Mechanical Transmission of Citrus Viroids

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ABSTRACT

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Preliminary transmission assays conducted under greenhouse conditions demonstrated that *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III), and *Citrus viroid IV* (CVd-IV) can be mechanically transmitted from citron to citron (*Citrus medica*) by a single slash with a knife blade. The impact of mechanical transmission of viroids by pruning and harvesting operations was also demonstrated in experimental and commercial field plots. Transmission efficiency under field conditions ranged from 4% in 'Nules' clementine to 10% in 'Navelina' sweet orange and 21% in 'Verna' lemon. Transmission efficiency varied only slightly with viroid and donor hosts. The impact of viroid transmission on tree height, canopy volume, and crop harvest was minimal. When the donor host was coinfecting with several viroids, the viroids were not necessarily cotransmitted. Considerations regarding viroid transmission in other climates are discussed. Measures to control viroid spread in nurseries should be mandatory in certification programs.

Additional keywords: cachexia, xyloporosis, exocortis

Exocortis disease of citrus was described in 1948 as a bark shelling or scaling disorder affecting trees grown on trifoliolate orange (*Poncirus trifoliata* (L.) Raf.) rootstock (12), and soon afterward it was demonstrated to be a graft-transmissible disease (4,5). The citrange hybrids (*Citrus sinensis* × *P. trifoliata*) and Rangpur lime (*C. limonia* Osb.) used as rootstocks, and some citrons, limes, and lemons, are also sensitive to exocortis. The disease was considered to be induced by infection with a virus until it was shown that *Citrus exocortis viroid* (CEVd) was the causal agent (41). Studies regarding the mechanical transmission of the exocortis agent were conducted before the agent of the disease was established (17) and demonstrated that it could be transmitted from citron (*C. medica* L.) to citron with 100% efficiency simply by slashing with a knife blade (17,19), which retained viable inoculum for several days (2). As a consequence, a

number of procedures to decontaminate cutting tools were proposed to avoid the spread of the disease (17,34).

Cachexia disease was described in 1948 as discoloration, gumming, and browning of phloem tissues, wood pitting and bark cracking on Orlando tangelo (*C. paradisi* × *C. reticulata*) (8). Most commercial species and cultivars are tolerant, but mandarins, some mandarin hybrids (tangors and tangelos), and kumquats (*Fortunella* sp.) are sensitive. Alemow (*C. macrophylla* Wester) and Rangpur lime used as rootstocks are also sensitive. Because the disease shares biological properties with exocortis, including efficient transmission by slashing with a knife blade, it was suspected to be caused by a viroid infection (34). Following the discovery that citrus are naturally infected with several viroids, the agent of the disease was identified (40) and characterized as a specific sequence variant of *Hop stunt viroid* (HSVd) (30). Furthermore, its identity to a similar disorder described earlier as xyloporosis in Palestine sweet lime (*C. limettioides* Tan.) (32) was demonstrated (31).

Viroids are small, covalently closed, single-stranded RNAs (246 to 401 nucleotides) that replicate in their host plants. In addition to CEVd and HSVd, three additional viroids were reported in citrus including *Citrus bent leaf viroid* (CBLVd), *Citrus viroid III* (CVd-III), and *Citrus viroid IV* (CVd-IV), all belonging to the

Pospiviroidae family comprised of species with a central conserved region (CCR) and without hammerhead ribozymes (13). CBLVd and CVd-III are mild pathogens that may cause a reduction in tree size and fruit harvest yields (21,38,42). In addition, CVd-IV also causes severe bark cracking on susceptible species (42). Two additional viroid variants, CVd-I-LSS (LSS = low sequence similarity) and CVd-OS (OS = original sample), have been reported in Japan (23,24). Since all citrus viroids induce symptoms on Etrog citron, biological indexing tests using this indicator are not specific for the exocortis agent as initially thought. The utilization of viroid indexing tests based on nucleic acid analysis have shown that samples collected from naturally infected trees usually contain mixtures of several viroids (1,9,11,25). Many field sources that were initially characterized as severe exocortis isolates based on the symptoms induced in Etrog citron have since been shown to contain other viroids or viroid mixtures in addition to CEVd, whereas sources characterized as mild or moderate isolates contain other viroids or viroid mixtures but not CEVd (10,20,26,36). Similarly, cachexia isolates may contain other viroids in addition to the cachexia agent (39). Because assays conducted on the transmission of what were initially considered exocortis and cachexia isolates most likely contained viroid mixtures, the information generated needs to be reassessed according to the present knowledge of viroids infecting citrus.

