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Keywords: CTV, viroids, citrus, nursery

Abstract: In Italy, Citrus tristeza closterovirus (CTV) was found several times in the last decades and Plant Protection Services (PPSs) are involved in monitoring plans and surveillance activity. Beside well-monitored orchards linked to citrus industry, the occurrence of virus or virus-like disease in ornamental nurseries may represent an under notice risk factor. A CTV monitoring program was modified with the aim to include other viruses (Citrus variegation virus, CVV; Citrus psorosis virus, CPsV) and viroids (Citrus exocortis viroid, CEVd; Hop stunt viroid, HSVd; Citrus bent leaf viroid, CBLVd; Citrus dwarfing viroid, CDVd; Citrus bark cracking viroid, CBCVd). Ornamental mother plants were monitored for four-years throughout application of cost-saving multiplex RT-qPCR protocols. CTV incidence was 1.6-13.5%, with an average distribution of 11.9%. CVV and CPsV average incidence was 6.3% and 2.7%, respectively. Higher CTV, CVV and CPsV incidences were observed in C. x paradisi, C. grandis and C. x clementina. The most widespread viroid is CEVd (32.9%), frequently observed in C. x limonia and C. limon. HSVd (10.5%) and CDVd (7.1%) were mostly found in C. x limonia. Lower infection rates were observed for CBLVd (2.0%) and CBCVd (1.4%). However, nurseries' response to PPS' virus alert seems partially effective, because CTV incidence was reduced in nurseries re-checked after first detection, but the pathogen was not eradicated from two nurseries out of three. Furthermore, occurrence of viroids was reduced in just one nursery. Concluding, multiplex diagnosis of viruses and viroids in ornamental citrus nurseries should offer useful alert to nurseries, due to repeated survey of harmful viruses and high widespread of viroids.

Plant health management for ornamental nurseries

Occurrence of viruses and viroids in ornamental citrus mother plants in Tuscany (Central Italy)

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Keywords

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Abstract

In Italy, Citrus tristeza closterovirus (CTV) was found several times in the last decades and Plant Protection Services (PPSs) are involved in monitoring plans and surveillance activity. Beside well-monitored orchards linked to citrus industry, the occurrence of virus or virus-like disease in ornamental nurseries may represent an under notice risk factor. A CTV monitoring program was modified with the aim to include other viruses (Citrus variegation virus, CVV; Citrus psorosis virus,

CPsV) and viroids (Citrus exocortis viroid, CEVd; Hop stunt viroid, HSVd; Citrus bent leaf viroid, CBLVd; Citrus dwarfing viroid, CDVd; Citrus bark cracking viroid, CBCVd). Ornamental mother plants were monitored for four-years throughout application of cost-saving multiplex RT-qPCR protocols. CTV incidence was 1.6-13.5%, with an average distribution of 11.9%. CVV and CPsV average incidence was 6.3% and 2.7%, respectively. Higher CTV, CVV and CPsV incidences were observed in *C.* x *paradisi*, *C. grandis* and *C.* x *clementina*. The most widespread viroid is CEVd (32.9%), frequently observed in *C.* x *limonia* and *C. limon*. HSVd (10.5%) and CDVd (7.1%) were mostly found in *C.* x *limonia*. Lower infection rates were observed for CBLVd (2.0%) and CBCVd (1.4%). However, nurseries' response to PPS' virus alert seems partially effective, because CTV incidence was reduced in nurseries re-checked after first detection, but the pathogen was not eradicated from two nurseries out of three. Furthermore, occurrence of viroids was reduced in just one nursery. Concluding, multiplex diagnosis of viruses and viroids in ornamental citrus nurseries should offer useful alert to nurseries, due to repeated survey of harmful viruses and high widespread of viroids.

Introduction

Citrus tristeza virus (CTV) is widespread throughout tropical citrus-growing areas but concerns are raising in Europe, where strict quarantine measures are necessary to avoid the introduction of CTV into countries where the virus is not present. Measures to control CTV damage include quarantine and budwood certification programmes and elimination of infected trees (Moreno et al., 2008), deeply involving Plant Protection Services (PPSs) for health check and post-diagnosis procedures.

