Recent advances in *Citrus psorosis virus*

Asmae Achachi · Essaïd Ait Barka ·
Mohammed Ibriz

Received: 1 August 2013 / Accepted: 31 January 2014 / Published online: 20 February 2014
© Indian Virological Society 2014

Abstract Psorosis is a globally devastating disease of citrus caused by an infectious filamentous ophiovirus, *Citrus psorosis virus* (CPsV), which causes annual losses of about 5 % and a progressive decline of trees by affecting the conductive tissues. The disease can be harboured asymptotically in many citrus species. In the field, the most characteristic symptoms of the disease in adult trees are bark scaling in the trunk and main branches and also internal staining in the underlying wood. The virus has a tripartite single-stranded RNA genome, and has been inadvertently spread to most citrus growing areas through the movement of citrus propagative material. No natural vectors have been identified except in limited citrus areas in some cases. Management strategies for CPsV involving shoot-tip grafting and thermotherapy or somatic embryogenesis from stigma and style cultures have been successfully used to eliminate CPsV from plant propagating material. Molecular pathogen-mediated strategies have been used to produce citrus plants. Such a strategy protects against infections by the virus from which the resistance gene and promising resistance may emerge from trials. Certification programs are among the best established means of increasing phyto-sanitary 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. In this review, we discuss an updated status of CPsV disease.

Keywords *Citrus psorosis virus* · *Ophiovirus* · Segmented negative-stranded RNA virus · Diagnostics · Viral resistance · Certification

Introduction

Psorosis is a graft-transmissible viral disease of citrus. The most characteristic symptoms of the disease in adult field trees are bark scaling in the trunk and main branches. Gum may accumulate below the bark scales and may impregnate the xylem producing wood staining and vessel occlusion [54]. Other symptoms including chlorotic flecking or spotting in young leaves, and ringspots in leaves or fruits are sometimes observed, particularly in the spring flush [33]. The disease has been reported from many citrus-growing areas all over the world [54]. Usually, infected trees are not killed by the virus but slowly decline and eventually become unproductive, causing damage to citrus industry in the Mediterranean basin, and in some areas of South America. In the 1980s, in Argentina and Uruguay it was a serious disease causing annual losses of about 5 % of trees [8] and the disease is still present as recently reported by Zane et al. [80]. Another earlier investigation of Paul W. Moore in California, published in 1957, was conducted in order to find out the approximate bearing efficiency of an orchard in which psorosis appears to be the principal cause of crop reduction. The yields-during the harvest season of a

A. Achachi (✉) · M. Ibriz
Laboratoire de Génétique et Biométrie, Département de
Biologie, Faculté des Sciences, Université Ibn Tofail, Kénitra,
Maroc
e-mail: Asmae.Achachi@univibntofail.ac.ma

E. Ait Barka
Laboratoire de Stress, Défenses et Reproduction des Plantes,
Unité de Recherche Vignes et Vins de Champagne, UFR
Sciences, Université de Reims Champagne-Ardenne,
Reims Cédex, France

good crop year-of 153 Valencia orange trees affected with psorosis were compared with the yields of 153 normal trees. The production of trees in progressive stage of psorosis was 28 % of a normal crop. Although the number of trees in the comparative study is relatively small and was taken in only one crop year, the data clearly point out the role that psorosis plays in reducing Valencia yields even in favorable crop years.

The causal agent of the psorosis disease is the *Citrus psorosis virus* (CPsV). CPsV is classified in the genus *Ophiovirus* (Greek ‘ophio’ = snake), indicating the elongated twisted and coiled appearance of the virus particles [33, 36, 73].

The last Psorosis review appearing 20 years ago focused on the identity of the pathogen, methods for indexing, cross-protection, elimination of the pathogen from propagative budwood, programs for certification and eradication of psorosis and relation-ship of the citrus ringspot to psorosis [54]. This summary of research may contribute to a more detailed understanding of CPsV and its relationships with other members of the genus *Ophiovirus*. In addition, an overview of recent advances in the use of CPsV for virus-induced gene silencing is presented.

Pathology and symptoms

The term “citrus psorosis group” has been used to refer to several widespread graft-transmissible disorders of citrus diseases that may not have a common causal agent. Names for virus-like pathogens causing psorosis-like symptoms on indicator plants include concave gum, impietratura and cristacortis, leading to emergence of the idea of a non-CPsV psorosis-like disease of unknown etiology [71].

Two types of psorosis has been described, psorosis A (PsA), the more common disease, characterized by the presence of bark-scaling in the trunk and limbs of infected field trees (Fig. 1a) and staining of interior wood. The more aggressive psorosis B (PsB), causes rampant bark scaling (Fig. 1b, c) even in fine twigs in field trees, chlorotic blotching in old leaves (Fig. 1d, f) with brownish gummy pustules in the leaf underside (Fig. 1g), and sometimes ringspots on fruits [33, 54, 77]. Bark scaling often appears in 10–12-year-old trees. However, in the absence of the severe B form of the disease, the bark-scaling symptom may be delayed or even absent [53, 54]. These symptoms appear to be caused by CPsV but in the former ‘psorosis group’ there are other non-CPsV diseases with symptoms such as chlorotic leaf-flecking and oak-leaf patterns; while graft-transmissible, these diseases have not yet yielded to further analysis.

The citrus species most severely affected are sweet orange, grapefruit, and mandarin. Certain other species show typical leaf symptoms when infected, but remain free of bark lesions without obvious ill effects. Sour orange and lemon fall in this category [37, 54].

Apart from CPsV, several other diseases can cause psorosis-like bark scaling on citrus, particularly, leprosis (most commonly caused by *Citrus leprosis virus-C*), Bahía bark scaling, foot rot caused by *Phytophthora*, Rio Grande gummosis, and genetic disorders including lemon shell bark and sunscald [33, 37], and psorosis-like bark scaling reported from Spain noted in some trees apparently free of CPsV by serology and psorosis-free by biological indexing [32].

Historical background

The literature on psorosis identification has become noticeably confused over the years. Since bark scaling was the first and characteristic symptom of the disease, some psorosis reports were based on the simple observation of this symptom without further testing for its transmissibility. After observation that grafts from bark-scaled trees were able to induce young leaf symptoms in sweet orange seedlings, graft inoculation of this indicator plants was used for quick psorosis diagnosis [53, 54], although other graft-transmissible diseases were later shown to induce similar symptoms in the same test [54]. The discovery that lesion bark inoculum was able to induce PsB in sweet orange seedlings, and that only PsA infected seedlings showed cross protection against PsB and they do not develop leaf pustules or twig blisters characteristic of the PsB, provided a specific diagnostic test [53, 54]. However, many subsequent reports on psorosis diagnosis failed to use it. Additional confusion was added by the consideration of ringspot-type psorosis as a different disease from psorosis [54]. Citrus ringspot have been described as a different bark scaling disease, inducing chlorotic flecking and spotting of young leaves with later developed into yellow blotches vein banding and/or distinct rings in mature leaves of several indicators species. Particularly, it was associated with severe PsB type bark scaling.

Later on the basis of cross-protection tests [10], the similarity of symptoms and filamentous morphology between ringspot and psorosis isolates [19, 42], and differences in number of particles of CPsV detected by immunosorbent electron microscopy (ISEM) using an antiserum to the isolate CPV-4 [43], it was suggested that ringspot and psorosis are variants of the same disease. The same conclusions were advanced by Da-Graça et al. [7] and

Fig. 1 Symptoms characteristic of the psorosis A (PsA) and psorosis B (PsB) syndromes in field trees in Northeast and Central Morocco (a–d) or in indicator plants in the greenhouse at the index facility at Kenitra, Morocco (e–g). PsA symptoms include bark scaling in the trunk of field trees (a), a shock reaction with leaf shedding of the first flush, and transient chlorotic flecking in young leaves of the following

flushes (e) in indicator sweet orange seedlings. PsB symptoms in the field include rampant bark scaling (b–c) and chlorotic blotching in old leaves (d). In indicator sweet orange seedlings PsB isolates incite the shock reaction observed with PsA isolates (e), chlorotic blotches in old leaves (f) with gummy pustules on the underside (g)

Rouag et al. [56] respectively, who used serological and molecular comparisons.

Geographical distribution

Psorosis is an ancient disease; bark-scaling of citrus was first observed in Florida and California in the 1890s [54]. The disease has been brought under control in most advanced citrus-growing countries due to rigorous indexing and quarantine [71]. Differences in the extent of infection are found between countries. For instance, In Italy, the spread of the disease is higher compared to other Mediterranean countries [2]. Nevertheless, these data may be explained by a lack of data in other Mediterranean countries, particularly those in North Africa.

Host-virus relationships

Histology

CPsV particles occur in phloem and parenchyma cells of citrus. No virus particles or any specific inclusion or cytopathology have been detected in thin sections of infected tissues [37]. However, a more recent study detected the presence of inclusion bodies in the intercellular spaces of infected leaves of grapefruit seedlings (25 days post inoculation). These structures could contain virus particles, but they are not the only structures to do so. It should also be noted that the study of anatomical and structural changes on grapefruit leaves infected with CPsV have demonstrated that a set of alterations appear in the microscopic structure of the leaves showing that CPsV infection clearly affected the conductive tissues. The main cytopathic changes in infected citrus cells were the presence of large number of abnormal chloroplasts, mitochondria and cellular abnormality and the presence of hypertrophied nuclei. Other morphological changes such as the presence of an unstructured parenchyma in the upper epidermis, reduction of the number of oil glands, the appearance of secondary growth in midvein and major lateral veins in smaller veinlets, can be attributed to different indirect effects occurring in the host plant, for instance the lower auxin level resulting from virus infection and not attributed to the virus itself [62].