Assays demonstrating that exocortis and cachexia were mechanically transmissible were mainly conducted from citron to citron under controlled greenhouse conditions; however, the possibility that their viroid agents might spread naturally in the field was considered. Additional transmission assays, also conducted under greenhouse conditions using species other than citron either as donor or as receptor hosts (18), further reinforced the risk that commercial cultivars growing in the field could become infected as a result of mechanical transmission during pruning and harvesting operations, as evidenced later in lemon orchards (6,7).

In many citrus-growing countries, trees are grown on viroid sensitive rootstocks such as Rangpur lime, *P. trifoliata*, citrange

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Carrizo, citrange Troyer, and alemow. The performance of trees grafted on these rootstocks relies on the propagation of virus-free certified budwood. However, newly established viroid-free trees are subjected to the risk of mechanical infection from symptomless infected trees growing in adjacent orchards or even within the same orchard.

In the present work, we describe the results of a preliminary assay conducted under greenhouse conditions demonstrating the transmissibility of all citrus viroids. Subsequent assays in experimental and commercial field orchards were designed to evaluate transmission efficiency in the field and its impact on the performance of trees under the growing conditions of eastern Spain.

MATERIALS AND METHODS

Greenhouse transmission assays. Preliminary experiments under greenhouse conditions were conducted prior to field experiments to verify that all citrus viroids described herein could be mechanically transmitted by slash wounding using a knife blade. Plants of the 861-S1 selection of Etrog citron grafted onto Rough lemon (*C. jambhiri* Lush.) rootstock were used as receptor plants when the scion reached at least 40 cm in height (4 to 6 months after grafting). Citrons infected with CEVd, CBLVd, HSVd, CVd-III, or CVd-IV were used as donor plants. Citron, 'Nules' clementine (*C. clementina* Hort. ex Tan.), and 'Verna' lemon (*C. limon* (L.) Burm. f.) infected with viroid mixtures were also used as donor plants. Citrons had been infected for several years and were periodically pruned to produce actively growing shoots under greenhouse conditions (28 to 32°C and a relative humidity around 60%). Shoots of 'Nules' clementine and 'Verna' lemon were collected in early summer from inoculated plants actively growing in the field. Viroids used as single viroid sources or as mixtures were: CEVd (CEVd-117) (16), CBLVd (CVd-Ia-117) (14), HSVd (CVd-IIa-117, CVd-IIb, CVd-

IIc) (29–31), CVd-III (CVd-IIIId) (14), and CVd-IV (CVd-IV-Ca) (15).

Mechanical viroid transmission was performed in the spring by the slash inoculation method using a sterile surgical blade. A single slash incision wound was made at an angle of 45° into the bark of the stem of the actively growing donor plants; and immediately thereafter, with the same blade, a similar single slash incision wound was made in the bark of the receptor citron. The incision wound made in the receptor stem was wrapped with Parafilm, and the plants were maintained in the greenhouse at 28 to 32°C. Three months later, receptor citrons were cut back 40 cm above the soil level, and the new shoots formed were analyzed by sequential polyacrylamide gel electrophoresis (sPAGE) analysis.

Experimental field plot. Following the demonstration that all citrus viroids were mechanically transmissible by knife blades, three experimental field plots were established in 1988 to evaluate the impact of mechanical transmission under field conditions. Trees of 'Verna' lemon (56 trees), 'Nules' clementine (50 trees), and 'Navelina' sweet orange (*C. sinensis* (L.) Osb.) (60 trees) grafted on Carrizo citrange

were propagated using viroid-free certified budwood. Half of the trees of each species were graft inoculated with a field isolate of citrus viroids containing CEVd, CBLVd, HSVd (CVd-IIa), and CVd-III. Trees were planted in the field in such a way that inoculated trees were always located next to noninoculated trees within rows as well as between rows. The plants were subjected to normal pruning and harvesting (with clippers) operations without disinfecting the tools. In 2002, all plants were indexed on the 861-S1 selection of Etrog citron grafted on Rough lemon rootstock. Each indicator plant was graft inoculated with three bark pieces collected from each of the main branches of the tree and maintained in the greenhouse at 28 to 32°C. After 6 months, the citrons were analyzed by imprint hybridization (27,28).

