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

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ABSTRACT

Citrus tristeza closterovirus (CTV) has been found several times in the last decades in Italy, and plant protection services are involved in monitoring and surveillance. Although orchards linked to the citrus industry are well monitored, there is an underestimated risk of viruses or virus-like diseases in ornamental nurseries. Our aim was to modify a CTV monitoring program 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 in 15 nurseries in two locations in central Italy using inexpensive multiplex RT-PCR protocols. CTV incidence was 1.6–13.5%, with an average distribution of 11.9%. The average incidence of CVV and CPsV 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 identified was 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, the nurseries' response to the virus alert by the protection services was only partially effective. Although the CTV incidence was lower in nurseries re-checked after the initial detection, it was not eradicated from two nurseries out of three, and the occurrence of viroids was reduced in just one nursery. Given that dangerous viruses along with the concomitant spread of viroids have unfortunately become a fact of every day life, multiplex RT-PCR diagnoses are likely to play an increasing role in warning nursery managers of possible infections.

1. Introduction

Citrus tristeza virus (CTV) is widespread throughout tropical citrus-growing areas. In Europe 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 the elimination of infected trees (Moreno et al., 2008). Plant protection services (PPSs) are heavily involved in health checks and post-diagnosis procedures.

In Italy, where the virus has been found several times (Djelouah et al., 2009; Davino et al., 2013), PPSs are involved in monitoring and surveillance. While citrus trees are mainly grown in southern Italy and are related to the citrus industry, several ornamental citrus plants are cultivated in nurseries that do not work directly for the citrus industry, such as central or northern Italy. In these areas, citrus diseases may go unnoticed due to limited cultivation, yet virus or virus-like infections of mother plants of ornamental citrus could lead to severe spread of diseases in importing countries within the European Union.

For PPSs, the main costs involved in diagnostic molecular testing (i.e. PCR, RT-PCR, qPCR) are sample collection (due to staff and travel costs), sample preparation, and the extraction of nucleic acids. These time-consuming tasks increase staff costs if carried out manually, and the use of semiautomatic grinders or automated DNA/RNA extraction systems increases the equipment costs. Thus, once the sample has been collected and prepared, the marginal

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costs of multiple pathogen recognition are reduced, which is the main reason for using multiplex detection protocols.

In addition to CTV, other viruses cause concern in citrus cultivation, such as Citrus variegation virus (CVV) or Citrus psorosis virus (CPsV) (Gonsalves and Garnsey, 1975; da Graça et al., 1991; Martín et al., 2004; Velázquez et al., 2016). When such viruses are monitored by PPSs, this helps in supporting nursery activities and in buidling trust among stakeholders. Multiplex reverse transcription quantitative PCR (RT-qPCR) protocols (Loconsole et al., 2010; Osman et al., 2015) have been developed to investigate the presence of various viruses.

Citrus plants are also the natural hosts of several viroid species (Flores et al., 2005; Ding, 2009) which may cause different types of disease symptoms (Murcia et al., 2015). Exocortis and cachexia are severe diseases caused by the Citrus exocortis viroid (CEVd) and Hop stunt viroid (HSVd), respectively. While viroids such as Citrus bent leaf viroid (CBLVd), Citrus dwarfing viroid (CDVd), or Citrus bark cracking viroid (CBCVd) may have a small effect on the fruits, the infection can reduce height and canopy volume (Bani Hashemian et al., 2010; Rizza et al., 2011; Murcia et al., 2015). Significant effects can be also observed on rootstocks (Polizzi et al., 1991).

Viroids are generally controlled through preventive measures, such as viroid-free budwood used as a propagation material followed by indexing (Eiras et al., 2009). Although such measures were initially designed for fruit trees, they can 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 report on the impact of viruses and viroids in ornamental nurseries in Tuscany (central Italy) using multiplex RT-PCR protocols. We modified the CTV monitoring program in order to include emerging but yet not regulated pathogens such as viroids.

