INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2007/09–1–27–30
http://www.fspublishers.org

Elimination of Citrus Tristeza Virus of Washington Navel Orange (*Citrus sinensis* [L.] Osbeck) Through Shoot-tip Grafting

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ABSTRACT

Shoot-tip grafting *in vitro* is used to recovery of pathogen-free plants, sanitation programs, quarantine procedures and separation of viruses in mixed infections. This study was carried out for producing citrus tristeza virus (CTV) free plants. Shoot-tip of infected Washington navel orange by tristeza containing meristem and two leaves primordia was excised. Then, it was grafted on Troyer citrange and Citrumello seedlings by two methods, inverted T-budding and in contact with the vascular ring. These seedlings had 3 - 5 cm length. Grafted seedlings were planted on liquid medium and incubated in growth chamber at 27°C and 16 h photoperiod with 1000 lux illumination. If grafting was successful, plant was transplanted in suitable soil. Shoot-tip grafted plants were tested by DAS ELISA against tristeza virus. None of them were infected. The results showed that inverted T-budding was more successful than other one. There was no significant differences between used rootstocks.

Key Words: *Citrus*; Washington navel orange; Shoot-tip grafting; Tristeza virus; Rootstock

INTRODUCTION

Citrus is one of the most important fruit crops in the world. In Iran, citrus occupies over 250,000 ha with approximately 50% oranges, 20% mandarins (*C. reticulata* Blanco) and the rest with miscellaneous cultivars of lemon (*C. limon* [L.] Burm. f.), etc. (FAO, 2004). Among the orange cultivars, the predominant one is navel. Commercial citrus production in Iran is located in the Mazandaran province with 90,000 ha. There has been a growing interest in recent years in substitution of local cultivars of sweet orange with Washington navel and Thomson navel oranges. To obtain virus-free propagation materials for planting in the new areas, it is very important to use sanitation programs, which eliminate the viruses and viroids (Rahimian *et al*., 2000).

Citrus plants are infected by many virus diseases and tristeza virus, which eradicates thousands of citrus trees in various countries especially grafted on bitter orange trees and causing big loss in agricultural economy. Virus and virus-like diseases, which result in decline of vigor, yield, quality, restriction the use of rootstocks and in some cases death of the trees, or make them completely un-productive, caused the high economic losses in citrus orchards. For example, more than 50 millions citrus trees on sour orange were lost, because of CTV infection in the world (Ferguson *et al*., 2000).

In the last years, growing nucellar seedlings was the only method available for producing disease free citrus cultivars from clones infected with virus or other graft-transmissible pathogens. The primary disadvantage of producing citrus budlines through nucellar embryony is the phenomenon of juvenility. Young nucellar seedlings exhibit excessive thorniness, vigorous and up-right habit of growth slowness to fruit, alternate bearing in early years and physical differences in fruit characteristics, which are often detrimental in marketing the fruit. These characteristics may persist for many years and over many budded generations. Nucellar budlines usually produce higher yields of fruit than their parental clones over a period of 8 - 10 years or more (Cameron *et al*., 1968; Nauer *et al*., 1983). The portion of this higher yield that can be attributed to elimination of virus and virus-like pathogens in the parental bud-line has not been determined. Variations among citrus nucellar budlines and differences, other than juvenility, from the parental bud-line have been reported (Frost *et al*., 1957; Nauer *et al*., 1983) in numbers indicating that genetic variants may occur more often during production of nucellar bud-lines than occur during standard nursery trees production by bud propagation.

Thermotherapy is highly effective for eliminating most citrus viruses. Thermotherapy was first demonstrated to be an effective means of eliminating tristeza and psorosis viruses from citrus bud-wood (Grant, 1967). Sub-sequent studies have shown that many other pathogens including concave gum, greening, impietratura, infectious variegation, psorosis-A, psorosis-B tatter leaf, tristeza, seedling yellows tristeza and vein enation could be eliminated from bud-wood by heat treatments, but that some including exocortis, cachexia stubborn and yellow vein were more difficult to eliminate (Rossetti *et al.*, 1965; Grant, 1967; Calavan *et al.*, 1972; Roistacher & Calavan, 1974).

Therefore, a method to recover citrus plants free of all virus and virus-like diseases and without juvenile characters was needed. The first attempts in this direction were made by shoot-tip culture *in vitro*, a technique widely used to

recover healthy herbaceous plants. However, attempts to develop citrus plants from shoot-tips failed (Edriss *et al*., 1984).

