Efficiency of *Citrus Tristeza Virus* Isolates in Inducing Early Systemic Infection in Different Citrus Hosts

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Summary

Citrus tristeza virus (CTV) is the most destructive citrus pathogen. The knowledge about how its infectivity is accomplished, time in which CTV causes systemic infection after transfer to the host plant and the relation between virus invasion and CTV pathogenicity is very scarce. The main objective of this study was to analyse whether different CTV genotypes vary in efficiency to cause systemic viral infection. Three weeks post-inoculation, set of seven host-plant citrus species were checked for CTV systemic infection. Among seven CTV isolates tested, six of them were monophyletic according to the p*25* gene and previously biologically characterised. For one polyphyletic virus isolate the pathogenicity was established in this study. Our results showed that the transmission of virus and systemic infection is correlated with pathogenicity of CTV isolates, namely stem-pitting syndrome (SP). In three weeks CTV-SP isolates achieved systemic infection detectible by DTBA serological test. Polyphyletic isolate (composed of Gp 3b and Gp 5) that caused 100% infection, after biological characterization showed severe form of SP-syndrome. Monophyletic isolates belonging to Gp4, Gp3a and Gp2, which were determined as SP-CTV isolates, also achieved high percentages of systemic infection, respectively. On the contrary to severe SP isolates, isolates that are not related to appearance of SP disease symptoms were less efficient in accomplishing systemic virus infection. These results lead us to consider the aggressiveness of SP-CTV isolates as an important factor in processes of accomplishing early systemic plant infection.

Key words

Citrus tristeza virus, systemic infection, bioindicators, stem-pitting symptoms

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Received: January 30, 2014 | Accepted: March 31, 2016

ACKNOWLEDGEMENTS
This work was supported by the Ministry of Science, Education and Sports of the Republic of Croatia (grant: 091-0910468-0279).

Introduction

Citrus tristeza virus (CTV) is the causal agent of tristeza, devastating citrus virosis manifested through severe or mild pathogenic changes (Bar-Joseph et al., 1979; Moreno et al., 2008). CTV is a member of the *Closterovirus* genus (Karasev et al., 1995). The virions are flexuous and measure about 2000 nm in length and 11 nm in diameter and they are limited to phloem-associated cells in citrus trees (Karasev et al., 1995). CTV contains highly divergent positive sense, single stranded RNA genome that contributes to differentiations of its pathogenicity. CTV is transmitted vegetatively via infected budwoods and by aphids (*Hemiptera, Aphididae*) in a semipersistent manner (Moreno et al., 2008).

Viral genomic RNA is one of the main criteria for grouping CTV isolates, and it is a good basis for predicting the pathogenic potential. Several molecular methods for the detection and characterization of the virus "strains" (Moreno et al., 1997) were developed, among them, the combination of methods SSCP (*Single-Strand Conformation Polymorphism*) (Rubio et al., 1996; Ayllon et al., 1999; Sambade et al., 2003) and analysis of the nucleotide sequences as the most reliable method.

In some cases, CTV can be asymptomatic in infected plant, while in others the virus is pathogenic and induces disease symptoms. There are also plants resistant to all or some CTV genotypes (Garnsey et al., 1987 and 2005). Pathogenic CTV isolates may induce three characteristic disease syndromes: quick decline (QD), stem pitting (SP) and seedling yellows (SY). In early 1900s QD isolates destroyed entire citrus industries in South America. Currently, CTV continues to limit citrus production in much of a citrus producing world (Moreno et al., 2008). The virus causes a range of disease, but the phenotype that is presently the most economically important is SP. Trees with severe SP grow poorly, have lack of vigour, diminished yield, and fruits are unmarketable as a result of disrupted cambium development (Wallace, 1978; Garsney et al., 1998). In areas of the pits the cambium appeared to be missing and the lack of new xylem formation results in a depression or pits in the surface of the stem, as the normal areas continue increasing the girth of the stem (Brlansky et al., 2002). SY syndrome is characterized by chlorosis, lack of chloroplast development, reduced photosynthesis and growth (Dawson et al., 2013).

