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Citrus tristeza virus indexing and elimination from Satsuma mandarin (*Citrus unshiu* Marc.) cv. Kuno

Indeksiranje i eliminacija virusa tristeza iz mandarine Unshiu (*Citrus unshiu* Marc.) cv. Kuno

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ABSTRACT

The main grown citrus crop in Croatia is Satsuma mandarin (*Citrus unshiu* Marc.). The preliminary surveys have shown widespread occurrence of the most devastating citrus pathogen, *Citrus tristeza virus* (CTV) in Croatia. In this research four Satsuma mandarin cultivars: Chahara, Ichimaru, Kuno and Zorica Rana were biologically tested for the presence of CTV on Mexican lime (*Citrus aurantifolia* Swingle) bioindicator. Displayed symptoms strongly suggest the existence of mild CTV strains in four tested mandarin cultivars. Laboratory diagnostic methods (DTBIA, DAS-ELISA, IC/RT-PCR) confirmed these findings. Plants of cv. Kuno mandarin underwent sanitation treatments which consisted of thermotherapy and shoot-tip grafting (STG; micrografting) on *in vitro* germinated Troyer citrange [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck)] rootstock. Tristeza-virus was eliminated from 42% of infected 'Kuno' mandarins (confirmed by DTBIA). Completion of the above mentioned procedures will help development and implementation of citrus certification program in Croatia which would ensure the production of high-quality citrus propagating material in both sanitary and pomological aspects.

Key words: bioindicator, micrografting, certification

SAŽETAK

Najčešće uzgajana i komercijalno najznačajnija vrsta agruma u Hrvatskoj je mandarina Unshiu (*Citrus unshiu* Marc.), ponajviše zbog njene otpornosti na niske temperature karakteristične za ovo geografsko područje. U većini područja gdje se uzgajaju agrumi, kako u svijetu, tako i u nas, česta je pojava bolesti 'tristeza', najopasnije i najrasprostranjenije virusne bolest agruma, a čiji je uzročnik *Citrus tristeza virus* (CTV). U ovo istraživanje bila su uključena četiri kultivara mandarine Unshiu: Chahara, Ichimaru, Kuno i Zorica Rana, koji su podvrgnuti biološkom indeksiranju na meksičkoj limeti (Mexican lime; *Citrus aurantifolia* Swingle), najpogodnijoj indikatorskoj vrsti za detekciju virusa tristeza. Rezultati biološkog indeksiranja ukazali su na postojanje blagih sojeva tristeza-virusa u testiranim kultivarima, što je potvrđeno i egzaktnim molekularno-biološkim metodama (DTBIA, DAS-ELISA, IC/RT-PCR). Zaraženi klonovi kultivara Kuno podvrgnuti su postupku eliminacije virusa primjenom

toplinskog tretmana te cijepljenjem malenog vegetacijskog vrška plemke na bezvirusni sjemenjak podloge Troyer citrange [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck], prethodno uzgojen u uvjetima *in vitro* (mikrocijepljenje). Nakon kultiviranja mikrocijepljenih biljaka i adaptacije na stakleničke uvjete, virus tristeza eliminiran je iz 42% zaraženih biljaka cv. Kuno, što je potvrđeno testom DTBIA.

Rezultati ovog istraživanja trebali bi potaknuti izradu certifikacijskog programa za agrume, ali i za druge voćne vrste, koji bi omogućili proizvodnju visoko kvalitetnog sadnog materijala, kako u zdravstvenom, tako i u pomološkom pogledu.

Ključne riječi: mikrocijepljenje, biološko testiranje, certifikacija

INTRODUCTION

The citrus planted area in Croatia is located in the southern part between 42^0 and 43^0 $30^{'}$ of the northern latitudes, on the Dalmatian coast and on the islands, with the acreage of more than 2000 ha. The main commercially grown citrus crop is the most frost-tolerant citrus - Satsuma mandarin (*Citrus unshiu* Marc.). The choice of cultivars has been made in such a way that the ripening season is expanded to four months (from mid September until mid January). Lemon and sweet orange can be grown in some warmer microlocations on the Dalmatian islands (Vis, Brač).

Since 'tristeza' caused by *Citrus tristeza virus* (CTV) is one of the most devastating citrus diseases, we focused primarily on this virosis.

