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PROPAGATION OF CTV-FREE SWEET ORANGE [*CITRUS SINENSIS* (L.) OSBECK] PLANTS THROUGH MICROBUDDING TECHNIQUE

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Abstract

Microbudding technique was used for the early propagation of sweet orange [*Citrus sinensis* (L.) Osbeck] cv. Musambi plants, free of citrus tristeza virus (CTV) using thermotherapy. Source plants of sweet orange, both infected and apparently healthy, were collected from the orchards at Sahiwal and Faisalabad and analyzed along with microbudded plants through ELISA. Budwood of 3 mm and 4 mm in size was microbudded at two different heights (15 cm and 23 cm). Maximum (50%) success of microbudding was obtained, when budwood of 4mm was microbudded at the height of 23 cm. It would suggest that microbudding could efficiently be used for the early propagation of CTV free Musambi plants.

Introduction

Citrus is a prized fruit of Pakistan and holds prominent position among all fruits both in area and production. Main citrus species grown in Pakistan are: Kinnow and Feutrell's early mandarins (*Citrus reticulata* Blanco.), Blood red and Musambi sweet orange (*Citrus sinensis* (L.) Osbeck), grape fruit (*Citrus paradisi* Macf.) and sweet lime (*Citrus limettioides* Tan.), whereas lemon and lime occupy relatively small area.

In citrus, the use of grafting material from infected trees for nursery propagation is primarily responsible for the dissemination of virus and virus-like diseases. Citrus growers are struggling to cope with severe out breaks of these diseases. Citrus plant in Pakistan is susceptible to a large number of virus and virus-like diseases, which are graft-transmissible and inflict heavy losses in production (Bove, 1995). Therefore, nursery propagation and operations are mainly responsible for the contamination and spread of these diseases, leading to deterioration of quality of fruit, short life span of citrus trees and slow or quick decline. Virus and virus-like diseases have a major constraint on successful citrus production. Plant viruses can spread in various ways, however, the principal means of transmission are infected propagative material, insects and contaminated tools (Ferguson & Garnsey, 1993).

Many techniques like serological reactions, electron microscopy, electrophoretic analysis, molecular probes and gene amplification by polymerase chain reaction (PCR), are now available for the efficient diagnosis of viruses and to obtain virus free citrus plants. Enzyme linked immunosorbent assay (ELISA) is a sensitive, accurate, economical and rapid serological technique for the detection of several pathogens (Clark & Adams, 1977, Roistacher, 1991). The presence of CTV was detected in mandarin and sweet orange at 14 localities of five districts of Pakistan through ELISA. It was found that Kinnow is more susceptible to CTV compared to Musambi sweet orange (Anwar & Mirza, 1992).

Table 1. Reaction of citrus tissue in CTV-ELISA tests.

Test plants	Reaction	ELISA Value (A_{405} nm)
Microbudded Musambi plants	Negative	0.033-0.037
Musambi mother plant at Sahiwal	Positive	0.147-0.258
Musambi mother plant at Faisalabad	Negative	0.027-0.076
Positive samples	Positive	0.172-0.257
Healthy	Negative	0.029
Buffer	Negative	0.016

Thermotherapy followed by a shoot-tip grafting (STG) was successfully used for the elimination of major citrus pathogens along with the diagnostic techniques (Roistacher, 1988). Shoot-tip grafting of very small shoot apices (0.1-0.3mm) on to the apical or lateral part of decapitated epicotyl seedling rootstocks, cultured *in vitro*, was very helpful to obtain citrus clones free from virus and virus-like diseases (Starrantino, 1992). Microbudding is a novel technique developed to produce citrus plants rapidly and inexpensively (Skaria, 2000). However, budwood size is big enough to eliminate the pathogens. Preliminary studies done in our laboratory have shown that microbudding was useful for the early propagation of Kinnow mandarin plants. The objective of this study was to produce and multiply CTV-free Musambi plants through thermotherapy followed by microbudding technique along with the ELISA indexing.

Materials and Methods

Microbudding: Four 6 months old seedlings of rough lemon (*Citrus jambhiri* Lush.) were grown in sterilized potting medium and used as a rootstock. The budwood taken from Musambi sweet orange (*C. sinensis*) was used as a scion material. Healthy budwood was collected from Faisalabad whereas infected source plants were collected from Sahiwal district. Source plants were maintained at greenhouse conditions for thermotherapy (Roistacher, 1977). The budwood of 3mm and 4mm in size was budded at two different heights (15 cm and 23 cm). The microbudding procedure (Skaria, 2000) was based on budding untapped small scionic tissue on 4-6 months old rootstocks. The scion buds were capped with tips of micropipette. In microbudding, budwood size is large, therefore budwood from thermotherapy applied source plants were used to eliminate the citrus tristeza virus in microbudded sweet orange plants.