Distribution of CPsV in the host

The heterogeneity of CPsV was established by Barragan-Valencia et al. [4]. An initial test was to determine the concentration of viral samples from field and it turned out that the presence of virus in the tree was not uniform since

it was detected in only 40 % of the analyzed samples. In a subsequent test, two assays were made to determine viral concentration in leaves of 10 Mars orange trees; in the first, quantitative detections were made in three different samples of a single leaf from each tree, whereas in the second, detection were made in three samples of different leaves from each tree. In the first assay, the virus was detected in four trees but in the second assay, the virus was detected in nine of the trees evaluated. Therefore, it is considered that the distribution of the virus in field trees is not uniform confirming thus the first observation. However, the distribution of virus in the greenhouse plant shows a greater homogeneity of viral concentration in the analyzed samples.

Virus transmission

The virus is graft-transmitted, but symptoms rarely appear before trees are 10 years old; consequently, growers often propagate psorosis infected buds from symptomless trees, explaining partly the high disease incidence in some areas [32]. Some virus isolates are, though sometimes not easily, mechanically transmissible to *Chenopodium quinoa* and produces necrotic local lesions, and systemically infected *Gomphrena globosa* [37, 54]. More recent tests of seed transmission provided no experimental evidence for the transmission of CPsV through the seed in the mandarin and sour orange [13].

However, several reports on the increase in incidence of citrus psorosis symptoms in the field suggested a possible natural spread of the disease. For instance, observation in Texas, in nucellar Redblush grapefruit trees and in originally virus-free Rio Red grapefruit trees supports the hypothesis that natural transmission occurs [8, 21, 54, 61]. Spatial and spatio-temporal analyses of data from surveys over a period of 3 years in four citrus orchards in Texas containing psorosis-infected trees were consistent with disease spread via vectors [21]. This information changed considerations in the context of phytosanitary controls, because, for the moment, psorosis was not considered as a quarantine pest by any regional organization for protection of plants [16].

At the present stage of research, it was suggested that the vector is a species of the Oomycete genus *Olpidium* [44]. Focusing on the Oomycete, Palle et al. [44] showed that an *Olpidium*-like species is associated with citrus roots (the study involved the grapefruit), and that CPsV could be detected in zoospores from infected roots. Further work is required to confirm if this Oomycete is able to transmit the virus to healthy citrus. In 1998, T. Natsuaki and T. Morikawa have indicated that the vector of *Tulip mild mottle mosaic virus* (TMMMV) is *Olpidium brassicae* (personnel

communication). *Lettuce big-vein associated virus* (LBVaV) (genus *Varicosavirus*) and *Mirafiori lettuce big-vein virus* (MiLBVV) (genus *Ophiovirus*) are transmitted by *Olpidium virulentus*, a noncrucifer strain of *O. brassicae* [60], and *Lettuce ring necrosis virus* (LRNV) (genus *Ophiovirus*) is also transmitted by *O. brassicae* [64]. A new soil-transmitted ophiovirus associated with freesia leaf necrosis disease [68], *Freesia sneak virus* (FreSV) is likely involved [69, 72]. In lettuce big-vein disease it has been shown that zoospores of *Olpidium* released from sporangia contain the virus in their protoplast and transmit to the roots. The virus is also found in dormant spores, which survive for years in the absence of host [30].

Diagnosis

Detection, identification and differentiation of viruses and viroids infecting various crops constitute the basic steps for the development of effective crop disease management systems. The tools used to detect CPsV in citrus trees are mainly based on using indicator plants and on laboratory tests including enzyme-linked immunosorbent assay (ELISA) [20, 33, 80]. After CPsV sequences became available, molecular tests based on RT-PCR and dot blot or tissue-print hybridization were also developed for CPsV detection [5, 20, 26, 28, 32, 55, 57, 67].

Biological indexing

Psorosis is currently diagnosed by biological indexing on sensitive indicator plants, usually sweet orange seedlings (*Citrus sinensis*) growing in a temperature-controlled (18–26 °C) greenhouse [53, 54]. Characteristic symptoms include a shock reaction with leaf fall and shoot necrosis in the first flush and/or chlorotic leaf flecking and spotting in the following flushes (Fig. 1e). Since the latter symptoms are also induced by other graft-transmissible diseases, specific diagnosis of psorosis additionally requires a cross protection test using PsB [53, 54]. In this test, sweet orange seedlings pre-inoculated with the candidate psorosis isolate are challenge-inoculated with PsB. The latter psorosis type additionally incite chlorotic blotching in old leaves with gummy pustules on the leaf underside (Fig. 1f, g), similar to symptoms observed in field trees, and blisters on the stem and twigs that usually evolve to produce twig necrosis [53, 54]. It is important to state that sweet orange varieties will differ in their ability to show shock or leaf symptoms [53]. Psorosis symptom expression is affected by temperature, with cool temperatures favouring appearance of the shock reaction in the first flush after inoculation, while warm temperatures often inhibit shock and mask leaf symptoms [42, 76]. Virus titre estimated by ELISA and by

northern and dot blot hybridization paralleled symptom intensity, with significantly higher virus accumulation in plants incubated at 26/18 °C [76]. The amount of CPsV-derived small RNAs (CPsV-sRNAs) slightly increased at 32/26 °C, with the ratio of CPsV-sRNA/vRNA being higher at 32/26 °C than at 26/18 °C. These results suggest that CPsV infection induces RNA silencing in citrus plants, that symptom intensity is associated with virus accumulation, and temperature increase enhances the RNA silencing response of citrus plants and decreases virus accumulation [76].

Immunological-protein based methods

Rapid tests for detecting CPsV incidence and severity have been developed using serology, and tested during CPsV regional, country and state surveys. Availability of an improved antiserum [20] and of monoclonal antibodies (MAbs) to the coat protein (CP) [1, 46], has allowed detection of CPsV by double and triple antibody sandwich (DAS- and TAS-) ELISA, in biologically characterized and non-characterized psorosis sources [2, 12, 32]. Monoclonal antibodies (24 MAbs) raised to the southern Italian CPsV isolate IAM-191Xa have been found to be useful to distinguish between CPsV isolates. These MAbs were tested singly by ELISA against a panel of 40 psorosis sources from different geographical areas (Italy, Lebanon, Spain, and USA). Sixteen different epitopes were identified and considerable serological variability, apparently associated with the geographical origin of the isolates was found [11], but no data are available on the sensitivity of detection achieved. Variation in the epitopes present in the CP of different CPsV isolates [3, 11] makes it advisable to use a mixture of MAbs [32].

The antisera produced against recombinant viral CP form an additional or alternative tool for manufacturing standardized kits for serological detection of viruses like CPsV that are difficult to purify. Further, in the indirect DAS-ELISA format, the polyclonal antibodies (PAb) developed against the recombinant CP protein was efficiently and successfully employed in place of monoclonal antibody used earlier for the detection of CPsV [27].

Alternative enzyme

Apart from AP and penicillinase (PNC), the enzymes horseradish peroxidase (HRP) and urease are also suitable for use in ELISA. Of these, HRP is the most widely used. Its substrate is the chromogenic 3, 3', 5, 5'-tetramethyl benzidine dihydrochloride (TMB) or *o*-phenylenediamine (OPD). Zaneck et al. [80] describe the use of HRP for detecting the presence of the virus in the field samples and

in the transgenic sweet oranges expressing very low amounts of viral CP. HRP is more reliable, faster and more sensitive than AP with no significant variability and has a lower detection limit, since the AP reaction takes from 17 to 24 h instead of 30 min for the TAS-ELISA-HRP. Using this improved method, the presence of psorosis has been confirmed in the northwest region of Argentina.

Tissue blot immunoassay

Tissue-blot immunoassay (TBIA) is a variant of ELISA has been developed for the detection of CPsV. Localization of plant viruses has differentiated the types of tissues that support virus replication/accumulation. Direct TBIA technique was employed for the detection of CPsV in the ovaries of infected flowers [15]. Interestingly, CPsV was detected in the ovary explants from closed flower more than ovary from open flowers. DTBIA also represents a very convenient and safe system for shipping blotted membranes from one place to another [15].

The comparative efficacy of DAS-ELISA, TAS-ELISA and DTBIA in detecting CPsV was assessed [32]. All the three assay procedures detected CPsV readily in young shoots and leaves. However, DTBIA was less efficient in detecting the virus in old leaves. The presence of CPsV in nine different citrus varieties was efficiently detected by ELISA formats and DTBIA, indicating that CPsV accumulated to equivalent levels in the varieties tested. There was good correlation between results of immunoassays and that of biological indexing [32]. The results showed that CPsV in infected trees was clearly detected in old hardened leaves in winter [32]. DTBIA is simpler, less expensive and faster than ELISA providing the same level of specificity, if young leaves are selected for testing. Furthermore, tissue prints can be prepared in the field and stored for long periods without loss of reactivity, making DTBIA more useful and convenient technique for epidemiological studies. In addition, several membranes can be printed with the same shoots for future processing with new antibodies and tissue-printed membranes may be failed to a laboratory where the required antibodies are available [32].

