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Citrus viroids: Characterization, prevalence, distribution and struggle methods

Bibliographic Review

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Abstract – *Citrus* is one of the most important horticultural crops, with a worldwide fruit production of over 110 million tons per year. The necessity of using rootstocks for citrus fruits is to have a profitable production against some limiting factors such as climate conditions, bad soil conditions and diseases. However, sour orange which the most commonly used rootstock even in the Mediterranean basin has the disadvantage to be highly susceptible to *Citrus tristeza virus* (CTV), the causal agent of Tristeza disease and strongly limits the use of this rootstock in many citrus growing countries. CTV- tolerant rootstocks have been selected in many citrus growing countries, but in some cases, they exhibited sensitivity to other diseases such as those caused by viroids. Seven viroids reported to infect *Citrus* spp belong to four genura of the family of *Pospiviridae.* Surveys have been carried out in many countries and showed that viroids are widespread in commercial citrus plantations, where they are perpetuated with the propagation of infected, symptomless budwood. Several trial fields were conducted under artificial inoculation by different species of viroids to evaluate the performance of some citrus varieties grafted on different rootstocks. The elimination of viroids from infected plants has been a challenging issue and different approaches have been studied to produce viroid-free planting material. Management strategies for viral sanitation involving shoot-tip grafting or somatic embryogenes have been successfully used to eliminate many viroids and virus from plant propagating material. One of essential elements for success to produce virus free propagation material is to implement a rigorous certification program of citrus budwood.

Keywords: Viroids, Characterization, Prevalence, Rootstocks, Certification program.

1. Introduction

The genus *Citrus* belonging to the family *Rutaceae*, includes several species that produce important fruits such as oranges, mandarins, limes, lemons, sour oranges, and grapefruits. *Citrus* is one of the most important horticultural crops, with a worldwide fruit production of over 110 million tons per year. Most of citrus orchards trees consist of two parts that combine favorable attributes of the scion and rootstocks. Selection of rootstocks is a major consideration in every citrus growing area (Ramin and Alirezanezhad, 2005). In fact, Rootstocks play an important role in the worldwide spreading citrus development. The necessity of using rootstocks for citrus fruits is to have a profitable production against some limiting factors such as climate conditions, bad soil conditions and diseases (Yildrim *et al*., 2010). In this respect, sour orange (*Citrus aurantium* L.) which is well adapted to calcareous and other common soil types is still the most commonly used rootstock even in the Mediterranean basin. However, sour orange has the disadvantage to be highly susceptible to *Citrus tristeza virus* (CTV), the causal agent of Tristeza disease that strongly limits the use of this rootstock in many citrus growing countries. Regarding this problem, researchers and citrus growers engaged consistent efforts to seek for alternative rootstocks. Indeed CTV-tolerant rootstocks, have been selected in many citrus growing countries, but in some cases, they exhibited sensitivity to other diseases such as those caused by viroids (Roistacher *et al.*, 1977; Roistacher, 1983).

Consequently, several investigations were conducted in order to characterize these pathogens, study their dissemination and propose struggle methods against them.

The present review aims to give some highlights on (i) Characterization of citrus viroids, (ii) importance and geographic distribution of viroids, (iii) the evaluation of the performance of certain varieties grafted on different rootstocks in presence of these pathogens (iii) the necessity of a certification schema to produce certified healthy plants.

2. Characterization of viroids

Viroids are small infectious agents of single stranded, unencapsided, non translated and circular RNA with self- complementary sequences (Flores et al., 1986). Citrus viroids have been classified into distinct groups based on their biological and physical properties: Seven viroids reported to infect Citrus spp. belong to four genera of the family of Pospiviridae (Duran-Vila *et al*., 1988): citrus exocortis viroid (CEVd), citrus bent leaf viroid (CBLVd), hop stunt viroid (HSVd), citrus dwarfing viroid (CDVd), citrus bark cracking viroid (CBCVd), citrus viroid V (CVd-V) and citrus viroid VI (CVd-VI) which has only been reported inJapan (Ito et al., 2003).These viroids have small genomes from 284 to 375 nucleotides (Duran-Vila *et al*., 1988a). CEVd is a causal agent of the exocortis disease. HSVd includes variants that induce cachexia disease as well as variants that do not induce cachexia (Semancik *et al.*, 1988; Duran-Vila and Semancik, 2003). These two viroids cause economically important losses. The other viroids cause minor effects (Vernière *et al*., 2006).

