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In vitro plantlet formation in Carrizo citrange: A promising citrus rootstock

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ABSTRACT

The shoot tip and nodal segment explants from three-year-old plants of Carrizo citrange were exposed to mercuric chloride (0.1%) for different durations. In case of nodal segments, maximum survival (39.00%) and minimum microbial contamination (60.80%) was observed with mercuric chloride treatment for 10 min., whereas, shoot tips failed to establish. The explants were established on Murashige and Skoog (1962) medium supplemented with different concentrations of BAP, NAA and IBA. Maximum culture establishment (97.20%) was observed highest on MS medium supplemented with BAP (2 µM) + NAA (1 µM). Season of culturing also effected the establishment of shoot cultures as maximum establishment (96.60%) was observed during April-May followed by August-September. The highest shoot proliferation (3.64 per explant) and shoot length (3.71 cm) were also recorded on MS medium supplemented with BAP (2 µM) + NAA (1 µM). The micro-shoots were rooted on half-and full-strength media supplemented with different concentrations of IBA and NAA. Earliest root induction (10.60 days) and maximum rooting (53.89%) were observed on MS medium supplemented with IBA (10 µM) + malt extract (500 mg/l). Maximum number of roots per shoot (1.37) and root length (5.0 cm) were observed on half-strength MS medium containing IBA (2.5 µM) + NAA (2.5 µM). The maximum *ex vitro* plantlet survival (81.4%) was observed in potting mixture consisting of cocopeat + vermiculite + perlite (2:1:1).

Key words: *In vitro* propagation, Carrizo citrange, citrus rootstock.

INTRODUCTION

In India, citrus is the third largest fruit industry after mango and banana, covering approximately 0.91 m ha area with an annual production of 7.9 MT. In Punjab, citrus ranks first with an area of 49, 244 ha and annual production of 10,15,628 MT (Anon, 1). Among citrus fruits, Kinnow ranks first with respect to area (45, 851 ha) and production (9,88,633 metric tonnes). At present, about 61 per cent area of fruit crops in the state is occupied by Kinnow mandarin. In Punjab, citrus is mainly propagated on rough lemon rootstock. It is a vigorous rootstock, induces large fruit size and higher yield; and also tolerant to drought, exocortis and tristeza viruses. Presently, citrus growers are facing the problem of *Phytophthora* root rot when plants are grown on rough lemon rootstock due to its susceptibility to *Phytophthora* fungus. Around 30-65 per cent incidence of *Phytophthora* root rot of citrus in Kinnow orchards is noted in Punjab. In central India, 6-9 million plants are propagated every year and out of this 20 per cent plants die due to *Phytophthora* in the nurseries. *Phytophthora* root rot can reduce citrus yield by 46 per cent and with losses upto $5 million (Naqvi, 9). Rootstock affects the tree performance, longevity and tolerance to various biotic and abiotic stresses. The use of resistant rootstocks is a major strategy to manage the soil-borne diseases like *Phytophthora*.

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Punjab Agricultural University has recommended the cultivation of Daisy and W. Murcott mandarins on Carrizo citrange rootstock for cultivation in Punjab. Daisy-an early maturing variety is a cross between Fortune and Fremont mandarin. Carrizo citrange, a citrus rootstock is tolerant to *Phytophthora*, tristeza and nematodes. Carrizo citrange is a hybrid of Washington navel orange (*Citrus sinensis* Osbeck) and *Poncirus trifoliata* L. (Raf.). Fruit quality on this rootstock is excellent; fruit size is medium with thin and smooth rind. Juice and sugar content are high and acidity level is medium to high. There is scarcity of Carrizo rootstock through there is huge demand of Daisy and W. Murcott mandarins. Micropropagation is a useful tool for the production of larger number of true-to-type planting material in short period of time with the year around availability. Tallon (12) was of the view that with the efficient micropropagation protocols for citrus rootstocks, production of plants will not be affected by availability of seed and uniform planting material can be produced. Keeping these facts in view, the present investigations was undertaken to develop a protocol for clonal propagation of Carrizo citrange.