Transversely cut tender shoots, or whole flowers, or rolled old and young leaf blades of citrus trees infected by CPsV were gently pressed onto nitrocellulose membranes. After adopting standard step of TBIA, the blotted membranes were incubated with alkaline phosphatase conjugated As-Ps.Rc1 IgGs at a dilution of 1:500. Membranes were incubated in substrate solution of BCIP-NBT Sigma fast in distilled water for the development of purple color. CPsV was readily detected in tissue samples from tender shoots, flowers and young leaves collected during spring, but not from hardened old leaves. Similar results were

observed with the various ELISA protocols and immunosorbent electron microscopy. All these tests showed that As-Ps.Rc1 antiserum was specific in detecting CPsV in citrus samples, irrespective of the technique applied [27].

Immunosorbent electron microscopy

Antisera have been used to detect CPsV particles using immunosorbent electron microscopy (ISEM) [9, 10, 19, 36, 43]. ISEM technique was also applied for the efficient detection of CPsV in cross-protected citrus plants [33]. Recently, CPsV was extracted from infected leaves of *C. quinoa*. Electron microscope grids were sensitized by floating them on drops of crude antiserum and then they were floated on plant extracts at 4 °C overnight. The adsorbed virus particles were stained with 2 % uranyl acetate and examined under the electron microscope. The CPsV particles trapped from crude plant extracts were observed in infected leaves of *C. quinoa* [27].

Molecular-nucleic acid based methods

Nucleic acid-based virus detection systems make use of cloned DNA probes in a dot-blot assay or specifically designed primers in PCR-test. Dot-blots are unlikely to differentiate by a single nucleotide and PCR may fail to detect an isolate where the 3'nt of the primer mismatches the template, thus failing to detect rather than differentiating isolates. These two approaches will be summarized in the following paragraphs.

Dot-blot assay

This development in nucleic acid hybridization technology offers a great potential for virus detection. CPsV can be detected by dot blot or tissue print hybridization using full-length ³²P-labelled riboprobes of the CPG [5]. Positive sense probes hybridized to several isolates in tissue print and dot-blots at 55 °C. At this temperature, non-homologous isolates could be detected without any appreciable hybridization to host RNA. However, only the homologous isolate CPV-4 was detected when hybridization temperatures was at or above 60 °C. Some isolates of CPsV that could be detected by RT-PCR could not be detected by hybridizations assays due to low titre of the virus. Nevertheless, these isolates were not detected at hybridization temperatures lower than 55 °C due to non-specific reactions with host nucleic acids [5].

Recently, a dot blot hybridization protocol using digoxigenin-labelled riboprobes was finalized for the detection of CPsV and *Citrus variegation virus* (CVV) [28]. In greenhouse analysis, CPsV detection rate in young leaves/flowers was similar by ELISA and dot blot hybridization

during spring. The higher sensitivity of the molecular with respect to the serological assay was more evident in aged leaves tested in winter, since CPsV was detected in 40 samples instead only 22 samples by ELISA. The higher efficiency of the dot blot hybridization assay over ELISA was confirmed by the results obtained over a 9 month period, testing different tissues (aged leaves, young leaves, flowers, fruit, peel and flavedo, juice and seeds) from selected field-grown CPsV infected plants, molecular detection was possible throughout the year from one organ/tissue or the other, although the highest detection rates were in spring from flowers and young leaves. It can be concluded that dot blot hybridization is more sensitive than ELISA and can be usefully applied for routine large-scale diagnosis, thus constituting a useful alternative to serological assays.

RT-PCR methods

The CPsV genome consists of three single-stranded RNAs of negative polarity. RNAs 1, 2 and 3 of the isolate CPV-4 have been sequenced [5, 39, 58, 59]. RNA extracted from citrus infected tissue is assayed for the presence of CPsV sequences by RT-PCR, cDNA copies are made and amplified for analyses. Based on increasing sensitivity criteria, RT-PCR methods are classified as conventional, nested, and real-time modes. Primer design must thus take into account both the mode (conventional, nested, or real-time) and the range of specificity of the RT-PCR to be applied.

Portions of RNA1 have been cloned and a consensus sequence was obtained from which primers were designed for RT-PCR [20]. The CPG has been used to develop RT-PCR [5]. In both cases, the RT-PCR was able to detect CPsV at concentrations substantially lower than those required for detection by immunoassays and electron microscopy [20]. However, the sensitivity has not been sufficiently high to detect the heterologous psorosis isolates using primers designed from CPV-4. Nested RT-PCR is usually applied when RNA sample concentration is low; it is based on the use of the first PCR product as starting material for the second PCR round. In some instances, a hemi-nested RT-PCR is used, where one of the second-round primers, usually the forward one, is the same as in the first-round RT-PCR. For the CPsV, first step of the hemi-nested RT-PCR is carried out as described above. The second step (hemi-nested) is carried out with internal forward (primer 6) and reverse (primer 7), yielding a 195-bp amplified fragment. This test is able to detect CPsV in an amount as small as an equivalent of 10^{10} of the original sample. This represents an improvement of 10^6 -fold from previously reported sensitivity [26]. Disadvantages of nested PCR include additional time and cost associated

with the two stages of RT-PCR, and the increased risk of contamination incurred during transfer of first-stage amplification products to a second tube.

Simplex PCR detects one target per reaction and requires different PCRs to detect multiple targets. Multiplex PCR (mPCR) is a useful technique since different RNA and DNA viruses may infect a single host like citrus. Seven viruses infecting citrus viz., *Citrus leaf rugose virus* (CLRv), *C. psorosis virus* (CPsV), *C. tatter leaf virus* (CTLV), *C. tristeza virus* (CTV), *C. variegation virus* (CVV), *C. yellow mosaic virus* (CYMV), and *Indian citrus ringspot virus* (ICRSV) belonging to six different virus genera were detected simultaneously by the multiplex PCR (mPCR) assays [57]. Degenerate primers were designed based on the sequences of respective virus isolates. The cDNA fragments (245-942 bp) specific to the viruses were simultaneously amplified using mPCR and they were identified on the basis of their molecular sizes. The consistent results of the mPCR were compared with simplex PCR for detection of each virus pathogen. In most cases the detection sensitivity for the mPCR at equivalent concentrations was less than for the simplex PCR assay. The detection sensitivity of multiplex PCR decreased for CLRv, CPsV and CTLV, but increased in case of CVV and CYMV detection. The reason for this decrease may be that in the multiplex assay the cocktail primers compete for all the seven viral templates rather than for one and therefore the detection end point is lower. This could pose a problem for some assays, the use of the mPCR system needs testing with naturally infected materials in conjunction with bio-indexing methods. The diagnostic technique reduces the risk of contamination, saves time and reduces the cost as compared to other conventional methods for citrus virus detection. Furthermore, the mPCR provides a useful rapid method for detecting multiple virus infections in citrus plants that will aid in the production of virus-free citrus plants for certification programs [57].

An improved one-step RT-PCR was also developed for the detection of CPsV in citrus trees. The test was more sensitive than the ELISA and as accurate as bioassays [55].

Real time RT-PCR

This approach has provided insight into the kinetics of the PCR reaction and it is the foundation of 'real time' PCR. According to recent research from Bari, Italy, rapid and reliable methods for detecting multiple pathogens are important for routine diagnosis by reducing time, labour and costs. To this end, primers and TaqMan probes for CPsV and CVV detection by singleplex realtime (q) RT-PCR were initially designed. Further optimizations included the development of a multiplex (m) RT-qPCR assay to detect simultaneously CPsV, CVV, and CTV in a single

reaction. When tenfold serial dilutions prepared using total RNAs from CPsV- and CVV-infected plants were tested, RT-qPCR assays proved to be 100 and 1,000 times more sensitive than conventional RT-PCR, respectively. The target viruses were effectively identified by mRT-qPCR in field infected clementine and sweet orange trees. A one step real-time RT-PCR protocol has been successfully developed and validated to detect CVV and CPsV in citrus trees. Rapid and accurate multiple detection of CTV, CPsV and CVV was achieved using the triplex one step real-time RT-PCR protocol. These assays may prove useful as a tool to support quarantine, eradication and certification programs, resulting faster, more sensitive, relatively easier to perform and less cost effective than the conventional detection procedures [29].

Single-strand conformational polymorphism (SSCP)

The Single-stranded conformation polymorphism (SSCP) of sub-isolates of PsA and PsB of CPsV showed identical SSCP profiles in homologous segments of the RNAs 1 and 3. Segment of the RNA2, representing distinct SSCP profiles. The SSCP analysis of RNA2 reveals two kinds of variants of CPsV. Analysis of the RNA2 population showed that PsA-inducing isolates contain PsB associated sequence variants at low frequency, the PsB-associated sequence variant is predominant in blisters twigs and gummy pustules affecting old leaves, characteristic of PsB isolates, and the PsB-associated sequence variant accumulates preferentially in bark lesions of the trunk and limbs. SSCP analysis of the RNA2 population also enabled monitoring of interference between PsA and PsB associated variants in plants co-inoculated with both psorosis types [77].

Citrus psorosis virus characterization

Many CPsV isolates have been described. The original isolate CPV-4 produces necrotic local lesions when upon mechanical inoculation of the older, fully expanded leaves of *C. quinoa* [53, 54]. Another factor is that CPV-4 can reach relatively high concentrations in infected tissues [20].

