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An Improved Protocol for Multiple Shoot Regeneration from Seedlings and Mature Explants of *Citrus macroptera* Mont.

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Abstract

Efforts have been made to establish a protocol for direct multiple shoot regeneration from both *in vitro* grown seedlings and mature plants of *Citrus macroptera*. Both nodal and shoot tip explants taken from *in vitro* grown seedlings were cultured in MS supplemented with different concentrations of BAP and Kn either singly or in combinations. Both these explants are capable to regenerate and produce *in vitro* multiple shoots. Maximum number of shoots were obtained from nodal explants in MS supplemented with 1.0 mg/l BAP. BAP alone was found superior to Kn. On the other hand, only nodal explants from mature plant**s** were used and 1.0 mg/1 BAP was also found best suitable for shoot induction and multiplication. *Ex vitro* rooting in pot soil (mixed with biogas slurry derived from cow-dung) was most successful compared to *in vitro* rooting in half strength of MS supplemented with different concentrations of NAA and IBA.

Introduction

Citrus macroptera (Sat Kara) fruits are well-known in the northern parts of the greater Sylhet district of Bangladesh and Assam in India. This citrus species is semi-wild and its fruits are used by local tribes of Assam for medicinal purpose and in cooking (Ghosh 1990). In Bangladesh, both green and mature fruits (even dried fruits) are used in cooking. The thick peel (pericarp) along with endocarp is used for flavoring curry, *dal* and vegetables etc. It is different from common citrus fruits. It is not consumed fresh like mandarin, Shaddak orange and lemon. The juice of the fruit is not used directly like other citrus fruits. Another major use of this fruit is in pickle preparation and the delicious pickles are now available in any big shop of Bangladesh. This fruit is so popular that it is exported to the U.K., the U.S.A., and the Middle East countries. Bangladesh earns a handsome amount of foreign currency every year.

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Unfortunately the production of this popular fruit is not satisfactory in Bangladesh and maximum fruits come from the neighboring States of India. In Bangladesh, propagation of this plant is usually done by traditional methods such as from seeds, grafting and budding. Because of its long juvenile period, heterozygosity, self-incompatibility and nucellar polyembryony, it is very difficult to grow rapidly from seeds on a large scale. The other methods are not satisfactory because of slow multiplication rate. So, it is required to develop techniques for rapid multiplication. Nowadays tissue culture techniques possess the potential for a very high multiplication rate as observed in the production of multiple shoots from apical parts of some citrus varieties (Baralass and Skene 1982). Sim et al. (1989) developed a micropropagation technique of *Citrus mitis* Blanco, which yielded a higher frequency (66 - 100%) of shoot regenerants from shoot tips and nodal segments. Marin and Duran-vila (1991) developed a tissue culture protocol using explants of *C. senesis.* Otoni and Teixeira (1991) used nodal segments of *Citrus sinensis* L. for their clonal propagation. They observed that shoot proliferation of axillary buds was better influenced on MS containing BAP than a combination of Kn, 2iP and zeatin.

In vitro development or propagation of *Citrus macroptera* has not yet been reported but other species of citrus (as mentioned above) have been used and regenerated *in vitro.* With the help of these findings, attempt has been made to develop protocol for direct regeneration and multiplication from the explants of seedlings and mature plant.

Materials and Methods

Nodal segments and shoot tips were collected from six - eight weeks old *in vitro* grown seedlings of *Citrus macroptera* Mont. var. *annamensis* and cut into 10-12 mm long tips. These segments were cultured on MS supplemented with different concentrations of of BAP and Kn. BAP and Kn alone at 0.5, 0.1 and 1.5 mg/1 and combinations BAP and Kn at 0.5 + 0.5, 1.0 + 0.5 and 0.5 + 1.0 were used for direct shoot regeneration. The regenerated shoots were cultured on the same medium for further multiplication. The data from both shoot induction and multiplication were recorded after four - five weeks interval.