Commercial field plot. A commercial field plot suspected of having been exposed to the mechanical transmission of citrus viroids was selected in the location of Benicarló (Castelló, Spain). The orchard was established in 1985 with noncertified 'Nules' clementine trees grafted on sour orange that were suspected to be infected with viroids. In 1995, this plot was interplanted with 'Nules' clementine trees

Fig. 1. Indexing by sequential polyacrylamide gel electrophoresis (sPAGE) analysis of a lemon donor and six receptor citron plants. Lane 1, lemon donor coinfecting with *Citrus exocortis* viroid (CEVd), *Citrus bent leaf* viroid (CBLVd), *Hop stunt* viroid (HSVd), and *Citrus viroid III* (CVd-III); lanes 2, 3, 7, three noninfected receptor citron plants; lanes 4, 5, 6, three infected receptor citron plants. The lemon donor was first graft inoculated on Etrog citron and subsequently analyzed by sPAGE.

Table 1. Mechanical transmission of citrus viroids by a single slash using a surgical blade under experimental greenhouse conditions

Species	Donor plant	Receptor citrons ^a				
		No. infected plants/no. slashed plants ^b				
	Inoculated viroids ^c	CEVd	CBLVd	HSVd	CVd-III	CVd-IV
Citron	None	0/8	0/8	0/8	0/8	0/8
Citron	CEVd	2/8	0/8	0/8	0/8	0/8
Citron	CBLVd	0/8	2/8	0/8	0/8	0/8
Citron	HSVd (CVd-IIa)	0/8	0/8	4/8	0/8	0/8
Citron	HSVd (CVd-IIb)	0/8	0/8	2/8	0/8	0/8
Citron	HSVd (CVd-IIc)	0/8	0/8	6/8	0/8	0/8
Citron	CVd-III	0/8	0/8	0/8	3/8	0/8
Citron	CVd-IV	0/8	0/8	0/8	0/8	1/8
Citron	CBLVd+HSVd+CVd-III	0/6	0/6	0/6	1/6	0/6
Clementine	CEVd+CBLVd+HSVd+CVd-III	0/6	0/6	0/6	0/6	0/6
Lemon	CEVd+CBLVd+HSVd+CVd-III	0/6	1/6	3/6	1/6	0/6

^a Receptor plants were indexed by sequential polyacrylamide gel electrophoresis (sPAGE) analysis.

^b Gray boxes designate positive transmissions of each viroid.

^c *Citrus exocortis* viroid (CEVd), *Citrus bent leaf* viroid (CBLVd), *Hop stunt* viroid (HSVd), *Citrus viroid III* (CVd-III), and *Citrus viroid IV* (CVd-IV).

grafted on Carrizo citrange purchased as certified virus-free plants. No measures were implemented to disinfect the tools during the pruning and harvesting operations.

A sample of 18 presumably infected trees grafted on sour orange were tested for viroid infection by biological indexing on Etrog citron, sPAGE, Northern blot hybridization, and reverse transcription–polymerase chain reaction (RT-PCR). Seventy trees grafted on Carrizo citrange were biologically indexed on Etrog citron, and viroid infection was determined by imprint hybridization of the inoculated citrons.

Viroid indexing. Citron (5 g of young leaves and stems) samples were homogenized in phenol containing extraction me-

dium (0.4 M Tris-HCl, pH 8.9, 1% [wt/vol] sodium dodecyl sulfate [SDS], 5 mM EDTA, and 4% [vol/vol] mercaptoethanol) (37). The total nucleic acids were partitioned in 2 M LiCl, and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, and 0.1 mM MgCl₂). Aliquots of these preparations were subjected to sPAGE, Northern blot hybridization against viroid-specific probes, or RT-PCR.