Ophiovirus taxonomy

Ophiovirus (Family: *Ophioviridae*) have single stranded negative/possibly ambisense RNA genome (11.3–12.5 kb size) divided into 3–4 segments, each encapsidated in a single CP (43–50 kDa) forming filamentous virions about 3 nm in diameter, in shape of kinked (probably internally coiled) circles of at least two different contour lengths

[19, 73]. Since only one genus is currently recognized, the family description corresponds to the genus description. The genus *Ophiovirus* is currently comprised of five recognized species: CPsV, TMMV [38], *Ranunculus white mottle virus* (RWMV) [66], MiLBVV [52], LRNV [63, 64] and FreSV [68]. The first ophiovirus described was discovered in citrus, but most of them has been found in ornamental plants as *ranunculus* (dicotyledonous), *freesia*, *tulips* and *lachenalia* (monocotyledonous), and *lettuce* (dicotyledonous). The true fungi of the species *O. brassicae* transmit viruses of the genera *Ophiovirus* and *Vari-cosavirus*. These viruses are transmitted in a mode referred to as ‘in vivo’, which resembles circulative transmission because the viruses enter into the cytoplasm of the vector, mostly zoospores from where they are released, presumably during germination [51].

Assessment of the biological activity

A comprehensive review of the conditions, husbandry, tools, grafting techniques, assessment of symptoms and indexing methods used for CPsV has earlier published by Roistacher [53]. Briefly, lateral buds or “blind buds or chip buds” from the donor plant are graft-inoculated onto the stem of a receptor or indicator plant, and usually this is repeated in quadruplicate. Uninoculated receptors are kept under the same conditions as control plants. Well characterized CPsV isolates can be used also as controls in tests. A panel of six citrus cultivar or species combinations is generally used for the strain characterization. Visual assessments of the subsequent growth from the receptor plants are noted into two flushes after inoculation for each of the six citrus cultivar or citrus species combinations. Symptom expression in each host-isolate combination was quantified by a pathogenicity index (PI) that considered symptom intensity and the number of plants showing each symptom. A general pathogenicity index (GPI) was defined for each isolate as a weighted mean of the different PI. Generally, the higher the cumulative GPI score, the more severe the CPsV isolate [42].

The *Citrus psorosis virus* genome

CPsV, the type species of the genus *Ophiovirus*, is the presumed causal agent of psorosis disease [33]. CPsV virions are kinked filaments, 3–4 nm in diameter [19] that separate into two components during sucrose gradient centrifugation [9, 18]. They consist of three single-stranded RNA (ssRNA) of negative polarity (vRNA) and a coat protein (CP) of ~ 48 kDa [5, 58]. RNA 1 is ~ 8,184 nt in size and its viral complementary strand (vcRNA) has two

open-reading frames (ORFs). The first ORF potentially encodes a 24 kDa protein of unknown function and, separated by an intergenic region of 109 nt, a second ORF potentially coding for a 280 kDa protein contains between positions 635 and 874 the core polymerase module with the five conserved motifs proposed to be part of the RNA dependent RNA polymerase (RdRp). vcRNA 2 (~1,644 nt) carries one ORF coding for a 54 kDa protein of unknown function, which includes a motif resembling a nuclear localization signal (NLS), and vcRNA 3 (~1,454 nt) codes for the CP of 48, 6 kDa [5, 39, 58, 59].

In the 3'-terminal untranslated region of RNA2 there is a putative polyadenylation signal [59], was also found in the 3'UTR of the positive strand of RNA3 [58] and RNA1 [39]. The 3'-terminal regions of the three CPsV RNAs are very similar, while their 5'-terminal regions in contrast, differ considerably in sequence.

Genetic variability of the coat protein gene

Variability of the CP gene of CPsV was assessed serologically and by sequence analysis of two genomic regions located in the 3' (region C) and 5' (region V) halves of the gene [3]. Serological analysis of the 53 CPsV field sources from Campania with 23 MAbs revealed nine reaction patterns (serogroups) and at least ten different epitopes. Reaction pattern was not strictly associated to location of the field trees or citrus cultivar. Phylogenetic analysis of the V and C regions showed that the CPV-4 isolate was clearly separated from Campania sources. Furthermore, a low value of ratio between nonsynonymous and synonymous substitutions (d_N/d_S) was estimated for all CPsV isolates, indicating negative selective pressure for amino acid changes, more intense in the C region [3].

The genetic variations of distinct populations of CPsV for 22 isolates from Argentina, California, Florida, Italy and Spain were studied and compared with the control isolate CPV-4 from Texas [35]. Sequence variability was studied using cDNA of regions R1, R2 and R3, located on genomic RNAs 1, 2 and 3, respectively. The three regions selected represented about 12 % of the genome and variations internal to each population were minimal, suggesting a genetic variation among three population groups. Group 1 is the group of isolates from Spain, Italy, Florida and California, group 2 including the population of Argentina, which form a genetically homogeneous population and clearly separated from other populations, and group 3, including the isolate CPV-4 from Texas was distant from both groups. Incongruent phylogenetic relationships in different genomic regions suggested that exchange of genomic segments may have contributed to CPsV evolution [35]. In general, genetic variation of plant negative-stranded RNA viruses have been less studied than those of

positive-strand RNA viruses. Knowledge of the structure and variation of viral populations is often necessary for diagnostic, epidemiological and control purposes [35]. These results point to the need to consider the research programs and control specifically regional.

Insights into virus biology

The molecular and cellular aspects of the infection cycle of ophioviruses have not been investigated yet. To gain insight into CPsV biology, Peña et al. [45] have studied the subcellular localization and functions of the proteins 24 K, 48 K and 54 K. The subcellular localization of the CP and 54 K proteins was analyzed by subcellular fractionation of infected *C. quinoa* and by confocal laser scanning microscopy (CLSM) using GFP fusion proteins expressed transiently in *Nicotiana benthamiana*. The 54 K protein was found in the nuclear, cytoplasmic, cell wall and microsomal fractions. By CLSM, the 54-GFP fusion protein was found in the nucleus, cytoplasm and in the plasmodesmata. Applying the same strategies, the CP was only localized to the cytoplasm but not in other subcellular fractions. The 54 K is probably involved in virion movement and suppression of post transcriptional gene silencing (PTGS), the antiviral defence mechanism of the plant. These two mentioned functions seem to be shared with the 24 K protein of CPsV, but not CP, have shown systemic suppressor activity [31, 45]. The results indicate that CP is limited to encapsidation while the 24 K and 54 K proteins may be involved in suppression of systemic RNA silencing and viral movement [31, 45].

Relationships with other members of the genus *Ophiovirus*

CPsV is the type species of the genus *Ophiovirus*, Family Ophioviridae [73]. The other species of this genus include TMMMV [38], (RWMV) [66], MiLBVV [30, 52, 74], LRNV [63, 64] and FreSV [68]. All these viruses have similar morphology: circular, filamentous, naked nucleocapsids of variable lengths and about 3 nm in diameter. RWMV has at least three RNAs of approximately 7.5, 1.8 and 1.5 kb coated by a 43 kDa protein [66], and its RNA1 has been partially sequenced. TMMMV has a 47 kDa CP [38], and MiLBVV contains four RNAs of 7.8, 1.7, 1.5 and 1.4 kb and a 48 kDa CP with similarity to the CP of CPsV [52, 74]. The complete sequence of MiLBVV RNAs, potentially encoding seven ORFs, has been determined [74]. LRNV contains four RNAs of 7.6, 1.8, 1.5 and 1.4 kb and a 48 kDa CP [64], RNA 2 is about 1.7 for FreSV and the RNA3 is 1.5 kb with a 48.4 kDa CP [70]. Negative and

positive stranded RNAs of ophioviruses are encapsidated in a single CP of 43–50 kDa [5, 18, 66].

The 3′-terminal regions of the three CPsV RNAs are very similar. In contrast, their 5′-terminal regions differ considerably in sequence. The circular morphology of CPsV virions suggests the possibility of a panhandle structure formed by base pairing between the two terminal regions of each RNA [19]. The first 75nt of the CPsV RNA1 terminal regions show 41 % complementarity, a value similar to that described for the RNAs of MiLBVV and of the lettuce big-vein varicosavirus [39]. However, Naum-Ongania et al. [39] observed that secondary structure predictions do not support significant complementarity between the termini of the viral RNAs to afford panhandle formation, or of a “Corkscrew” structure as proposed for Influenza A virus and MiLBVV. None of these structures were predicted by the MFOLD program for any of the CPsV RNAs [39]. On the contrary, in MiLBVV, the terminal sequences do carry partial inverted repeats, potentially allowing panhandles to form; the terminal sequences also contain palindromes potentially able to fold into stem-loops, not found for CPsV sequences [74]. Alternative explanations for the observed circular structures of CPsV are required [39].

At their 3′ termini, the three CPsV viral RNAs have a conserved 12 nt sequence, A₇GUAUC, similar to the A_{4,6}UAAUC found in MiLBVV RNAs [74], and A₇GUAUCA and A₃UA₃GUAUCA for LRNV [67]; which might be involved in the recognition of the RNAs by the RdRp [39].

