SHORT COMMUNICATION

CHERRY CHLOROTIC RUSTY SPOT AND CHERRY LEAF SCORCH: TWO SIMILAR DISEASES ASSOCIATED WITH MYCOVIRUSES AND DOUBLE STRANDED RNAs

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SUMMARY

Cherry chlorotic rusty spot (CCRS) is a disease of unknown etiology affecting sweet and sour cherry in Southern Italy. CCRS has constantly been associated with the presence of an unidentified fungus, double-stranded RNAs (dsRNAs) from mycoviruses of the genera *Chrysovirus*, *Partitivirus* and *Totivirus* and two small circular RNAs (cscRNAs) that may be satellite RNAs of one of the mycoviruses. The similarity of CCRS and Cherry leaf scorch (CLS), a disease caused by the perithecial ascomycete *Apiognomonia erythrostoma*, is discussed in the light of symptomatology, fungal fructifications, nucleotide sequence analysis of fungal genes, including the 18S rDNA amplified by PCR from infected leaves, and isolated mycelia. Comparison of mycoviral dsRNAs isolated from plants affected by both diseases further supports the view that CCRS and CLS are closely related. This is the first report showing the presence of CCRS-like mycoviral dsRNAs in CLS-infected cherry trees from Spain, indicating that CCRS-associated mycoviruses are more widely spread than though before.

Key words: dsRNAs, mycoviruses, *Apiognomonia erythrostoma, Diaporthales, Phomopsis.*

Cherry chlorotic rusty spot (CCRS), was first observed in sweet cherry trees in Campania (Southern Italy) in 1996 (Di Serio *et al*., 1996) and, two years later, in sour cherry (Di Serio *et al*., 1998). In spring, the disease is characterized by translucent-chlorotic spots on which rusty freckles appear later. Over time, the lesions cover the entire leaf surface causing their premature shedding. Fruits of affected plants show irregular reddish lines on the skin, are deformed and smaller than those from healthy looking trees and do not mature properly. This disorder may have a fungal etiology since mycelium-like structures are consistently associated with it, plus 10 double-stranded RNAs (dsRNAs) identified as genomic components of mycoviruses in the genera *Chrysovirus*, *Partitivirus* and *Totivirus* (Alioto *et al*., 2003; Di Serio, 1996; Covelli *et al.,* 2004; Coutts *et al*., 2004; Kozlakidis *et al*., 2006), and two closely related small circular RNAs (cscRNAs). CscRNAs possess ribozymatic activity mediated by hammerhead structures in both polarity strands (Di Serio *et al*., 1997), thus they were proposed to be mycovirus satellites (Di Serio *et al*., 2006). Two recently sequenced smaller dsRNAs of unclear nature (Covelli *et al*., 2008) are also associated with the disease.

Based on symptom expression, the presence of mycelial structures, the size, number and nucleotide sequence of the mycoviral dsRNAs, CCRS appears similar to Amasya cherry disease (ACD) reported from Turkey in 1970 (Blodgett *et al*., 1970; Citir, 1987; Açikgöz *et al*., 1994; Di Serio *et al*., 1996, 1998; Coutts *et al*., 2004; Covelli *et al*., 2004, 2008; Kozlakidis *et al*., 2006). The mycete supposed to be the aetiological agent of both diseases has never been isolated on artificial media, thus it is still unidentified.

A recent PCR analysis of the r18S fungal ribosomal gene has disclosed the presence of a fungus of the order Diaporthales in the leaves of CCRS-affected sour and sweet cherry trees (Carrieri, 2009). This prompted us to investigate whether CCRS could be connected with *Apiognomonia erythrostoma,* family Gnomoniaceae (order Diaporthales), the causal agent of a cherry disease known as leaf scorch (CLS) (Arnaud and Arnaud, 1931; Frank, 1891; Goidanich, 1964; Sànchez Sànchez and García Becedas, 2007; Chalkley *et al*., 2009). In fact, some CCRS symptoms, especially those shown late in the season by leaves and fruits, are strikingly similar to those caused to cherry by *A. erythrostoma*, even though the persistence through winter of dead withered leaves and fruits, reported as distinctive CLS features (Arnaud and Arnaud, 1931; Ferraris, 1938), has never been observed in CCRS-affected trees. This type of symptoms, however, was reported as typical of Amasya disease

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(Blodgett *et al*., 1970).