For sPAGE analysis, samples (20 µl equivalent to 300 mg fresh weight) were initially subjected to nondenaturing 5% PAGE at 60 mA for 2.5 h. After ethidium bromide staining, a segment (1.5 cm) of the gel between CEVd and 7sRNA was

excised and subjected to a second 5% PAGE containing 8 M urea (33). Viroid bands were viewed by silver staining (22). For Northern blot analysis, nucleic acids were transferred electrophoretically to positively charged Nylon membranes at 80 V for 1 h directly from the urea-containing gel obtained after sPAGE using a modified TBE buffer (40 mM Tris, 40 mM boric acid, 1 mM EDTA, pH 8.3) for blotting. For imprint hybridization analysis, stems were freshly cut transversely and firmly pressed onto the surface of positively charged Nylon membranes (27,28). In all instances, the nucleic acids were immobilized by UV cross-linking. Prehybridization and hybridization were carried out in 50% formamide using digoxigenin (DIG)-labeled DNA probes (27,28).

RT-PCR was performed essentially as described by Yang et al. (43) using two contiguous primers of opposite polarity specific for each viroid (28). In order to increase the specificity of the reaction (L. Bernad, *unpublished results*), first strand cDNA was synthesized at 60°C using 15 U of ThermoScript RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA).

Tree growth and fruit yield. Tree height, canopy size, and fruit yield were measured at the end of 2003. Canopy size was calculated as $V_c = 4/3\pi R^3$, where R is the radius of the canopy. R was estimated as one-half the average of canopy height and two perpendicular width measurements. Data from each citrus species were analyzed by ANOVA.

RESULTS

Mechanical transmission under greenhouse conditions. The results of a small-scale preliminary transmission assay using citron as the receptor host conducted under greenhouse conditions demonstrated

Table 2. Mechanical transmission of citrus viroids by standard agronomic practices in ‘Nules’ clementine, ‘Navelina’ sweet orange, and ‘Verna’ lemon trees growing adjacent to infected trees under experimental field conditions^a

Species	No. infected plants/no. tested plants ^{b,c}				Transmission ^d
	CEVd	CBLVd	HSVd	CVd-III	
‘Nules’ clementine					
Infected donor	25/25	25/25	25/25	25/25	
Receptor	1/25	0/25	0/25	0/25	1/25 (4%)
‘Navelina’ sweet orange					
Infected donor	30/30	30/30	30/30	30/30	
Receptor	0/30	1/30	0/30	2/30	3/30 (10%)
‘Verna’ lemon					
Infected donor	28/28	28/28	28/28	28/28	
Receptor	5/28	0/28	1/28	0/28	6/28 (21%)
Viroid transmission ^e	6/83 (7.2%)	1/83 (1.2%)	1/83 (1.2%)	2/83 (2.4%)	

^a All trees were subjected to standard fertilization and phytosanitary treatments. Pruning and harvesting were conducted without disinfecting the tools.

^b Receptor plants were biologically indexed on Etrog citron and by imprint hybridization analysis. Gray boxes highlight positive transmissions of each viroid.

^c *Citrus exocortis* viroid (CEVd), *Citrus bent leaf* viroid (CBLVd), *Hop stunt* viroid (HSVd), and *Citrus viroid III* (CVd-III).

^d Number of infected receptors over total number of receptor plants of each species. Percentages were compared using normal approximation and a *t* test.

^e Number of receptor plants infected with each viroid over total number of receptor plants. Percentages were compared using normal approximation and a *t* test.

Fig. 2. Statistical analysis by ANOVA of height, canopy volume, and yield parameters collected in three experimental field plots of A, ‘Nules’ clementine, B, ‘Navelina’ sweet orange, and C, ‘Verna’ lemon grafted on Carrizo citrange. Bars show the mean standard error of infected (nonshaded) and healthy (shaded) trees. Asterisks (*) show values from individual receptor trees that succumbed to infection with the viroid indicated during the trial.

that all citrus viroids could be mechanically transmitted from citron to citron by slash inoculation with a knife blade. This confirms that in addition to the exocortis and cachexia agents, CBLVd, CVd-III, CVd-IV, and non-cachexia variants of HSVd can also be transmitted to citron by a single slash (Table 1).

Transmission assays using citron, clementine, and lemon coinfectd with several viroids as donor hosts showed that viroids are not necessarily cotransmitted (Table 1).