Aside from the viral genotype that is major factor influencing the disease outcome, it is also affected by environmental conditions and rootstock/scion combination (Moreno et al., 2008). CTV is an obligate intracellular parasite that depends on host cells for their genome replication and protein synthesis. Interference with differentiation and plant development results in disease symptoms appearance (Dawson et al., 2013). Understanding the processes of CTV-plant host interactions which lead to symptoms expression and viral spread could be crucial for predicting the type and intensity of disease outcome. In order to infect a plant, the virus first needs to enter in the cell and to overcome the constitutive and/or inducible plant defences to direct the plant cellular machinery for its viral multiplication (Whitman et al., 2006) The ability of viruses to cause systemic infection in host-plant and realize full pathogenic potential depends on virus ability to multiply, the possibility of interaction of viral proteins with complementary proteins in plant and the suppression of the host-plant defence responses to the viral infection (Culver and Padmanabhan, 2007; Folimonova et al., 2008; Tatineni et al., 2012). This processes trigered by the virus infection determine the gradient of susceptibility of different citrus species. Citrus genus contains many species, varieties, and intergenic hybrids with which CTV could interact causing a range of physiological and biochemical responses (Dawson et al., 2013).

In Croatia the citriculture is mainly oriented towards the production of Satsuma mandarins grafted on *Poncirus trifoliata* (L.) Raf. rootstock. This scion/rootstock combination usually does not display tristeza symptoms. Today, the presence of CTV is established in arround 40% of the tested Satsuma samples (Černi et al., 2009a). Moreover, genomic variants from the phylogenetic groups causing SP and SY have been found along with mild ones (Černi et al., 2009b). These findings were also confirmed by direct observation of SP symptoms in a Satsuma orchard (Černi et al., 2005). Considering all the facts, the Satsuma mandarin is a good candidate for the symptomless CTV reservoir host except in the case of severe SP isolates when disease symptoms can be displayed.

Although there have been considerable advances in the study of the CTV genetics the virus-plant interaction and in particular the mechanisms involved in the development of disease symptoms or plant resistance, are still poorly understood. The aim of this experiment was to determine the success of CTV systemic infection in different citrus hosts and by different CTV isolates under the same environmental conditions. The transfer of CTV isolates in the host-plant in relation to type and intensity of disease symptoms is discussed in the paper.

Material and methods

Prior to virus transmissions, the capsid gene of CTV isolates was molecularly characterized using *Single-Strand Conformation Polymorphism* analysis and sequencing to confirm their monophyletic status as previously reported (Hančević et al., 2013a). Isolates used in this study: 3, 440, 8, 57, 2 and 176 were representatives of the phylogenetic groups: Gp1, Gp2, Gp3a, Gp4, Gp5, and Gp M, respectively (Hančević et al., 2013a) based on the sequence analysis of p*25* capsid gene and their sequences were previously deposited in the GenBank under accession numbers: JQ655276-JQ655293. One virus isolate, isolate 32, was

Table 1. Origin of the genotypes used in this study and known syndrome expression

Phylogenetic group (Gp)	Virus isolate	Country of Origin	Syndrome expression*
1	3	Albania	QD, SY
2	440	Croatia	SP
3a	8	Madeira Island	SP, SY
4	57	Egypt	SP
5	2	Madeira Island	SY
M	176	Spain	SY
32	3b and 5	Italy	?

*QD-quick decline; SY-seedling yellows; SP-stem pitting

previously shown to be polyphyletic, composed of Gp3b and Gp5 CTV variants (Mounia, 2006). The list of the genotypes used in this study with basic information about its geographic origin and known symptomatology is given in the Table 1. Isolate 32 was also included in the study to explore its symptomatology on different citrus hosts.