Sequence analysis of CPsV RNA1 showed in its complementary strand two ORFs potentially encoding proteins of 24 kDa and 280 kDa, separated by an intergenic region. Downstream the 5′-UTR, the first ORF potentially encodes a 24 kDa protein of unknown function is encoded. The 109-nt intergenic region following this ORF, (isolate CPV-4 from Florida), is rich in A + U (88.3 %) and contains an 18-nt sequence (UUAAAA)₃ that could form a hairpin loop. Near the end of the intergenic region, a typical AAUAAA polyadenylation signal is found 12 nt upstream of the CA start polyadenylation site [39]. However, these sequences were not found for the CPsV Spanish isolate P-121 [34], neither for MiLBVV (intergenic region of 147 nt, 66 % of A + U), and LRNV (intergenic region of 80 nt, 65 % of A + U) [67, 74]. A second ORF potentially coding for a 280 kDa protein of 2416 amino acid residues was found after the intergenic region [39].

Interestingly, RNA1 of MiLBVV, the other ophiovirus sequenced, also has two separate ORFs of similar size in its complementary strand [74]. This is a taxonomically significant feature because none of the segmented negative-stranded RNA viruses possess such a genomic organization [39]. The 24 kDa protein has no significant similarity with other entries in data banks, including the 25 kDa ORF

encoded in the complementary strand of MiLBVV RNA1, whereas the 280 kDa protein, which contains the five conserved motifs proposed to be part of the RdRp, shows a significant similarity with the 263 kDa protein also encoded in the complementary strand of MiLBVV RNA1 [39]. These RdRps contain the SDD sequence which is a signature for segmented negative-stranded RNA viruses (*Orthomyxoviridae*, *Arenaviridae* and *Bunyaviridae* families) as opposed to the GDNQ characteristic of negative-stranded non segmented RNA viruses (*Rhabdoviridae* and *Paramyxoviridae* families). These data support grouping both viruses in the same genus *Ophiovirus* [39] previously advanced on the basis of their similar virion morphology and other molecular characteristics [36]. Vaira et al. [67] designed primers for an-ophiovirus-specific RT-PCR test based on sequence similarities in conserved portions of the ophiovirus RNA-1 polymerase encoding region. Phylogenetic studies based on comparisons of amino acid sequences of the conserved portions of the polymerase of MiLBVV, as well as two other ophioviruses, RWMV and CPsV, suggest that ophioviruses represent a monophyletic group that is separated from the other negative-stranded RNA viruses, reinforcing the taxonomic relatedness of the group [39]. Furthermore, RNA1 s of MiLBVV, CPsV and RWMV are similar in size and those of MiLBVV and CPsV also in genomic organization and sequence [39].

A protein about 50–55 kDa of unknown function is encoded by RNA 2 in the positive strand of the ophioviruses CPsV, MiLBVV and LRNV [59, 64, 74]. The 54 kDa protein of CPsV has been detected in infected tissue confirming its size and coding assignment [45]. It is probably involved in virion movement and suppression of post transcriptional gene silencing (PTGS), the antiviral defence mechanism of the plant [31, 45]. These two mentioned functions seem to be shared with the 24K protein of CPsV.

On the other hand, the nuclear localization signal (NLS) present in the 280 kDa protein was also found in the 54 kDa protein encode in the complementary strand of CPsV RNA2 [59]. Naum-Ongania et al. [39] have observed that a similar motif is present in the 263 kDa and 55 kDa proteins of MiLBVV (between amino acids 222–238 and 275–291, respectively), and in the RWMV amino acid sequence (positions 368–384), suggesting that in Ophioviruses, as in plant nucleorhabdoviruses, replication could occur in the nucleus. In any case, gold immunolabeling and electron microscopy of RWMV-infected tissues showed accumulation of CP in the cytoplasm [66].

In the viral complementary RNA 2 of MiLBVV an additional minor ORF encodes a putative protein of about 10 kDa [74], but its function is unknown. In the RNA 2 of CPsV and LRNV this small protein is absent, and so far, it is unknown whether this putative protein is present in

RWMV and FreSV genomes. In case the 10 kDa polypeptide were not present in these viruses, MiLBVV would be the unique ophiovirus with ambisense RNA 2 [74].

RNA3 contains one ORF in the vcRNA in all three viruses which has been identified as coding for the CP [38, 58, 64, 66, 70, 74]. Although the morphology of ophioviruses resembles that of the nucleocapsids of bunyaviruses and tenuiviruses, their RNAs 3 and 2 have no sequence homology with them or with any other negative or ambisense viruses and their CP have no serological relationship [59, 66]. CPsV is serologically unrelated to other ophioviruses, but RNA3 and its product, the CP, share 46,5 % and 30,9 % nucleotide and amino acid sequence identities, respectively, with a Japanese isolate of MiLBVV [22]. FreSV CP protein (Freesia isolate) shows 48.8 % identity (70.5 % similarity) with MiLBVV CP, 49 % identity (68.5 % similarity) with LRNV CP and 30.6 % identity (53.9 % similarity) with CPsV CP [70].

The CPs of MiLBVV and TMMMV are closer with 80 % homology. Attempts to find similarities, with the exception of CPsV, some serological relationship between TMMMV and MiLBVV, and between RWMV and MiLBVV, have been found indicating that some epitopes in the CP among most of the ophiovirus are conserved [52]. In general, different isolates of the same ophiovirus species showed highly conserved amino acid sequences in the CP as showed for CPsV [35] and MiLBVV [40], and less conserved among the different ophiovirus species.

MiLBVV and LRNV present a fourth RNA of negative polarity [64, 65, 74]. The RNA 4 of LRNV encodes a potential protein of 38 kDa, and the RNA 4 of MiLBVV one of 37 kDa (p37). MiLBVV has an additional ORF of 10.6 kDa with a 38 nt overlapping sequence with the p37. This second ORF is proposed to be expressed by a + 1 translational frameshift of p37, but lacks an initiation codon [74]. So far, the functions of these putative proteins are unknown.

Control methods

Healing methods

Since the establishment of control programs based on the elimination of sources of infection is primordial, the fight against CPsV when the tree is infected is not a priority. Indeed, preventing infection seems more appropriate than eliminating the virus when the tree is already infected.

Sanitation technique based on the heat treatment combined with the culture of meristems or grafting onto healthy rootstocks can be adopted. Nevertheless, Roistacher [54] indicated that shoot-tip-grafting (STG) or thermotherapy, alone or in combination, is no guarantee of the elimination

of psorosis. On the other hand, Carvalho et al. [6] showed that STG associated to thermotherapy are necessary to assure a complete virus-free condition. In this experiment, nursery trees were maintained in a climatic chamber at 38 °C under 16 h of light and 32 °C under 8 h of darkness for 60 days, and the meristematic apices were used to shoot tip graft. After the shooting of the meristematic part, the shoot tip graft was over-grafted on 7-month-old Rangpur lime (*Citrus limonia*) seedlings, and developed under greenhouse conditions. Shoot tip grafted clones of sweet orange (*C. sinensis*) cultivars Viz., Lima, Rubi, Piralima, Salustiana, Joao Nunes, Rosa and Pera Caire, subjected to thermotherapy, had 100 % success in elimination of psorosis virus as reported by biological index using cv. Do Ceu sweet orange as indicator plant.

Somatic embryogenesis was used to eliminate CPsV from three citrus species (common mandarin, sweet orange and Dweet tangor), all of which regenerated somatic embryos with different embryogenic potential from stigma and style explants. CPsV was detected by DAS-I-ELISA in explants and embryogenic callus, but was not detected in any plants obtained from somatic embryos, even 24 months after regeneration. Loss of juvenile characters (disappearance of thorns) was observed in the first year of growth and was retained in plants propagated by grafting from thornless stems. Somatic embryogenesis appears to be a very promising technique for the production of healthy citrus stocks [14].

Cross protection

The test of cross-protection is an alternative method of indirect biological indexing in the case of infection by latent or moderate strains. This is a double inoculation of indicator plants with a virulent strain and the inoculum to be tested. The absence of the pathogen in question is confirmed by the development of symptoms due to the lack of cross-protection. Thus, it was determined that lesion bark inoculum was able to induce PsB in sweet orange seedlings, and that only PsA-infected seedlings showed cross protection against PsB [54]. This feature is exploited as a method of disease control [79], consisting to inoculate the plant with a less virulent strain to induce the protection against severe strains. In the case studied, the severe strain is PsB and moderate strain is PsA, which causes less severe symptoms and leads to a slower decline of the tree.

Genetic engineering and disease control in citrus

Because of its economic impact, disease control is often the objective of plant improvement programs. Hence, resistance and defence genes isolated from well studied plant species have been successfully incorporated into other

species to generate pathogen-resistant plants. Recent advances in genomics in citrus and other species have made available an abundance of genes that can be easily cloned and used in transformation process [17].

Whilst retaining desirable characteristics of yield and quality, genetic crossing methods to incorporate CPsV resistance genes into citrus cultivars have proved to be very lengthy and difficult in citrus. Several citrus species have been transformed with the CPG of a severe strain of CPsV but presently there is little published information on the resistance of such plants to CPsV infection [23, 48, 49, 50, 81].