Citrus exocortis disease was described in 1948 as a bark shelling or scaling disorder affecting trees grown on trifoliate orange *(Poncirus trifoliata* L. Raf.) rootstock (Fawcett and Klotz, 1948). Once graft transmission was demonstrated (Benton et al; 1950), the disease was considered to be of viral etiology. With the discovery of viroids as a new class of plant pathogens, the exocortis disease was demonstrated to be associated with the *citrus exocortis viroid* (Semancik and Weathers., 1972). When Etrog citron (*Citrus medica* L.) was used as an indicator for indexing of exocortis in field trees, it displayed a variety of symptoms ranging from severe to very mild (Calavan, 1968), which were erroneously considered for many years as evidence for existence of CEVd strains. Schlemmer *et al* (1985) provided the first evidence indicating that viroids other than CEVd were responsible for the mild and moderate symptoms observed on inoculated citrons, and subsequently a number of viroids RNAs with electrophoretic mobilities faster than CEVd were consistently identified in field isolates (Duran-Vila et al, 1986; Duran-Vila *et al*., 1988). With tomato as an experimental host, Visvader and Symons (1985, 1986) proposed a classification of CEVd sequences based on their biological properties into severe ‘‘Class A’’ and mild ‘‘Class B’’, which differ by a minimum of 26 nucleotides, mainly affecting two regions (PL and PR) located respectively in the P and V domains of the viroid secondary structure. Infectivity assays conducted with chimeric cDNA clones suggested that the changes in the PL region were responsible for symptom modulation (Visvader and Symons., 1986), but the role of PR as well as of two class-specific changes located in the lower strand of the C domain remained undetermined. The role of individual structural domains was also studied by infectivity assays of chimeras of CEVd and tomato apical stunt viroid (TASVd), which showed that TL and P domains modulate symptom severity, whereas V and TR domains are involved in the level of replication and/or accumulation (Sano et al.,1992).

HSVd is a typical viroid with CCR (central conserved region) and without hammerhead self cleavage, that belong to genus *Hostuviroid* (Flores et al, 1998*)*.Two HSVd distinct strains have been reported: (1) non pathogenic strain (formerly included as CVd-IIa) that infect sensitive hosts without inducing symptoms and (2) pathogenic strains (formerly referred as CVd-IIb and CVd-IIc) that infect the same sensitive hosts, but inciting, cachexia disease. The causal agents of cachexia disease can incite severe gumming, discoloration and wood pitting symptoms in alemow (*Citrus macrophylla* webster), clementines (*C. clementina* Hor .Ex.Tan), mandarins (*C. reticulata Blanco*), satsumas (*C.unshiu* (Macf.) Marc.), Rangpur lime (C. *limonia* Osb.) kumquats (*Fortunella* spp) and hybrids like tangelos (Eiras *et al*, 2013). The 'Parson's Special mandarin' has been used as experimental host for biological indexing to the presence of cachexia-inducing isolate (Reanwarakorn and Semancik, 1999).

In HSVd RNA molecule, there are five highly conserved residues (Cachexia expression motif) located in the upper (3 nucleotides) and the lower (2 nucleotides) strands for 'V' domain that determine the expression cachexia symptoms (Palacio-Bielsa et al., 2004). In an exhaustive work evaluating the individual effect of each citrus viroid on symptom expression on clementine grafted on trifoliate orange, confirmed that only CEVd and specific variants (CVd-IIb and CVd-IIc) included exocortis and cachexia, respectively (Vernière *et al*., 2004).