'Tristeza' (meaning sadness in Spanish and Portuguese) was the name originally used to describe rapid and widespread decline and death of millions of citrus trees on sour orange rootstock in Argentina and Brazil following introduction and spread of CTV in the 1930s (Wallace, 1978). Depending on a rootstock/scion combination, the most severe CTV strains can cause quick decline of citrus trees and/or stem pitting (SP) symptoms. As a consequence, the tree vigor and fruit size are reduced, which adversely affects fruit production (Wallace, 1978; Garnsey *et al*., 1998).

CTV is a member of the family *Closteroviridae* (Martelli *et al*., 2002), and the complete sequence and genetic organization of several CTV isolates have been determined (Karasev *et al*., 1995; Suastika *et al*., 2001)

The traditional method for detection of CTV infection in symptomless hosts is graft inoculation of Mexican lime plants followed by vein clearing symptoms observation in new flushes of lime tissue (Roistacher, 1991). Serological methods, especially ELISA (*Enzyme Linked Immunosorbent Assay*) and DTBIA (*Direct Tissue Blot Immunoassay*), have been used increasingly for CTV detection (Garnsey and Cambra, 1991; Garnsey *et al*., 1993; Djelouah *et al*., 2003). Recently, PCR (*Polymerase Chain Reaction*) is becoming a widely used method whenever higher level of sensitivity or specificity is needed (Niblett *et*

al., 1993). As a consequence of the high economic losses caused by virus and virus-like diseases, a shoot-tip grafting in vitro (STG, micrografting) has been developed for citrus.

Cv. Kuno is a highly yielding cultivar of midseason maturing period. With its high quality organoleptic characteristics, primarily with harmonious soluble solids to titratable acids ratio, it is distinguished among the other cultivars of proximately the same maturing period.

In this paper, we report the sanitary status of *C. unshiu* with regard to CTV presence, and sanitation of cv. Kuno Satsuma mandarin representing one of the first steps in obtaining high-quality citrus propagation material.

MATERIALS AND METHODS

Satsuma germplasm cultivar collection was established on the experimental field of the "Institute for Adriatic Crops and Karst Reclamation" in Kaštel Stari.

Four chosen cultivars of Satsuma mandarin (Chahara, Ichimaru, Kuno and Zorica Rana) were CTV-positive after testing by DTBIA (*Direct Tissue Immunoblotting Assay*; Garnsey *et al.*, 1993), DAS-ELISA (*Double Antibody Sandwich Enzyme Linked Immunosorbent Assay*; Clark and Adams, 1977) and IC/RT-PCR [(*Immunocapture Reverse Transcription Polymerase Chain Reaction*; Zemzami *et al.*, 2002); Table 1]. Plants were also subjected to biological indexing. Two-year-old seedlings of Mexican lime (*Citrus aurantifolia* Swingle), CRC grapefruit (*Citrus paradisi* Macf.), sour orange (*Citrus aurantium* L. and Madame Vinous sweet orange [*Citrus sinensis* (L.) Osbeck] were used for CTV-biological indexing. For symptoms severity, plants were scored using following scale according to Bonacalza (1998): 0- no symptoms, 1- mild, 2- moderate, and 3- severe symptoms.

Table 1. CTV-infected Satsuma mandarins (*C. unshiu* Marc.) detected by IC/RT-PCR DAS-ELISA and DTBIA in Croatia.

Tablica 1. Zaraženost kultivara mandarine Unshiu (*C. unshiu* Marc.) virusom tristeza u Hrvatskoj, testiranih metodama DAS-ELISA, IC/RT-PCR i DTBIA.

Cultivar/Kultivar	Method/Metoda		
	DAS-ELISA	DTBIA	IC/RT-PCR
Chahara	+	+	n.t.*
Ichimaru	+	+	+
Kuno	+	+	n.t.*
Zorica Rana	+	+	+

*not tested/nije testirano

Sanitation of Satsuma mandarin cv. Kuno was performed by using shoot-tip grafting *in vitro* (STG; micrografting) modified method of Navarro *et al.* (1975) combined with thermotherapy. Troyer citrange [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck] seeds were used as pathogen-free rootstocks for micrografting. Shoot-tips (0.3 mm) were grafted on decapitated two-weeks-old Troyer citrange seedlings grown *in vitro*. Micrografted plants were cultivated in Murashige and Skoog (1962) liquid medium supplemented with sucrose (30%). Cultures were incubated for two to three months at 25±2 ^{0}C under artificial illumination with light intensity of 15-30 $\mu Em^{-2}s^{-1}$, and 16 hrs photoperiod.