Transgenic lines are propagated by bud grafting onto seedlings used as rootstocks generating replicates of each line. For challenge, transgenic scions are infected by grafting using infective tissue. With respect to CPsV, Kayim et al. [23] have obtained transgenic citrus plants expressing the CP of CPsV. In this study, Grapefruit (*Citrus paradisi* Macf. cv Duncan), sweet orange (*C. sinensis* Osbc. cv Hamlin) and Carrizo citrange [*C. sinensis* (L.) Osbc. x *Poncirus trifoliata* (L.) Raf.] plants were transformed with the CPG of a severe strain of CPsV. Southern, Northern and Western blot analyses of transgenic plants demonstrated that stably transformed grapefruit, sweet orange and Carrizo citrange plants were obtained and that all transgenic plants had at least one copy of the transgene and showed transgene expression. Individual transgenic lines from “Duncan” grapefruit and “Hamlin” sweet orange were propagated for virus challenge by budwood grafting onto Carrizo. Ten plants from each transgenic line of Hamlin sweet orange and Duncan grapefruit were graft inoculated with a severe strain of CPsV. One transgenic line from Hamlin sweet orange was resistant for the virus although two different transgenic lines of Duncan grapefruit showed shock symptoms.

The same strategy was used by Zane et al. [81] producing 21 independent lines of transgenic sweet orange (CP-lines) expressing low and variable amounts of CPsV CP (isolate CPV-4). In these lines no correlation was found between copy number and transgene expression and no significant differences were observed in the response to virus challenge among the lines or among the replicates. Although two different viral loads were evaluated to challenge the transgenic plants, no resistance or tolerance was found in any line after 1 year of observations. On line “23” containing three copies of the transgene, only small amounts of its mRNA is expressed and negligible amounts of CP accumulated. Although the transformant failed to protect against the viral load used, southern blot indicating methylation of the ORF origin, suggests PTGS mechanism of the CP transgene [81].

Recently, Reyes et al. [48] showed that the resistance mechanism is the post transcriptional gene silencing

(PTGS). A mechanism of RNA degradation involved in plant protection against viruses and can be induced by transgenic expression of pathogen-derived sequences encoding hairpin RNAs. Using this strategy, a herbaceous model plant, *Nicotiana benthamiana* was transformed with hairpin constructs obtained from two different genome regions of the Argentine CPsV 90-1-1 isolate, the CP from RNA3 and the 54K gene from RNA2. The degree of resistance obtained varied according to the chosen viral sequence. The analysis of the levels of small interfering RNA (siRNA) accumulation by Northern blot and viral RNAs analysis by RT-PCR indicated that the construct derived from CP gene was better at inducing PTGS than that originating from the 54K gene. The dependence of PTGS induction on the degree of identity between the target and the inducer transgene sequences was tested using sequences derived from CPV-4, a more distant isolate of CPsV, as PTGS targets. Efficient silencing induction was also obtained to this isolate through the expression of the CP-derived hairpin. Furthermore, alignment between CPV-4 and the RNA2-hairpin fragment (54K-fragm) of CPsV 90-1-1 sharing 81 % identity, a good degree of gene silencing is achieved by means of genes sequences having less than 100 % identity between the inducer siRNA segment and the target sequence making unlikely the explanation of the limited resistance observed in these intron-hairpin-54K (ihp54K) lines by means of lack of sequence identity between isolates [48].

Transgenic sweet orange plants transformed with hairpin constructs corresponding to viral CP, 54K or 24K genes has been also reported [49]. After challenge with the virus, the CP transgenic plants were more effective in controlling the CPsV and consistently showed lower virus levels and no symptom development compared to 54K and 24K transgenic plants. The study reported that the observed CPsV resistance was due to preactivated RNA silencing rather than the siRNA accumulation levels in the ihp-CP transgenic sweet orange plants prior to virus challenge [49]. Virus induced gene silencing (VIGS) is evidenced by the presence of specific siRNA molecules for RNA1, RNA2, and RNA3 of CPsV in infected plants [76]. Reyes et al. [49] observed also VIGS induction through CP specific siRNA accumulation in the inoculated plants. From sweet orange transgenic lines previously obtained, fourteen lines were selected containing 1, 2 or 3 copies of the transgenes. These plants were evaluated for their acquired resistance against two isolates, PsA (CPV-4) and PsB (CPsV 189-34), which differ in symptoms severity. These lines were susceptible to both isolates when graft-infected, although one of the lines carrying the CPG (CP-96 line) containing two copies of the transgene and expressing a low level of the CP showed a delay in symptom expression when inoculated with the PsB isolate

[50]. These studies represent a first step to a possible control of CPsV, but the implementation of the technique requires time consuming in transformation procedure and multiplication. To date, control methods do not appear to be effective solutions or sustainable and only the prevention could contain the dispersion of the virus, through certification programs which are crucial to update.

Certification program

Certification programs introduce regulatory or legal requirements and restrictions that enforce to some degree the use of clean material. These regulations are based upon local conditions and may not regulate all pests and pathogens [25, 41]. 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 [24].

In California, the Citrus Clonal Protection Program (CCPP) carries out a comprehensive biological indexing program to detect graft-transmissible diseases that may arrive in an imported budline. The tissue of the imported budline is grafted onto citrus indicator seedlings, used to detect specific diseases. The indicator varieties are selected because of their sensitivity to diseases and their ability to express symptoms. Laboratory tests are also used for disease diagnosis. If any disease or pathogen is found, various therapies are used and if successful, the budline enters a full-scale variety introduction index [78]. Other well-established program includes those of Spain, Australia, South Africa and Florida [24, 75].

A successful certification programs actually comprises three linked sub-programs: a quarantine programs, a clean stock programs and a certification programs [75]. The quarantine programs are designed to ensure the safe introduction of new types of germplasm, while clean stock programs have the aim of producing and maintaining healthy, true-to-type local cultivars, and the certification programs ensures that the already quarantined and tested plants are distributed and tracked after distribution.

The most important components of certification programs are the sources of propagative material. The bases for all further propagations are foundation blocks. These include both protected foundation blocks of initial material and field-planted or protected foundation blocks usually located at the nurseries [24]. Certification programs may be either mandatory or voluntary. Mandatory programs create precise requirements and obligations to prevent the introduction and spread of the relevant diseases. By distributing and requiring the use of pathogen-tested and pathogen-free budwood, mandatory certification decreases the ability of

pathogens to infiltrate citrus crops [75]. In voluntary programs, the initially higher investment in certified materials may lead some nurserymen to use non-certified materials despite the long-term negative economic impact of using this inferior material [24].

Germplasm banks may have a very important role in certification programs by supplying the initial materials for propagation, particularly if they maintain healthy plants of commercial varieties [24].

Almost every country facing the threat of citrus disease has adopted a certification program to rebuild or preserve its citrus industry [75], but they do not yet take in account the recent data on the natural spread of the viruses mentioned above. The need for the updating the criteria is evident as shown by the following examples.

The occurrence of CPsV in Campania, southern Italy, was studied by Alioto et al. [2]. The results show that CPsV is widespread in Campania with an especially high incidence in younger plantings, suggesting that infected propagating material has been increasingly used in this area, in the absence of certification.

A study conducted by Powell et al. [47] showed that for the variety of grapefruit ruby red, 79 % trees of orchards were infected with psorosis and 31 % trees used to propagation were contaminated (in seven centers of propagation). The fact that there are fewer individuals affected in the center of propagation than in orchards could indicate a natural spread. These results were quite unintended, because California has developed a program in 1952 (based on the first program «Psorosis Free Citrus») to eliminate CPsV of the region. The development of a control program should consider the scientific advances and technical on knowledge of the virus and the detection means.

Concluding remarks

CPsV is the causal agent of old disease of citrus. Interactions between the different CPsV strains and their citrus hosts assembled a complicated plant pathosystem. Most of the ophioviruses are soil-transmitted by a root-infecting Oomycete genus *Olpidium*, and in the case of CPsV an aerial vector is also suspected, although there are no evidences so far. The virions are circles with 3–4 nm in diameter of at least two different contour lengths, particles can form pseudolinear duplex structures, and the coiled filamentous are about 9–10 nm in diameter [36]. The 3'-terminal regions of the three CPsV RNAs are very similar. In contrast, their 5'-terminal regions differ considerably in sequence and they do not anneal to perfect panhandle structures, which is expected to form according to the circular morphology and a “corkscrew” conformation was not found. Although a remarkable advance in the

knowledge of CPsV genetics and the diversity of CPsV viral populations have been achieved. Therefore, further attention needs the study of the interactions between viral products, the different citrus hosts and the vector transmission factors, which are the basis of pathogenicity, host resistance and viral epidemiology. The success of the citrus management strategies depends on a deep understanding of these interactions.

Recently, biotechnological approaches of viral control, which exploit virus plant-host interactions for viral control, such as sequence-based control strategies were developed. Resistant transgenic plants based on PTGS, against specific viral sequences, are already developed and promising resistance may emerge from trials. This strategy retains high possibilities of success in the proper management of CPsV disease.