Stem twigs of mature plants were collected from the field and washed thoroughly under running tap water for 30 min to remove dust and surface contaminants. Then they were treated in 70% alcohol for five min and were surface sterilized in 4% Na(OCL) solution with two - three drops of Tween 20 for 15 min. During this process the liquid was continuously stirred on a magnetic stirrer under a vacuum pump to facilitate expulsion of all air bubbles followed by surface sterilization of all exposed surfaces. Finally they were washed four times in sterile distilled water under laminar airflow cabinet. Then the stem was

cut into 10-15 mm segments each containing a single node. The prepared explants were cultured on MS containing BAP and Kn for axillary bud initiation at 0.5, 1.0, 1.5 and 2.0 mg/1. The regenerated shoots were transferred into the same media for further multiplication.

Each regenerated shoot was cultured on a freshly prepared medium containing half strength of MS with NAA and IBA alone at 0.1, 0.5 and 1.0 mg/1 for adventitious root initiation. At the same time, a number of shoots were cultured on MS without growth regulator for further elongation. Thereafter, elongated shoots were directly transferred to sterilized plastic posts filled with sterile soil and cow-dung derived from biogas slurry (1 : 1) for adventitious root formation.

Results and Discussion

The results of shoot regeneration of nodal and shoot tip explants (Fig. 1a and 1b) from *in vitro* grown seedlings of *Citrus macroptera* (Table 1) suggest that both these explants are capable of producing multiple shoots. Proliferation efficiency of nodal explants was higher than that of shoot tip. In case of 1.0 mg/1 BAP on MS the highest shoot bud initiation (93.33%) and number of shoots per explant (4.88) were recorded for nodal explants (Fig. 1c) as against shoot tip (75 % and 2.84).

Similar trend i.e. performance of nodal explants was found superior to shoot tip in other supplemented media singly or in combination. BAP and Kn were tested alone or in combinations and in all cases BAP was found superior to Kn and the combination of BAP and Kn. BAP also stimulated shoot induction in different citrus species such as *C. madarensis* (Grinblat 1972), *C. grandis* (Chaturvedi et al. 1974). *C. sinensis* (Altman and Goren 1974), *C. paradisi* (Raj Bhansali and Arya 1978a) and *C. aurentifolia* (Raj Bhansali and Arya 1978b). Amin and Akther (1993) obtained the best proliferation of shoots from pummelo seedling explants and the optimum concentration range of BAP was 1.0 - 2.0 mg/1. Otoni and Teixeira (1991) obtained 3.5 shoots per explant from *C. sinensis* in 0.75 mg/1 BAP. They also noted higher shoot lengths with BAP more than Kn. In the present experiment, Sat Kara seedling also showed almost similar results and the optimum concentration of BAP was 1.0 - 1.5 mg/1. Kn also showed moderate results in this concentration followed by the combination of 0.5 mg/l BAP and 0.5 mg/l Kn.

Table 1. Effects of different combinations and concentrations of BAP and Kn in MS on direct shoot regeneration from *in vitro* grown seedling explants of *C. macroptera*. Data (Mean ± S.E) were recorded after five weeks of culture.

Growth regulators (mg/1)	% of explants responded	Days to shoot initiation	No. of shoots/ explant	Av. length of microshoots
BAP				
0.5	NS = 46.66	7 - 10	1.60 ± 0.37	13.97 ± 0.68
	ST = 33.33	6 - 70	1.12 ± 0.42	14.20 ± 0.56
1.0	NS = 93.33	6 - 10	4.88 ± 0.34	19.35 ± 1.20
	ST = 75.00	7 - 11	2.84 ± 0.38	22.20 ± 0.86
1.5	NS = 53.33	7 - 12	2.30 ± 046	17.65 ± 1.32
	ST = 41.66	6 - 10	2.00 ± 0.26	18.20 ± 1.21
Kn				
0.5	NS = 46.66	10 - 13	1.24 ± 0.34	9.00 ± 0.88
	ST = 33.33	10 - 12	0.85 ±0.22	10.02 ± 0.53
1.0	NS = 73.33	9 - 11	1.52 ± 0.30	15.25 ± 1.25
	ST = 66.66	7 - 9	0.96 ± 0.46	17.20 ± 098
1.5	NS = 40.00	9 - 13	1.84 ± 036	13.30 ± 0.68
	ST = 46.66	8 - 12	1.52 ± 0.32	15.22 ± 0.86
BAP + Kn				
0.5 + 0.5	NS = 66.66	10 - 12	2.00 ± 0.44	16.20 ± 0.66
	ST = 46.66	9 - 11	1.45 ± 0.28	12.42 ± 0.73
1.0 + 0.5	NS = 53.33	10 - 13	1.85 ± 0.34	15.25 ± 0.55
	ST = 41.66	10 - 12	0.86 ± 0.53	13.20 ± 0.48
0.5 + 1.0	NS = 46.66	10 - 13	1.64 ± 0.36	12.38 ± 0.38
	ST = 41.66	10 - 12	1.12 ± 0.22	11.32 ± 0.26

NS = Nodal segments, ST = Shoot.