The objective of this study was to investigate the biological, morphological and molecular properties of the fungus putatively involved in CCRS and to compare them with those of the causal agent of CLS. In addition, the molecular characteristics were investigated of mycovirus dsRNAs present in CLS symptomatic leaves.

Leaves were collected from CLS-affected sweet cherry trees (cvs Ambrunés and Pico Colorado) in Copa Barrado, San Jorge de Navaconcejo, Bahonal de Cabrero and San Jorge de Piornal (Spain), in different locations of the Valle del Jerte (Barrado, Navaconcejo, Cabrero y Piornal, Cáceres, Spain) and from CCRS-affected sour and sweet cherry trees (cvs Napoleon Bigarreau and La Signora) in Ariano Irpino (Avellino, Italy), showing mild or severe chlorotic spots on the leaves.

The presence of fungal fruiting bodies was monthly looked for in symptomatic CCRS leaves on the tree canopy and soil. Symptomatic leaves of all examined trees, prior to shedding in October, bore pycnidia 100-160 µm in size containing colourless, single-celled, filiform and slightly curved conidia 14-25×1-1.3 µm in size (beta conidia). From November onwards perithecia were found on the leaves fallen to the ground. These fruiting bodies were hypophyllous, partially immersed, globose, apically reddish-brown, and measured 200-350 µm in diameter (Fig. 1a). The asci were cylindrical, with a tapering base and apical ring, and contained eight ascospores arranged in two rows. The ascospores (15-20×4-6 µm) were hyaline, bicellular, with a smaller lower cell (Fig. 1b). The morphological characteristics, size and period of appearance of perithecia, pycnidia and respective spores, agree with *A. erythrostoma* description and are identical to those observed on CLS-affected cherry leaves from Spain (Monod, 1983). Inoculation of healthy cherry seedlings with ascospore and conidial suspensions failed, so that the *A. erythrostoma* role in the aetiology of CCRS disease could not be determined. Attempts to isolate the fungus on artificial media using infected leaves, beta conidia and ascospores were unsuccessful, confirming what reported by Monod (1983). Thus, a molecular approach was followed starting from infected plant samples.

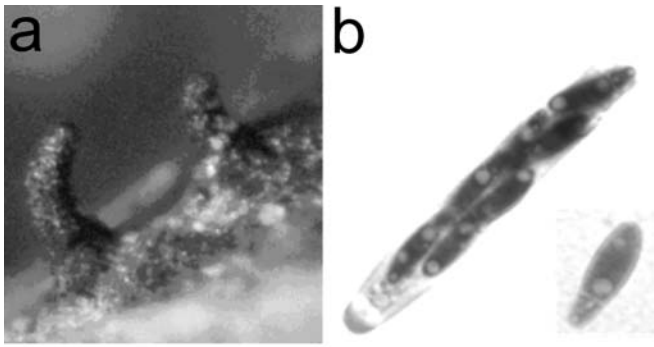

Fig. 1. *Apiognomonia erythrostoma*: a) perithecia with the typical reddish apex; b) asco with ascospores. The inset shows a detail of the two-celled ascospore.