References

1. Alioto D, Gangemi M, Deaglio S, Sposato S, Noris E, Luisoni E, Milne RG. Improved detection of *Citrus psorosis virus* using polyclonal and monoclonal antibodies. *Plant Pathol.* 1999;48: 735–41.
2. Alioto D, Troisi A, Peluso A, Quatrano G, Masenga V, Milne RG. Occurrence of *Citrus psorosis virus* in Campania, Southern Italy. *Eur J Plant Pathol.* 2000;106:795–9.
3. Alioto D, Malfitano M, Troisi A, Peluso A, Martín S, Milne RG, Guerri J, Moreno P. Variability of the coat protein gene of *Citrus psorosis virus* in Campania, southern Italy. *Arch Virol.* 2003;148:2155–66.
4. Barragan-Valencia G, Morales-Loredo A, Alvarez-Ojeda MG, Rio MAPD, Quintero-Zapata I. Quantitative diagnosis of *Citrus psorosis virus* by real time RT-PCR. *Agrociencia.* 2008;42(2): 225–32.
5. Barthe GA, Ceccardi GL, Manjunath KL, Derrick KS. *Citrus psorosis virus*: nucleotide sequencing of the coat protein gene and detection by hybridization and RT-PCR. *J Gen Virol.* 1998;79: 1531–7.
6. Carvalho SA, Santos FA, Machado MA. Psorosis virus complex elimination from citrus by shoot-tip-grafting associated to thermotherapy. *Fitopatologia Brasileira.* 2002;27(3):306–8.
7. Da Graça JV, Bar-Joseph M, Derrick KS. Immunoblot detection of citrus psorosis in Israel using citrus ringspot antiserum. *Proceedings of the 12th International Organisation of Citrus Virologists (IOCV) Conference, Riverside.* 1993, pp 432–434.
8. Danos E. La psorosis de los cítricos: la epidemia en curso en Argentina y el desafío de su control. *Revista de Investigaciones Agropecuarias.* 1990;22:265–77.
9. Derrick KS, Brlansky RH, da Graça JV, Lee RF, Timmer LW, Nguyen TK. Partial characterization of a virus associated with citrus ringspot. *Phytopathology.* 1988;78:1298–301.
10. Derrick KS, Lee RF, Hewitt BG, Barthe GA, da Graça JV. Characterization of citrus ringspot virus. In: Timmer LW, Garnsey SM, Navarro L, editors. *Proceedings of the 11th International Organisation of Citrus Virologists (IOCV) Conference, Riverside.* 1991, pp 386–390.
11. Djelouah K, Potere O, Boscia D, D'Onghia AM, Savino V. Production of monoclonal antibodies to citrus psorosis associated virus. In: da Graça JV, Lee RF, Yokomi RH, editors. *Proceedings of the 14th International Organization of Citrus Virologists (IOCV) Conference, Riverside.* 2000, pp 152–158.
12. D'Onghia AM, Djelouah K, Alioto KM, Castellano A, Savino V. Elisa correlates with biological indexing for the detection of *Citrus psorosis virus* associated virus. *J Plant Pathol.* 1998;80: 157–63.
13. D'Onghia AM, Djelouah K, Savino V. Serological detection of *Citrus psorosis virus* in seeds but not in seedlings of infected mandarin and sour orange. *J Plant Pathol.* 2000;82(3):233–5.
14. D'Onghia AM, Carimi F, De Pasquale F, Djelouah K, Martelli GP. Elimination of *Citrus psorosis virus* by somatic embryogenesis from stigma and style cultures. *Plant Pathol.* 2001;50(2): 266–9.
15. D'Onghia AM, Djelouah K, Frasheri D, Potere O. Detection of *Citrus psorosis virus* by direct tissue blot immunoassay. *J Plant Pathol.* 2001;83:139–42.
16. EPPO. Data Sheets on Quarantine Pests: Citrus ringspot virus. Prepared by CABI and EPPO for the EU under Contract 90/399003. 2005. http://www.eppo.org/QUARANTINE/virus/Citrus_ringspot_virus/CIRSV0_ds.pdf.
17. Febres V, Fisher L, Khalaf A, Moore AG. Citrus transformation: challenges and prospects. *Genetic transformation.* 2011;101–22.
18. Garçia ML, Grau O, Sarachu AN. Citrus psorosis is probably caused by a bipartite ssRNA virus. *Res Virol.* 1991;142:303–11.
19. Garçia ML, Dal Bó E, Grau O, Milne RG. The closely related citrus ringspot and *Citrus psorosis viruses* have particles of novel filamentous morphology. *J Gen Virol.* 1994;75:3585–90.
20. Garçia ML, Sánchez de la Torre ME, Dal Bó E, Djelouah K, Luisoni E, Milne RG, Grau O. Detection of citrus psorosis-ringspot using RT-PCR and ELISA. *Plant Pathol.* 1997; 46:830–6.
21. Gottwald TR, Palle SR, Miao H, Seyran M, Skaria M, da Graça JV. Assessment of the possibility of natural spread of citrus psorosis disease. *Proceedings of the 16th International Organization of Citrus Virologists (IOCV) Conference, Riverside.* 2005, pp 240–250.
22. Kawazu Y, Sasaya T, Morikawa T, Sugiyama K, Natsuaki T. Nucleotide sequence of the coat protein gene of *Mirafiori lettuce virus*. *J Gen Plant Pathol.* 2003;69:55–60.
23. Kayim M, Barthe G, Beretta J, Derrick K. Transgenic citrus plants expressing the coat protein gene of *Citrus psorosis virus*. *Phytopathology.* 2005;95(6):S52–S52 Suppl.
24. Khan IA. Citrus genetics, breeding and biotechnology. Cambridge: CABI Wallingford; 2007.
25. Lee RF, Lehmann P, Navarro L. Nursery practices, budwood and rootstock certification programs. In: Timmer LW, Duncan L, editors. *Citrus health guide.* St. Paul: APS Press Minn; 1999. p. 35–46.
26. Legarreta GG, Garcia ML, Costa N, Grau O. A highly sensitive heminested RT-PCR assay for the detection of *Citrus psorosis virus* targeted to a conserved region of the genome. *J Virol Methods.* 2000;84:15–22.
27. Loconsole G, Castellano MA, Dell'Orco M, Boscia D, Savino V. Serological detection of *Citrus psorosis virus* using a polyclonal antiserum to recombinant virus coat protein. *J Plant Pathol.* 2006;88:171–3.
28. Loconsole G, Fatone MT, Savino V. Specific digoxigenin-labeled riboprobes for detection of *Citrus psorosis virus* and *Citrus variegation virus* by molecular hybridization. *J Plant Pathol.* 2009;91(2):311–9.
29. Loconsole G, Saponari M, Savino V. Development of real-time PCR based assays for simultaneous and improved detection of citrus viruses. *Eur J Plant Pathol.* 2010;128:251–9.
30. Lot H, Campbell RN, Couche S, Milne RG, Roggero P. Transmission by *Olpidium brassicae* of *Mirafiori lettuce virus* and

