

Cherry Chlorotic Rusty Spot: Description of a New Viruslike Disease from Cherry and Studies on its Etiologic Agent

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ABSTRACT

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A novel cherry disease that has been observed in southern Italy is characterized by chlorotic leaf spots that later develop a rusty appearance, small and deformed fruits with color alterations, and tree decline. We propose to call it cherry chlorotic rusty spot disease. The disease spreads naturally in the field, but the vector is not known. A bacterial or fungal etiology is unlikely based on testing that indicates the probable involvement of a virus- or viroidlike agent. In agreement with this hypothesis, 12 double-stranded (ds) RNAs and one or two small, circular RNAs have been consistently isolated only from symptomatic cherry plants. The pattern of dsRNAs is reminiscent in its complexity, but not in the size of the components, of that characteristic of phyto-reoviruses. This pattern could also be produced by the genomic and subgenomic dsRNAs resulting from the infection of one, or a mixture of more than one, single-stranded RNA virus. The close association in symptomatic tissue between the small, circular RNAs and the dsRNAs suggests that the former could be viroidlike satellite RNAs rather than autonomously replicating viroids, although definitive conclusions must await infectivity bioassays.

Avellino province is a mountainous area with a few small valleys in the Campania region of southern Italy. Cultivation of cherry (*Prunus avium* (L.) L.) was introduced into this area by the middle of the last century by planting cultivars from the Naples province. Intensive research on virus and viruslike diseases of cherry began in Avellino province in the early 1970s. At that time, a decline of cherry trees was recognized and associated with a double infection by strawberry latent ring-spot virus (SLRV) and prune dwarf virus (PDV) (17). Additional surveys for virus infections carried out since then revealed that a number of diseases described elsewhere were also present (16). However, some cherry trees showing symptoms of an apparently undescribed disease were found in 1990. In this paper, we provide a description of this new cherry disease together with some studies about its etiology.

MATERIALS AND METHODS

Field observations. Surveys have been made in different cherry-cultivated areas of

the Campania region since 1990. The inspections were more frequent in the orchard where the disease was first detected, and they were repeated each year to observe changes in symptoms.

Isolation of fungi and bacteria. Attempts to isolate fungi and bacteria were made by plating aseptically collected pieces of symptomatic leaf tissue onto potato-dextrose agar (PDA) medium with or without lactic acid. In other experiments, the tissue was crushed in a small volume of sterile water, and the resulting suspension was streaked on the same media.

Infectivity assays. Leaves, buds, and petals were collected from 20 affected and 10 asymptomatic trees. These materials were ground with suitable additives (10), and the sap was inoculated onto the herbaceous hosts *Chenopodium quinoa* Willd., *Chenopodium amaranticolor* Costa & Reyn., *Cucumis sativus* L., and *Nicotiana tabacum* L. in order to detect mechanically transmissible viruses, which were then identified by the test of double diffusion in gel and by immunoelectron microscopy with decoration (12).

Detection of viruses by ELISA. Petals and young leaves from symptomatic trees were tested by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (6) for detecting PDV, SLRV, *Prunus necrotic ringspot*

virus (PNRV), and apple chlorotic leaf spot virus (ACLSV) using commercial kits.

Extraction and fractionation of nucleic acids. Total nucleic acids from leaves collected from affected and healthy-looking cherry trees were extracted with buffer-saturated phenol, as reported previously (15). In some cases, samples from the chlorotic rusty areas and from the asymptomatic surrounding tissue of the same leaves were extracted independently. Nucleic acid preparations enriched in double-stranded (ds) RNAs were obtained by chromatography on nonionic cellulose (CF11, Whatman) with STE (100 mM NaCl, 50 mM Tris-HCl [pH 7.2], and 1 mM EDTA) containing 16% ethanol (13). Nucleic acid preparations enriched in viroidlike RNAs were obtained using the same approach, with the exception that binding to CF11 and subsequent washes were performed with STE containing 35% ethanol (11). In both cases, nucleic acids were released from CF11 by washing with STE without ethanol. Polysaccharides present in nucleic acid preparations enriched in viroidlike RNAs were removed with methoxyethanol (2).

Polyacrylamide gel electrophoresis (PAGE). Nucleic acid preparations enriched in dsRNAs were analyzed in non-denaturing 5% gels (39:1 acrylamide:bis-acrylamide) with TAE buffer. In some experiments, the preparations were treated prior to electrophoresis with RNase A in either 2× SSC or 0.1× SSC (1× SSC is 15 mM sodium citrate [pH 7.0] containing 150 mM NaCl) and with DNase I in sodium acetate 0.1 M (pH 5) containing 5 mM magnesium sulfate. dsRNAs from *Nicotiana glauca* L. infected by cucumber mosaic virus (CMV) and *N. tabacum* infected by tobacco mosaic virus (TMV), as well as dsRNAs from purified virions of maize rough dwarf virus (MRDV), were used as molecular weight markers.

