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ELIMINATION OF CITRUS TRISTEZA CLOSTEROVIRUS FROM CITRUS BUD-WOOD THROUGH THERMOTHERAPY

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Abstract

Citrus tristeza closterovirus (CTV) was eliminated from citrus bud-wood through thermotherapy in temperature controlled chamber (TCC). Three citrus species, sweet orange (*Citrus sinensis*), sour orange (*C. aurantium*) and Eureka lemon were mechanically and graft inoculated with CTV. The inoculated plants were indexed by double antibody sandwich-enzyme-linked immuno-sorbent assay (DAS-ELISA) to ensure the infection of the test plants. CTV was successfully eliminated from citrus bud-wood by incubating citrus germplasm at 35/30°C (day/night) for two weeks, followed by 40/35°C for another one week in preconditioning and then same germplasm was incubated at higher temperature 50/40°C for one week in TCC. CTV was not detected by DAS-ELISA or back inoculation of leaf extracts and graft inoculation on diagnostic plant species 3-4 months after inoculation. However, at higher temperature (50/40°C) treatment in TCC, 50% of plants were lost during one week of incubation. The plants kept at 50/40°C without preconditioning (35/30°C for two weeks, followed by 40/35°C for another one week) did not survive at higher temperature in TCC. CTV was completely eliminated from citrus plants after a week incubation period at 50/40°C.

Introduction

Citrus tristeza closterovirus (CTV) is an economically destructive pathogen of citrus fruits in many areas of the world. The virus is transmitted by aphid species in a non-persistant manner (Bar-Joseph & Lee, 1989; Bar-Joseph *et al*., 1989). Aphid species, *Toxopetera citricida* (Kirkaldy), *Aphis gossypii* (Glover) and *A. spiraecola* (Patch) are the most important virus vectors (Bar-Joseph *et al*., 1979; Raccah *et al*., 1989; Yokomi *et al*., 1994; Rocha-Pena *et al*., 1995). CTV is a monopartite virus with flexuous-filamentous particles of approximately 2000 x 11nm (Gonsalves *et al*., 1978; Bar-Joseph *et al*., 1979; Bar-Joseph and Lee, 1989). The virus genome contains plus sense single-stranded RNA of about 19,296 nucleotides (Karasev *et al*., 1995) encapsidated in 25kDa coat protein (Bar-Joseph & Lee, 1989; Sekiya *et al*., 1991; Karasev *et al*., 1995). The virus has numerous isolates differing in biological (symptoms and aphid transmissibility) and serological properties (Roistacher & Bar-Joseph, 1984; Ballester-Olmos *et al*., 1993; Cambra *et al*., 1989; 1993) and genome sequence (Karasev *et al*., 1995; Vives *et al*., 1999).

The incidence of CTV in citrus species has widely been reported from NWFP and the Punjab provinces of Pakistan (Catara *et al*., 1988). Recently, the incidence of CTV has gone up to 30% in citrus fruits in the NWFP and is the main cause of citrus decline in the province (Arif *et al*., 2001, 2004).

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The control of this economically important virus is achieved elsewhere by preventive methods such as quarantine, use of disease free bud-wood and CTV-tolerant rootstocks. Eradication programmes and the use of cross-protection strategies with mild isolates can also contribute to control of the disease (Roistacher & Moreno, 1991). The pathogen-free bud-wood could be produced through micro-propagation or *in vitro* shoot tip propagation, and elimination of virus and virus-like pathogens from infected bud-wood by thermotherapy (Roistacher & Calavan, 1972; Roistacher, 1977; Navarro & Juarez, 1977, Navarro *et al*., 1981; Nauer *et al*., 1983). Thermotherapy is a suitable and practicable technology and was first demonstrated successfully by Grant (1967) for the elimination of tristeza and psorosis viruses from citrus bud-wood and also for many other pathogens. Other pathogens, effectively eliminated were: concave gum, greening, infectious variegation, psorosis A and B, tristeza, seedling yellows-tristeza, vein enation (Lin & Lo, 1965; Calavan *et al*., 1972; Muller & Costa, 1974), stubborn (Roistacher, 1977) and greening (Schwarz & Green, 1972).

Production and distribution of CTV-free citrus bud-wood is extremely needed to save citrus plantation and associated industry. Infection of mother trees and bud-wood with virus, viroids and virus-like organisms reduce life expectancy or even kill trees, reduces fruit yield, size and number, induces nutritional and bud union problems and predisposes trees to frost injury. These pathogens spread via infected plants, bud-wood or vectors to regions previously free of them, become endemic and a hazard to citrus plantation (Roistacher, 1977). The present report describes the technique to produce CTV-free bud-wood through thermotherapy in temperature controlled chamber (TCC).