- Lettuce big-vein virus* and their roles in lettuce big-vein etiology. *Phytopathology*. 2002;92:288–93.
31. Luna GR, Peña EJ, Borniego MB, Heinlein M, Garcia ML. Ophioviruses CPsV and MiLBVV movement protein is encoded in RNA 2 and interacts with the coat protein. *Virology*. 2013;441(2):152–61.
 32. Martín S, Alioto D, Milne RG, Guerri J, Moreno P. Detection of *Citrus psorosis virus* in field trees by direct tissue immunoassay in comparison with ELISA, symptomatology, biological indexing and cross-protection tests. *Plant Pathol*. 2002;51:134–41.
 33. Martín S, Alioto D, Milne RG, Garnsey SM, García ML, Grau O. Detection of *Citrus psorosis virus* by ELISA, molecular hybridization, RT-PCR and immunosorbent electron microscopy and its association with citrus psorosis disease. *Eur J Plant Pathol*. 2004;110:747–57.
 34. Martín S, López C, García ML, Naum-Onganía G, Grau O, Flores R. The complete nucleotide sequence of a Spanish isolate of *Citrus psorosis virus*: comparative analysis with other ophioviruses. *Arch Virol*. 2005;150(1):167–76.
 35. Martín S, García ML, Troisi A, Rubio L, Legarreta G, Grau O, Alioto D, Moreno P, Guerri J. Genetic variation of populations of *Citrus psorosis virus*. *J Gen Virol*. 2006;87:3097–102.
 36. Milne RG, Djelouah K, García ML, Dal Bo E, Grau O. In: Structure of citrus-ringspot psorosis-associated virus particles: implication for diagnosis and taxonomy. Proceedings of the 13th International Organization of Citrus Virologists (IOCV) Conference, China. 1996, pp 189–97.
 37. Milne RG, García ML, Moreno P. *Citrus psorosis virus*. In: AAB descriptions of plant viruses. 2003. No. 401.
 38. Morikawa T, Nomura Y, Yamamoto T, Natsuaki T. Partial characterization of virus-like particles associated with Tulip mild mottle mosaic. *Ann Phytopathol Soc Jpn*. 1995;61:78–581.
 39. Naum-Onganía G, Gago-Zachert S, Peña E, Grau O, García ML. *Citrus psorosis virus* RNA 1 is of negative polarity and potentially encodes in its complementary strand a 24K protein of unknown function and 280K putative RNA dependent RNA polymerase. *Virus Res*. 2003;96:49–61.
 40. Navarro JA, Torok VA, Vetten HJ, Pallas V. Genetic variability in the coat protein genes of *Lettuce big-vein associated virus* and *Mirafiori lettuce big-vein virus*. *Arch Virol*. 2005;150: 681–94.
 41. Navarro L. Citrus sanitation, quarantine and certification programs. Moreno P, da Graça JV, Timmer LW, editors. Proceedings of the 12th International Organization of Citrus Virologists (IOCV) Conference, Riverside. 1993, pp 383–91.
 42. Navas-Castillo J, Moreno P. Biological diversity of citrus ring-spot isolates in Spain. *Plant Pathol*. 1993;42:347–57.
 43. Navas-Castillo J, Moreno P. Filamentous flexuous particles and serologically related proteins of variable size associated with citrus psorosis and ringspot diseases. *Eur J Plant Pathol*. 1995;101:343–8.
 44. Palle SR, Miao H, Seyran M, Louzada ES, da Graça JV, Skaria M. Evidence for association of *Citrus psorosis virus* with symptomatic trees and an *Olpidium*-like fungus. Proceedings of the 14th International Organisation of Citrus Virologists (IOCV) Conference, Riverside. 2005, pp 423–6.
 45. Peña E, Luna GR, Zaneck MC, García ML. *Citrus psorosis virus*: insights into virus biology. Proceedings of the 18th International Organisation of Citrus Virologists (IOCV) Conference, Riverside. 2010, p 52.
 46. Potere O, Boscia D, Djelouah K, Elicio V, Savino V. Use of monoclonal antibodies to *Citrus psorosis virus* for diagnosis. *J Plant Pathol*. 1999;81:209–12.
 47. Powell CA, Pelosi RR, Sodona RM. A psorosis like agent prevalent in Florida's Grapefruit groves and budwood sources. *Plant Dis*. 1998;82(2):208–9.
 48. Reyes CA, Peña EJ, Zaneck MC, Sanchez DV, Grau O, García ML. Differential resistance to *Citrus psorosis virus* in transgenic *Nicotiana benthamiana* plants expressing hairpin RNA derived from the coat protein and 54K protein genes. *Plant Cell Rep*. 2009;28:1817–25.
 49. Reyes CA, De Francesco A, Pena EJ, Costa N, Plata MI, Sendin L, Castagnaro AP, Garcia ML. Resistance to *Citrus psorosis virus* in transgenic sweet orange plants is triggered by coat protein-RNA silencing. *J Biotech*. 2011;151(1):151–8.
 50. Reyes CA, Zaneck MC, Velázquez K, Costa N, Plata MI, García ML. Generation of sweet orange transgenic lines and evaluation of *Citrus psorosis virus*-derived resistance against psorosis A and psorosis B. *J Phytopathol*. 2011;159:531–7.
 51. Rochon D, Kakani K, Robbins M, Reade R. Molecular aspects of plant virus transmission by *Olpidium* and *Plasmodiophorid* vectors. *Annu Rev Phytopathol*. 2004;42:211–41.
 52. Roggero P, Ciuffo M, Vaira AM, Accotto GP, Masenga V, Milne RG. An Ophiovirus isolated from lettuce with big-vein symptoms. *Arch Virol*. 2000;145:2629–42.
 53. Roistacher CN. Graft-transmissible diseases of citrus. Handbook for detection and diagnosis. Rome: Food and Agriculture Organization of the United Nations (FAO); 1991. p. 115–26.
 54. Roistacher CN. Psorosis-A Review. In: Moreno P, da Graça JV, Timmer LW, editors. Proceedings of the 12th International Organisation of Citrus Virologists (IOCV) Conference, Riverside. 1993, pp 139–54.
 55. Rosa C, Polek M, Falk BW, Rowhani A. Improved efficiency for quantitative and qualitative indexing for *Citrus tristeza virus* and *Citrus psorosis virus*. *Plant Dis*. 2007;91:1089–95.
 56. Rouag N, Kichi A, Lousini E, Melin R. Etude comparative des relations sérologiques, morphologiques et moléculaires entre le Citrus Ringspot et le *Citrus psorosis virus*. In: Rapport du 9ème Congrès Arabe pour la Protection des Végétaux, Damas, Syrie. 2006.
 57. Roy A, Fayad A, Barthe G, Brlansky RH. A multiplex polymerase chain reaction method for reliable, sensitive and simultaneous detection of multiple viruses in citrus trees. *J Virol Methods*. 2005;129:47–55.
 58. Sánchez de la Torre ME, Riva O, Zandomeni R, Grau O, Garcia ML. The top component of *Citrus psorosis virus* contains two ssRNAs, the smaller encodes the coat protein. *Mol Plant Pathol*. 1998. <http://www.bspp.org.uk/mppol/1998/1019sanchez>.
 59. Sánchez de la Torre ME, López C, Grau O, Garcia ML. RNA 2 of citrus psorosis virus is of negative polarity and contains a single open reading frame in its complementary strand. *J Gen Virol*. 2002;83:1777–81.
 60. Sasaya T, Koganezawa H. Molecular analysis and virus transmission tests place *Olpidium virulentum*, a vector of Mirafiori lettuce big-vein virus and tobacco stunt virus, as a distinct species rather than a strain of *Olpidium brassicae*. *J Gen Plant Pathol*. 2006;72:20–5.
 61. Skaria M, Miao H, Avila E. Post-freeze status of *Citrus psorosis virus* in Texas. Proceedings of the 15th International Organisation of Citrus Virologists (IOCV) Conference, Riverside. 2002, pp 366–7.
 62. Sofy AR, Moussa AA, Fahmy H, Ghazal SA, El-Dougoudg KA. Anatomical and ultrastructural changes in Citrus leaves infected with *Citrus psorosis virus* Egyptian isolates. *J Appl Sci Res*. 2007;3(6):485–94.
 63. Torok VA and Vetten HJ. Characterization of an ophiovirus associated with lettuce ring necrosis. Joint Conference International Working Groups on Legume and Vegetable Viruses (Abstract), Bonn. 2002, p 4.
 64. Torok VA and Vetten HJ. Identification and molecular characterization of a new ophiovirus associated with lettuce ring necrosis disease. Proceedings of Arbeitskreis Viruskrankheiten der Pflanzen, Heidelberg, Germany. 2003.

65. Torok VA, Vetten HI. Ophiovirus associated with lettuce ring necrosis (Abstract). Cernobbio: European Society for Virology Meeting; 2010. p. 282.
66. Vaira AM, Milne RG, Accotto GP, Luisoni E, Masenga V, Lisa V. Partial characterization of a new virus from ranunculus with a divided RNA genome and circular supercoiled thread-like particles. *Arch Virol*. 1997;142:2131–46.
67. Vaira AM, Accotto GP, Costantini A, Milne RG. The partial sequence of RNA1 of the ophiovirus *Ranunculus white mottle virus* indicates its relationship to rhabdovirus and provides candidate primers for an ophiovirus-specific RT-PCR test. *Arch Virol*. 2003;148:1037–50.
68. Vaira AM, Lisa V, Costantini A, Masenga V, Rapetti S, Milne RG. Ophioviruses infecting ornamentals and a probable new species associated with a severe disease in freesia. *Acta Hort*. 2006;722:191–9.
69. Vaira AM, Kleynhans R, Hammond J. First report of Freesia sneak virus infecting *Lachenalia* cultivars in South Africa. *Plant Dis*. 2007;91(6):770.
70. Vaira AM, Hammond J. An update on Freesia sneak virus, a new species in the Ophiovirus genus. 12th ISVDOP, Haarlem, The Netherlands. 2008.
71. Vaira AM, Milne RG. Ophiovirus. In: Mahy BWJ, Van Regenmortel M, editors. *Encyclopedia of Virology*. Oxford: Elsevier; 2008. p. 447–54.
72. Vaira AM, Hansen MA, Murphy C, Reinsel MD, Hammond J. First report of Freesia sneak virus in *Freesia* sp. in Virginia. *Plant Dis*. 2009;93(9):965.
73. Vaira AM, Gago-Zachert S, García ML, Guerri J, Hammond J, Milne RG, Moreno P, Morikawa T, Natsuaki T, Navarro JA, Pallás V, Torok V, Verbeek M, Vetten HJ. Ophioviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*. 2011. p. 743–8.
74. Van der Wilk F, Dullemans AM, Verbeek M, van den Heuvel JM. Nucleotide sequence and genomic organization of an Ophiovirus associated with lettuce bug-vein disease. *J Gen Virol*. 2002;83:2869–77.
75. Vapnek J. Legislatively establishing a health certification programme for Citrus. FAO legal papers online. 2009. <http://www.fao.org/lega/prs-ol/1po81.pdf>.
76. Velázquez K, Renovell M, Comellas P, Serra M, García ML, Pina JA, Navarro L, Moreno P, Guerri J. Effect of temperature on RNA silencing of a negative stranded RNA plant virus: *Citrus psorosis virus*. *Plant Pathol*. 2010;59:982–90.
77. Velázquez K, Pina JA, Navarro L, Moreno P, Guerri J. Association of citrus psorosis B symptoms with a sequence variant of the *Citrus psorosis virus* RNA2. *Plant Pathol*. 2012;61:448–56.
78. Vidalakis G, da Graça JV, Dixon WN, Ferrin D, Kesinger M, Krueger RR, Lee RF, Melzer MJ, Olive J, Polek M, Sieburth PJ, Williams LL, Wright GC. Citrus Quarantine Sanitary and Certification Programs in the USA. *Citrograph*. 2010;3:26–35.
79. Voinnet O. RNA silencing as a plant immune system against viruses. *Trends Genet*. 2001;17(8):499–500.
80. Zane MC, Peña E, Reyes CA, Figueroa J, Stein B, Grau O, Garcia ML. Detection of *Citrus psorosis virus* in the northwestern citrus production area of Argentina by using an improved TAS-ELISA. *J Virol Methods*. 2006;137:245–51.
81. Zane MC, Reyes CA, Cervera M. Genetic transformation of sweet orange with the coat protein gene of *Citrus psorosis virus* and evaluation of resistance against the virus. *Plant Cell Rep*. 2008;27:57–66.