2. Materials and methods

Leaf samples were collected in 2012–2015 from ornamental mother plants of *Citrus* spp. (19 species), *Fortunella* spp. (six species), *Microcitrus* spp. (three 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 areas of Tuscany. In each nursery, sampling was representative of each lot of grown plants.

The occurrence of virus and viroids was also analyzed in three nurseries where CTV had been detected. In these nurseries, CTV-infected plants were destroyed within six months of diagnosis and farmers were informed about the health status of all the mother plants tested. Two years after the PPS had first alerted nursery owners to CTV infection, different lots of mother plants were checked for viruses and viroids. The results of the two health checks were then compared.

Samples consisted of four young shoots with leaves collected around the canopy during late summer of each year. Each sample was processed independently. Total RNAs (TNAs) from citrus tissues were extracted from 0.2 g of leaf petioles after homogenization with a 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.

Multiplex RT-qPCR reactions for viruses (CTV, CVV, CPsV) were performed in 1X IQ-Multiplex power mix (Biorad) with 15U of Multiscribe-RT (Applied Biosystem). As reported by Loconsole et al. (2010), the following concentrations of primers and probes were used: for CTV, 0.16 μM of forward primer and probe, 0.32 μM of reverse primer; for CVV, 0.16 μM of primers, 0.08 of probe; for CPsV, 0.32 μM of primers, 0.16 μM of probe. The final volume of was 25 μl. Amplifications were carried out on the CFX96TM Real time System (Biorad) using the following conditions: 5 min at 50 °C and 10 min at 95 °C, followed by amplification of 40 cycles of 95 °C for 20 s, 58 °C for 40 s, 60 °C for 40 s and 62 °C for 40 s. Data analysis and Ct calculations were carried out using SDS 1.2 (Applied Biosystems, USA).

Multiplex RT-PCR reactions for viroids (CEVd, HSVd, CBCVd, CBLVd, CDVd) were performed in a SuperScript™ one-step RT-PCR system with a Platinum Taq DNA polymerase kit (Invitrogen). As reported by Wang et al. (2009), the following concentrations of primers were used: for CEVd, 0.50 μM; for HSVd, 0.10 μM; for CBCVd, CBLVd, CDVd, 0.20 μM. Amplifications were carried out on the GeneAmp PCR System (Thermo Fisher Scientific) using the following conditions: 5 min at 95 °C, followed by amplification of 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 68 °C for 46 s, followed by a final extension at 68 °C for 7 min. Products were stored at 4 °C until used. PCR products were analyzed on 2% agarose gels stained with ethidium bromide.

3. Results

The distribution of viruses and viroids from 2012 to 2015 is reported in Table 1. The incidence of virus or viroid infection in analyzed species/hybrids is reported in Table 2.

CTV, the main target of monitoring, was widespread in the monitored area. Although CTV was not very frequent during the first year of monitoring (1.6%), more than 13% of the plants were found to be infected in the third year. In 2012–2015, the virus was found in more than 21% of the *C.* x *paradisi* plants tested, and high infection rates were also observed in *C. deliciosa* (18.2%), *C. limon* (12.8%), and *C.* x *Limonimedica Florentina* (12.5%). The virus was found in six species/hybrids out of more than 25 tested.

The distribution of CVV was 3.6–10.5% and involved 10 different species/hybrids. The virus was particularly frequent (>25% of infected plants) in *C. grandis*, *C. bergamia* and *C. aurantifolia*. On the other hand, CPsV, whose overall infection rates in 2012–2015 were below 5%, was limited to four species/hybrids, with a quite high frequency in *C.* x *clementina* (17.6%).

Mixed infections of viruses only involved just over 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 the viroids investigated were detected during the surveys. CEVd was the most widespread viroid, with an incidence of over 30% in three years of monitoring (average infection of 32.9%), CEVd was detected in almost all the species/hybrids analyzed, with more than half of the *C. limon* and *C.* x *limonia* plants infected.

