

Rapid Propagation and Transplantation of Disease-Free Mandarin Plants Grafted on Tissue Cultured Rootstocks of Trifoliate Orange in Gorkha and Lamjung District

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ABSTRACT

Protocols for in vitro propagation of disease-free trifoliate orange and Madame Vinous sweet orange, an indicator plant for indexing against citrus greening disease (CGD) have been developed. Hardened plants of trifoliate orange have been made available to the nurserymen of Gorkha and Lamjung districts for tip shoot grafting with virus-free budwoods of mandarin inside insect-proof screen tunnels. Successful grafts have been monitored against citrus tristeza virus using DAS-ELISA and CGD using biological indexing. Completely disease-free saplings of grafted mandarins have been made available for transplanting in the orchards for the first time in Nepal.

Key words: In vitro propagation, indexing, citrus greening disease, citrus tristeza virus, DAS ELISA and tip shoot grafting

INTRODUCTION

Mandarin orange (*Citrus reticulata* Blanco) is the most important fruit crop in the hills of Nepal. It is considered as an indigenous citrus fruit of Nepal (Shrestha and Verma, 1998). It has recently become a commercial crop in 49 out of 55 hill districts (NCDP, 1995). According to Agriculture Statistics Division, Ministry of Agriculture, the total area, productive area and production under mandarin are estimated at 9,146 hectares, 5,423 hectares and 57,425 metric tons respectively in 1997. This productivity (10.5 mt/ha) of mandarin is considered as very small compared to 50-90 mt/ha in the developed countries. This is due to citrus decline which is a complex problem caused mainly by various diseases such as citrus greening disease (CGD), citrus tristeza virus (CTV), phytophthora root rot and insect pests such as citrus brown aphids (*Toxoptera citricida*) and citrus psylla (*Diaphorina citri*).

Widespread use of susceptible seedlings and faulty cultivation practices in the hill terraces are mainly responsible for root rot in citrus (Ranjit and Gharti-Chhetri, 1997). It is suspected that CTV and CGD were inadvertently introduced to Nepal by lack of strict quarantine during importation of the planting materials. These diseases are now widespread throughout the major citrus growing belts in the country. Most of the citrus nurseries in the country are located below 1000m asl altitude. In lower altitude areas, insect vectors of many diseases are considered active because of favorable environment. The citrus psylla and brown citrus aphid are the vectors transmitting CGD and CTV respectively. Although the actual health status of the saplings

produced in lower altitudes has not been well studied, it is most likely that the saplings are infected by insect and graft transmissible diseases (Roistacher, 1991). A current serological study on incidence of CTV on different plants of citrus including the nursery saplings in Gorkha, Lamjung and Kathmandu has determined that more than 96% and 62% of the plants tested in Lamjung and Gorkha were infected (Ranjit *et al.*, 1998).

Citrus greening disease was first identified in Nepal by Knorr *et al.*, 1970 by thin layer chromatography using bark extracts. Different methodologies have since been employed for CGD diagnosis such as electron microscopy, immunoaffinity chromatography and double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using monoclonal antibody (Villechanoux *et al.*, 1990). Deoxyribonucleic acid (DNA) probes have been developed to detect different strains of CGD (Villechanoux *et al.*, 1992) and used to diagnose citrus samples from Nepal (Regmi, 1994). The latest method of CGD diagnosis was the molecular detection of causative agents of Asian greening as *Liberobacter asiaticum* using DNA amplification fingerprinting, DAF (Tamot and Gresshoff, 1999).

This paper aims to (1) develop protocols for tissue culture propagation of trifoliate orange (TO) and Madame Vinous sweet orange (SO), an indicator plant for testing CGD (Roistacher, 1991) (2) employ double antibody sandwich enzyme linked immuno-sorbent assay (DAS-ELISA) technique (Clark and Adams, 1977) to test CTV, (3) employ biological indexing using indicator plants derived from tissue culture to test CGD and 4.) produce disease-free grafted plants of mandarin inside screen

tunnels in Gorkha and Lamjung districts.