Materials and Methods

Plant culture and growth conditions: Young plants of sweet orange-Red blood (*Citrus sinensis* (L.) Osbeck) grafted on sour orange (C. *aurantium* L.) rootstock and sour orange plants were obtained from the Department of Horticulture, NWFP Agricultural University, Peshawar. Plants were transplanted in to 36 cm diameter clay pots using sterile potting mixture consisting of field soil, peat, sand and farm yard manure (1:1:1:1 v/v). Seeds of sour orange and indicator plants were also germinated in big tray containing above potting mixture and 1-2 month seedlings were transplanted in 26 cm diameter clay pots and kept in insect free screen house. Plant-lets of Eureka lemon were generated from cuttings in above soil mixture.

CTV-isolates: collection, preservation and inoculation: Leaf samples from CTV (DAS-ELISA) positive citrus trees from different orchards of 10 districts in the NWFP were collected in polythene bags. The samples were homogenized in pestle and mortar with 1:5 ratio of 0.1 M Potassium Phosphate buffer, pH 7.0 separately as described by Arif *et al*., 2005. Sufficient amount of carborundum powder was added during crushing to make a fine homogenate, passed through double layer of muslin cloth and immediately used for mechanical inoculation as reported earlier by Garnsey *et al*., (1977). Eighteen plants of each of sour orange, sweet orange and Eureka lemon were mechanically inoculated from the leaf extract from known infected trees. Similar number plants were inoculated with healthy plant sap or even with buffer. Inoculated plants were incubated in insect free screen house at 25°C for symptom development for maximum four months.

Detection of CTV through DAS-ELISA: DAS-ELISA was used for the indexing of citrus trees during field surveys and confirmation of CTV infection in sour orange and sweet orange mechanically inoculated test plants. The coating and labelled antibodies were obtained from Agdia, Elkhart, Indiana, USA. The procedure used has already been reported by Arif *et al*., (2005). The reaction was assessed visually or measured at 405 nm times with Titertek Multiskan, Model MC (Flow Laboratories Inc.). Samples were considered to be positive when the A_{405nm} values exceeded the mean of the virus-free samples by at least a factor of three.

Thermotherapy: CTV positive three batches (one batch consisting of six plants) each of *C. sinceses* (three years old) and *C. aurantium* (two years old) plants were incubated in growth chamber at 35/30°C for two weeks (16h day and 8h night temperature) with 1200 lux light. Plants were removed and indexed (by ELISA) and re-incubated at 40/35°C for one more week and re-indexed for virus infection. These plants in three batches were further incubated at high temperature (50/40°C) for one week in temperature controlled chamber. Additional water was applied through a tray kept beneath the pots to protect the plants from desiccation. The treated and untreated plants were returned to the screen house for further investigations.

Results

Sweet orange (*Citrus sinensis*) and sour orange (*C. aurantium*) were indexed for citrus tristeza virus (CTV) through DAS-ELISA. Presence CTV was confirmed by ELISA in the bark and leaves of selected sour orange and sweet orange plants 4-6 months after inoculation. The titre of CTV before and after thermotherapy is given in Table 1.

CTV was eliminated from infected plants by heat treatment. Preconditioning at 35/30°C for two weeks followed by another one week at 40/35°C could not eliminate CTV and other viruses in *C. sincencis* and *C. aurantium* (Table 2). Plants incubated directly at high temperature without preconditioning were desiccated and did not survive (Table 2). None of Eureka lemon plant survived during high temperature treatment (results not shown). However, CTV was successfully eliminated from citrus bud-wood by incubating citrus plants at 35/30°C for two weeks, followed by 40/35°C for another one week and finally incubating at 50/40°C for 1 wk in TCC (Table 2). Approximately, 50% of plants were lost during one week of incubation at 50/40°C treatment in TCC. The plants kept at 50/40°C at 35/30°C for two weeks and 40/35°C for another one week did not survive at high temperature in TCC (Table 1 & 2). CTV was completely eliminated from citrus plants after one week of incubation period at 50/40°C (Table 2).

Discussion

The control of viruses infecting citrus fruits is a challenge. Once a tree is infected with a virus species it remains infected and after certain period of time, the virus can kill the tree. There is no direct control method like chemotherapy to cure an infected plant/tree. However, certain alternative measures such as thermotherapy has been used to eliminate virus from the infected trees and to produce virus-free bud-wood in citrus fruits. This technique has been successfully used by several investigators for the elimination of CTV and other viruses and for the establishment of healthy clones (Desjardins *et al*., 1957; Calavan *et al.,* 1972; Roistacher & Calavan, 1974b). In this study, CTV-free citrus bud-wood has been produced using high temperature in temperature controlled chamber.

Table 1. Cirus tristeza virus concentration in citrus germplasm before and after thermotherapy treatment.