MATERIALS AND METHODS

The present investigations were conducted during 2012-2013 in the Department of Fruit Science, PAU, Ludhiana. The citrus rootstock, Carrizo citrange was used as an explants source for *in vitro* clonal

propagation. Two explants (nodal segment and shoot tip) were obtained from the nine-month-old seedlings grown under screen-house conditions as well as three-year-old field grown plants. The basal nutrient medium used in these experiments was of Murashige and Skoog (8) inorganic salts and vitamins. The explants were cultured on MS medium supplemented with different growth regulators for the development of shoot cultures (Table 1). The *in vitro* shoots obtained by culturing the nodal segments on MS medium (supplemented with various growth regulators) served as material for proliferation. After 2-3 weeks, the sprouted segments were inoculated on shoot proliferation media (Table 3) for regeneration of shoots (direct organogenesis). Ten cultures with one and two shoots, respectively, in each concentration formed one replication. The number of replications varied with each experiment so as to keep the error degree of freedom in analysis of variance at or above 12. Observations were recorded 4 week after culture initiation, on number of cultures proliferated and number of shoots per culture.

After four weeks of culturing on shoot proliferation medium, the regenerated shoots were transferred to root induction media (Table 4). All cultures were kept in incubation room at 25 ± 2ºC temperature under well illuminated white florescent tube lights (2000 lux), maintaining 16 h continuous light and 8 h dark regime. The *in vitro* rooted plantlets were removed from the culture tubes and thoroughly washed under running tap water for 1-2 h to remove the traces of nutrient media from the roots. Dead and decayed parts of the plantlets were removed. The plantlets were then transplanted in plastic cups containing different substrates, *viz*. soil + FYM (2:1), soil + FYM + cocopeat (2:1:1) and cocopeat + perlite + vermiculite (2:1:1) to study the comparative survival of micropropagated plantlets. The constituents of different substrates were mixed volume / volume. Bavistin® (0.1% carbendazim) was applied during the first watering of plants transferred to the plastic cups. There were 50 cultures in each replication. Strength and composition of media formed one replication. The number of replications varied with each experiment so as to keep the error degree of freedom in analysis of variance at or above 12. Observations were recorded on days taken for root initiation, rooting percentage, number of roots and longest root length (cm). All the experiments were was laid as per the Completely Randomized Design (CRD). The mean separation was done using least significant difference (Fisher's LSD) at $P \leq 0.05$ following significant F test. Student's t-test was used to ascertain the significance of effect of age of mother plant on explant establishment.

Table 1. Effect of different levels of growth regulators on explant establishment.

Medium	Establishment (%)
MS Basal	75.20 (8.67)*e
MS + 2 µM BAP	86.40 (9.29) c
MS + 4 µM BAP	66.40 (8.15) f
MS + 2 µM BAP + 1 µM NAA	97.20 (9.86) a
MS + 2 µM BAP + 2 µM NAA	85.20 (9.23) c
MS + 4 µM BAP + 1 µM NAA	60.00 (7.74) g
MS + 4 µM BAP + 2 µM NAA	56.80 (7.53) h
MS + 2 µM BAP + 1 µM IBA	92.80 (9.63) b
MS + 2 µM BAP + 2 µM IBA	80.80 (8.99) d
MS + 4 µM BAP + 1 µM IBA	58.80 (7.67) gh
MS + 4 µM BAP + 2 µM IBA	48.80 (6.98) i

The values for the treatments and means followed bythe same letter do not differ significantly at P ≤ 0.05. Value in parenthesis indicates transformed value.