MATERIALS AND METHOD

Tissue Culture Propagation

Tissue culture propagation of TO (*Poncirus trifoliata*) and SO (*Citrus sinensis*) was initiated from meristematic shoot tips of seedlings grown in vitro on MS basal media (Murashige and Skoog, 1962) at high temperature, 38 ± 1 °C for 3 weeks. Seeds of TO were obtained from Horticulture Development Project, Kirtipur, Nepal and those of SO from University of California, Riverside, USA. Shoot tips of TO and SO were rapidly proliferated in multiplication media containing different concentrations of benzyl adenine (BA) and naphthalene acetic acid (NAA) according to Niroula, 1994 and Ranjit and Karki, 1999. Elongated shoot tips were induced to root in vitro at different concentrations of NAA in presence or absence of BA.

Transplanting in the Screen-House

Rooted shoot tips of TO and SO were transplanted on sterile mixture of soil, sand and compost (1:1:1) at the spacing of 10 cm X 10 cm inside insect-proof screen house. They were irrigated with UV-sterilized water and put under plastic cover for 2 weeks to maintain high humidity. After the plants were well established, they were supplemented with N, P and K fertilizers at the rates of 10, 10 and 5 g/m². When the basal stem size has attained at least 5.0 mm in diameter, the plants of TO were used for shoot tip grafting and those of SO were transferred to individual polythene pots for indexing.

DAS-ELISA

Leaf and pedicel samples of tissue cultured plants of TO and SO and mother plants of mandarin were collected from the screen house of GREAT and orchards of Gorkha and Lamjung and tested for CTV using DAS-ELISA according to Ranjit *et al.*, 1998. The antibody and conjugated antibody for CTV and the substrate were received from Bioreba, Switzerland.

Biological Indexing

In order to use the tissue cultured plants of SO as indicator plants for CGD, they were individually transferred in polythene bags filled with sterilized mixture of soil and compost (1:2) enriched with N,P,K and micro-nutrients and grown for six months. The mother plants were selected from only those plants that were found free from CTV (Ranjit *et al.*, 1998). The scions from CTV-free trees were collected and side-grafted on to the indicator plants according to the method described by Roistacher, 1996. The grafts were kept for observation inside the screen house for three months. The effect of grafting on the new leaves of SO was monitored by examining the typical symptoms of CGD such as zinc deficiency like interveinal chlorosis and small green blotches between veinlets.

Shoot Tip Grafting

CTV and CGD-free mother plants of mandarin were identified from various orchards of Gorkha and Lamjung districts (Ranjit *et al.*, 1998). Certified budwoods were collected from these trees and used for tip shoot grafting onto the tissue cultured rootstocks of TO. The rootstocks were first cut about 10 to 15 cm above the root-shoot

interface with a sterilized secateur and a short cleft was made on the top a little bit sideways from the center with a sterilized grafting knife. The budwood was selected with two to three buds and cut slantingly at one end. A short opposite cut was made to smother the tip. The scion was immediately inserted on the cleft of the rootstock making sure that the cambia of the stock and the scion were well connected at least at one side. Then the union was wrapped with a plastic strip and the graft was transplanted on a nursery bed inside a screen tunnel. A total of 3,500 grafts were made in this way and enclosed in four insect-proof screen tunnels set up at two locations namely Prithivinarayan and Tanglichok in Gorkha district and two namely Mohariyakot and Udipur in Lamjung district.

RESULTS AND DISCUSSION

Seedlings of trifoliate orange and Madame Vinous sweet orange were exposed to high temperature (38 ± 1 °C) for 3 weeks in a growth chamber to discourage viral replication and promote apical shoot tip elongation. Meristems were then excised using sterile techniques under stereoscopic microscope and established on filter paper bridge in liquid medium. Shoot tips derived from such meristems were cultured on MS multiplication medium supplemented with different concentrations of BA and NAA. The best combination of NAA for both species was found to be 0.1 mg/l whereas the optimum concentration of BA was 1.0 mg/l for TO and 0.5 mg/l for SO. As shown in Table 1, the proliferation rates were 6.8 and 6.6 for TO and SO in 6 weeks at these concentrations. Increasing the concentration of NAA had an adverse effect on shoot proliferation in both species. Similar results were previously reported by Niroula, 1994 and Ranjit and Karki, 1999.