Total nucleic acids (TNA) were extracted from 200-500 mg of healthy and symptomatic CCRS and CLS leaf tissues and from 50-100 perithecia according to Foissac *et al*. (2001) and resuspended in a final volume of 300 µl of sterile water. Aliquots (4 µl) of TNA extracts were analyzed by PCR using primers EF4-EF3 (Smit *et al*., 1999), specific for the fungal 18S rDNA region and primers ApioF2-ApioR1 (Bahnweg *et al*., 2005), specific for the ITS regions of members of the family Gnomoniaceae. The PCR master mix, containing 50 ng of DNA, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 400 nM of forward and reverse primers and 2.5 U of *Taq* DNA polymerase (Sigma-Aldrich, USA), was maintained at 95°C for 3 min. Thirty-five cycles of PCR were done by heating at 95°C for 30 sec, 48°C (primers EF4-EF3) or 55°C (primers ApioF1-ApioR2) for 30 sec, and 72°C for 1 min, followed by final extension at 72°C for 10 min. PCR products of the expected size (1600 and 320 bp, respectively) were separated by agarose gel electrophoresis and purified from the excised gel bands using NucleoSpin Extract kit (Macherey-Nagel, Germany). In the case of PCR with EF4-EF3, the purified products were ligated in pDRIVE vector (Qiagen, USA) and used to transform competent *E. coli* TG1 cells by thermal shock. After transformation, bacterial cells were spread to LB-agar plates in the presence of 100 µg/ml ampicillin and grown overnight at 37°C. For each experiment 10 positive colonies were investigated whose inserts were sequenced (BMR Genomics, Italy). Sequences from selected clones and purified PCR products were compared with those deposited in databases using the BLASTn program with default parameters (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was constructed using the MEGA 3.1 program (Kumar *et al*., 2004), and the Neighbor joining (NJ) method of phylogenetic reconstruction (Saitou and Nei, 1987) with 1000 bootstrap replicates under the conditions of uniform rates among sites and pairwise deletion of gaps.

PCR with primers EF4 and EF3, which generically detects fungi, amplified a 18S rDNA fragment, *ca.*1600 bp in size, from symptomatic areas of both CCRS- and CLS-affected leaves. The alignment of nucleotide sequences of 10 selected clones obtained from the two samples, generated two consensus sequences (accession Nos HQ651154 and HQ651153), showing an extensive identity (99.9%) to each other. This result confirmed the presence of the same fungus in the symptomatic CLS and CCRS leaf tissues but, contrary to expectations, this fungus appeared more related to the family Diaporthaceae than Gnomoniaceae, as shown by the comparison of the 18S rDNA sequenced fragment with