Acclimatization of grafted plants was performed by their direct transfer to pots containing sterilized soil mix suitable for citrus growing. Pots were covered with polyethylene bags and placed in a shaded area. After few months of acclimatization in the greenhouse conditions, 'Kuno' mandarins were tested for the presence of virus tristeza using DTBIA laboratory diagnostic method.

RESULTS

Two months after grafting four Satsuma cultivars (Chahara, Ichimaru, Kuno and Zorica Rana) on two-year-old Mexican lime seedlings, typical symptoms of 'tristeza' were observed (Table 2). Tested cultivars expressed mild to severe symptoms of leaf cupping, chlorosis, vein clearing, stem-pitting and stunting. The strongest reaction was observed in lime indicator which had been grafted with cv. Ichimaru (Table 2). Results of biological indexing on Mexican lime seedlings have been correlated with CTV infection diagnosed by DTBIA, DAS-ELISA and IC/RT-PCR (Table 1). Biological indexing on CRC grapefruit, sour orange and Madame Vinous sweet orange for detail characterization of CTV strains which are present in Satsuma mandarins is ongoing.

CTV-free plants of cv. Kuno mandarin were obtained by thermotherapy in combination with shoot-tip grafting *in vitro*. After sanitation, plants were tested for the presence of tristeza-virus using DTBIA test. Results of this test showed that 42% of 'Kuno' mandarins were CTV-negative.

Micrografted plants cv. Kuno were successfully acclimatized. Ninety percent survival with excellent subsequent growth was obtained and all plants displayed a normal phenotype.

Table 2. Biotest for CTV detection of four Satsuma mandarin (*C. unshiu* Marc.) cultivars from germplasm collection two months after grafting on Mexican lime (*Citrus aurantifolia* Swingle) bioindicator.

Tablica 2. Detekcija virusa tristeza u četiri kultivara mandarine Unshiu (*C. unshiu* Marc.) iz kolekcijskog nasada dva mjeseca nakon biotesta na bioindikatoru meksičkoj limeti (*Citrus aurantifolia* Swingle)

ultivar ultivar	Iexican lime/ Meksička limeta				
	*LC	CHl	VC	SP	RG
Chahara	[a]1/3	1/3	1/3	0/3	0/3
Ichimaru	2/3	2/3	2/3	2/3	2/3
Kuno	2/3	1/3	2/3	1/3	0/3
Zorica Rana	2/3	1/3	2/3	1/3	0/3

*LC: leaf cupping; CHl: chlorosis; VC: vein clearing; SP: stem-pitting; RG: retarded growth
LC savijanje listova; CHl: kloroza; VC: prosvjetljenje žila; SP: jamičavost drva; RG: kržljavost rasta
[a] According to Bonacalza (1998)
[a] Način praćenja simptoma: prema Bonacalza (1998)

DISCUSSION

The aim of this work was to determine the presence of CTV in four Satsuma cultivars from germplasm collection (Chahara, Ichimaru, Kuno and Zorica Rana) and CTV elimination from the cultivar Kuno. The assessment of cultivars sanitary status, before and after sanitation treatments were based on different types of assays. DAS-ELISA and DTBIA are based on CTV coat protein detection, while IC/RT-PCR higher detection sensitivity is due to virus capture by specific coat protein antibodies combined with subsequent genome fragment detection. In addition, strains from infected Satsuma mandarins, including cv. Kuno, were biologically tested on Mexican lime bioindicator. 'Tristeza' symptoms suggested the existence of mild CTV strains in investigated cultivars.

'Tristeza' is nowadays present in all citrus-growing regions. Previously performed laboratory tests showed that 40% percent of Croatian samples taken from the field were tristeza-positive (Černi *et al.*, 2005). Symptoms of tristeza disease are rarely visible on Satsuma mandarin in the field. This fact might be attributed to the tolerance of trifoliate rootstocks [e. g. *Poncirus trifoliate* (L.) Raf.] that are widely used for Satsuma grafting in Croatia. The existence of symptomless but CTV infected Satsuma trees, coupled with the illegal grafting practices infers high risk of CTV spread in the area. These features make the Satsuma mandarin the best candidate for the CTV reservoir host in the region.

The sanitation procedure resulted in 42% of 'Kuno' plants tristeza-negative, as determined by DTBIA test. Micrografted plants were transferred to pots and grown under greenhouse conditions with 90% survival rate which is in agreement with the 95% survival rate obtained by Navarro (1992).