Germinating and inoculation of plants

Healthy plants of seven virus-free citrus varieties (Mexican lime (*Citrus aurantifolia* L.), CRC grapefruit (*Citrus paradisi* Macf.), sour orange (*Citrus aurantium* L.), Madame Vinous sweet orange [*Citrus sinensis* (L.) Osbeck], Satsuma mandarin (*Citrus unshiu* Mac. Mak.), *Citrus wilsonii* Tan. and sour orange/ sweet orange were germinated from the seeds and cultivated in an insect-proof screen house for 18 months before the graft inoculation with the source tissue containing CTV-isolates. Except for Satsuma plants and *C. wilsonii* seedlings for which we hypothesis its bioindicative properties (Hančević et al., 2013b), all other citrus species were standard indicator plants used for diagnosis and biological characterization of tristeza disease. The inoculation was done as described by Roistacher (1991), with two bark and bud chips per each seedling. In total, 201 host plants were inoculated, Mexican lime, sour and sweet orange 42 plants each, 21 plants of *Citrus wilsonii*, grapefruit and Satsuma mandarin 20 plants each and 14 plants of sweet /sour orange.

Inoculated plants were maintained in shaded greenhouse with average temperature arround 27ºC. All plants were treated with N:P:K fertilizer (18:18:18) and urea when neccessary.

Testing inoculated plants for the virus presence

Three weeks post inoculation plants were tested for the virus presence using *Direct Tissues Blot Immunoassay* (DTBIA Agritest, Bari, Italy; Garsney et al., 1993). Crossection of young shoots were used to blot the plant juice to the nitrocellulose membrane. To establish the successful virus transmission, all samples were blotted in duplicate and checked for stained areas under the same stereomicroscope, at a 20x magnification which would indicate the presence of virus particles.

Observing symptoms for biological characterization of mix CTV isolate

Leaf changes were observed in the next vegetation season and SP was inspected one year post-inoculation. For the biological characterization of mix isolate 32, changes in inoculated lime plants were used to initially distinguish wheatear the isolate is mild or severe. Plants were inspected for vein clearing, leaf cupping and SP that, if observed indicated the presence of severe CTV isolate, especially if SP was one of the symptoms detected. SP potential of isolates was assessed in seedlings of Madam Vinous sweet orange (Rostacher, 1991; Garsney et al., 2005). Sweet orange plants were additionally observed for vein clearing of young leaves indicative of severe SP isolate (Roistacher, 1991). SP in seedlings of grapefruit was used to detect SP syndrome and measure the ability of isolates to cause SP in grapefruit, but also served as an additional indicator for SY in case of foliar changes appearance (McClean, 1960; Garsney et al., 2005). Seedlings of sour orange were primarily used to detect the SY syndrome induced by certain CTV isolates (McClean, 1960; Wallace, 1978; Garsney et al., 2005). SY-pathogenic potential in the most severe form can cause fully retarded plant growth of sour orange, followed by chlorotic changes in leaves and their reduced surface (Saustika et al., 2001). Plants composed of sweet orange scions grafted on sour orange seedling rootstocks were used to evaluate changes that reflect a CTV-induced injury to the phloem at the bud union which is associated with typical tristeza decline syndrome (Schneider, 1959).

Results and discussion

In this experiment we have determined the success of early systemic infection in the host plant, e.g. the transfer of CTV only three weeks after inoculation with isolates belonging to different phylogenetic groups based on p*25* gene. Beside isolates previously determined as monophyletic and with already described biological properties (Hančević et al., 2013a), in this study we also introduced polyphyletic isolate and explored its pathogenicity on standard bioindicator plants (Table 1), so the induced symptoms could be compared to the efficiency of virus transmission and systemic infection caused by heterogeneous CTV isolate.

Three weeks after inoculation the success of the virus transmission in inoculated plants was checked by DTBIA serological test.