RESULTS AND DISCUSSION

The minimum explant microbial contamination (26.40%) was recorded when the explants were exposed to maximum duration (20 min.) of mercuric chloride (Fig. 1). There was no survival of shoot tip explants following surface sterilization at any treatment duration. Highest survival of 39.00 per cent was recorded when the explants were exposed to 0.1 per cent mercuric chloride for 10 min. duration, which significantly gave higher survival (Fig. 1). It was followed by survival of 27.60, 14.40 and 10.00 per cent when the explants were exposed for 15, 5 and 20 min., respectively. The proportion of aseptic cultures increased with an increase in the duration of exposure of nodal segments to mercuric chloride. The lowest contamination was recorded at 20 min. exposure. However, maximum survival of nodal segment explants was recorded at exposure duration of 10 min. (Fig. 1). Lower survival percentages were recorded at treatment

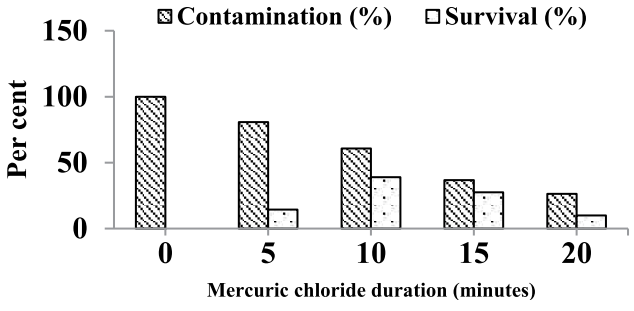

Fig. 1. Effect of surface sterilant mercuric chloride and its duration of exposure on explant contamination and survival.

durations lesser or longer than 10 min. The death of explants at longer exposure durations to mercuric chloride may due to the phytotoxicity caused by mercury (Hg^{2+}) or as shoot tip explants were tender.

Addition of growth regulators in the establishment media significantly increased the establishment in comparison to 75.20 per cent establishment in MS basal medium (Table 1). Lower levels (2 µM) of cytokinin (BAP) resulted in higher explant establishment (86.40%) in comparison to 66.40 per cent with higher level of BAP (4 µM). Explant establishment was higher in MS medium having BAP along with auxin (NAA or IBA) than BAP alone. In general, lower auxin levels (1 µM) along with BAP resulted in higher explant establishment than higher level of 2 µM. NAA was more effective in increasing explant establishment than IBA. MS medium containing BAP (2 µM) + NAA (1 µM) resulted in maximum establishment (97.20%) followed by 92.80 per cent explant establishment in MS medium with BAP (2 µM) + IBA (1 µM), 86.40 per cent in MS with BAP (2 µM) and 85.20 per cent in MS with BAP (2 µM) + NAA (2 µM). The shoots grew from pre-existing axillary buds in the explant without any visible deformities or callus formation. Cytokinins induce bud break by activation of meristems. Outgrowth of axillary buds is well correlated with cytokinin level in the bud. The efficacy of BAP was further enhanced, when small amount of NAA was added in medium.

The highest explant establishment frequency (96.60%) was recorded during April-May which declined significantly during the other parts of the year (Fig. 2). During August-September explant establishment frequency was 88.00 per cent, which was followed by 80.00 and 75.20 per cent during rainy season (July-August) and autumn (October-November), respectively. The high explant establishment during April-May may be due to the presence of high levels of growth promoting substances and low growth inhibitors in actively growing shoots. In grapes, highest explant establishment was found in April and it declined considerably with the progress of season (Singh *et al.*, 11). The establishment percentage (97.20) of nodal segment explants from nine-month old seedlings grown in screenhouse did not differ significantly from 91.6 per cent establishment from three-year-old field grown mother trees (Table 2).

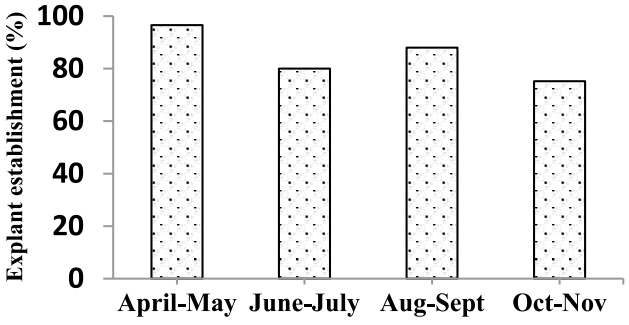

Fig. 2. Effect of different seasons of explant collection on explant establishment.