In fact, when lemon coinfectd with CEVd, CBLVd, HSVd, and CVd-III was used as a donor host, three of the six citrons used as receptor hosts became infected. These infected citrons presented mild and moderate symptoms compared with the severe symptoms observed in citrons that were graft inoculated from the donor lemon. The results of sPAGE analysis of donor and receptor plants are shown in Figure 1.

Mechanical transmission under experimental field conditions. Three ex-

perimental plots of 'Verna' lemon, 'Nules' clementine, and 'Navelina' sweet orange were designed to mimic the number of growers who renew their orchards by planting young, certified virus-free plants in between old viroid-infected plants. The results demonstrate that citrus viroids are also transmissible under field conditions (Table 2). The number of infected plants as a result of mechanical transmission ranged from 21% in lemon to only 4% in clementine. Comparisons of transmission

Fig. 3. Indexing of 15-year-old 'Nules' clementine trees grafted on sour orange sampled in a commercial field plot. Plants were graft inoculated on Etrog citron. Figures show results of **A**, sequential polyacrylamide gel electrophoresis (sPAGE), **B**, Northern blot hybridization against viroid-specific probes, and **C**, reverse transcription-polymerase chain reaction (RT-PCR) using viroid-specific primers. Lane 1, positive control infected with *Citrus exocortis viroid* (CEVd) and *Citrus viroid IV* (CVd-IV); lanes 2 and 3, two of 16 indexed clementine trees; lane 4, positive control infected with *Citrus bent leaf viroid* (CBLVd) (variant CVd-Ib), *Hop stunt viroid* (HSVd), and *Citrus viroid III* (CVd-III).

percentages in the three species were calculated using normal approximation and a *t* test. Differences in transmission were found (10% significance level) between lemon and clementine ($P = 0.0856$), but not between clementine and sweet orange ($P = 0.5378$) or between sweet orange and lemon ($P = 0.3194$).

All transmissions were of a single viroid, confirming that the four viroids coinfecting the donor plants were not necessarily cotransmitted, results that were consistent with greenhouse transmission assays. Comparisons of transmission percentages of different viroids were calculated as above. Differences in transmission were only found between CEVd and CBLVd ($P = 0.0822$) and between CEVd and HSVd ($P = 0.0822$).

Donor trees presented bark scaling symptoms that were only perceptible below the soil level; however, none of the trees infected as a result of mechanical transmission showed symptoms. Infected donor clementine trees were significantly smaller than the noninfected receptor trees in terms of height, canopy volume, and yield (Fig. 2A). The size and yield of the single receptor clementine tree that succumbed to CEVd infection was within the range of the noninfected trees, suggesting a recent infection (Fig. 2A). No significant differences were found between donor and noninfected receptor sweet orange trees (Fig. 2B), whereas highly significant statistical differences were found between noninfected receptor and infected donor lemon trees (Fig. 2C). The height and canopy volume of the six lemon trees that became infected as a result of mechanical transmission ranged from 1.7 to 2.3 m (height) and 6.46 to 16.80 m³ (canopy volume), falling within the range of infected as well as noninfected trees (Fig. 2C). These differences suggest that the trees became infected at different times.

Transmission under commercial field conditions. Transmission results obtained in the experimental field assay were further confirmed by analysis of plants collected from a commercial orchard of non-certified 'Nules' clementine trees grafted on sour orange that had been interplanted with young certified trees of the same cultivar grafted on Carrizo citrange. Eighteen samples of the old trees suspected of being infected, were subjected to biological indexing in Etrog citron and sPAGE analysis. All trees were infected with the same viroids (CEVd, CBLVd, HSVd, and CVd-III) (Fig. 3), indicating that they had been propagated from the same scion source. Identities of the viroids identified by sPAGE were confirmed by Northern hybridization analysis using viroid-specific DIG-labeled DNA probes (27) and RT-PCR (Fig. 3).

Seventy clementine trees grafted on Carrizo citrange were indexed biologically on Etrog citron and by imprint hybridization

analysis. As summarized in Table 3, 10% of the trees succumbed to infection as a result of mechanical transmission. Receptor trees were infected either with a single viroid or with combinations of two to three viroids. No significant differences were found among the frequencies of transmission of different viroids when comparisons of transmission percentages were calculated using normal approximation and a *t* test.

