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ORIGINAL ARTICLE

In vitro micrografting for production of *Indian citrus ringspot virus* (ICRSV)-free plants of kinnow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenora)

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Abstract Production of *Indian citrus ringspot virus* (ICRSV)-free plants from an infected plant of kinnow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenora) is reported. The shoot apices of different sizes (0.2–1.0 mm) excised from the ICRSV-infected plant were micrografted onto decapitated rootstock seedlings of rough lemon (*C. jambhiri*). Micrograft survival depended on the size of shoot apex and the sucrose concentration of the culture medium. Increase in scion size from 0.2 to 0.7 mm resulted in an increase in micrografting success rate from 30.55 to 51.88%. Further, micrograft survival obtained with 0.2 mm was improved from 30.55 to 38.88% by increasing sucrose concentration in the culture media from 5 to 7.5%. The micrografted plants were tested for ICRSV using ELISA and RT-PCR. All plants raised from 0.2-mm scion were found negative with both ELISA and RT-PCR whereas only 20% of the ELISA negative plants raised from 0.3-mm scion were found negative for ICRSV with RT-PCR. The outcome of this research is the successful establishment, acclimatization and virus testing of micrografted plants.

Keywords Kinnow (*Citrus nobilis* × *C. deliciosa*) · ICRSV (*Indian citrus ringspot virus*) · Micrografting

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Introduction

Indian citrus ringspot virus (ICRSV) was first reported in India by Byadgi et al. (1993) from Delhi and is now placed in the genus *Mandarivirus* of Flexiviridae family (Adams et al. 2004; Sharma et al. 2007). ICRSV is widely distributed in orchards of different Indian states such as Punjab, Haryana, Delhi, Uttarpradesh, Orissa, Maharashtra, Karnataka and Andhra Pradesh (Byadgi and Ahlawat 1995; Thind et al. 1995; Sharma et al. 2004). Commercial cultivars of citrus in India, like Khasi mandarin (*C. reticulata*), lemon (*C. aurantifolia*), sweet orange (*C. sinensis*) and kinnow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenora), are susceptible to this viral infection (Pant and Ahalawat 1998). Maximum disease incidence was recorded by Byadgi and Ahlawat (1995) in kinnow mandarin orchards (83.8%) followed by sweet orange (70%) and lime (20%).

In India, kinnow is widely cultivated and is the major fruit crop. However, in recent years, ICRSV infection has resulted in tremendous loss in the yield and quality of this fruit crop (Thind et al. 2005). The leaf lamina of the infected plant exhibits chlorotic flecks, mottling, ringspots and irregular chlorotic patterns on mature leaves. The virus is graft transmissible and it is disseminated primarily by infected budwood. Insect vectors, soil, seed and nematodes are not known to transmit the virus (Pant and Ahalawat 1998; Thind et al. 1999). The health of the infected trees deteriorates year after year and leads to yield losses of between 20 and 98%. A decrease in fruit granulation, weight, size, juice content, total soluble solids (TSS), TSS/acid ratio, vitamin C and reduced sugars content was observed in fruits of virus-infected plants (Thind et al. 2005). So far, the only effective measure to overcome ICRSV infection is to eradicate virus-affected plants.

Micrografting of shoot tips has been successfully employed to produce virus-free plants in citrus (Navarro et al. 1975; Navarro and Juarez 1977; Navarro 1984; Nicoli 1985; Carvalho et al. 2002), peach (Mosella-Chancel et al. 1980; Navarro et al. 1982), cherry (Deogratias et al. 1986), grapevine (Cupidi and Barba 1993) and sweet pepper (Katoh et al. 2004). It has also been employed to: eliminate *huanglongbing liberibacter* from citrus (Navarro et al. 1991); *phytoplasma* from almond (Chalak et al. 2005); study graft incompatibilities (Jonard et al. 1990; Cantos et al. 1995); detect viral infection (Tanne et al. 1993; D'Khili and Grenan 1995; Pathirana and McKenzie 2005); rejuvenate mature shoots material (Ponsonby and Mantell 1993; Perrin et al. 1994; Mneney and Mantell 2001; Onay et al. 2004; Thimmappaiah et al. 2002); recover plants from non-rooting shoots (Lou and Gould 1999), genetically transformed cultures (Pena et al. 1995; Pena and Navarro 1999) and somatic embryos (De Pasquale et al. 1999; Raharjo and Litz 2005).