Bioindicators significantly differed in a systemic infection degree depending on virus isolates and type of plant (Table 2). For the most efficient virus detection, the choice of plant tissue and time of sampling are important and should be considered when a certain protocol is applied. As DTBI technique is equally

Table 2. The ratio of infected and inoculated host- plants expressed in percentages, three weeks after inoculation with different CTV isolates

CTV-isolate/ phylogenetic group	Mexican lime	Sour orange	Sweet orange	Grapefruit	Sweet on sour orange	Satsuma mandarin	*Citrus wilsonii*	Percentage of infection
2/5	33.3	17.7	50.0	66.7	50.0	66.7	66.7	44.9
3/1	50.0	33.3	33.3	66.7	100	66.7	66.7	51.7
8/3a	100	83.3	83.3	100	100	100	100	96.5
57/4	100	66.7	66.7	100	100	100	100	82.5
176/M	50.0	50.0	50	100	100	100	100	69
440/2	66.7	66.7	83.3	100	100	100	66.7	79.3
32/3b + 5	100	100	100	100	100	100	100	100
Total percent of infection	71.4	59.6	66.7	90.5	92.9	90.5	85.7	

Figure 1. Foliar symptoms of *Citrus tristeza virus* caused by polyphyletic isolate 32:a) vein clearing and chlorosis on Mexican lime, b) chlorosis and seedlings yellows on grapefruit seedling, c) chlorosis and stunting of sour orange, d) local chlorosis on sweet orange.

efficient as ELISA test for routine CTV detection (Hančević et al., 2012), we used it to check virus presence by blotting young shoots on nitrocellulose membrane. According to the positive DTBI reactions, our results showed that early transfer of virus and its sufficient multiplication to the level of DTBI detection in high percentages occurred in sweet on sour orange, grapefruit, Satsuma mandarin and *C. wilsonii*. Although Mexican lime is considered as a general and most susceptible bioindicator of tristeza disease and presumably good host for virus multiplication (Folimonova et al., 2008), its susceptibility to CTV infection was mild.

In this study, we used fresh buds and bark of infected material in order to inoculate bioindicators and investigate the virus transfer and the appearance of systemic infection short after inoculation, e.g. three weeks. Although the reception of buds and bark was 100%, the efficiency of certain virus isolate to provoked systemic infection in three weeks differed significantly not only among the plant hosts but also among the CTV isolates. The largest proportion of infected plants was recorded after viral inoculation with polyphyletic isolate 32 (composed of variants belonging Gp 3b and Gp5). This virus isolate achieved 100% of infection. CTV isolate 8 (Gp3a) was successful in infecting 96.5% of inoculated plants, followed by isolate 57 (Gp4, 82.4%) and isolate 440 (Gp2, 79.3%), while the isolate 2 was the least efficient (Gp5, 44.9%) (Table 2). The test was conducted in order to make sure that the hosts are really infected with the virus. Such test could be used in the future as an indicator of virus invasion even in the symptomless cases.

The results of serological analysis of the CTV infection in this study for almost all isolates showed the proportional relationship between the percentages of plant systemic infection with the intensity of developed symptoms. The latter was analyzed in this study for the polyphyletic isolate and by Hančević et al. (2013a) for the monophyletic isolates with regard to p*25*. Taking into account that virus hosts used here beside Satsuma mandarin, are all well-known bioindicators that have been evaluated in the terms of developing specific tristeza symptoms, results of our study showed correlation between early transfers of virus with the severity of stem-pitting syndrome (SP). The ability of viruses to cause systemic infection in some host depends on the compatibility interactions between viral and host factors when the expression of plant and virus genes are differentially altered resulting in physiological and cellular plant changes. When the virus evolves to acquire virulence factors to counteract plant basal defence it is in the position to trigger the viral infection. The firm connection between specific viral genes and efficiency of virus to cause systemic infection has not yet been established. The three-kb-terminal region that includes CP-gene sequences also contains other genes whose products have been proven to modulate the host response through post-transcriptional gene silencing (Lu et al., 2004) and those are also the pathogenicity determinants, e.g. p*23* (Ghorbel et al., 2001; Fagoaga et al., 2005; Albiach-Marti et al., 2010). Also the gene p*25* was suggested to be one of the pathogenicity determinants (Nolasco et al., 2009), and for the SP symptom development genes p*13*, p*18* and p*33* are considered to be involved in enhancement or attenuation of the tristeza disease in some hosts (Tatineni and Dawson, 2012). The same authors suggested that SP phenotype can result not from a specific sequence or protein but from a balance between the expressions of different viral genes. In that sense, the combination of virus genotype, their expression and citrus host direct the final outcome of tristeza disease. The polyphyletic isolate did not only displayed appreciated invasion potential independently of citrus host (100% transfer to tested hosts), but the developed symptoms and the final disease outbreak was very severe (Figures 1 and 2). Mexican lime, a general indicator of tristeza disease showed the symptoms of vein clearing (Figure 1a) and leaf cupping even in old leaves, addressing to the presence of severe CTV isolate. Young leaves of grapefruit were chlorotic and new shoots were stunted (Figure 1b). The same reaction was observed in sour orange (Figure 1c) leading us to the conclusion about the SY-potential of mix isolate. Even the leaves of sweet orange plants, which usually very rare display leaf symptoms, were locally chlorotic (Figure 1d). Stem pitting was found in Mexican