Micrografting allows quite a satisfactory rate of successful grafts followed by recovery of pathogen-free plants. Several reviews, including different aspects of micrografting technique on other citrus species, have been previously published (Jonard, 1986; Navarro, 1988; Manganaris *et al.*, 2003; Hančević *et al.*, 2006). By using the shoot-tip grafting technique it is possible to recover plants free of pathogens that cannot be eliminated by thermotherapy, and the resulting plants do not have juvenile characteristic. Micrografting may also have an important application for basic research in the field of graft incompatibility, physiology of the graft union and in the research on plant aging.

Table 3a. Citrus cultivars grafted on different rootstock species.
Tablica 3a. Kultivari agruma cijepljeni na različite podloge.

Cultivar / Kultivar	Rootstock / Podloga			
	Poncirus trifoliata (Kaštela)	*P. trifoliata* 'Roubidoux'	lying Dragon trifoliate	Citrumelo
Owari	x	X		x
Armstrong	x	x	x	x
Aoshima	x	x		x
Kuno		x		x
Kawano Wase		x		x
Miho		x	x	x
Dobashi Beni		x		x
Nepolitana		x		x
Oroval Clementine				
Nules Clementine				
Sun Chu Sha mandarin		x		
Allen Eureka				
Frost Eureka				
Improved Meyer	x		x	x
Volkameriana				
Fukumoto	x		x	x
Skaggs Bonanza	x		x	x
Pineapple sweet orange				
Standard Seville sour orange				x

Table 3b. Citrus cultivars grafted on different rootstock species.
Tablica 3b. Kultivari agruma cijepljeni na različite podloge.

Cultivar / Kultivar	Rootstock / Podloga			
	Cleopatra mandarin	Sour orange (Vis)	icilian sour orange	Schaub rough lemon
Owari	x			
Armstrong	x			
Aoshima	x			
Kuno	x			
Kawano Wase	x			
Miho	x			
Dobashi Beni	x			
Nepolitana	x			
Oroval Clementine			x	x
Nules Clementine			x	x
Sun Chu Sha mandarin	x			
Allen Eureka	x		x	x
Frost Eureka	x		x	x
Improved Meyer	x		x	x
Volkameriana		x		
Fukumoto	x		x	x
Skaggs Bonanza	x		x	x
Pineapple sweet orange	x	x		x
Standard Seville sour orange	x			
Mexican lime	x	x		x
Marsh grapefruit	x	x		x
CRC grapefruit	x	x		x
Citrumelo		x		
Carrizo citrange	x	x		
Troyer citrange	x	x		

The highest rate of successful grafts was obtained with Troyer citrange two-week-old seedlings (Navarro *et al*., 1975; Edriss and Burger, 1984; Starrantino and Carruso, 1988) which is in agreement with our results. Grafting success depends on the degree of tissue differentiation of the rootstock, which is affected by light and age (Navarro, 1992).

The frequency of successful grafts increases with the size of shoot-tip, but the frequency of recovery of healthy plants decreases (Navarro *et al.*, 1975).

In this research, a method for elimination of virus tristeza from Satsuma mandarin cv. Kuno was established, with the eradication of 42%. The described method would be applicable for elimination of other citrus viruses. The technique, besides its utilization in viral infection control, is also valuable in

bacterial and fungal disease eradication that could be transmitted through traditional clonal propagation *in vitro*.

Different citrus rootstocks were successfully introduced primarily with the purpose of determining new compatible grafting combinations for Satsumas, oranges, lemons and grapefruits. The grafting of certified virus-free buds of 25 citrus cultivars on some of 8 different citrus rootstocks in overall 79 combinations (Tables 3a,b) was made with the purpose of finding the most suitable rootstocks for different citrus species and cultivars. The grafting plan was made due to chosen cultivars and rootstock seedlings and on the basis of the previously gained cognitions about the incompatibilities among some rootstocks and cultivars. Grafting was made on completely randomised design, with uneven variables in different combinations.

Further sanitation of infected important cultivars of Satsuma mandarin, as well as other citrus species, is a prerequisite for obtaining high quality foundation plant material. Implementation of an increase method will ensure healthy true-to-type nursery stock, as well as quick production of new cultivar budwoods for commercial nurseries.

CONCLUSION

The appearance of different symptoms on biological indicators suggests biological diversity among detected CTV isolates in the investigated 'Kuno' mandarin plants. *In vitro* grafting of small (0.3 mm) shoot tips on two-week-old Troyer citrange rootstock seedlings enables successful grafts were transplanted to soil with 90% survival rate. The efficiency of virus tristeza eradication from cv. Kuno Satsuma mandarin was tested by using the laboratory diagnostic method DTBIA. CTV elimination was confirmed in 42% of tested plants.

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