Viroidlike RNAs were analyzed by two consecutive electrophoreses under non-denaturing and denaturing conditions (8,18) using as markers citrus exocortis viroid (CEVd) and in some cases avocado sunblotch viroid (ASBVd).

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RESULTS

Symptom description. Some trees of the cherry cultivars Imperiale and La Signora displaying symptoms of an apparently uncharacterized disease were found in an orchard in May 1990. The trees were about 20 years old, and the symptoms appeared on fully developed leaves. The early symptoms consisted of roundish leaf spots, 1 to 2 cm diameter, with a translucent or greasy aspect (Fig. 1A), that developed into yellow spots with small rusty areas (Fig. 1B). The rusty areas enlarged with time and finally covered the entire surface of the spots. These leaves soon abscised. Most fruits remained small, were elongated, and did not ripen (Fig. 2A); others that did ripen exhibited reddish and irregular lines on the skin (Fig. 2B). The affected trees showed a slow decline.

The same symptoms were detected in 1992 in some young trees of the cultivar Bigarreau Napoleon, imported from Tuscany (northern Italy) and planted in 1987 in a field located 10 km from the one where the disease was first observed. By visual inspection, it was estimated that about 40% of the trees were affected, but in 1993 the percentage increased to 80% and in 1994 to 100%.

Infectivity assays. Attempts to isolate pathogenic fungi or bacteria were always negative. Out of 20 affected and 10 asymptomatic cherry trees, three and one, respectively, appeared infected by PDV. No other viruses were recovered from the mechanically inoculated herbaceous hosts.

ELISA. DAS-ELISA of petals and young leaves from symptomatic trees detected PDV but infrequently. This virus was not consistently associated with the new cherry disease.

Electrophoretic analyses. Nondenaturing PAGE of dsRNA preparations from symptomatic cherry trees of different cultivars showed a typical pattern formed by 10 bands, which ranged in size between approximately 1,700 and 5,500 bp (Fig. 3A, lanes 3 and 4, and Fig. 3B, lane 2), and by two other dsRNAs with approximate sizes between 500 and 650 bp (Fig. 3A, lanes 3 and 4). These dsRNAs were never detected in asymptomatic trees (Fig. 3A, lane 2). When the dsRNA preparations were treated with DNase I or with RNase A (in the latter case in a buffer of high ionic strength), the electrophoretic pattern was unaltered (Fig. 4, lanes 3 and 4). However, treatment with RNase A in a buffer of low ionic strength led to the degradation of the dsRNAs (Fig. 4, lane 5). These results

confirmed the dsRNA nature of the nucleic acids giving rise to the 12 bands associated with the observed cherry syndrome.

In addition to the dsRNAs, the presence of a band corresponding to a circular RNA was detected by double-PAGE in all the RNA preparations enriched for viroidlike RNAs in extracts from symptomatic cherry trees (Fig. 5, lane 2). This circular RNA was not observed in control extracts from asymptomatic cherry trees (Fig. 5, lane 3). In some preparations from symptomatic trees, a second circular RNA with a slightly higher electrophoretic mobility was also detected (Fig. 5, lane 2). The lower levels at which this second viroidlike RNA accumulates did not allow a definitive conclusion on whether it is present in all symptomatic samples. Approximate sizes between 450 and 425 bases were inferred for the two cherry viroidlike RNAs on the basis of comparisons of their mobilities in denaturing gels with those of CEVd and ASBVd (371 and 247 bases, respectively). Sensitivity to RNase confirmed the RNA nature of the two small, circular nucleic acids (data not shown).

When tissue from symptomatic cherry leaves was separated prior to extraction into two fractions formed by the chlorotic rusty areas and by the symptomless ad-