One of the important objectives of plant tissue culture is to maintain the existing elite cultivers through *in vitro* culture from vegetative explants of mature plants. Many researchers developed successful protocols from different fruit trees like jackfruit (Amin and Jaisal 1993). However, limited research has been carried out in the regeneration of shoots and establishment of plantlets from some mature citrus species such as *C. mitis* Blanco (Sim et al. 1989) and *C. grandis* (Begum et al. 2001) with inconclusive results. In this investigation only 9 - 12 nodal explants were used in each experiment . Like *in vitro* grown seedling nodal explants of mature plant also showed better results in BAP than Kn supplemented medium (Table 2). Shoot induction (Fig. 1d) required 6-13 days in all cases.

In BAP supplemented medium, the highest percentage of shoot induction was (91.66) recorded in 1.0 mg/1 followed by 1.5 mg/1 (53.33). Similarly in Kn supplemented medium, the highest percentage of shoot induction was recorded (75.00) in 1.0 mg/1 followed by 1.5 mg/1 (50.00). The

highest number of shoots (3.78) was also recorded in 1.0 mg/1 BAP (Fig. 1e) followed by 1.0 mg/1 Kn (2.45).

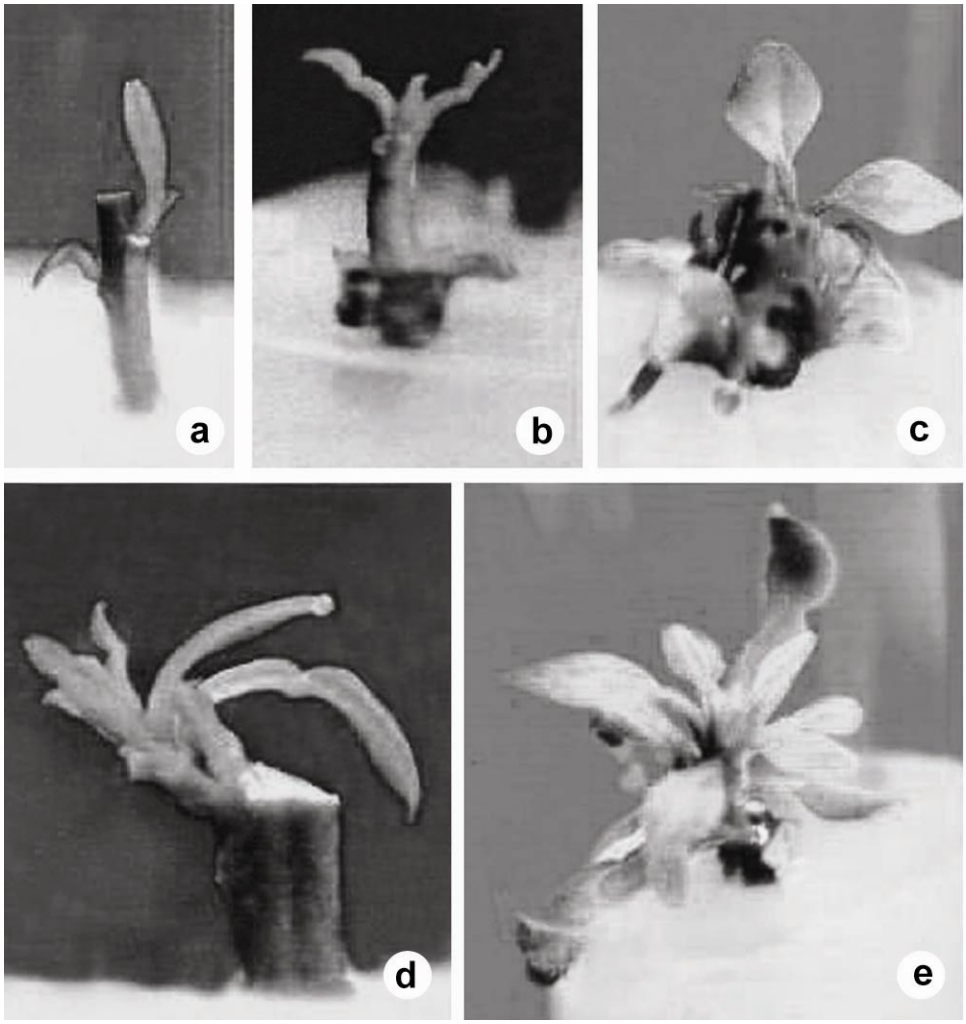

Fig. 1. *Citrus macroptera* Mont: Direct shoot regeneration. (a) Shoot induction from nodal segment of *in vitro* grown seedling after two weeks of culture in MS + 1.0 mg/l BAP. (b) Shoot initiation from shoot tip explant of *in vitro* grown seedling after two weeks of culture in MS + 1.0 mg/l BAP. (c) Multiple shoot development from nodal explant of *in vitro* grown seedling in 1.0 mg/l supplemented MS medium. (d) Shoot induction from nodal segment of mature plant after two of culture in MS + 1.0 mg/l BAP. (e) Multiple shoot developed from nodal explant of mature plant in 1.0 mg/l BAP supplemented MS medium.

It was noted that 1.0 mg/1 was found superior to 1.5 and 0.5 mg/1 in both BAP and Kn supplemented medium. From these results it is clear that BAP was superior to Kn and similarly the concentrations of cytokinin were also important for shoot proliferation. These results are in close conformity with the findings of Begum et al. (2001) reported in *C. grandis.* In case of *C. sinensis,* Otoni and

