Close similarities between Cherry chlorotic rusty spot disease from Italy and Cherry leaf scorch from Spain.

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Abstract

Cherry chlorotic rusty spot (CCRS), a disease affecting sweet and sour cherry in Southern Italy was regularly found associated with an unidentified fungus and with a complex pattern of viral-like double-stranded RNAs as well as with two small circular RNAs (cherry small circular RNAs, cscRNAs). Further studies revealed that i) the ds-RNAs correspond to the genome of different mycoviruses belonging to the genera *Chrysovirus*, *Partitivirus* and *Totivirus* and ii) the two viroid-like RNAs consist of two groups of variants with similar sequences but differing in size (394–415 and 372–377 nt for cscRNA1 and cscRNA2, respectively). Here we report that the dsRNAs of *Chrysovirus* and *Partitivirus* have been detected by RT-PCR analysis with CCRS specific primers in nucleic acid preparations from cherry leaves affected by cherry leaf scorch (CLS) in Spain, a disease whose etiological agent is the ascomycetes *Apiognomonia erythrostoma*, order *Diaporthales*. Moreover, Northern-blot hybridization assays showed that a viroid-like RNA co-migrating and sharing high sequence similarity with the cscRNA1 previously reported in Italy, accumulate in leaves from CLS affected trees in Spain. These data, together with other evidence showing similar symptoms, disease cycle and fungal fructifications in CCRS and CLS affected trees, suggest a close relationship between the two cherry disorders.

Keywords: dsRNAs, cscRNAs, *Apiognomonia erythrostoma*, Diaporthales

Introduction

Cherry chlorotic rusty spot (CCRS), was firstly described affecting sweet cherry trees in Campania (Southern Italy) in 1996 (Di Serio et al., 1996). Two years later, sour cherry plants showing the same disorder were reported in the same area (Di Serio et al., 1998).The disease, similar to Amasya cherry disease (ACD) described in Turkey in 1970 (Blodget 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), causes chlorotic and rusty spots on leaves and deformation, color alteration and premature dropping of fruits (Figure 1).

Fig. 1 Chlorotic rusty spots on leaves affected by CCRS disease.

Since CCRS was found regularly associated to 10-12 double-stranded RNAs (dsRNAs) and to 2 small circular RNAs (cherry small circular RNAs, cscRNAs), a viral agent of unknown nature was initially proposed as a possible causal agent (Di Serio et al., 1996).

However, further studies revealed that:

i) the disease is not graft transmissible to cherry seedlings and peach GF 305 (Alioto et al., 2003);
ii) mycelium-like structures are constantly associated to symptomatic tissues of leaves and fruits (Alioto et al., 2003);
iii) the two cscRNAs consist of two groups of variants with similar sequences but differing in size (394–415 and 372–377nt for cscRNA1 and cscRNA2, respectively) (Di Serio et al., 2006);
iv) The largest dsRNAs correspond to the genome of a putative new species of mycoviruses belonging to the genera *Chrysovirus*, *Partitivirus* and *Totivirus* (Covelli et al., 2004; Coutts et al., 2004; Kozlakidis et al., 2006);
v) The smallest dsRNAs do not show sequence similarities neither with CCRS-associated mycoviruses or with the two small circular RNAs or with the sequences deposited in databases, so their nature remains to be clarified (Covelli et al., 2008).

Most of these findings suggested that a fungus could be the etiological agent of the disease but, no fungus was isolated on artificial media and identified. Recently, molecular analysis of 18S fungal ribosomal gene has shown that CCRS symptomatic tissues of infected sour and sweet cherry leaves are closely associated with a fungus belonging to the order *Diaporthales* (Carrieri, 2009). A bibliographic research on *Diaporthales* infecting cherry trees has suggested the involvement of *Apiognomonia erythrostoma* (*Diaporthales*, *Gnomoniaceae*), in etiology of CCRS (Sánchez Sánchez and García Becedas, 2007). This fungus is reported as the agent of a disease named Cherry leaf scorch (CLS) inducing symptoms resembling those of CCRS.

In this paper, we report data showing a close similarity between the Italian CCRS and the Spanish CLS diseases.

Materials and methods

Diseased and healthy sample sources: CLS-affected leaves were collected from sweet cherry (*Prunus avium* L.) trees of the local variety “Ambrunés” from fields of three different areas of Plasencia (Cáceres, Spain). All leaves showed mild or severe chlorotic spots (Figure 2). CCRS-affected leaves, showing the typical symptoms of the disease, were sampled from sour and sweet cherry trees at Ariano Irpino (Avellino, Italy). Healthy leaves were collected from sour and sweet cherry trees in Ariano Irpino and from sweet cherry in Cáceres.

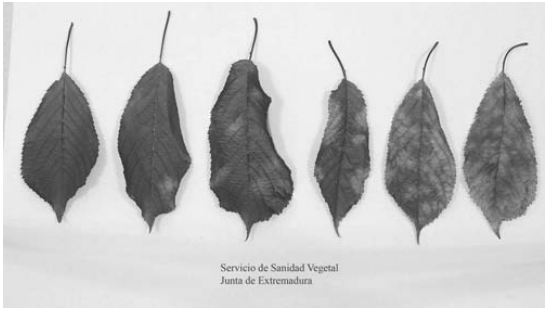

Fig. 2 Chlorotic spots on leaves from Spain affected by CLS disease caused by *Apiognomonia erythrostoma.*

RNA extraction and RT-PCR: Total RNA was extracted from 200-500 mg of healthy and CCRS and CLS infected leaf tissues as described by Foissac et al. (2001). A two-step RT-PCR was performed according to the protocol described by Covelli (2004) and Covelli et al. (2004), with minor modifications, using specific internal primers derived from the *Chrysovirus*-dsRNA4 (RF195/RF270/RF105) *Partitivirus*-dsRNA1 (RF480/RF481). The PCR amplicons, expected to be 180 bp (*Chrysovirus*) and 280 bp (*Partitivirus*) in size, were purified and sequenced (BMR Sequencing Service, Padova, Italy).