CDVd is a member of the genus Apscaviroid of the family Pospiviroidae with a ‘‘central conserved region’’ (CCR) and lacking RNA self-cleavage activity (Flores et al., 2005). The rod-like secondary structure of viroids can be divided into five structural-functional domains: P (pathogenicity), C (central), V (variable), TL (terminal left) and TR (terminal right) (Keese and Symons., 1985). In the Etrog citron

(Citrus medica) indicator, CDVd causes mild stunting and a ''leaf-dropping pattern'' due to a moderate epinasty resulting from petiole and mid-vein necrosis. In the field, CDVd has been shown to induce different degrees for dwarfing in certain rootstock citrus combination (Vidalakis et al, 2011; Murcia et al., 2015). It was later characterized as three distinct sequence variants (CVd-IIIa, CVdIIIb and CVd-IIIc) (Rakowski et al., 1994; Semancik et al., 1997). These variants differ in size by 18 nucleotides located in the left and right regions flanking the CCR; behave like distinct strains of CDVd with different levels of severity (Murcia et al., 2009). The involvement of the TL domain in the pathogenicity of CDVd was also reported (Serra et al., 2009).

CBCVd (formerly named Citrus viroid IV) consists of 284 nucleotides that can be arranged into the viroid-specific rod-like secondary structure model as depicted below in which 63 G:C, 32 A:U and 8 G:U pairs are present so that 71% of all its nts are base-paired. Sequence comparison revealed that CVd IV is a novel mosaic type chimeric viroid, and that most of its right hand part and of its central region (CR) resembles CEVd, whereas the left terminal region is very similar to that of HSVd (Puchta et al., 1991). 'Etrog' citron plants inoculated with CVd IV alone displayed only transiently mild symptoms of leaf epinasty after hot summer weather. Severe bark cracking symptoms were consistently found in the Citrange carrizo and trifoliate orange rootstocks infected with this viroid (Vernière et al;, 2004). The severe cracking was more perceptible by observing the characteristic green streaks apparent after scraping the bark (Murcia et al., 2015).

Vd-V and CVd-VI are a two newly reported viroid species (Owen et al., 2011). Examination of the primary structure of the CVd-V revealed the presence of the TCR characteristic of the genus *Apscaviroid*. The reference variant, had a predicted rod-like secondary structure of minimal free energy with 68.7% of the nucleotides paired (71.3% G–C, 22.8% A–U and 5.9% G–U pairs). The transition C197→U in the lower CCR strand resulted in the change of a canonic base pair (G–C) between the upper and lower strands into a wobble base pair (G-U). The conserved nucleotides of the CCR upper strand and the flanking inverted repeats can form a thermodynamically stable hairpin (hairpin I), which is like in all members of the family *Pospiviroidae* includes a terminal tetraloop, an adjacent 3-bp stem and a long stem at the base (Serra et al., 2008a). CVd-V has been reported in California (USA),Spain, Nepal, Sultanate of Oman, Iran, China, Japan and Pakistan (Serra et al; 2008b; Bani- hashemian *et al*., 2010, Ito and Ohta., 2010; Cao *et al*., 2010; Cao et al., 2013) and recently in Tunisia (Hamdi *et al*., 2015).

CVd-VI, former named Citrus viroid-original source (CVd-OS), was first identified from a citrus cultivar 'Shiranui' [(Citrus reticulata Blanco × C. sinensis (L.) Osb.) × C. reticulata] in Japan (Ito et al. 2001) and was later detected from Japanese persimmon (Nakaune and Nakano, 2008). Its genomic RNA ranges from 326 to 331 nt consisting of the CCR and terminal conserved region (TCR) identical to those of the genus Apscaviroid (Flores et al. 1997; 1998). However, the highest sequence similarities between the CVd-VI with other viroids were only 68%, which is much less than 90% of the species criterion. Indicator Arizona 861-S1 Etrog citrons (C. medica L.) infected with CVd-VI alone showed mild leaf bending and petiole necrosis. Thus, CVd-VI is a new species of the genus Apscaviroid, due to low sequence identity and distinct biological properties. Its structure and nucleotide composition are identical to that proposed for the Apple scar skin viroid (ASSVd), the type species of the genus *Apscaviroid* (Koltunow and Rezaian, 1989; Flores et al., 1997). CVd-VI seems to be restricted to Japan (Ito et al., 2003).