Shoot-tip grafting currently appears to be the most useful method for producing pathogen-free citrus propagative stock. There is the most extensive citrus sanitation program based on STG in some countries such as Spain, Italy, France and millions healthy trees, originally recovered by this technique, have been already planted in the field (De Lang, 1978; Navarro *et al.*, 1995). In Iran, citrus planting area is more than 250000 ha with 3.7 millions ton fruit production annually and so, using the sanitation programs could be very important role on fruit production. For the first time, shoot-tip grafting was carried out by Murashigue *et al*. (1972). Then Navarro *et al.* (1975) developed this technique and it was used instead of nucellar, thermotherapy, apical bud culture and etc. Currently, this method is used to produce virus and virus-like free plants. This study reports the use of STG in eliminating the CTV to obtain disease-free mother trees of Washington navel orange in Iran.

MATERIALS AND METHODS

This research was done in a complete randomized design with 4 treatments, 4 replications and 10 plantlets in a replication and two factors included different rootstocks and methods of grafting were studied.

The standard procedure by Navarro *et al.* (1975, 1982 & 1995) was used in this research. It comprises the following steps: rootstock preparation, scion preparation, grafting, culture *in vitro* of grafted plants and transfer to soil.

Rootstocks are obtained by seed germination *in vitro*. Seeds of Troyer citrange [*Poncirus trifoliata* (L.) raft. × *Citrus sinensis* (L.) Osb.] and Citrumello [*Poncirus trifoliata* (L.) raft. × *Citrus paradisi* Macfad.] are peeled, removing both seed coats, surface sterilized by immersion for 10 min in a 0.6% sodium hypochlorite solution containing 0.1% Tween-20 wetting agent and rinsed three times with sterile distilled water. Seeds are individually planted in 25 × 150 mm culture tubes containing 25 mL of the plant cell culture salt solution of Murashigue and Skoog (1962), solidified with 1% bacto agar. Culture tubes are incubated at constant 27°C in continuous darkness for 2 - 3 weeks.

Infected Washington navel orange plants growing inside pots in the greenhouse were completely defoliated by hand and placed in a warm greenhouse. 3 cm long or shorter flushes were used (Fig. 1). They were stripped of larger leaves, cut to about 1 cm long, surface sterilized by immersion in a 0.3% sodium hypochlorite solution containing 0.1% Tween-20 wetting agent and rinsed three times with sterile distilled water.

The rootstock seedling was removed from the test tube under aseptic conditions and it was decapitated, leaving about 1.5 cm of the epicotyl the root was cut to a length of 4 – 6 cm and the cotyledons and their axillary buds removed (Fig. 2). Grafting could be done by two methods, top of the decapitated epicotyl, placing the shoot tip in contact with the vascular ring, or in an inverted-T incision (Navarro *et al.*, 1995). The shoot tip composed of the apical meristem and three leaves primordia and measuring 0.1 - 0.2 mm, was excised and used to grafting (Fig. 3). Grafted plants were cultured in a liquid nutrient medium composed of the plant cell culture salt solution of Murashigue and Skoog (1962), modified white's vitamins and 75 g/l sucrose (Navarro *et al*., 1995). The nutrient medium was distributed into 25 × 150 mm test tubes in 25 mL aliquots. The cultures were kept at constant 27°C and exposed 16 h daily to 40 - 50 $\mu e/m^2s$ illumination.

Scions of successful grafts with at least two expanded leaves were transplanted to pots containing steam-sterilized artificial soil mix suitable to grow citrus (Fig. 4). Pots were enclosed in polyethylene bags and placed in a shaded area of a greenhouse. After 2 to 3 weeks the bags were removed and the plants were grown under regular greenhouse conditions (De Pasquale *et al*., 1999). The produced plants from STG technique were tested by biological and serological indexing and these were indicated that free of