Since kinnow mandarin is susceptible to ICRSV infection and there is a continuous decline in production of this fruit crop due to virus infection, the present study was therefore planned to standardize in vitro micrografting for production of ICRSV-free kinnow plants.

Materials and methods

Selection of mother plant

The field grown 5-year-old kinnow tree naturally infected with ICRSV was selected for scion collection by screening for the symptoms of the disease from citrus orchard of Govt. Nursery, Department of Horticulture, Attari, Amritsar, Punjab (India). Virus infection was further confirmed by ELISA and RT-PCR in the selected plant before using it as source of explant for in vitro micrografting. Bud flushes were induced by defoliating and pruning shoot branches of the infected plant. These bud flushes were harvested after 10–15 days of pruning and transported to the laboratory in moist bags.

Virus detection

Indirect ELISA and RT-PCR were performed on the following: (1) leaves from the infected field tree used as a source of explant; and (2) leaves from young acclimatized plantlets produced through in vitro micrografting. These plants were routinely checked for the presence/absence of ICRSV after every 6 months until 2.5 years of age. The procedures adopted for indirect ELISA and RT-PCR have been described in our earlier reports (Singh et al. 2005, 2006).

Micrografting

Seeds for raising rootstock were obtained from the mature fruits harvested from rough lemon (*C. jambhiri*) plants growing in orchards of Govt. Horticulture Department, Attari (Punjab), India, and were soaked in water overnight. After removing both seed coats, they were rinsed in tap water, surface-sterilized aseptically in laminar cabinet with 0.05% mercuric chloride for 6 min and rinsed three times with sterilized double-distilled water. These surface sterilized seeds were cultured at the rate of one seed/tube in MS (Murashige and Skoog 1962) medium (25 ml/tube) containing sucrose (3%) and agar (0.8%), pH adjusted to 5.6 with 1 N NaOH and autoclaved at 121°C and 15 lb in^{-2} pressure for 20 min. The cultures were maintained at 26 ± 1°C in complete darkness.

The young bud flushes collected from virus infected plant were stripped off larger leaves, cut to about 1–2 cm of stem segments, surface sterilized aseptically in laminar cabinet with 0.05% mercuric chloride for 4 min and rinsed three times with sterilized double-distilled water. Shoot apices of different sizes (0.2–1.0 mm) were excised with the help of sterilized sharp fine scalpel blade using a stereoscopic microscope set up inside a sterile laminar flow cabinet.

Two-week-old etiolated seedlings of rough lemon were removed from the test tubes and trimmed by removing two cotyledons and decapitated by cutting terminal portions of epicotyl and hypocotyl aseptically in laminar cabinet (Fig. 1a). These terminal cut portions were immediately dipped in sterilized MS liquid medium to prevent oxidation of cut ends. Shoot apices of different sizes (0.2–1.0 mm) excised aseptically with a sharp fine scalpel blade were placed at the top of the decapitated epicotyls with their cut surfaces in contact with the cortex of seedlings. The diameter of epicotyls was greater than that of shoot apices so they were placed off-center and in contact with the vascular ring. This entire procedure was done rapidly under stereoscopic microscope in a laminar cabinet. In order to prevent cross-contamination between different grafts, scalpel blade was dipped in 95% ethanol and flamed after every graft. All the grafted seedlings were placed in perforated platform of Whatman paper (M strip with central hole) in culture tubes containing sterilized MS liquid medium (25 ml/tube) with 5% sucrose at pH 5.6 and were maintained at 26 ± 1°C with a luminous intensity of 40-μmole m^{-2} s^{-1} and 16-h photoperiod. The effect of shoot apex size on percentage micrograft survival was observed 4 weeks after micrografting. For each shoot apex size, 24 seedlings were micrografted and the experiments were repeated thrice.