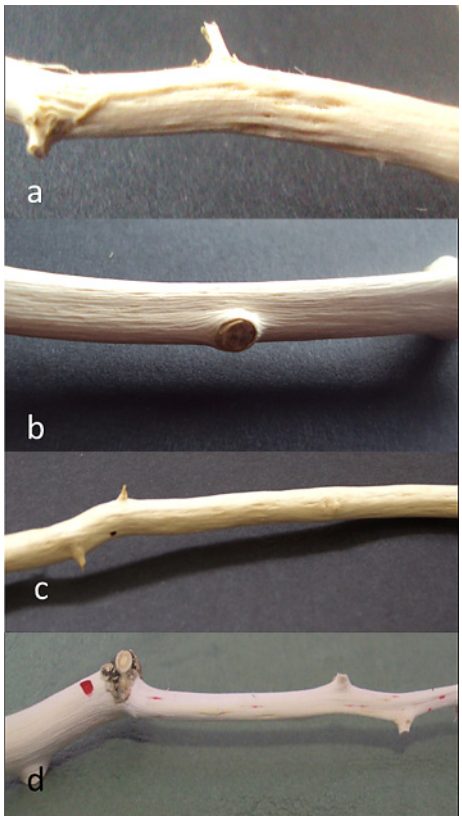

Figure 2. Stem-pitting (SP) symptoms caused by *Citrus tristeza virus* polyphytetic isolate 32. a) severe SP on Mexican lime branch, b) mild to severe symptoms of SP on sweet orange, c) mild to severe SP on grapefruit stem, d) mild SP on lateral branch of sweet orange on the sour orange rootstock

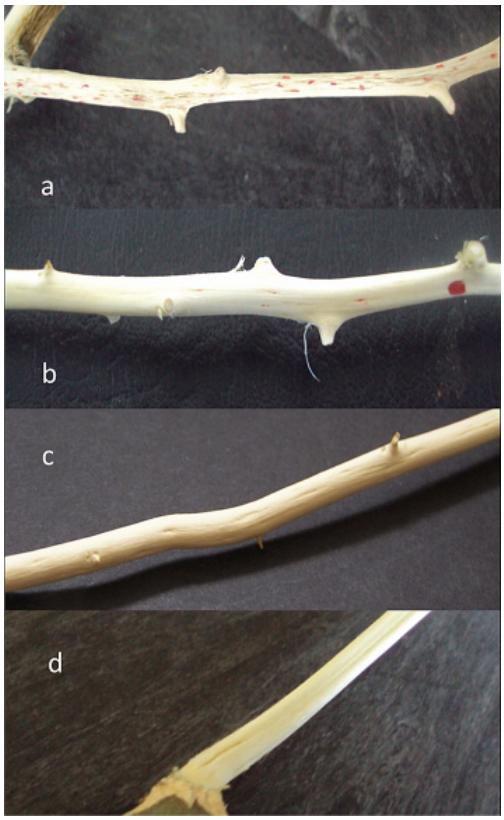

Figure 3. Stem-pitting (SP) symptoms on citrus stems caused by different *Citrus tristeza virus* isolates: a) severe SP on Mexican lime one year after inoculation with Gp4, b) mild to severe SP symptom on sweet orange caused by isolate belonging to Gp3a, c) mild to severe SP on grapefruit seedling provoked by Gp3a isolate, d) mild SP on sweet on sour orange caused by Gp2.