3. Occurrence, prevalence and distribution of viroids

Except for countries in which sanitation programs have been implemented, viroids are widespread in commercial citrus plantations, where they are perpetuated with the propagation of infected, symptomless budwood. Usually, field trees are co-infected with several viroids, a situation that for many years impaired the understanding of the effect of single or multiple infections on the performance of host. A procedure for viroid detection from field grown citrus trees originally described by Murcia et al.(2009) and successfully applied elsewhere (Mohamed et al; 2009) has been found to be highly sensitive, specific and an efficient alternative to biological indexing procedures because the time required for diagnostic is markedly shortened. Briefly, RNA preparations extracted from bark tissues of field grown citrus were subjected to polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions and Northern blot hybridization analysis with viroid specific digoxygenin (DIG-labelled) probes.

Prevalence of viroid infections was studied in some countries where surveys have been carried out.

In Italy: results of survey conducted in Campania region showed that HSVd, CDVd and CEVd were found to be the most widespread viroids infecting 87%, 85% and 68% of the tested samples and were detected in almost citrus species and cultivars. They were frequently found in mixed infection, as in

43% of tested trees (Malfitano and al, 2005). CBCVd and CBLVd were found only in 24 and 13% of the analysed trees but may be considered significantly high since in other countries these viroids, particularly CBCVd, have been reported as the least widespread (Duran -Vila and Semancik, 2003; Najar and Duran-Vila, 2004).

In Sudan: The citrus industry is based mainly on the cultivation of old-line cultivars of grapfruit (*Citrus paradisi* Macf.), sweet orange (*C. sinensis* L.) and willow leaf mandarin (*C.reticulata* Blanco), with sour orange (*C.aurantium* L.) as the almost exclusive rootstock. Gummy bark (Nour-Eldin, 1956; 1968) and Kassala disease (Bové, 1995) on sweet orange on grapefruit, respectively have been observed in Sudan. Both disorders are of unknown etiology, but since their symptoms resemble those of cachexia on mandarin, the possibility of a viroidal etiology was proposed but not demonstrated (Onelge et al., 1996, 2004). All samples collected were found to be infected with three, four or five viroids. CEVd was detected in 87% of samples, HSVd in 92%, CBLVd in 91%, CDVd in 96% and CBCVd in all the samples (Mohamed et al., 2009). Attempts to uncover the relationship between viroid infection and the "gummy bark" disease of sweet orange gave the following results: one sweet orange with most gummy bark symptoms was free from CEVd, while another sweet orange accession with mild symptoms was free from both CDVd and CBLVd. Therefore, CBCVd remains the only candidate for a viroid etiology of gummy bark assuming that the disease was indeed caused by a signle, known viroid species. However, the possibility cannot be ruled out that Sudanese gummy bark might be (i) the result of a specific synergistic interaction between two or several viroids co-infection the same tree, as describe by Vernière et al (2006) or (ii) a consequence of the high temperatures and very low relative humidity characteristic of the regions where the disease has been described., or (iii) Sudan, where old cultivars are grafted on sour orange citrus viroids represent a real threat, in the future when rootstocks like trifoliate orange, Carrizo citrange, Ranpur lime, sensitive to exocortis and/or cachexia, will have to be used to control tristeza (Mohamed et al., 2009).

In Uruguay: from 84 field grown citrus trees analysed, 62% were infected at least one viroid or combinations of CEVd, CBLVd, HSVd and CDVd. CBCVd and CV-VI were not detected (Pagliano et a., 2013). Single viroid infections were only observed for CBLVd and HSVd, with HSVd being the most commonly detected (40% of infected plants). Mixed infections with two , three or four viroids were found in 19%, 10% and 5% of the tested plants respectively. The most frequent mixed infection was HSVd+CDVd , followed by CEVd+HSVd+CDVd and CBLVd+HSVd+CDVd. HSVd was the most frequently detected viroid either in single or mixed infections with 92% of the total number of infected trees, while CDVd, CEVd and CBLVd were detected in 50%, 23% and 21% of the positive samples respectively. Viroid infection was found in all the commercial varieties tested: lemon (81%), grapefruit (71%), mandarine (62%) and sweet orange (55%) (Pagliano et al., 2013). This recent survey in Uruguay complements previous data regarding viroid occurrence, prevalence and distribution in commercial citrus orchards in Uruguay in six provinces sampled (Pagliano et al., 1998; 2000). The failure to detect CBCVd and CVd-VI , confirms other reports indicating a more restricted distribution worldwide (Duran-Vila et al, 1988; Ito et al., 2001).