DISCUSSION

Early reports demonstrated that the exocortis and cachexia agents were mechanically transmitted by slashing with a knife blade (17,19,34). In spite of the small number of receptor plants used in the preliminary greenhouse transmission study, the results showed that CBLVd, CVd-III, CVd-IV, and all strains of HSVd were also mechanically transmissible by a single slash. The apparent discrepancy between the frequency of transmission from citron to citron found in the present study and the near 100% transmission reported earlier (17,19,34) was likely due to the fact that we used a single slash versus 10 slashes in earlier studies.

Viroid transmission has also been demonstrated among trees growing in the field, with rates lower than anticipated when taking into consideration the high number of slashes performed during pruning and harvesting operations without disinfecting the tools. Only slight differences in terms of transmission efficiencies of different viroids and within different host species were found. These differences may result from the differences in viroid titers found among different infected species (3,28).

The results of the three assays have demonstrated that viroids coinfecting do-

nor hosts are not necessarily mechanically cotransmitted. Transmission assays conducted by Roistacher et al. (35) showed that receptor citrons presented a great variability of symptoms, ranging from mild to severe, when compared with the severe reaction induced by the donor plant. Those observations were considered to be due to separation of strains differing in virulence contained in the severe donor isolate. The results reported here have demonstrated that the mild and moderate reactions thought to be the result of infection with mild exocortis are in fact the result of different viroid species present as mixtures in field isolates.

These results confirm that, in addition to the exocortis and cachexia agents, all known citrus viroids are mechanically transmissible. Under the environmental conditions in which the assay was performed, rate of transmission in the field and impact on the performance of trees that became infected was lower than anticipated. However, measures to control the spread of viroid agents should still be recommended in areas in which viroid-free plants are grown close to infected plants. These strategies should be reinforced in those orchards planted with trees of which either the rootstock, the scion, or both are viroid-sensitive species. Even though the results of the present study indicate that transmission rates in field grown trees are fairly low, this information should not be extrapolated to trees growing under other environmental conditions. In temperate climates such as those of Mediterranean countries, harvesting and pruning operations take place in late autumn and winter when trees are dormant and contain low viroid titers (28), a situation that probably does not favor mechanical transmission.

Table 3. Mechanical transmission of citrus viroids by standard agronomic practices in 'Nules' clementine trees growing adjacent to infected trees under commercial field conditions^a

'Nules' clementine	No. infected plants/no. tested plants ^{b,c}				Transmission ^d
	CEVd	CBLVd	HSVd	CVd-III	
Infected donor	18/18	18/18	18/18	18/18	
Receptor	1/70	4/70	4/70	3/70	7/70 (10%)
Receptor plants infected/ receptor plants tested	Viroids detected in receptor plants				
	CEVd	CBLVd	HSVd	CVd-III	
2/70	—	+	+	—	
1/70	+	—	—	—	
1/70	—	+	+	+	
1/70	—	—	—	+	
1/70	—	—	+	+	
1/70	—	+	—	—	
63/70	—	—	—	—	
Frequency ^e	1.4%	5.7%	5.7%	4.3%	

^a All trees were subjected to standard fertilization and phytosanitary treatments. Pruning and harvesting were conducted without disinfecting the tools.

^b Receptor plants were biologically indexed on Etrog citron and by imprint hybridization analysis. Gray boxes highlight transmission frequencies of each viroid.

^c *Citrus exocortis* viroid (CEVd), *Citrus bent leaf* viroid (CBLVd), *Hop stunt* viroid (HSVd), and *Citrus viroid III* (CVd-III).

^d Number of infected receptors over the total number of receptor plants.

^e Number of receptor plants infected with each viroid over the total number of receptor plants. Percentages were compared using normal approximation and a *t* test.

However, the high relative humidity and elevated temperatures of tropical climates, similar to the greenhouse conditions where our transmission assays were performed, prevent dormancy, and therefore trees may be more susceptible to infection.

Control of viroid spread is critical in nurseries where the unfortunate contamination of a single mother tree may result in the dissemination of many infected plants. For this reason, disinfection of tools used in pruning and harvesting operation should be mandatory in certification programs.

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