lime inoculated with isolate 32 (Figure 2a) and in sweet orange (Figure 2b) in severe form. In grapefruit plants (Figure 2c) it was also mild to severe. In sweet on sour orange (Figure 2d), and *C. wilsonii* SP was observed in mild form suggesting the severe SP pathogenic potential of 32 polyphyletic isolate. The remarkable feature of stem pitting syndrome is the high degree of specificity. For example, some CTV isolate can provoke SP reaction in sweet orange, but not in grapefruit (Garsney, 2005; Hilf et al., 2005). In our study polyphyletic isolate caused SP in all relevant SP bioindicators as well as in *C. wilsonii* and sweet/sour orange. Isolate 32 caused the most severe SP and other symptoms comparing to all tested isolates. As stated before, the mechanism of symptoms expression is not known, but the interaction between proteins of different CTV isolates present in the inoculum, could affect the symptoms appearance (Nchongboh et al., 2014).

The isolates 8 (Gp3a), 57 (Gp4) and 440 (Gp2) achieved in this study virus transmission in more than 79% cases and all of them are already known to cause severe tristeza symptoms (Černi et al., 2009b; Hančević et al., 2013a). The large proportion of infected plants (96.5%; Table 2) was recorded after viral inoculation with isolate 8 (Gp3a), a monophyletic isolate that causes serious SP in sweet orange (Figure 3b) and grapefruit (Figure 3c) and SY in grapefruit plants, as was previously demonstrated in different studies (Yang et al., 1999; Ruiz-Ruiz et al., 2006; Černi et al., 2009b). Our results also showed that 57 (Gp4) and 440 (Gp2) achieved virus transmission in 82.5 and 79% cases, respectively (Table 2), which correlates with its SP severity (Garsney 2005). For the virus isolate belonging to Gp4 it is already known that it induces SP symptoms in Mexican lime (Figure 3a), sweet orange, sweet on sour orange, grapefruit, and *C. wilsonii* (Garsney 2005; Hilf et al., 2005; Hančević et al., 2013a). Isolate 440 (Gp2) induces SP symptoms in all relevant indicators except sweet orange plant (Figure 3d; Hančević et al., 2013b). In the case of mild isolate 176 (GpM), only 69% of inoculated plants were tested positive to systemic infection and for isolate 2 (Gp5), successful infection of healthy plants was 44.9%. Regarding isolate 3 (Gp1) we expected greater efficiency in virus transmission since it is potentially highly pathogenic virus isolate, causing SY and QD syndrome, but systemic infection has been achieved in only 51% of the plants. Although it is considered as one of severe isolates due to SY and QD (Hilf et al., 2005; Nolasco et al., 2009) the absence of SP potential might be related to lower success of early CTV transmission.

Conclusions

There is an elevated complexity in the *Citrus*-CTV interactions that generate processes like pathogenesis or plant host resistance. Although there have been considerable advances in the study of the CTV genetics, there is essentially no understanding of how combinations of genotypes affect symptom expression and disease severity (Harper, 2013). This study provides the evidence that appearance of early CTV systemic infection is closely related to the stem-pitting CTV isolates. The relationship between the percentages of plant infection and severity of SP is proportional. We can assume that different manners of SP-CTV isolates are caused by the different virus-host interactions. In that way this study offers a new perspective and starting point for further examination of interactions between viruses and their hosts, the pathways of their infection and changes associated with early and late viral infection. Special attention should be given to the reactions associated with tolerant or resistant plants which can induce severe SP symptoms, as Satsuma mandarin on trifoliate rootstock. Better understanding of mechanisms of viral infection in test-indicators but also in plants of commercial value can lead to the improvement of citrus production.

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acs81_05