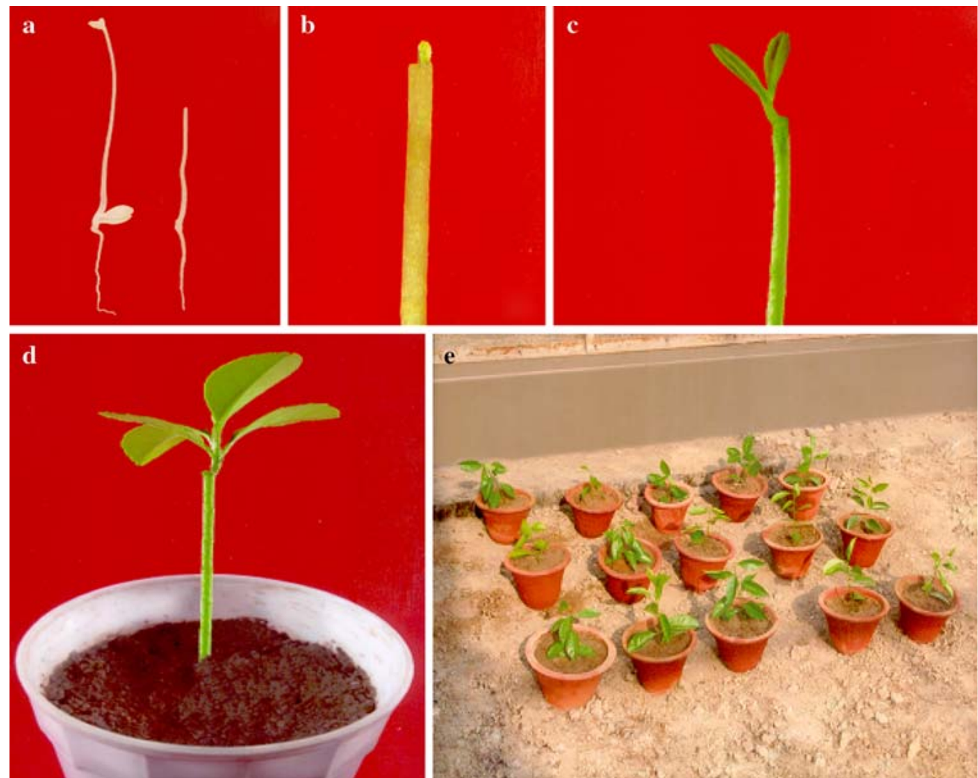

Fig. 1 **a** Etiolated seedlings of rough lemon (*C. jambhiri*) prepared for micrografting. **b** Rootstock after 12 days of micrografting. **c** Rootstock with scion showing two expanded leaves after 30 days of micrografting. **d** Micrografted plantlet after acclimatization. **e** Micrografted plantlets growing in screen house

Acclimatization of micrografted plants

Five to six weeks after micrografting, plantlets with scions having at least two expanded leaves were transferred to autoclaved plastic pots containing a sterilized mixture of garden soil, sand and vermiculite in the ratio of 3:1:1. Hardening of micrografted plantlets was accomplished in an incubation room set at 26 ± 2°C, 16-h day-length (40 μmole m^{-2} s^{-1}) and covered with polyethylene bags to maintain high humidity. After 12–15 days, polyethylene bags were removed initially for a short duration (15–30 min) daily for about 1 week. Gradually, the daily exposure time was increased by 30 min for each day and after 20 days polyethylene bags were completely removed. Subsequently, the plantlets were transferred to the earthen pots containing only garden soil and transferred to the glasshouse. Acclimatized plantlets were indexed by indirect ELISA and ELISA negative plants were again indexed by RT-PCR. It is necessary to choose a shoot tip size that gives a realistic degree of grafting success and provides a reasonable number of virus-free plants. So the effect of different sucrose concentrations (4–8%) was studied on survival of micrografts obtained from appropriate size of shoot apex (the one giving maximum number of virus-free plants).

Statistical analysis

Data pertaining to effect of sucrose concentration and shoot apex size on percentage survival of micrografted plants were subjected to one-way analysis of variance (ANOVA) test, and the differences among means were compared by high-range statistical domain (HSD) using Tukey's test (Meyers and Grossen 1974).

Results

Micrografting

After micrografting, most scions remained green although some turned brown within 4–5 days. These brown scions failed to grow further. Successful micrografts were established (Fig. 1b) after 12–15 days of culture in MS liquid medium with 5% sucrose and formed 2–3 expanded leaves after 28–30 days (Fig. 1c). Micrografting success increased from 30.55 to 51.88% with an increase in scion size from 0.2 to 0.7 mm while further increase in scion size reduced micrografting success rate. Micrograft success depended on the continuity developed between the vascular region of root stock with scion.