Citrus germplasm	Before treatment	40/35°C at		50/45°C
		1 week	2 week	1 week
C. sinensis				
1	++	+	Not survived	-*
2	++	++	+	Not survived
3	++	++	+	Not survived
4	+	+	+	Not survived
5	++	++	++	Negative
6	+	+	+	Not survived
7	+	+	+	Negative
8	+	+	+	Not survived
9	++	++	+	Not survived
10	+	+	+	Not survived
11	+	+	+	Not survived
12	++	+	Not survived	-
13	++	+	+	Negative
14	+	+	+	Not survived
15	++	++	+	Not survived
16	++	++	+	Not survived
17	+	+	+	Not survived
18	++	+	+	Negative
C. aurantium				
1	++	++	+	Not survived
2	+	+	Not survived	-
3	+	+	+	Not survived
4	++	+	+	Not survived
5	++	++	Not survived	-
6	++	++	+	Not survived
7	++	+	+	Not survived
8	++	++	+	Not survived
9	++	++	+	Not survived
10	+	+	Not survived	-
11	++	++	+	Negative
12	+	+	Not survived	-
13	++	++	+	Not survived
14	++	++	+	Not survived
15	+	+	+	Not survived
16	+	+	Not survived	-
17	++	++	Not survived	-
18	++	++	+	Negative

*- not treated; + = Positive; ++ = strong positive

Our results indicated that CTV was eliminated in *Citrus* plants when preconditioned at 35/30°C for two weeks, followed by 40/35°C for another one week and treatment at 50/40°C for another one week in TCC. The study showed that CTV was not eliminated

at low temperature and neither by direct high temperature, which is lethal for plants. CTV elimination requires gradual preconditioning for certain period of time with gradual increase in temperature. Similar observations have been made by Roistacher & Calavan, (1974a) where they mentioned that CTV can not be eliminated at preconditioning temperature but preconditioning plus supplemental heat is required to eliminate the virus from infected citrus budwood.

Table 2. Effect of thermotherapy for the elimination of citrus tristeza virus from sour and sweet orange plants.

Citrus species	Pre-conditioning			Temperature Controlled Chamber	
	Total plants tested	35/30°C		40/35°C	50/40°C
		1 week	2 week	1 week	1 week
Experiment-1					
Citrus sinencis Blood Red	18	18/0[a]	18/0	18/0	–
Citrus aurantium	18	18/0	18/0	10/2	–
Experiment-2					
Citrus sinencis Blood Red	18	–	–	–	0/-
Citrus aurantium	18	–	–	–	0/-
Experiment-3					
Citrus sinencis Blood Red	18	18/0[a]	18/0	12/4	4/4
Citrus aurantium	18	18/0	18/0	10/2	2/2

[a]Total number of surviving plants/ number of pathogen-free plants.

The mechanism of virus inactivation with heat therapy is not well understood. However, in some investigations it has been reported that heat treatment effect viral replication in plant cell system. Thermotherapy has been extensively used for the elimination of different viruses from various crop plants and in different viruses/plant infection. Different incubating temperatures and time period has been used depending upon infected plant species and the infecting virus. Sustained temperatures of 37°C or above completely inhibit multiplication of many viruses (Kassanis, 1957). In studies with tobacco mosaic virus (TMV), no evidence of viral RNA synthesis was detected when inoculated plants were maintained at 40°C for several hours (Dawson, 1976). However, when infected plants were moved from 25°C to 40°C, synthesis of viral dsRNA continued at high temperature for a period, slowed and then stopped (Dawson, 1978). During the slowing period, if plants were returned to 25°C, synthesis of virus RNA resumed. However, once dsRNA synthesis stopped at 40°C, the ability to resume viral RNA synthesis at 25°C was temporarily lost (Wu & Wallner, 1984). New synthesis occurred only after a 12 hours lag period. *In vivo* synthesis of three TMV-specific proteins (160, 110 and 17.5 kDa) was inhibited at 40°C in a pattern similar to high temperature inhibition of dsRNA synthesis (Dawson, 1983). The studies conducted on cowpea chlorotic mottle virus (CCMV) indicated that RNA synthesis was inhibited at 40°C. In contrast to TMV where dsRNA synthesis continued only briefly at 40°C,

synthesis of CCMV dsRNA declined gradually (Dawson *et al*., 1978). For both TMV and CCMV, synthesis of viral ssRNA ceased immediately at 40°C but resumed upon return to 25°C (Dawson, 1978, 1983). Increase in temperature above 35°C resulted in inhibition of synthesis of most pre-existing plant proteins followed by synthesis of a set of a new proteins referred to as heat shock proteins (hsp). *In vivo* synthesis of TMV specific protein was found to proceed independently of heat shock effects (Dawson & Boyd, 1987). When shifted from 40°C to 25°C, host RNA synthesis resumes immediately. However, there was a 4-8 hour delay before CCMV RNA synthesis resumed (Dawson *et al*., 1978) and a 16-20 hour delay before TMV RNA synthesis resumed. The exposure of infected plants to high temperature could eliminate synthesis of both coat protein and movement proteins. This would likely restrict cell-to-cell movement of pre-existing virus (Hull, 1989; Mink *et al.,* 1998).

In this preliminary investigation, a technique was developed for the elimination of CTV from infected plants using heat therapy. However, a comprehensive program for the elimination of CTV and other viruses and prokaryotic pathogens and for the production and distribution of certified pathogen-free bud-wood needed to be initiated and developed in order to protect the citrus industry from total collapse in NWFP, Pakistan.

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