Fig. 1. Source of scion preparation

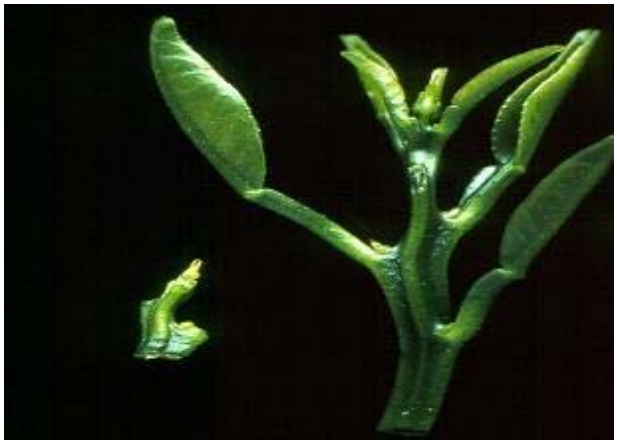

Fig. 2. Seedlings preparation for grafting

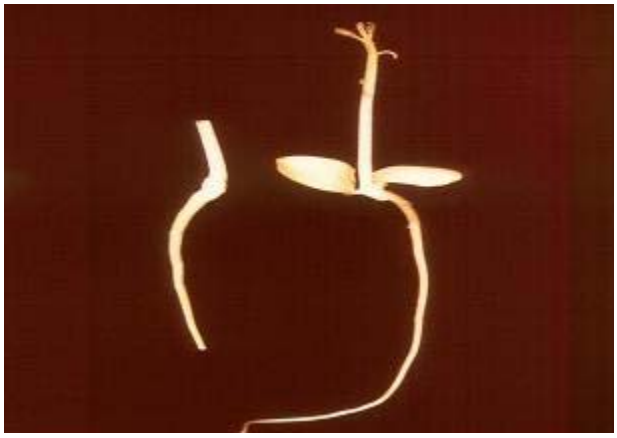

Fig. 3. Two methods of grafting

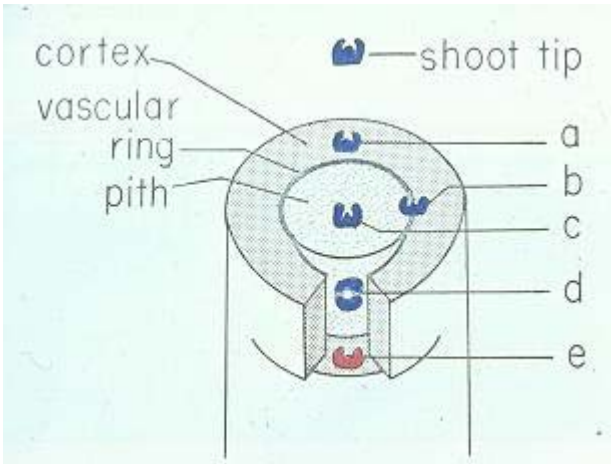

Fig. 4. Transfer to soil

citrus tristeza virus.

RESULTS AND DISCUSSION

Data analysis showed that there were significant differences between methods of grafting in 0.01 and 0.05 levels and inverted T-budding method, was the best. Also rootstocks had no effects on grafting success (Table I & II).

There were no differences between rootstocks (Troyer citrange & Citrumello). They had no effect on success of micropropagation. These are like Navarro *et al*. (1992, 1995) results. Navarro *et al*. (1992, 1995) has normally used Troyer citrange in his studies. It is advantage in trifoliate orange rootstocks and bud-wood could be distinguished easily (Zarei *et al*., 1997; Shahsavar *et al*., 1994).

Data analysis indicated that, inverted T-budding is better than placing the shoot tip on decapitated epicotyl in contact with the vascular ring. There were no differences between these methods in Navarro's studies. But inverted T-budding is a standard method, because bud grows simply in this method and there is no mistake in distinction between it

Table I. Analysis of variance on under studied character

Source	Degrees of freedom	Sum of square	Mean square	F value	Prob
Rootstock	1	506.250	506.250	3.5217	0.0851
Grafting	1	1406.250	1406.250	9.7826	0.0087
Interaction	1	156.250	156.250	1.0870	0.3177
Error	12	1725.000	143.750	-	-

Table II. Mean compare of under studied character by Duncan

Original order	Treatments	Mean	D.M.R.T. 5%	Ranked order	D.M.R.T. 5%
1	A1B1	72.500	A	1	A
2	A1B2	47.500	B	3	AB
3	A2B1	55.000	AB	2	B
4	A2B2	42.500	B	4	B

and adventitious shoots. Skaria *et al*. (1996) and Roistacher (1991) used a new method in grafting (wedge grafting) and compared it with inverted T-budding method but there was no significant differences between them.

According to some studies Troyer citrange was the best among Sour orange, Poncirus trifoliata and Rough lemon. Also inverted T-budding was the best method. Our results are similar them. Using bud-woods with two leaves primordia is effective to produce plants without concave gum disease (Chalavi & Rahimian, 1992).

In this study, plants transfer to soil (transferring from *in vitro* to *in vivo*) was very successful. More than 90% of transferred scions grew well and stayed alive. This method needs a greenhouse with optimum condition and continual treatment. The most of un-successfully in transferred scions is for absence of greenhouse optimal condition.

ELISA test and biological indexing of transferred scion showed that all of them are free from CTV. According to results, STG with inverted T- budding on trifoliate orange rootstocks was more effective in CTV elimination and old methods like using nucellar thermotherapy and so on could be replaced by it.

Besides, this technique could be employed for sanitation, quarantine of imported cultivars and isolation of viruses in complex infection. These plants usually set flowers and fruits two years after grafting.

In this research, STG was used to produce some scions free of CTV. It could be applied to produce health seedlings free of virus and virus-like diseases commercially. STG with thermotherapy are more effective to produce healthy scions as well, but eradication of infected trees must be done simultaneously.

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(Received 27 December 2005; Accepted 20 August 2006)