Table 1 Effect of shoot apex size on micrografts survival in MS liquid medium containing 5% sucrose and on elimination of ICRSV

Shoot apex size (mm)	Successful micrografts (%)	ELISA negative/ tested	RT-PCR negative/ tested
0.2	$30.55 \pm 1.39^{a,b}$	22/22	22/22
0.3	$31.94 \pm 2.80^{a,b}$	20/23	4/20
0.4	$38.88 \pm 1.40^{a,b}$	16/29	0/16
0.5	41.66 ± 2.43^{a}	2/30	0/2
0.6	44.44 ± 1.38^{a}	0/32	–
0.7	51.38 ± 2.76^{a}	0/37	–
0.8	49.99 ± 2.41^{a}	0/36	–
0.9	48.61 ± 1.39^{a}	0/35	–
1	43.05 ± 2.79^{a}	0/31	–
	$F_{8,18} = 11.775$*, HSD = 9.867		

Means followed by the same letter are not significantly different using HSD multiple comparison test. Values represent mean ± SE of three experiments recorded after 28 days of micrografting; each experiment consisted of 24 replicates

* $P \leq 0.05$

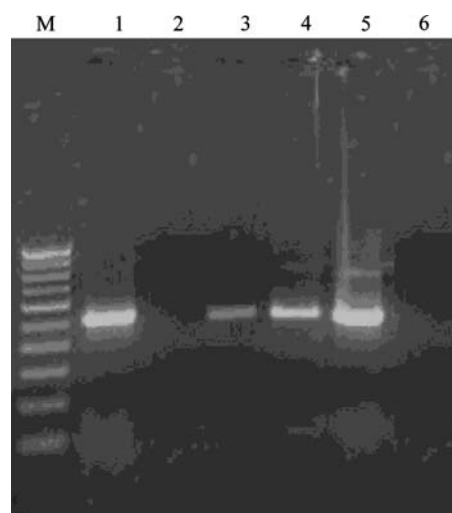

Fig. 2 Agarose gel electrophoresis of RT-PCR products from in vitro micrografted plants to detect presence or absence of ICRSV (Lane M: 100 bp ladder as marker; Lane 1: virus-infected mother plant as control; Lane 2: 0.2-mm scion micrografted plant; Lane 3: 0.3-mm scion micrografted plant; Lane 4: 0.4-mm scion micrografted plant; Lane 5: 0.5-mm scion micrografted plant, Lane 6: negative control)

Virus indexing

The parent plant from which the shoot apices were taken for micrografting was found positive for ICRSV by indirect ELISA. RT-PCR of the same plant showed amplification of a 539-bp fragment corresponding to partial coat protein gene of ICRSV indicating the presence of this virus. Micrografted plantlets (Fig. 1d) raised from each size of shoot apex were indexed by indirect ELISA after acclimatization. All micrografts raised using 0.6–1.0 mm shoot apices were found positive for ICRSV with indirect ELISA whereas some plants obtained from 0.5 (2/30), 0.4 (16/29), 0.3 (20/23) and 0.2 mm (22/22) shoot apices were found negative for ICRSV (Table 1). RT-PCR were performed on ELISA negative micrografted plants raised from 0.2–0.5 mm size scions. All micrografts raised from 0.2-mm scions were found to be free from virus (Fig. 2) whereas only 20% (4/20) of ELISA negative plantets obtained from 0.3-mm scions were negative for virus in RT-PCR (Table 1). This confirms the sensitivity of RT-PCR method over ELISA. The effect of different concentrations of sucrose on survival of micrografts obtained from 0.2-mm scions is shown in Table 2. Maximum graft survival (38.88%) in 0.2-mm scions was observed in MS liquid medium containing 7.5% sucrose. Survival rate decreased with either decrease (7–4%) or increase (8%) in sucrose concentration from this optimal value. So micrografting success of 0.2-mm size shoot apex was improved from 30.55 to 38.88% by increasing sucrose concentration from 5 to 7.5%.

Micrografted plantlets transferred to the screen house (Fig. 1e) have low graft union and grow very slowly. So to overcome this problem micrografted plants were grafted again on to 3-month-old seedlings of rough lemon (*C. jambhiri*) by the cleft graft method in the screen house. The graft unions were wrapped with parafilm and the plantlets were covered with polythene bags, which were gradually opened after 2 weeks. Using this procedure, rapid acclimatization and development of the plants was observed under screen house conditions.