Extraction and purification of cscRNAs and Northen blot hybridization: Nucleic acid preparations enriched in viroid-like RNAs were obtained from leaves of healthy and CCRS-affected (*Prunus avium* L. 'La Signora') and CLS-affected (*Prunus avium* L. 'Ambrunés') sweet cherry trees as reported previously (Di Serio et al., 1997). These preparations were

separated by two consecutive electrophoreses under non-denaturing and denaturing conditions, electroblotted to nylon membranes (Amersham), fixed by UV irradiation (Di Serio et al., 1997) and, finally, hybridized with a cscRNA1 specific digoxigenine-labelled riboprobe following the protocol reported by Lolic et al. (2007). The riboprobe was generated by *in vitro* transcription from a linearized plasmid containing the full-length cDNA of cscRNA1 (Di Serio et al, 2006) using a commercial Dig-labelling kit (Roche Diagnostics GmbH, Germany).

Fruiting body observations: To evidence the presence of fungal fruiting bodies or conidia on CCRS infected trees, the symptomatic tissues of infected leaves and fruits were marked. Ten leaves were monthly removed from June to October and examined under a stereomicroscope (WILD Heerbrugg M35, Switzerland) and images were recorded by digital camera. During September and October, marked symptomatic leaves were also collected and placed in terylene mesh bags (20 per bag). Samples were placed on the soil surface or buried at depths of 0.1-0.5 cm and left overwintering under the infected trees. Sufficient bags were included to allow monthly sampling. The leaves were examined for the presence of ascocarps under a stereomicroscope and images were recorded by digital camera.

Microscopy: When conidia and perithecia were firstly observed, squash preparations were made to have an indication of the morphology of conidia and asci and ascospores. Mounts were made in lactophenol Cotton Blue and observed under a Leica DMR optical microscope.

Results and discussion

The RT–PCR amplifications using nucleic acid preparations from symptomatic leaf tissues of CLS affected sweet cherry trees of 'Ambrunés' yielded the expected fragments of 180 and 280 bp (Figure 3), suggesting the presence of mycoviruses of the genera *Chrysovirus* and *Partitivirus* respectively, that was further confirmed by sequence analysis of the RT-PCR amplicons showing high similarity (ranging from 97.3 to 100 % for *Chrysovirus* and from 98.6 to 100 % for *Partitivirus*) with the corresponding genomic sequences of the two mycoviruses constantly associated to CCRS disease. Moreover, in RNA extracts from CLS samples, a viroid-like RNA co-migrating and sharing high sequence similarity with the cscRNA1 previously reported from CCRS diseased trees, was detected by Northern-blot hybridization assays (data not shown).

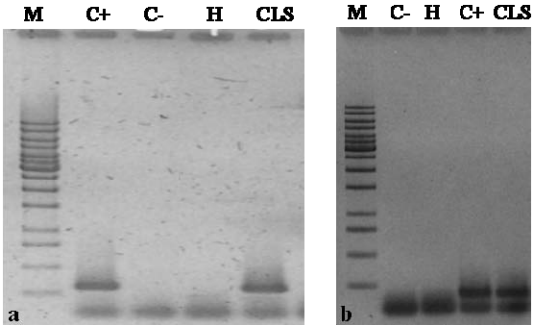

Fig. 3 Analysis by agarose gel electrophoresis of the RT-PCR products from CLS samples (a) *Partitivirus* and (b) *Chrysovirus.* Lane M, DNA marker 1Kb; Lane C+, cherry chlorotic rust spot affected samples; C-, negative control; H, Healthy sweet cherry sample;. CLS, Cherry leaf scorch affected sample.

Finally, orange pycnidia containing β-like conidia (Fig. 4a) were observed on the lower surface and in symptomatic areas of CCRS infected leaves that fell, at the beginning of October. Necked perithecia containing asci with bicellular ascospores (Fig. 4b) were also observed on the lower surface of the fallen leaves on the soil from month of November. These perithecia and pycnidia present the same morphological characters of fruiting bodies produced by *Apiognomonia erythrostoma* on CLS affected leaves.

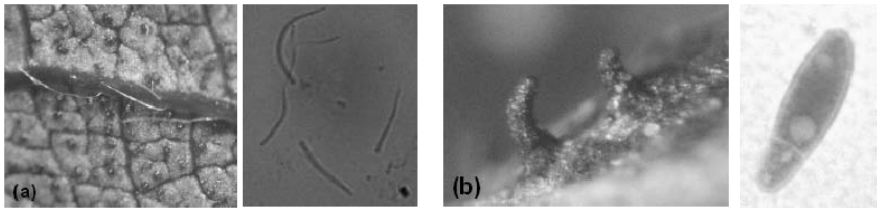

Fig. 4 (a) Picnidia (left) and β-like conidia (right) and (b) perithecia (left) and ascospora (right) observed on CCRS affected leaves.

These data, together with other evidence showing similar symptoms and a similar disease cycle in CCRS and CLS affected trees, suggest a close relationship between the two cherry disorders.

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