Teixeira (1991) also noted that BAP is superior to other cytokinins for shoot proliferation from nodal explants.

Experiments were conducted to initiate roots in shoots on half MS with or without hormone. Similarly, 2 - 3 cm long microshoots were directly transferred into plastic pot soil for root induction. Direct rooting in soil was found more effective than *in vitro* rooting.

Table 2. Effects of different concentrations of BAP and Kn supplemented MS on direct shoot regeneration from nodal explants of mature plants of *C. macroptera*. Data (Mean ± S.E.) were recorded after five weeks of culture.

Growth regulators (mg/1)	Days to shoot induction	% of explants responded	No. of shoots/ explant	Av. length of shoot (mm)
BAP				
0.5	6 - 10	33.33	1.28 ± 0.22	14.05 ± 1.69
1.0	6 - 9	91.66	3.78 ± 0.34	20.18 ± 0.72
1.5	6 - 10	53.33	2.20 ± 0.33	15.80 ± 0.74
2.0	6 - 11	44.44	1.45 ± 0.64	0.00
Kn				
0.5	8 - 13	33.33	1.33 ± 0.27	11.86 ± 0.94
1.0	8 - 12	75.00	2.45 ± 0.34	18.65 ± 1.45
1.5	9 - 13	50.00	1.88 ± 0.26	13.50 ± 0.87
2.0	10 - 13	33.33	1.62 ± 0.37	12.48 ± 1.25

In this experiment, poor induction and slow growth of seedlings were obtained in half strength of MS supplemented with NAA. Long roots (10 - 12 mm) were noted after seven weeks of culture in 0.1 mg/1 NAA supplemented medium. Begum et al. (2001) observed that the best root induction occurs in *C. grandis* in 0.1 mg/1 NAA supplemented half MS, whereas, Amin and Akhtar (1993) found best root induction for the same species in 0.4 mg/1 IBA supplemented half MS.

However, in this investigation direct rooting into pot soil was found best. After four weeks a single root developed that was 2.5 - 3.5 cm long (Figs. 2a, b); and after eight weeks two - three adventitious roots (5 - 7 cm) developed with several branching (Figs. 2c, d). So far there is no report on direct rooting of citrus in *in vitro* grown microshoots into soil. The induction of *ex vitro* rooting from cultured shoots may be more economical besides producing better and strong roots.