The shoot multiplication became evident within 8-10 days after inoculation (Table 3). The lower BAP level (2 µM) resulted in significantly higher shoot proliferation than the higher level (4 µM) of BAP (Table 3). Addition of 1 µM NAA significantly increased the shoot proliferation but only with lower levels of BAP (2 µM). The highest shoot proliferation (3.64 per explant) and maximum shoot length (3.71 cm) were recorded with MS medium containing 2 µM BAP + 1 µM NAA. It was followed by MS medium supplemented with 2 µM BAP, where 1.56 shoots/ explant were recorded with 2.30 cm shoot length. The MS medium with 4 µM BAP produce1.27 shoots per explant, which did not differ significantly from control (1.11 shoots per explant). Shoot multiplication has been significantly affected by the concentration of BAP. Cytokinins overcome the apical dominance and induce shoot proliferation. BAP has also been reported to enhance shoot proliferation in citrus (Parthasarthy *et al.*, 10). Similarly, a promotive effect of BAP on shoot regeneration at low concentrations and a toxic effect at higher concentrations have also been described for different citrus genotypes (Costa *et al*., 4). Higher levels of BAP (4 µM) suppressed the shoot proliferation and also resulted in callus formation. Tallon *et al.* (12) reported that the highest BAP concentration (3 mg/l) reduced shoot proliferation in all the three rootstocks, *viz*., Alemow, sour orange and Cleopatra mandarin.

Table 2. Effect of age of mother plant on *in vitro* explant establishment.

Age of explant	Establishment (%)
Seedling (9-month-old)	97.20a
Mature Plants (3-year-old)	91.60a
Student's t-test (p = 0.05)	NS

Table 3. Effect of different media on the shoot proliferation.

MS medium	No. of shoots/ explant	Length of longest shoot (cm)
Control	1.11c	2.20c
2 µM BAP	1.56b	2.30b
4 µM BAP	1.27c	1.45d
2 µM BAP + 1 µM NAA	3.64a	3.71a
4 µM BAP + 1 µM NAA	1.20c	1.47d

The values for the treatments and means followed by the same letters do not differ significantly at P ≤ 0.05

Addition of 1 µM NAA significantly increased the number of shoots per culture at lower BAP levels (Table 3). One of the possible roles of auxin during shoot proliferation is to nullify the suppressive effect of higher cytokinin concentrations on axillary shoot elongation and restore normal shoot growth. The increase in shoot length following addition of auxin in the shoot multiplication media may also be due to the nullification of suppressive effect of high cytokinin concentrations on axillary shoot elongation by auxin and restoration of normal growth (Lundergan and Janick, 7).

The earliest root initiation within 10.60 days was observed with MS medium fortified with IBA (10 µM) + malt extract (500 mg/l) followed by 16.20, 19.80 and 22.00 days in half-strength MS medium fortified with 2.5 µM IBA, 5.0 µM IBA and 10 µM IBA + 2.5 µM NAA, respectively (Table 5). Rooting was not observed in half-strength medium fortified with IBA (10 µM) and NAA (2.5 µM) and in control medium without any auxin. The rooting percentage ranged from 0.00 to 53.89 for different media (Table 4). The highest rooting (53.89%) was recorded on MS medium fortified with IBA (10 µM) + malt extract (500 mg/l) followed by 37.22 per cent rooting in half-strength MS medium containing IBA (10 µM) + NAA (2.5 µM) and 31.67 per cent rooting in half-strength medium containing IBA (2.5 µM). However, no rooting was observed on half-strength media containing IBA (10 µM) and NAA (2.5 µM) alone.