As far as in vitro rooting of the shoot tips was concerned, NAA at 1.0 mg/l was most optimum compared to other concentrations for both species (Table 2). Six roots appeared in TO shoot tips whereas only 3.4 roots appeared in case of SO shoot tips. When the

Table 1. Shoot proliferation of Trifoliate orange and Madame Vinous sweet orange in MS medium with different plant growth regulators in 6 weeks

BA mg/l NAA mg/l	Trifoliate Orange		Madame Vinous sweet orange	
	0.5	1.0	0.5	1.0
0.1	4.2 $\pm$ 1.2	6.8 $\pm$ 1.2	6.6 $\pm$ 1.1	4.8 $\pm$ 0.8
0.5	2.8 $\pm$ 0.7	4.6 $\pm$ 1.0	2.8 $\pm$ 0.8	3.4 $\pm$ 1.0

Table 2. In vitro rooting (# of roots) per shoot tip of Trifoliate orange and Madame Vinous sweet orange as affected by auxin and cytokinin

NAA mg/l BA mg/l	Trifoliate Orange		Madame Vinous sweet orange	
	0.5	1.0	0.5	1.0
0	3.4 $\pm$ 0.5	6.0 $\pm$ 0.9	1.8 $\pm$ 0.8	3.4 $\pm$ 1.0
0.1	0	0	0	0

Table 3. Citrus tristeza virus (A 405±SD) in plantlets of Trifoliate orange and Madame Vinous sweet orange and mother plants of mandarin from Gorkha and Lamjung, 1998

Location	Total Sample	Virus-free sample	Infected sample	% Infection
Plantlets	10	10 (0.158±0.015)	0	0
Gorkha	140	53 (0.176±0.010)	87 (0.894±0.327)	62.1
Lamjung	156	5 (0.167±0.010)	151 (0.913±0.398)	96.8
A 405	-	< 0.200	> 0.200	-

concentration of NAA was reduced by 50%, the number of roots was also reduced almost by one half. Incorporation of cytokinin, BA even at 0.1 mg/l in the medium prohibited rooting completely in both species. Since the rooted plantlets should be completely free from all known pathogens, in vitro rooting was preferred to other means of rooting.

Before the in vitro plantlets were rapidly propagated, five samples each of TO and SO were used for CTV test using DAS-ELISA. Similarly, 296 samples of mother plants of mandarin from Gorkha and Lamjung were also tested. The virus test (Table 3) indicated that all the in vitro plants of TO and SO were free from CTV whereas 62.1% of Gorkha samples and 96.8% of Lamjung samples were infected with CTV. The absorbance values at 405 nm of less than 0.200 were considered as negative. Though the percent infection in Gorkha was less than that in Lamjung, the A 405 values for the negative and positive samples from both districts were not significantly different from each other.

The CTV-free mandarin plants from both districts were further tested for CGD using tissue cultured indicator plants of SO. The SO plants were raised and well protected inside screen as described by Ranjit and Karki, 1999. Scion wood was collected in winter and side grafted on the indicator plant as described by Roistacher, 1991. The symptoms were monitored on the new flush the following spring. The results (Table 4) indicated that 55% of the trees were infected with CGD in Gorkha compared to only 20% in Lamjung. The biological indexing was just a preliminary test for diagnosis of CGD. It could only be confirmed by the use of either DNA probes (Regmi, 1994) or DNA amplification fingerprinting (Tamot and Gresshoff, 1999). Since these tests are not yet routinely available in Nepal, biological indexing of SO against CGD should be promoted.

Table 4. Citrus Greening Disease in Gorkha and Lamjung districts, 1999

District	Total Sample	CGD +ve	% Infection
Gorkha	53	29	55
Lamjung	5	1	20

By performing these tests of CTV and CGD, it was finally possible to identify some mother plants that were free from both of these pathogens. Scion woods were collected from these disease-free mother plants and used for tip shoot grafting on TO inside the screen tunnels. As shown in Table 5, altogether 3,500 plants were grafted at 4

nurseries and 2,850 successful grafts were prepared in 1999. A total of 1,759 disease-free grafted plants of mandarin were made available for planting permanently in the orchards for the first time in Nepal.

Table 5. Production and distribution of disease-free mandarin grafts in Gorkha and Lamjung districts, 1999

Location of Nurseries	# Grafted	# Successful	# Planted
Prithivinarayan	1,000	700	415
Tanglichok	1,000	800	432
Mohariyakot	1,000	900	762
Udipur	500	150	150
Total	3,500	2,850	1,759

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