In Italy, where the virus was found several times in the last decades (Djelouah et al., 2009; Davino et al., 2013), PPSs are involved in monitoring plants and surveillance activity. While citrus trees are mainly grown in southern Italy and are related to citrus industry, several ornamental citrus plants

52 are cultivated in areas not thoroughly involved in citrus industry, such as Central or North Italy. In
53 these areas, the occurrence of citrus diseases may be under notice due to limited cultivation but
54 virus or virus-like infections of mother plants of ornamental citrus could lead to severe widespread
55 of diseases in importing countries within the European Union (EU).
56 For PPSs, the main component of costs for diagnostic molecular test (i.e. PCR, RT-PCR, qPCR) are
57 sample collection (due to staff and travel costs), sample preparation and extraction of nucleic acids.
58 These time-consuming tasks increase staff costs if carried out manually, whereas the use of
59 semiautomatic grinders or automated DNA/RNA extraction systems increase equipment costs.
60 Thus, once sample was collected and prepared, the marginal cost of multiple pathogen recognition
61 is reduced: this is the main reason of using multiplex detection protocols when available. Besides
62 CTV, other viruses cause concern in citrus cultivation, such as Citrus variegation virus (CVV) or
63 Citrus psorosis virus (CPsV) (Gonsalves and Garnsey 1975; da Graça et al. 1991; Martín et al.
64 2004; Velázquez et al., 2016) which monitoring by PPS should support nursery activities and
65 improve trust policies among stakeholders. The opportunity to investigate the presence of various
66 viruses is underlined by development of multiplex reverse transcription quantitative PCR (RT-
67 qPCR) protocols (Loconsole et al., 2010; Osman et al., 2015).
68 Citrus are also natural hosts of several viroid species (Flores et al., 2005; Ding, 2009), wh role
69 on symptoms expression was investigated (Murcia et al., 2015). Exocortis and cachexia are severe
70 diseases caused by Citrus exocortis viroid (CEVd) and Hop stunt viroid (HSVd), respectively.
71 While viroids such as Citrus bent leaf viroid (CBLVd), Citrus dwarfing viroid (CDVd), or Citrus
72 bark cracking viroid (CBCVd) may cause little effects on fruits, the infection may reduce height and
73 canopy volume (Bani Hashemian et al., 2010; Rizza et al., 2011; Murcia et al., 2015). Significant
74 effects can be also observed on rootstocks (Polizzi et al., 1991). Commonly, their control is based
75 on preventive measures as availability of viroid-free budwood as a source of propagation material
76 follow by adequate indexing procedures (Eiras et al., 2009). Even if studies are more oriented to

fruit trees, these alteration may also play a significant role in ornamental citrus and many molecular diagnostic techniques for viroids are available (Luigi and Faggioli, 2013; Gucek et al., 2017).

In this paper we investigated the impact of viruses and viroids in ornamental nursery in Tuscany (Central Italy) using multiplex RT-qPCR protocols. Thus, in order to provide enhanced service to farmers, the CTV monitoring program was modified with the aim to include emerging but yet not regulated pathogen such as viroids and evaluate farmers' response to PPS pest alerts.

Materials and Methods

Leaf samples were collected in 2012-2015 from ornamental mother plants of *Citrus* spp. (19 species), *Fortunella* spp. (6 species), *Microcitrus* spp. (3 species), *Poncirus trifoliate* and hybrids (23 *Citrus* spp. hybrids, *C. aurantifolia* x *F. margarita*, *C.* x *sinesis* x *P. trifoliata* x *C.* x *paradisiaca*, *Citrange Morton*, *Eremocitrus glauca* x *C.* x *sinensis*, *F. margarita* x *C.* x *clementina*, *M. australasica* x *F. margarita*). Plants (124 in 2012, 228 in 2013, 193 in 2014 and 169 in 2015) were grown in open field conditions in 15 nurseries located in two Tuscan districts (Central Italy). In each nursery, sampling was representative of each lot of grown plants.

Occurrence of virus and viroids was also analyzed in three nurseries in which CTV was detected. In these nurseries, CTV-infected plants were destroyed within 6 months from diagnosis and farmers were informed about whole health status of tested mother plants. Two year later since first PPS' alert to CTV infection, different lots of mother plants were checked for viruses and viroids, comparing results of the two health check.