The shoots cultured on medium containing either IBA or NAA alone produced only one root per shoot at all concentrations, except in half-strength MS medium fortified with IBA (10 µM) or NAA (2.5 µM). The micro-shoots cultured on medium fortified with IBA and NAA in combination produced more roots per shoot. Maximum number of roots per shoot (1.37) was observed on half-strength medium fortified with IBA (2.5 µM) + NAA (2.5 µM) followed by half-strength medium fortified with IBA (10 µM) + NAA (2.5 µM) with 1.13 roots per shoot. Root length was significantly influenced by different concentrations of IBA and NAA (Table 4). Root length ranged from 2.98 to 5.0 cm with maximum (5 cm) on half-strength medium fortified with IBA (10 µM) + NAA (2.5 µM). Half-strength MS medium fortified with 5 µM IBA resulted in 4.16 cm long roots, which was not significantly different from media fortified with 5 µM NAA and 2.5 µM each of IBA and NAA. The present findings are in line to the results obtained by Kour *et al.* (6) in rough lemon. The half-strength MS medium fortified with IBA (10 µM) + malt extract (500 mg/l) resulted in the highest rooting but lowest root length (2.98 cm) was recorded with this treatment.

The addition of auxins (IBA or NAA) in the rooting medium is necessary to promote *in vitro* rooting in citrus (Carimi and De Pasquale, 2). In general, there was a poor rooting response of Carrizo citrange to all the rooting medium. Duran-Vila *et al.* (5) has previously reported low rooting efficiency as a

Table 5. Relative efficacy of different potting mixture on *ex vitro* plant survival of Carrizo citrange.

Potting mixture	Survival (%) (After 2 weeks)
Soil + FYM (2:1)	0.00 (0.00) c
Soil + FYM + cocopeat (2:1:1)	17.20 (3.69) b
Cocopeat + vermiculite + perlite (2:1:1)	81.40 (9.02) a

The values for the treatments and means followed by the same letter do not differ significantly at P ≤ 0.05. Value in parenthesis indicates transformed value.

Table 4. Effect of media and auxin concentration on rooting behaviour.

Medium	Days to root initiation	Rooting (%)	No. of roots / shoot	Length of longest root (cm)
½ MS + 2.5 µM IBA	16.20 d	31.67 (5.62)*b	1.00 c	3.00 c
½ MS + 5 µM IBA	19.80 c	25.00 (4.98) c	1.00 c	4.16 b
½ MS + 10 µM IBA	-	0.00 (0.00) e	0.00 d	0.00 d
½ MS + 2.5 µM NAA	-	0.00 (0.00) e	0.00 d	0.00 d
½ MS + 5 µM NAA	28.20 a	10.00 (3.09) d	1.00 c	3.90 b
½ MS + 2.5 µM IBA + 2.5 µM NAA	23.00 b	11.11 (3.29) d	1.37 a	3.76 b
½ MS + 10 µM IBA + 2.5 µM NAA	22.00 bc	37.22 (6.09) b	1.13 b	5.00 a
MS + 10 µM IBA + malt extract (500 mg/l)	10.60 e	53.89 (7.34) a	1.00 c	2.98 c
Control	0.00 f	0.00 e	0.00 d	0.00 d

The values for the treatments and means followed bythe same letter do not differ significantly at P ≤ 0.05. Value in parenthesis indicates transformed value.

major problem for *in vitro* production of citrus plants. Better rooting response was observed with IBA in comparison to NAA. The results are contrary to the earlier findings in several citrus species, where NAA has been reported to usually induce higher rooting percentages than IBA or IAA (Tallon *et al.*, 12). NAA alone was also effective in rooting, but to a less extent than the combination with IBA (Table 4). Best rooting response has been observed when IBA (10 µM) along with malt extract was added in the medium (500 mg/l). Chandra *et al.* (3) observed good rooting response in the presence of auxins in the culture media. There is no evident explanation for the lack of effectiveness of some treatments that have proved to be valuable in inducing rooting in citrus (Thomas, 13). Specific behaviour of this genotype is important to take into consideration in future research.