Another widespread viroid was HSVd, which was present in 10.5% of analyzed plants. This viroid was frequently found in *C.* x

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

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

Although infection by just one viroid was most frequently observed, mixed infections were found in almost 40% of the plants tested (Table 4). The most frequent combinations of mixed infections involved the most frequent viroids, CEVd, HSVd and CDVd, in fact more than 10% suffered from a CEVd/HSVd/CDVd mixed infection.

In relation to species/hybrids, *C. limon* hosted all the viruses or viroids tested, and *C. medica* and *C.* x *sinensis* were found to be common hosts for all viruses and most viroids.

The occurrence of CTV was analyzed in nurseries subjected to repeated health checks (Table 5). Although the nurseries were warned of risks by the PPS, CTV was not eradicated in two out of three nurseries, though its incidence was reduced. A similar behavior in nursery response was observed for CVV and CPsV (Table 5). On the other hand, the frequency of viroids was lower in one nursery (N1), but increased in two other nurseries.

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

4. Discussion

In Spain, citrus cultivars free of virus and virus-like pathogens have become available thanks to the implementation of a citrus improvement program, which included the recovery of local cultivars, the application of quarantine procedures, the establishment of a germplasm bank and the application of a certification program for nurseries. This program covers more than 70% of the country's citrus-growing regions (Bani Hashemian et al., 2010), confirming the role of quarantine, certification programmes and the elimination of infected trees in protecting citrus production. Based on the current legislation, finding CTV in a nursery would involve the mandatory destruction of all the plants.

Consequently, repeated findings of CTV from 2012 to 2015 highlight the worrying health status of ornamental citrus mother

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

plants in Tuscany, even though CTV is less widespread than in other producing regions in Italy (Abbas et al., 2015). We found that CTV incidence was lower in nurseries re-checked two years after the first detection, however the pathogen had not been eradicated from two in three nurseries. Furthermore, the health status is being threatened not only by CTV, but by two further viruses and a plethora of viroids. Unlike viruses, which tend to be strongly regulated (such as CTV) or whose diseases are well known by nursery managers, viroids may be an unnoticed menace.

Widespread cases of citrus viroids have been found in commercial fruit trees in Greece (Barbarossa et al., 2007), Uruguay (Pagliano et al., 2013) and low performing orchards with a high incidence of viroids have also been observed in Spain (Bani Hashemian et al., 2010). However, few data are available for ornamental plants. The pospiviroid status of ornamental plants has been verified in Italy for solanaceous ornamental genera, but beside the high incidence of the Potato spindle tuber viroid (PSTVd), their incidence was low (Luigi et al., 2011). In Tuscany, CEVd and HSVd have been detected in 45% and 9% of plants in citrus orchards (Ragozzino et al., 2005), while higher incidence was found in Lazio (also in central Italy) (Ragozzino et al., 2005).

Our findings indicate that viroids are widespread in Tuscan ornamental citrus tree nurseries, reaching almost 50% for CEVd. In addition we found a high frequency of mixed viroid infections in ornamental citrus trees. Interactions among viroids co-infecting the same tree could affect symptom expression and field performance (Vernière et al., 2004). However, long-term field assays have also revealed that viroid-induced effects might be greater when trees are exposed to mixed viroid infections (Vernière et al., 2004; Vidalakis et al., 2010).

Since the primary mode of transmission of most viroids is through mechanical means, consideration must be given to the potential spread of the viroid by the equipment used in farm operations (Barbosa et al., 2005; Eastwell and Nelson, 2007). The current control methods for viroid diseases include detection and eradication, and cultural controls, as well as CTV protection programmes (Kovalskaya and Hammond, 2014).

In conclusion, the multiplex diagnosis of virus and viroids in Tuscan ornamental citrus nurseries should help to warn nurseries of potentially harmful viruses and of the possible spread of viroids.

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