.1±0.35
1.0	4.0±0.41	2.8±0.11	2.5±0.14
2.0	2.6±0.15	1.8±0.22	2.1±0.28
Var.-3			
BAP			
0.5	2.0±0.05	1.5±0.11	2.0±0.35
1.0	4.1±0.14	3.2±0.08	2.5±0.08
2.0	3.1±0.17	2.0±0.22	1.8±0.20
Kn			
0.5	1.5±0.01	1.1±0.44	1.0±0.27
1.0	3.5±0.13	2.6±0.01	2.1±0.02
2.0	2.1±0.34	1.5±0.42	2.0±0.05

half-strength MS medium supplemented with the same concentrations of Kn, the shoot proliferation rate was lower considerably. It was to be noted that regeneration of adventitious shoots from the calli of Var.-1, Var.-2 and Var.-3 was remarkably influence by type and concentration of the cytokinin used. The cytokinin BAP at most of the concentrations was more effective in proliferating adventitious shoot, whilst Kn was considerably less effective (Table 2). Similar observations were made on epicotyl segment of *C. **depressa*** and *C. **jambhiri*** by (Rahman ***et al.***, 1987).

Table 3: Effects of different concentrations of auxin in MMS_1 medium for root induction of ***in vitro*** regenerated shoots of three varieties pummelo. There were 15 micro-cuttings were cultured of each treatment and data ($\bar{x}$±S.E) were collected after 8 weeks of culture

Types of auxin	Conc. of auxins	% of micro-cutting rooted	No. of root/ microcutting	Average length of roots (cm)	Days to emergence of roots
Var.-1					
	0.1	73.3	3.0±0.01	3.0±0.42	25-30
IBA	0.2	53.3	2.0±0.03	2.1±0.31	22-28
	0.5	26.6*	1.0±0.14	2.0±0.16	25-36
	0.1	100.0	5.0±0.06	3.5±0.31	20-25
NAA	0.2	80.0	4.0±0.07	3.1±0.46	24-30
	0.5	53.3	2.0±0.16	2.5±0.06	26-38
	0.1	33.3	2.0±0.11	2.0±0.16	35-48
IAA	0.2	26.6	1.5±0.06	1.5±0.31	28-43
	0.5	20.0*	1.0±0.05	1.2±0.40	25-36
Var.-2					
	0.1	66.6	2.5±0.08	2.8±0.22	22-30
IBA	0.2	40.0	1.5±0.23	1.5±0.11	22-28
	0.5	13.3*	1.0±0.44	1.1±0.06	28-37
	0.1	86.6	4.0±0.26	3.3±0.21	25-28
NAA	0.2	73.3	3.4±0.07	3.1±0.26	28-35
	0.5	46.6	1.8±0.46	2.2±0.16	26-38
	0.1	26.6	1.5±0.21	2.1±0.06	30-48
IAA	0.2	20.0	1.2±0.16	1.8±0.11	25-43
	0.5	13.3*	1.0±0.25	1.3±0.20	35-40
Var.-3					
	0.1	40.0	2.2±0.01	2.5±0.42	15-30
IBA	0.2	33.3	1.4±0.03	1.2±0.31	22-25
	0.5	13.3*	1.1±0.14	1.1±0.16	28-34
	0.1	66.6	3.7±0.01	3.1±0.31	20-25
NAA	0.2	53.3	3.2±0.07	3.0±0.46	23-35
	0.5	33.3	1.5±0.16	2.1±0.06	26-38
	0.1	20.0	1.4±0.11	2.0±0.16	35-47
IAA	0.2	13.3	1.1±0.06	1.5±0.31	28-43
	0.5	6.6*	1.0±0.05	1.2±0.40	32-40

(*) indicates slight callusing from the cutting base of microshoots

In vitro rooting

Although in most of the cases regenerated shoots produced roots simultaneously, it was necessary to culture the regenerated shoots in rooting medium for better rooting and easy

Fig. 1: A_1-D_3 : Regeneration of ***in vitro*** plantlets from cotyledon explants of three varieties pummelo.

A_1, A_2 and A_3: Callus initiation from cotyledon explant on MS + 1.0 mg l^{-1} BAP + 5.0 mg l^{-1} NAA of Var.-1, Var.-2 and Var.-3.

B_1, B_2 and B_3: Development of multiple shoots on half-strength MS medium containing 1.0 mg l^{-1} BAP of Var.-1, Var.-2 and Var.-3.

C_1, C_2 and C_3 : Root formation in regenerated shoots in half-strength MS medium containing 0.1 mg l^{-1} NAA of Var.-1, Var.-2 and Var.-3.

D_1 andD_2: Hardened plantlets established on the soil of pummelo.

transplantation. Different concentrations of NAA, IBA or IAA (0.1-1.0 mg l^{-1}) were used in half-strength of MS medium for root formation (Table 3). Cent percent regenerated shoots initiated roots on half strength MS + 0.1 mg l^{-1} NAA (Fig. 1 C_1, C_2 and C_3). Root induction was observed within 15-18 days of culture. On the other hand, good response was also obtained when regenerated shoot were cultured on half strength MS medium containing 0.1 mg l^{-1} IBA. The findings are in agreement with those observed in ***Citrus jambhiri*** by (Rahman ***et al.***, 1987); ***Punica granatum*** (Khantharaj ***et al.***, 1998); ***Citrus grandis*** (Begum ***et al.***, 2001).

Handling of *in vitro* raised plants

In the present experiment regenerated plants of "pummelo" were taken out from ***in vitro*** condition and planted into plastic pots containing garden soil, sand and compost in a ratio of 2:1:2. Survival frequency of the plantlets under ***ex vitro*** condition on soil was 95% (Fig. 1 D_1 and D_2).

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