Total RNAs (TNAs) from citrus tissues were extracted from 0.2 g of leaf petioles after homogenization with the Mixer Mill MM 400 (Retsch, Germany), following Foissac et al. (2001). TNAs were then eluted in 150 µl of RNase free water and their concentration was determined using a UV-vis Spectrophotometer. RT-qPCR reactions were performed in a final volume of 25 µl on the CFX96TM Real time System (Biorad). Loconsole et al. (2010) was followed for TaqMan probes,

primers and amplification conditions of CTV, CVV and CPsV. Viroids were assessed following Wang et al., 2009.

Results

Viruses and viroids distribution during 2012-2015 was reported in Table 1. Incidence of virus or viroid infection in analyzed species/hybrids was reported in Table 2.

CTV, the main target of monitoring, was widespread in the monitored area. Even if CTV was not so frequent during the first year of monitoring (l.6%), the pathogen was retrieved in the following years, reaching more than 13% of infection rates in the third year. In 2012-2015, the virus was found in more than 21% of tested plants of *C.* x *paradisi*, but high infection rates were also observed in *C. deliciosa* (18.2%), *C. limon* (12.8%) or *C.* x *Limonimedica Florentina* (12.5%). However, the virus was found in six species/hybrids out of more than 25 tested. Distribution of CVV was 3.6-10.5% but it is more widespread among species, involving 10 different species/hybrids. The virus was particularly frequent (>25% of infected plants) in *C. grandis*, *C. bergamia* and *C. aurantifolia*. Conversely, CPsV, which infection rates in 2012-2015 were globally below 5%, was limited to four species/hybrids, with a quite high frequency in *C.* x *clementina* (17.6%).

Mixed infections of viruses were quite uncommon, involving just more than 8% of infected plants (Table 3). However, CTV was found in mixed infection with both CVV and CPsV. Triple mixed infection was not observed.

All investigated viroids were found during survey. CEVd was the most widespread viroids, which incidence was over 30% in three different years of monitoring (average infection of 32.9%). The pathogen was detected in almost all analyzed species/hybrids, with more than half of plants infected for *C. limon* and *C.* x *limonia*. Another widespread viroid was HSVd, which presence was confirmed in 10.5% of analyzed plants. This viroid, highly widespread among species, was

frequently found in *C.* x *limonia* (42.9%), *C.* x *Limonimedica Florentina* (37.5%) and *C.* x *lumia* (33.3%). CDVd was found in more than 7% of analyzed plants and infections were observed in 15 different species/hybrids. High infection rate was observed in *C.* x *limonia*, *C.* x *lumia* and *F. margarita* x *C.* x *clementina*. Distribution of CBLVd and CBCVd was lower than other viroids, 2.0% and 1.4%, respectively. CBLVd was observed in *C. limon* and two hybrids, while CBCVd infected mainly *C.* x *Limonimedica Florentina*.

Even if single infection of viroid was the most frequent status for tested plants, mixed infections were also quite common, with almost 40% of incidence (Table 3). The most frequent combinations of mixed infections involved the most frequent viroids, CEVd, HSVd and CDVd. This finding was underlined by the high frequency of CEVd/HSVd/CDVd mixed infections, which incidence was higher than 10%.

In relation to species/hybrids, a worrisome health status was observed in *C. limon* which was the host for all tested viruses or viroids. Furthermore, *C. medica* and *C.* x *sinensis* seems common hosts for all viruses and most of viroids.

Occurrence of CTV was analyzed in nurseries subjected to repetitive health check (Table 5). Nurseries' response to PPS alert seems partially effective, because CTV incidence was reduced in each farm, even if the pathogen was not eradicated from two nurseries out of three. A similar behavior in nursery response was observed for CVV and CPsV (Table 5). Conversely, occurrence of viroids was lower for in one nursery (N1) while increase in the other two farms.