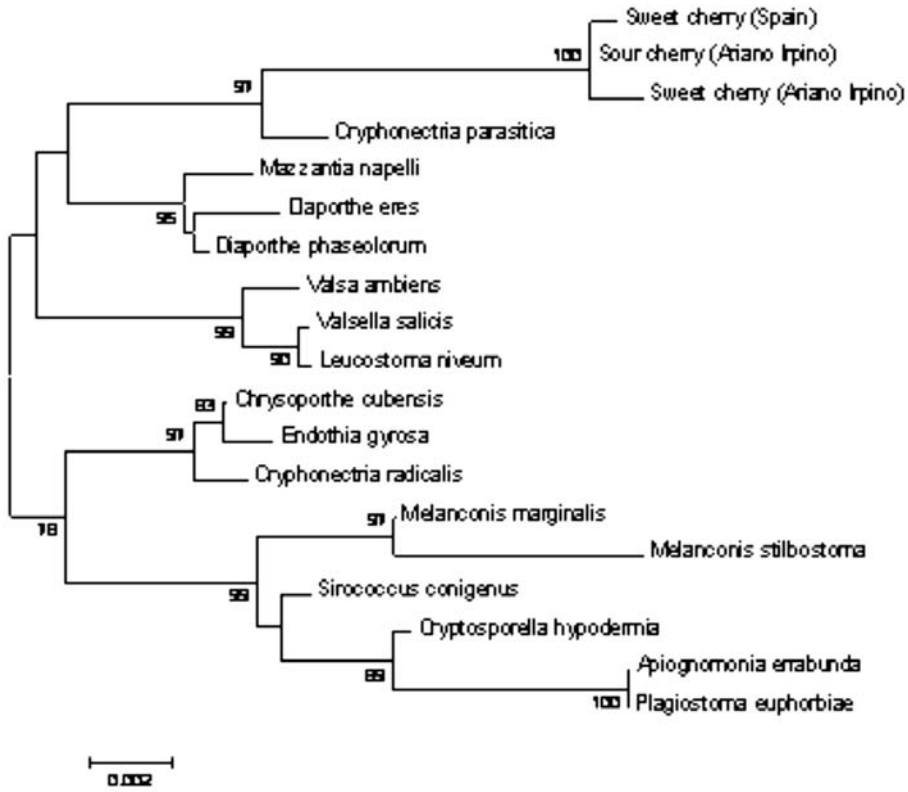

Fig. 2. Dendrogram based on 18S rDNA gene sequences of the fungus identified in CCRS and CLS infected leaves (Sweet and Sour cherry from Ariano Irpino and Sweet cherry from Spain) and other *Diaporthales* fungi.

those of other fungi in the order Diaporthales (Fig. 2).

PCR amplifications using TNA from CCRS- and CLS-affected leaves and Gnomoniaceae-specific primers (ApioF1 and ApioR2), were negative. In contrast, DNAs of the expected size (350 bp) were amplified when the same PCR protocol was applied to TNA preparation from walnut and plane leaves affected by *Ophiognomonia juglandis* and *Apiognomonia veneta*, two fungi of the family Gnomoniaceae used as positive controls. Therefore, despite the presence of *Apiognomonia*-like pycnidia and perithecia on CCRS-affected leaves, no fungus belonging to the genus *Apiognomonia* was amplified from symptomatic leaves by PCR. Likewise, no *Apiognomonia* species was identified by PCR in CLS leaf samples from Spain. Unfortunately, no specific product was obtained with PCR assays using perithecia content, which did not allow to ascertain whether the fungus producing *Apiognomonia*-like perithecia and the 18S rDNA fragment constantly amplified by PCR from CCRS -and CLS-affected leaves are correlated.

To gain additional information on the fungi involved in CCRS aetiology, infected leaves were incubated in a moist chamber at room temperature. After 2-3 weeks, black pycnidia, immersed in the leaf epidermal tissue were observed, that oozed yellowish drops containing *Phomopsis* like-pyriform or elliptical, colourless conidia (7-10×1-2µm in size) (alpha-conidia). Identical fruiting bodies and conidia were also observed on CLS-affected leaves incubated in a moist chamber.

Belonging of this fungus to the genus *Phomopsis* was confirmed analysing its ITS1-5.8S-ITS2 region (primers ITS4-ITS5; White *et al*., 1990) and the EF-1α gene (EF1-728F/EF1-986R; Carbone and Kohn, 1999), which showed identity values of 100 and 98% with the corresponding *Phomopsis* sequences deposited in databases, respectively.

On one hand, these data further support the correlation between CCRS and CLS, on the other, they suggest that more than one fungus may be associated with both diseases. In fact, unlike the Diaporthaceae species reported above, the *Phomopsis*-like fungus was readily isolated on PDA from alpha conidia. Furthermore, its 18rDNA region, amplified with primers EF4 and EF3 using TNA preparations from mycelium isolated on PDA, shared only 98% sequence identity with the corresponding sequences of the unidentified Diaporthaceae species constantly detected in CCRS- and CLS-infected tissues. In addition, none of the 10 analyzed colonies obtained from single alpha-conidia harboured any mycovirus constantly associated with CCRS and CLS diseases (see below), as shown by RT-PCR using specific primers (not shown).