Since the *in vitro* rooting of the plantlets was very poor they were not suitable for transfer to soil for establishment. However, successful achievement of direct *ex vitro* rooting encouraged transfer of the shoots directly into soil for their establishment. The survival rate of the transferred shoots (Fig. 2e) was also

high (85 - 90%). Better survival rate was observed in case of more aged shoots. Belarmino and Posas (1991) made similar observation in pummelo *in vitro* rooting. They observed that eight-week-old shoots efficiently produced roots in MS containing 0.5 to 1.0 mg/1 IBA compared to four-week-old shoots. The rooted plantlets also showed healthy and steady growth (Fig. 2f).

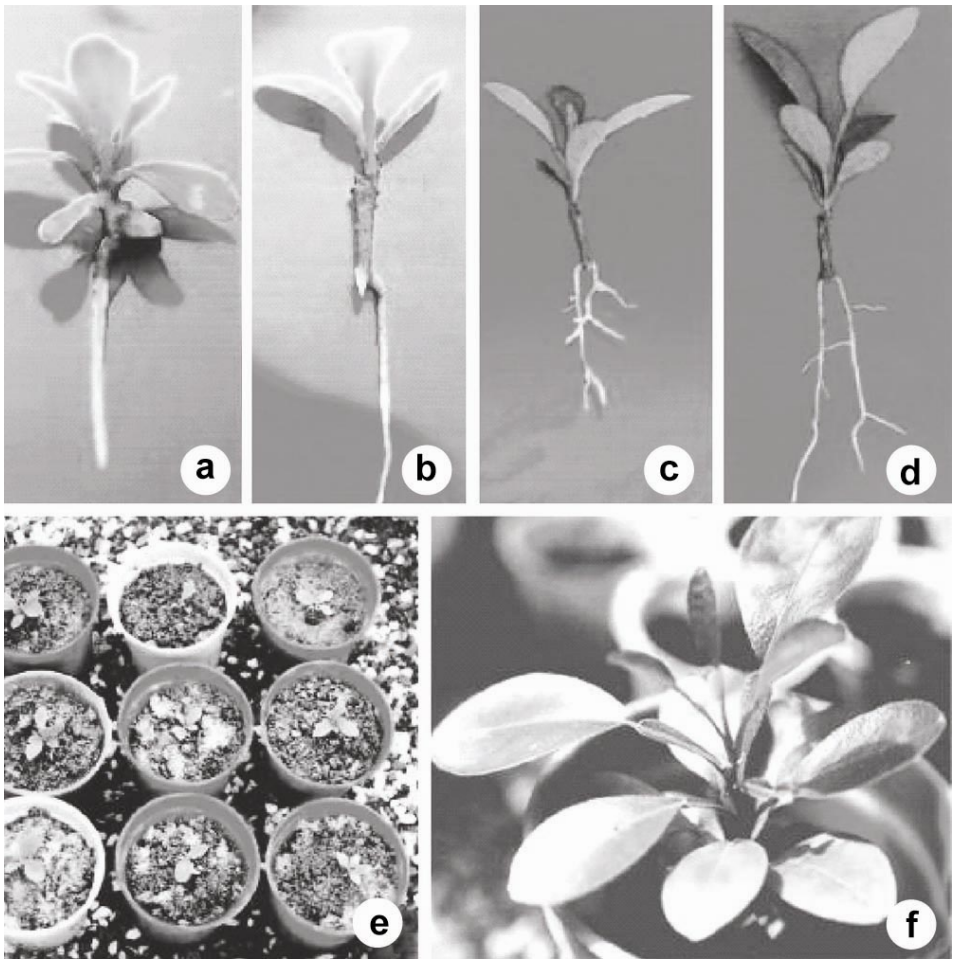

Fig. 2. *Citrus macroptera* Mont. regeneration of roots. (a) *Ex vitro* development of root in shoot directly from nodal explant of seedling after four weeks. (b) *Ex vitro* development of root in shoot directly from nodal explant of mature plant after four weeks. (c) *Ex vitro* development of multiple adventitious root in shoots directly from nodal explant of seedling after eight weeks. (d) *Ex vitro* development of multiple adventitious root in shoots directly from nodal explant of mature plant after eight weeks. (e) Established plantlets in pots. (f) Single established plant in pot soil after three months.

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