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Detection and Distribution of *Citrus exocortis* viroid and *Hop stunt viroid* in Citrus Orchards of Central Italy as Revealed by One-tube One-step RT-PCR

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ABSTRACT. A survey was carried out in citrus fields of Latium and Tuscany regions (central Italy), especially those located near Aprilia (Rome province) and Pescia (Pistoia province), the most important areas of citrus growing in Central Italy. The samples were tested to determine the presence of *Citrus exocortis* viroid (CEVd) and *Hop stunt viroid* (HSVd), both causing several citrus diseases. In order to make the diagnosis efficient and easier, the common procedure of viroid-RNA extraction using phenol, was compared with one based on the use of commercial kit. Besides, a one-tube one-step RT-PCR was developed for detection of CEVd and HSVd in citrus-tissues. This technique, used in the viroid detection of all collected samples, proved to be very sensitive, reducing the possibility of false positive tests because all steps are performed in the same tube thus avoiding cross-contamination. Moreover, our results showed that the diagnosis with the one step RT-PCR using commercial kit is effective and, in some cases, more efficient than the current technique which is more time-consuming and includes the use of organic solvents. The result of our investigation showed the presence of CEVd and HSVd, respectively, to be 100% and 30% of the samples from Latium and 40% and 11% from Tuscany. Specifically, among 21 cultivars belonging to orange, lemon and mandarin species, 15 were CEVd positive and four HSVd positive.

Citrus is an important fruit crop in Italy where the estimated acreage of its cultivation is about 132,500 ha. Citrus has been grown in southern Italy, particularly in Sicily, Apulia and Calabria, and during the last several years different studies on citrus viruses and viroids were carried out in these regions (1, 3, 5).

Until a few years ago citrus production in central Italy was limited to small areas. However, in recent years there has been an increased interest in ornamental citrus varieties, especially at the nursery level. Currently, there is no information available regarding the phytosanitary status of citrus in central Italy, in particular on the presence of viroids.

In Italy, *Citrus exocortis* viroid (CEVd), which is the causal agent of citrus exocortis disease and *Hop stunt viroid* (HSVd), which is the causal agent of citrus cachexia and non-cachexia diseases are the two most important viroids that occur in citrus orchards. Both of these viroids are regulated by the phytosanitary

rules and must be absent from commercial plantings that belong to “CAC” (*Conformitas agricola communitatis*) and thus are in a certified category. Because of their economic importance, in the framework of the national Project “Agricoltura,” which is supported by the Italian Ministry of Agriculture, a survey was carried out to establish a distribution map of CEVd and HSVd and to evaluate their incidence in citrus orchards of central Italy.

A one tube one-step RT-PCR protocol was utilized to test 105 leaf and bark samples representing citrus cultivars that are different species and hybrids (Table 1) which are grown in several citrus orchards and nurseries in the Latium and Tuscany regions. No specific symptoms were observed in trees from which samples were collected.

Viroid RNA isolation was performed from leaf and bark tissues using the RNeasy Plant Mini Kit, according to the manufacturer’s protocol (Qiagen GmbH, Hilden, Ger-

TABLE 1
LIST OF CITRUS SPECIES AND VARIETIES ANALYZED FOR CEVd AND HSVd

| Sweet Orange | Mandarin | Satsuma | Clementine | Lemon |
|------------------------------|-----------------------------------|-----------------|------------------------------------|----------------------------|
| Navelina ¹ | Beatrix | Miho | Clementina | Femminello carru- |
| Tarocco | San Francesco ² | Miyagawa | Hernandina | baro |
| Tarocco nucellare | Tardivo di Ciaculli | | Rufina | Femminello comune |
| Sanguinello | Primo Sole | | | Variegato |
| Citron | Lime | Limetta | Kumquat | Lemon × Citron (Hybrid) |
| Etrog ⁴ | Pinocchio | Pursha | <i>Fortunella</i> spp ³ | Cedrato |

¹Bold font indicates varieties infected by CEVd.

²Bold-italicized font indicates varieties infected by CEVd and HSVd.

³Italicized font indicates varieties infected by HSVd.

⁴Standard font indicates uninfected varieties.

many). RNA was finally eluted with 50 µl of RNase-free deionized water. The one tube one-step reverse-transcription polymerase chain reaction (RT-PCR) protocol, which used specific primers that amplified the full-length genome of each viroid (2, 4) was performed as previously reported (6).

The results confirmed that CEVd and HSVd are widespread in citrus orchards of central Italy. CEVd was most common, with an average incidence of infection of 71%, and reaching 100% incidence in Latium, whereas samples collected in Tuscany showed no more than a 45%

incidence of infection (Table 2). The average incidence of infection of HSVd was 19%, with a peak of 30% in the Latium samples and 9% in samples from Tuscany (Table 3).

In summary, among 21 cultivars analyzed, 13 cultivars were infected by CEVd, one by HSVd, three by mixed infection and four were free of both viroids.

The one tube one-step RT-PCR protocol, when compared with other protocols used for citrus viroid diagnosis, was very convenient in detecting the two most widely distributed and important viroids affecting citrus germplasm, making their detec-

TABLE 2
DISTRIBUTION OF CEVd IN TUSCANY AND LATIUM REGIONS

| | Varietal Incidence ¹ | | Sample Incidence ² | |
|----------------|---------------------------------|--------|-------------------------------|--------|
| | Tuscany | Latium | Tuscany | Latium |
| Sweet Orange | 1/2 | 2/2 | 5/10 | 10/10 |
| Mandarin | 0/1 | 3/3 | 0/5 | 15/15 |
| Clementine | 0/1 | 2/2 | 0/5 | 10/10 |
| Lemon | 2/2 | 1/1 | 10/10 | 5/5 |
| Citron | 0/1 | | 0/5 | |
| Limetta | 0/1 | | 0/5 | |
| Lime | 1/1 | | 5/5 | |
| Kumquat | 0/1 | | 0/5 | |
| Lemon × Citron | 1/1 | | 5/5 | |
| Satsuma | | 2/2 | | 10/10 |
| Total | 5/11 | 10/10 | 25/55 | 50/50 |

¹No. of infected varieties/total varieties tested.

²No. of infected samples/total samples tested.

TABLE 3.
DISTRIBUTION OF HSVd IN TUSCANY AND LATIUM REGIONS

| | Varietal Incidence ¹ | | Sample Incidence ² | |
|----------------|---------------------------------|--------|-------------------------------|--------|
| | Tuscany | Latium | Tuscany | Latium |
| Sweet Orange | 0/2 | 0/2 | 0/10 | 0/10 |
| Mandarin | 0/1 | 2/3 | 0/5 | 10/15 |
| Clementine | 0/1 | 1/2 | 0/5 | 5/10 |
| Lemon | 0/2 | 0/1 | 0/10 | 0/5 |
| Citron | 0/1 | | 0/5 | |
| Lime | 0/1 | | 0/5 | |
| Limetta | 0/1 | | 0/5 | |
| Kumquat | 1/1 | | 5/5 | |
| Lemon × Citron | 0/1 | | 0/5 | |
| Satsuma | | 0/2 | | 0/10 |
| Total | 1/11 | 3/10 | 5/55 | 15/50 |

¹No. of infected varieties/total varieties tested.

²No. of infected samples/total samples tested.

tion and control easier and more rapid. Additional tests, including spot-blot hybridization analysis of total nucleic acids using specific CEVd and HSVd cRNA probes, a

two-step RT-PCR protocol and biological assays on the specific indicator Etrog citron have confirmed the results obtained with our one-tube one-step RT-PCR.

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