The maximum *ex vitro* survival rate of 81.4 per cent of the plantlets (Table 5) was in potting mixture consisting of cocopeat + vermiculite + perlite (2:1:1). It is evident from the data that amongst all the media tried, the medium consisting of cocopeat + vermiculite + perlite (2:1:1) is best medium for survival. The better aeration and water holding capacity of the medium may be responsible for better survival of plantlets. The protocol developed and the stages are shown in Fig. 1.

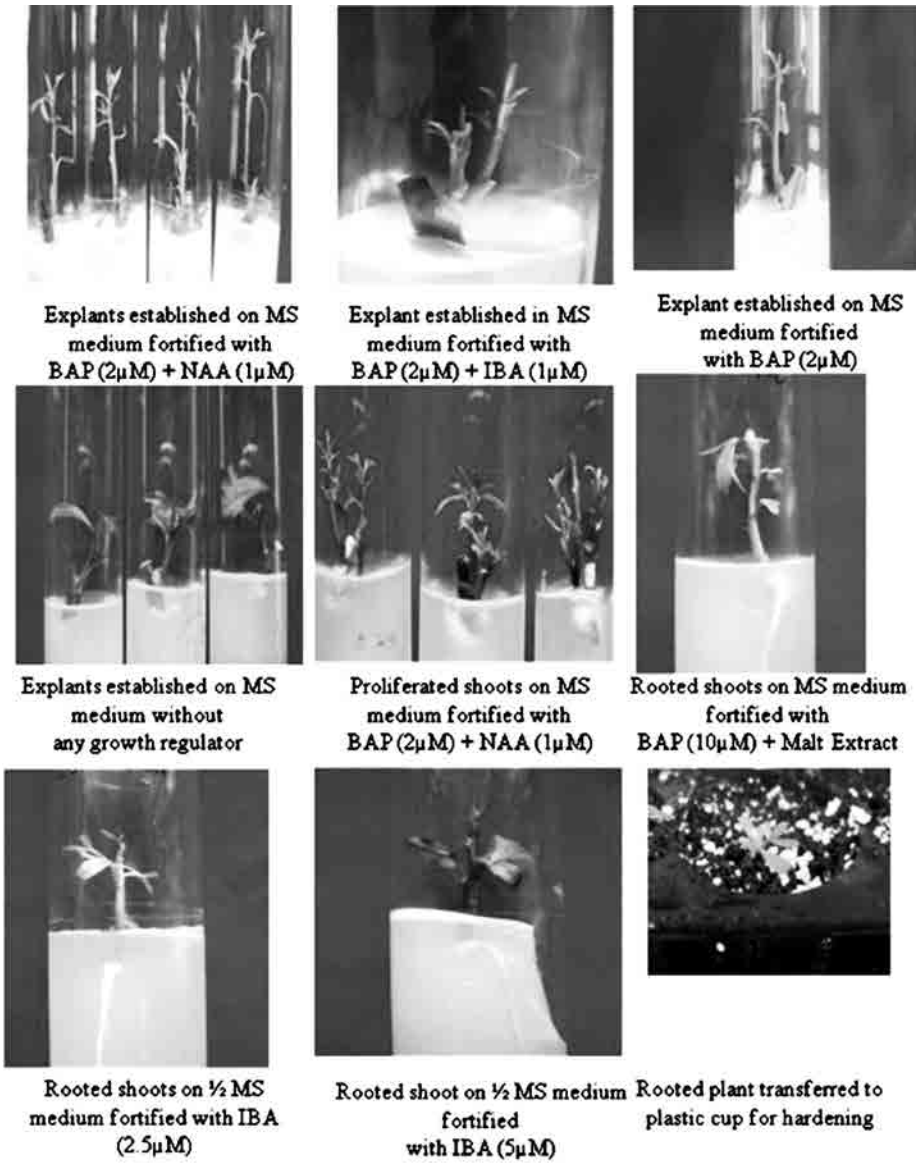

Fig. 1. *In vitro* plantlet multiplication in Carrizo citrange.

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