In Costa Rica: The survey conducted on sweet orange and lemon in the main citrus growing area located in the northern part of the country revealed that 69% of the samples analysed were positive at least one of the 4 viroids. CEVd, CBLVd, HSVd and CDVd were found widespread in the three principal regions of commercial citrus production with contamination rate of 29%, 15%, 85% and 25% respectively. CBCVd was not detected in any of the samples. Mixed infections of two, three and four viroids, whereas only 15% were infected with a single viroid (CEVd, HSVd and CDVd) (Villalobos *et al*. 1997).

In Tunisia: The five viroid species described by Duran-Vila *et al*. (1988) were identified in Tunisian citrus by sPAGE and molecular hybridization. CEVd, HSVd and CDVd were highly widespread accounting respectively for 70.3, 72.3 and 78.2% of the sources tested. CBLVd and CBCVd were only found in 28.2% and 3.0% of the tested trees. CEVd, HSVd and CDVd were found in almost all the cultivars analyzed, whereas CBCVd was only detected in 2 Maltaise sweet orange trees, 1 Common mandarin and 3 Eureka lemon. The most frequent viroid combinations were CEVd+HSVd+CDVd (34.6%) and HSVd+CDVd (22.3%). Other combinations such as CBLVd+HSVd+CDVd (12.9%), CEVd+CBLVd+HSVd (11.9%) and CEVd+CDVd (10.9%) were less frequent, whereas HSVd+CBLVd (4.9%) and HSVd+CBLVd+CDVd+CBCVd (2.5%) were rather infrequent (Najar *et al*., 2017).

The high frequency of single and mixed viroid infections in these countries probably occurred long time ago and that the use of infected budwood, contamination tools and top-grafting may have been responsible for viroid spread and accumulation in individual trees. Furthermore, it is important to mention that the high viroid contamination rate should be taken into consideration when new rootstocks will be chosen to manage the tristeza disease.

4. Field performance of rootstocks to the viroid infections

Several trial fields were conducted under artificial inoculation by different species of viroids to evaluate the performance of certain varieties grafted on different rootstocks.

Clementine Nules grafted on Citrange carrizo: this experiment was initiated in 1988 in Valencia, Spain. The most important results revealed after 13 years that, in general infected trees were significantly smaller than the non inoculated controls for size parameters (height, canopy volume and rootstock and scion circumference). Cumulative yield of infected clementine was 57% of those of non infected controls. Viroid infection did not have any significant effect on the quality of fruit. Observations of histological sections of fibrous roots of Citrange carrizo rootstock showed that cortex cells of healthy tress contain dark stained bodies that are very scarce in roots of infected trees (Bani Hashemian *et al*., 2009).

Washington Navel sweet orange grafted on Citrange carrizo: this trial was established in 1990**.** The results of the present study showed that vegetative growth was affected by viroid infection with height and canopy volume being significantly reduced. Unexpectedly, CBCVd infection caused the most significant reduction of tree size (height and canopy) that was associated with a small root system (Murcia et al., 2015). CBCVd infected trees, in spite of producing smaller yields than the non infected controls, resulted in a good efficiency (yield/canopy volume). Further assays with larger numbers of trees would be necessary to confirm the sensitivity of Citrange carrizo to CBCVd infection and to postulate its usefulness to control tree size in high density plantation. With the exception of fruits harvested from HSVd-infected trees that presented a small caliber, viroid infection did not affect any of fruit quality parameters (Murcia *et al.*, 2015).

'**Marsh seedless' grapefruit grafted on trifoliate orange**: The experiment carried out in Sao Paulo State, Brazil. Two mixed viroid treatments were evaluated: HSVd+CDVd and CEVd+HSVd+CDVd. A reduction of nearly 44% and 25% relative to absolute control was observed after 12 years for the isolates with and without CEVd, respectively. The average productivity of fruits compared to the control was nearly 44% for trees infected by isolates without CEVd and 33% for the isolates with CEVd. The fruit quality of the cultivar 'Marsh seedless' showed the lowest values of juice and weight fruit for the treatment with CEVd, while the viroid infection without CEVd was not different for the controls (Sanchez et al., 2007).