Discussion

In Spain, citrus cultivars free of virus and virus-like pathogens became available thanks to implementation of a citrus improvement program, and they cover today more than 70% of the citrus-growing regions of the country (Bani Hasheimian et al., 2010), confirming the role of quarantine, certification programmes and elimination of infected trees in protecting citrus

155 production. Based on the current legislation, the finding of CTV in a nursery would implicate the
156 mandatory destruction of the entire lot of plants. Thus, repetitive findings of CTV during 2012-
157 2015 underline a worrisome health status of ornamental citrus mother plants in Tuscany, even if
158 CTV was less widespread than in other producing regions (Abbas et al., 2015). Our observation
159 indicates as CTV incidence was reduced in nurseries re-checked two years after first detection, but
160 the pathogen was not eradicated from two nurseries out of three. Furthermore, the health status
161 seems threaten not only by CTV, but by two further viruses and a plethora of viroids. Conversely
162 from viruses, which may be strongly regulated (as CTV) or which diseases are well known by
163 nurseryman, viroids may represents an unnoticed - but harmful - menace.
164 High widespread of citrus viroids was previously assessed in commercial fruit trees in Greece
165 (Barbarossa et al., 2007), Uruguay (Pagliano et al., 2013) and low performing orchards with high
166 incidence of viroids were also observed in Spain (Bani Hasheimian et al., 2010). However, few data
167 is available for ornamental plants. The pospiviroid status of ornamental plants was assessed in Italy
168 for solanaceous ornamental genera, but beside high incidence of Potato spindle tuber viroid
169 (PSTVd), their incidence was low (Luigi et al., 2011). In Tuscany, CEVd and HSVd occurred in 45
170 and 9% of plants in citrus orchards during a survey carried out about ten years ago (Ragozzino et
171 al., 2005), while higher incidence was absorbed in the close region of Lazio (Central Italy)
172 (Ragozzino et al., 2005).
173 Our findings indicate as viroids are commonly widespread in Tuscan ornamental citrus tree
174 nurseries, reaching almost 50% of incidence for CEVd. Furthermore, our findings indicate high
175 frequency of mixed infections of viroids in ornamental citrus trees. Interactions among viroids co-
176 infecting the same tree could affect symptom expression and field performance (Vernière et al.,
177 2006). However, long-term field assays also revealed that viroid-induced effects might be
178 attenuated or enhanced when trees were exposed to mixed viroid infections (Vernière et al., 2006,
179 Vidalakis et al., 2010). Since the primary mode of transmission of most viroids is through
180 mechanical means, consideration must be given to the potential spread of the viroid by equipment

used in farm operations (Barbosa et al., 2005; Eastwell and Nelson, 2007). As reviewed by Kovalskaya and Hammond (2014) current effective control methods for viroid diseases include detection and eradication, and cultural controls, as well as CTV protection programme.

Concluding, multiplex diagnosis of virus and viroids in Tuscan ornamental citrus nurseries should offer useful alert to nurseries (not just in relation to quarantine pests) due to repeated survey of harmful viruses and high widespread of viroids.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Highlights (for review)

- CTV infections were repeatedly observed in ornamental nurseries
- CTV eradication was not always accomplished in 2 years of monitoring
- High virus incidences were observed in *C*. x *paradisi*, *C*. *grandis* and *C*. x *clementina*
- The most widespread viroid is CEVd frequently observed in *C*. x *limonia* and *C*. *limon*.

Table 1 Distribution of viruses and viroids in Tuscan nurseries during four years of monitoring. Infected samples out of analyzed samples are reported.

Viruses		**I**	**II**	**III**	**IV**	**Total**
	CTV	2/124	14/228	63/193	6/169	85/714
	CVV	7/124	24/228	7/193	7/169	45/714
	CPsV	0/124	10/228	6/193	3/169	19/714
Viroids		**I**	**II**	**III**	**IV**	**Total**
	CEVd	6/124	84/228	89/193	56/169	235/714
	HSVd	5/124	34/228	36/193	0/169	75/714
	CBCVd	0/124	10/228	0/193	0/169	10/714
	CBLVd	0/124	3/228	11/193	0/169	14/714
	CDVd	5/124	24/228	22/193	0/169	51/714

Table 2 Incidence (%) of Citrus tristeza closterovirus (CTV), Citrus variegation virus (CVV), Citrus psorosis virus (CPsV), Citrus exocortis viroid (CEVd), Hop stunt viroid (HSVd), Citrus bent leaf viroid (CBLVd), Citrus dwarfing viroid (CDVd) and Citrus bark cracking viroid (CBCVd) in mother plants of ornamental citrus, according to species (consistency of three or more plants).