Discussion

One limitation of use of shoot tip grafting/micrografting is the browning of cut surfaces due to oxidation of phenolic compounds, which reduces the success of micrografts. In the present study, cut surfaces of epicotyls were kept moist by dipping them in MS medium and micrografting was done rapidly to establish successful micrografts. Navarro (1988) also recommended rapid handling during micrografting to avoid problems associated with the oxidation of phenolic compounds. Size of the scion has been shown to have a significant effect on graft success with higher survival rate with larger scion size and lower with smaller scion (Gabova 1988; Juàrez et al. 1988, 1992; Navarro 1984; Lou and Gould 1999; Thimmappaiah et al. 2002; Onay et al. 2004; Suarez et al. 2005). Increased success observed in the larger size scion may be due to better cambial contact of the scion with rootstock. In the present

Table 2 Effect of different concentration of sucrose in MS liquid medium on micrografting success using shoot apex of 0.2 mm size

Sucrose concentration (%)	Successful micrografts (%)
4.0	18.05 ± 1.39[a,b]
4.5	24.99 ± 2.40[a,b]
5.0	30.55 ± 1.40[a]
5.5	31.94 ± 1.38[a]
6.0	33.33 ± 2.41[a]
6.5	34.72 ± 2.78[a]
7.0	36.10 ± 2.77[a]
7.5	38.88 ± 1.39[a]
8.0	37.49 ± 2.40[a]
	$F_{8,18}$ = 9.690*, HSD = 9.642

Means followed by the same letter are not significantly different using HSD multiple comparison test. Values represent mean ± SE of three experiments recorded after 28 days of micrografting, each experiment consisted of 24 replicates

* $P \leq 0.05$

study, micrograft success was found to increase with the size of shoot apex from 0.2 to 0.7 mm which decreased further by increasing shoot apex size from 0.8 to 1 mm. So an optimum size of 0.7 mm was found to give the maximum survival. The relationship between grafting success and optimum scion size has also been reported by Onay et al. (2004) while working on *Pistacia vera*. For micrografting, rootstock seeds were germinated in darkness as reported by Navarro et al. (1975) and Navarro (1984). It stimulates etiolation resulting in increased length of epicotyl which makes the micografting easier and promotes the survival of the scions, possibly by limiting photo oxidation at the grafting point and minimizing the destruction of the auxins synthesized in the scion. Liquid medium was used in micrografting mainly because it was easy to culture micrografted seedlings without disturbance and damage to micrografts and better availability and absorption of nutrients (Thimmappaiah et al. 2002).

Virus infection of the mother plant and the micrografted plants was checked by using indirect ELISA and RT-PCR. Earlier reports on virus and viroid elimination in citrus (Murashige et al. 1972; Navarro et al. 1975, 1976, 1980; Navarro 1984; Carvalho et al. 2002) by micrografting have been reported by using indexing procedures which are either of low sensitivity or time consuming. In our work, it was observed that even indirect ELISA lacks the sensitivity to detect viruses when present in low concentrations in plant tissues. In an attempt to overcome this problem more sensitive indexing procedure (RT-PCR) was used to confirm presence/absence of virus in micrografted plants. RT-PCR has been widely used for indexing of plants raised through thermotherapy and chemotherapy (Manganaris et al. 2003; Verma et al. 2005), but has been very rarely used to index micrografted plants (Suarez et al. 2005). Our results confirm the sensitivity of RT-PCR over ELISA. It was observed during micrografting and indexing of grafted plants that larger size shoot apices are likely to provide a large number of grafted plants, but the possibilities of recovering virus-free plants was reduced. Navarro et al. (1976) have also reported that frequency of grafting success increases with the size of the shoot tip, but the number of pathogen-free plants obtained decreases. In the present study, use of shoot apex of 0.2 mm gave virus-free micrografts. In an attempt to increase the percentage survival of micrografts with this size of shoot apex, increasing the concentration of sucrose from 5 to 7.5% helped. Our results are in conformity with those of Navarro et al. (1975) and Ioannou et al. (1991) who had shown that increasing concentration of sucrose increased percent survival of micrografts. Many other workers have also used sucrose at 7.5%, especially in citrus, for growing micrografted seedlings (Navarro 1984; Vijaykumari et al. 1999; Vijaykumari and Singh 1999, 2000; Kobayashi et al. 2003). However, Thimmappaiah et al. (2002) showed no beneficial effect of increasing sucrose concentration.

Kinnow is vegetatively propagated by bud grafting, and use of infected budwood results in widespread occurrence of ICRSV in India. The means to reduce loss caused by viral infection once an orchard was established by infected budwood are limited. The major advantage of micrografting is that viral diseases, which are carried by infected budwood, are eliminated at the initial stage of planting. As the ICRSV is only graft transmissible and not transmitted by insects vectors, soil, and nematode species in the field, it is useful to produce virus-free plants by this technique as the plants will not get infected again if proper phytosanitary conditions are maintained. Although the results of micrografting are encouraging, it remains to be assessed whether RT-PCR-negative plants will remain negative until fruiting after successful establishment in field conditions.

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