"Maltaise demi Saguine" grafted on different rootocks: Under the growing conditions of Tunisian citriculture, viroid infection with CEVd and HSVd caused a slight reduction of tree size when Maltaise tunisian cultivar grafted on Cleopatra Mandarin, Swingle citrumelo, Volkamer lemon, sour orange and Rangpur lime. However, in the case of trees grafted on trifoliate orange, tree size and canopy volume were significantly reduced as compared to the non-inoculated trees (Najar *et al*., 2017). These results are in agreement with those reported earlier by Vernière *et al.* (2004; 2006) who observed a severe stunting of clementine trees grafted on the trifoliate orange rootstock. The most relevant effect resulting from HSVd infection was observed on tree height and canopy volume of trees grafted on Alemow that were significantly smaller and in a state of general decline. The sensitivity of Alemow was reported by Aubert and Vullin (1998). Viroid infection did not affect the cumulative yield of trees grafted on Volkamer lemon, Alemow, Rangpur lime or sour orange. In the case of trees grafted on the other rootstocks tested, the registered values for this parameter revealed specific tendencies, as follows: In the case of trees grafted on Alemow, the only effect observed was due to infection with HSVd which caused a reduction of more than 70% of the cumulative yield. In contrast, deviation from a cumulative effect for the same roostock was observed in the case of the combination of HSVd with CBLVd, CDVd and CBCVd which expressed a significant antagonism. In fact, the cumulative yield of mixed-infected trees was almost similar to that of the non-inoculated trees (Najar *et al*., 2017). It seems that there is a sort of cross protection effect or interference among the different viroid species like previously reported by Niblett *et al.* (1978) and Semancik *et al.* (1992). Citrumelo swingle rootstock appeared to be very susceptible, particularly to the mixed viroid infection CBLVd+HSVd+CDVd+CBCVd, which induced a significant reduction of cumulative yield reaching 55% as compared to the non-inoculated controls (Najar *et al.*, 2017).

5. Elimination of viroids

Viroids cannot be controlled by therapeutic treatments in fields. Hence, the elimination of viroids from infected plants has been a challenging issue and different approaches have been studied to produce viroid-free planting material. Management strategies for viral sanitation involving shoot-tip grafting or somatic embryogenesis have been successfully used to eliminate many viroids and virus from plant propagating material (Navarro et al. 1980, Achachi et al. 2014). The production of healthy material has been obtained by in vitro shoot tip grafting alone or in combination with heat therapy (Navarro et al. 1980). However, this technique represents some disadvantages related to the difficulties to eliminate some viruses (Carvalho et al. 2002). Looking for other alternative techniques, researches proposed somatic embryogenesis from different floral parts (Carimi et al. 1994, Carimi et al. 1998, Carimi et al. 2005), but style and stigma gave better results and became more and more useful for their specific advantages concerning sanitation and juvenility traits (D'Onghia et al. 2000, Meziane et al. 2012). Certification programs are among the best established means of increasing phytosanitary health, and some of those for citrus are among the oldest in the world. In conjunction with quarantine and clean stock programs, they remain important weapons in the ongoing fight against citrus diseases. One of the elements essential for successful certification programs to produce such propagation material is the availability of sensitive and effective diagnostic methods (Achachi et al. 2014).

6. Conclusion

Viroids can cause diseases of economic importance in several crops mainly in citrus. Surveys conducted in many countries in citrus orchards showed a high contamination with these pathogens.
This frequency is probably due to the use of infected budwood, contamination tools and top-grafting. It is important to mention that the high viroid contamination rate should be taken into consideration when new rootstocks will be chosen to manage the tristeza disease because the Sour orange which is the most commonly used rootstock has the disadvantage to be highly susceptible to *Citrus tristeza virus* (CTV). In parallel, the control of foundation blocks and nurseries through a rigorous certification schema of citrus budwood should be implemented.

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