At each watering, 5ml L^{-1} Hoagland solution was administered as a nutrient source in irrigating water. Data was collected for microbudding success percentage, number of days for successful union, time taken to sprout first leaf, number of leaves and plant height of microbudded citrus plants. Means for the data collected were compared by standard error (Steel & Torrie, 1984).

Indexing: Biological indexing using an indicator plants is a reliable technique, however, it is time consuming and require proper greenhouse facilities. Compared to biological indexing, ELISA is especially effective where results are needed rapidly and where suitable indicator plants and/or greenhouse facilities are not available. Test samples were randomly collected from the microbudded plants as well as from source plants (Table 1). Controls consisted of positive samples of CTV, healthy tissue and buffer. Successfully

microbudded Musambi sweet orange plants were indexed only for citrus tristeza virus (CTV) through DAS-ELISA (CTV Antibodies: IgG and Conj IgG A 0323 and B 1808 from Agdia). The ELISA results were assessed by visual observation and absorbance was measured at 405 nm in EL_X 800 Universal Microplate Reader (BIO-TEK Instruments Inc. USA).

Results and Discussion

Microbudding success percentage: In all, 40 microbudded Musambi plants were obtained. The microbudded plants after 42 days were growing normally and manifested good health (Table 2). Results indicated that 4 mm of scion bud microbudded at 23 cm height was more successful (50%) whereas, scion bud of 3 mm or 4 mm in size microbudded at 15 cm height depicted less success percentage (Table 2). The results of our present study are in close conformity with the results obtained by Ochoa *et al*., (2000) which showed 61% success in Hamlin sweet orange, when microbudded on sour orange under the temperature range of 26.6-32.2ºC.

Table 2. Success of microbudding in Musambi sweet orange.

Plant growth parameters	Microbudding height			
	15 cm		23 cm	
	Bud size (3mm)	Bud size (4mm)	Bud size (3mm)	Bud size (4mm)
Success of microbudding in Musambi (%)	20	10	30	50
Number of days for successful union	34±1.00	17±0	15.75±0.946	19.67±1.61
Time taken to sprout first leaf	33±6	20±0	25±5.43	26.5 ±2.62
Number of leaves on scion of microbudded plants (After 42 days)	5±0	4±0	6±0	5.6±0.87
Scion length of microbudded plants (After 42 days)	4±0	5.10±0	7.0±3.20	7.42±3.04

Number of days for successful union: Significant interactions were also observed between cultivar and budding height, plant height and bud size. The Musambi scion buds of 3 mm budded on 23 cm took lesser time (15.75) whereas 3 mm scion buds budded on 15 cm height took maximum (34) days for successful union (Table 2). The results reported by (Mazhar *et al.,* 2004) showed that 4 mm Kinnow scion buds microbudded at the height of 15 cm took fewer days (12.75) for successful union.

Time taken to sprout first leaf: The interaction between budding height and scion bud size did not significantly effect the time (days) taken by microbudded Musambi plants to produce their first leaf. The scion buds of 4 mm budded on 15 cm took minimum time (20) and 3 mm scion buds budded on 15 cm height took maximum time (33) to sprout first leaf (Table 2).

Number of leaves on scion of microbudded plants: The number of leaves on the scions of microbudded established plants of Musambi were counted at 7-day intervals. Maximum number of leaves (6) was observed after 42 days in scion buds of 3 mm microbudded at 23 cm rootstock height and the minimum (4) was in 4 mm scions, budded on 15 cm of height (Table 2).

Plant height of microbudded plants: Maximum mean plant height (7.42 cm) was observed after 42 days in scion buds of 4 mm microbudded at 23 cm rootstock height (Table 2). The scion buds of 3 mm microbudded on 15 cm of rootstock height depicted minimum mean plant height (4 cm). The results documented by Mazhar *et al.* (2004) indicated that Kinnow scion buds of 4 mm size microbudded on 15 cm rootstock seedlings show maximum height of 10.65 cm after 42 days.

ELISA indexing: The Musambi budwood collected from Sahiwal district showed positive reaction for CTV infection. These plants had been grafted on sour orange rootstock and manifested clear CTV symptoms. Negative reaction was observed in the budwood taken from Faisalabad, which showed that the source trees were free of CTV. Axillary buds used from these plants were microbudded on rough lemon and found to be free of CTV in ELISA (Table 1). The results of our study for the detection of CTV in microbudded Musambi plants, are in full agreement with those reported by Al-Senan *et al.* (1997), Alioto *et al*., (2000), Besoain *et al*., (2000) and Solis-Gracia *et al*., (2001).

Microbudding technique and thermotherapy combined with virus indexing based on ELISA may significantly reduce the time to develop CTV-free Musambi plants. The approach may also facilitate and enhance citrus budwood certification programme.

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