Mother plant	**CTV**	**CVV**	**CPsV**	**CEVd**	**HSVd**	**CBCVd**	**CBLVd**	**CDVd**
C. aurantifolia	-	28.6	-	28.6	14.3	-	-	28.6
C. aurantifolia x *F.* margarita	-	-	-	-	-	-	-	-
C. bergamia	-	33.3	-	33.3	-	-	-	-
C. deliciosa	18.2	-	-	9.1	13.6	-	-	4.5
C. deliciosa x *C.* x *paradisi*	-	-	-	5.3	-	-	-	-
C. grandis	-	50.0	-	25.0	-	-	-	-
C. hystrix	-	-	-	-	-	-	-	-
C. limetta	-	-	-	28.6	14.3	-	-	14.3
C. limon	12.8	9.0	5.6	56.4	17.0	4.3	6.9	11.2
C. macrophylla	-	-	-	25.0	-	-	-	12.5
C. medica	2.5	15.0	2.5	37.5	17.5	-	-	12.5
C. mitis	-	-	-	15.4	-		-	-
C. myrtifolia	-	14.3	-	14.3	-	-	-	-
C. unshiu	-	-	-	-	-	-	-	-
C. volkameriana	-	12.5	-	-	12.5	-	-	-
C. x *aurantium*	-	-	-	33.3	5.6	-	5.6	-
C. x *clementina*	-	-	17.6	29.4	11.8	-	5.9	11.8
C. x *limonia*	-	-	-	57.1	42.9	-	-	57.1
C. x Limonimedica Florentina	12.5	-	-	25.0	37.5	12.5	-	-
C. x *lumia*	-	-	-	33.3	33.3	-	-	33.3
C. x *paradisi*	21.7	13.0	-	17.4	13.0	-	-	8.7
C. x *paradisi* x *C.* x *sinensis*	-	-	-	-	-	-	-	-
C. x *meyeri*	-	-	-	20.0	-	-	-	-
C. x *sinensis*	8.2	4.1	1.4	19.2	9.6	-	-	8.2
F. margarita	-	20.0	-	20.0	-	-	-	20.0

F. margarita x *C.* x *clementina*	-	-	-	-	-	-	-	33.3
M. australasica	-	-	-	-	25.0	-	-	25.0
Others	-	-	-	0.2	0.3	-	-	0.1

Table 3 Incidence (%) of single or mixed infection of virus.

Infection		**Incidence (%)**
Single infection		91.67
Mixed infection – 2 viruses		8.33
	CVV/CPsV	3.13
	CTV/CPsV	3.13
	CTV/CVV	2.08

Table 4 Incidence (%) of single or mixed infection of viroids.

Infection		**Incidence (%)**
Single infection		61.86
Mixed infection – 2 viroids		21.40
	CEVd/HSVd	11.16
	CEVd/CDVd	4.65
	CEVd/CBLVd	2.33
	HSVd/CDVd	1.86
	CBLVd/CDVd	0.47
	HSVd/CBLVd	0.47
	CEVd/CBCVd	0.47
Mixed infection – 3 viroids		14.88
	CEVd/HSVd/CDVd	10.23
	CEVd/CBLVd/CDVd	1.86
	CEVd/HSVd/CBCVd	1.86
	CEVD/HSVd/CBLVd	0.93
Mixed infection – 4 viroids		1.86
	CEVd/HSVd/CBLVd/CDVd	1.40
	CEVd/CBLVd/CBCVd/CDVd	0.47

Table 5 Occurrence of viruses and viroids in nurseries (N1, N2, N3) re-checked two years after first detection of CTV. T1 = infected samples out of analyzed samples observed during first detection of CTV; T1 = infected samples out of analyzed samples two years after CTV detection.

		N1		N2		N3	
		T1	**T2**	**T1**	**T2**	**T1**	**T2**
Viruses							
	CTV	12/72	3/38	2/48	3/73	10/10	0/5
	CVV	2/72	0/38	4/48	3/73	0/10	1/5
	CPsV	3/72	0/38	0/48	1/73	2/10	0/5
Viroids							
	CEVd	52/72	2/38	0/48	30/73	5/10	5/5
	HSVd	26/72	0/38	1/48	0/73	0/10	0/5
	CBCVd	0/72	0/38	0/48	0/73	1/10	0/5
	CBLVd	7/72	0/38	0/48	0/73	0/10	2/5
	CDVd	10/72	0/38	0/48	0/73	0/10	3/5