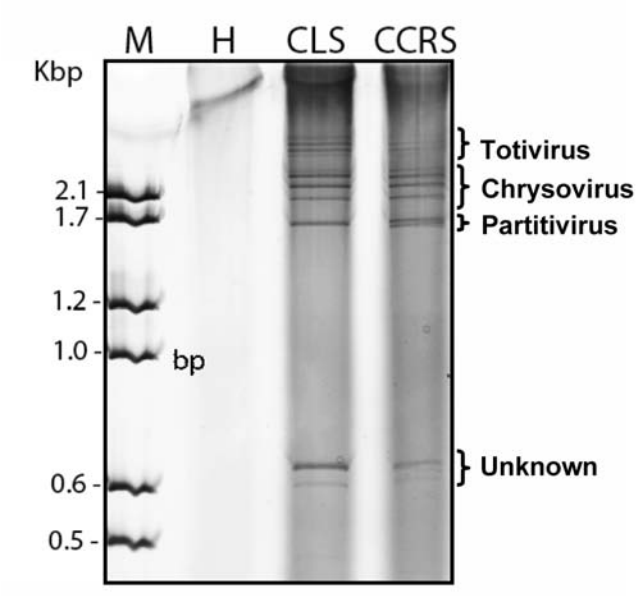

Fig. 3. Silver staining of nucleic acids separated by polyacrilamide-gel electrophoresis. Preparations of dsRNAs from CLS (lane 3) and CCRS (lane 4) affected leaves and control preparation obtained under the same conditions from leaves of asymptomatic cherry tree (lane 2) are compared with DNA of known molecular sizes indicated to the left in Kbp (lane 1). The genomic dsRNAs of totivirus, chrysovirus, partitivirus species and two dsRNAs of unknown nature characterized previously from CCRS affected leaves (Covelli *et al.,* 2004; Coutts *et al*., 2004; Kozlakidis *et al*., 2006; Covelli *et al*., 2008) are indicated on the right.

Interestingly, biological assays in greenhouse using *Phomopsis*-like mycelium isolated on PDA, showed that this fungus induces necrosis on the leaves of cherry seedlings. However, this symptomatology clearly differs from the chlorotic spots generally observed on CCRS-affected leaves, suggesting that this fungus cannot be considered as the unique, direct agent of the disease.

Finally, the similarity between CCRS and CLS was further supported by the finding, in symptomatic CLS leaves, of the same combination of mycoviruses regularly associated with CCRS. In fact, polyacrylamide gel electrophoresis (PAGE) of dsRNA preparations (Di Serio *et al*., 1996) from CLS-affected leaves of cv. Ambrunès, showed a dsRNA pattern similar to that of CCRS (Fig. 3). This result was further confirmed by RT-PCR amplification using the specific primers for detection of dsRNA 4 of the chrysovirus, dsRNA 1 of the partitivirus and dsRNA 3 of the totivirus, previously characterized, and associated with CCRS (Covelli *et al*., 2004). cDNAs of the expected sizes (180 bp, 280 bp and 1200 bp for dsRNA 4-chrysovirus, dsRNA1-partitivirus and dsRNA3-totivirus, respectively) were amplified from CLS-affected leaves of sweet cherry cvs Ambrunés and Pico Colorado collected in four different areas of Spain, indicating that the corresponding mycoviruses are widespread in the region. Sequencing of the amplified cDNAs showed high sequence similarities (ranging from 97.3 to 100% for dsRNA 4-chrysovirus, from 98.6 to 100% for dsRNA 1-partitivirus and 100% for dsRNA 3-totivirus) with the corresponding genomic sequences of mycoviruses associated with CCRS disease, conclusively showing that the same mycoviruses are associated with both CLS and CCRS. This is the first time that mycoviruses similar to those associated with CCRS have been observed in cherry samples from Spain.

This study provides, for the first time, convincing evidence of the similarity between CCRS and CLS based on: (i) similarity of symptoms; (ii) presence of the same fungi and of identical fungal fructifications in diseased leaves; (iii) close association with the same mycoviruses.

However, the role of each fungus reported herein in the aetiology of the two diseases remains to be defined. On one side, the fungus with the morphological characters of *A. erythrostoma* associated with both CCRS and CLS could not be isolated on artificial media and was not detected by PCR, on the other, the *Phomopsis*-like fungus isolated on PDA, did not cause the typical symptoms of CCRS or CLS when artificially inoculated to healthy cherry leaves and did not harbour any of the mycoviruses constantly associated with the two diseases.

Finally, the presence of *Apiognomonia*-like fruiting bodies on diseased leaves, together with the impossibility to amplify any DNA fragment from a fungus belonging to this genus, calls for further investigations for a better definition of the taxonomic status of *A. erythrostoma.*

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