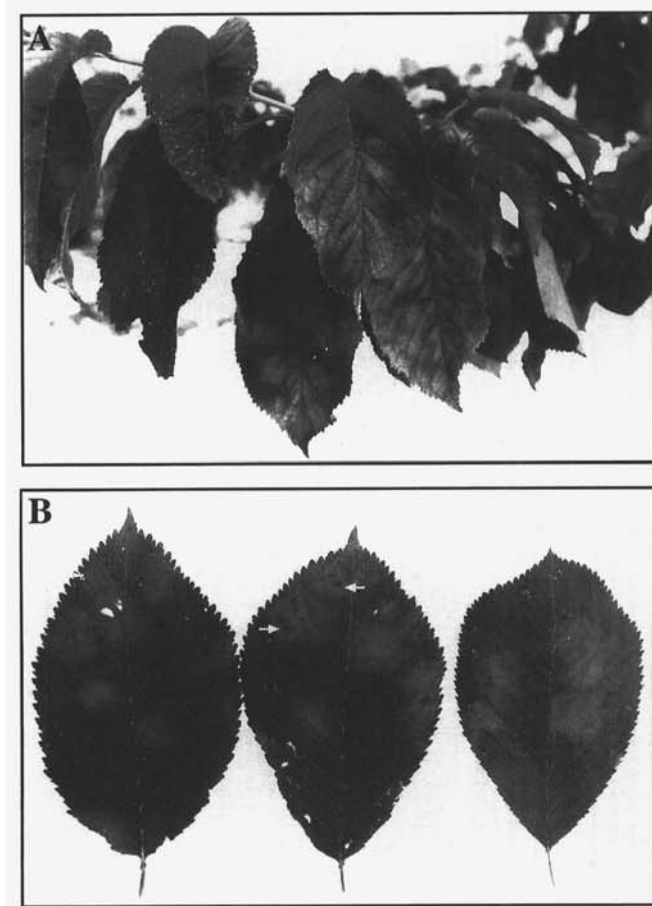


Fig. 1. Symptoms of cherry chlorotic rusty spot disease on leaves. (A) Early symptoms consisting of translucent spots 1 to 2 cm in diameter. (B) In later stages, the spots develop a yellow color, and small, necrotic areas with a rusty appearance (indicated by arrows) are observed.

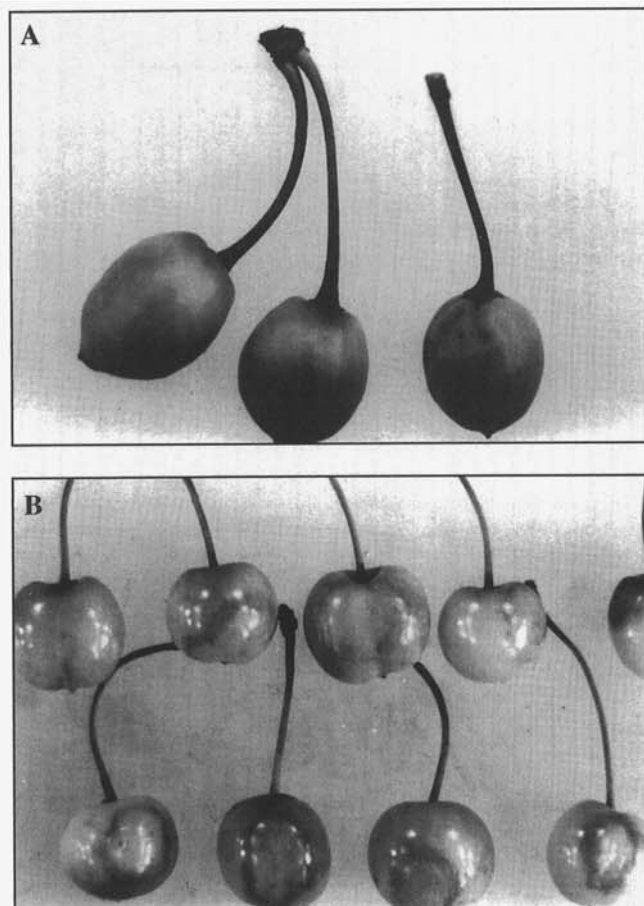


Fig. 2. Symptoms of cherry chlorotic rusty spot disease on fruits. (A) Elongated fruits that do not ripen. (B) Ripe fruits with color alterations in the skin.

joining zones, both the dsRNAs and the viroidlike RNA were found predominantly in the first fraction (data not shown). This observation indicates a close association of the dsRNAs and the viroidlike RNA with the disease and between each other.

DISCUSSION

The new cherry disease detected in Italy appears to be incited by a virus- or viroidlike agent that is spreading naturally in infected areas. The presence of a vector can be presumed. The disease has been observed on different cherry cultivars, and experiments to transmit its etiologic agent by grafting to cherry seedlings are currently in progress. None of the known cherry viruses appear to be consistently associated with the disease, which we propose to call cherry chlorotic rusty spot (CCRS) disease, basing the name on its most conspicuous symptoms.

We have found a close association of the disease with a set of dsRNAs, as well as with one or two small, circular RNAs, isolated from infected plants. The dsRNAs could represent either the components of a virus with a multipartite dsRNA genome or the genomic and subgenomic dsRNAs accumulating in the tissue as a result of the infection by a single-stranded (ss) RNA virus. A concurrent infection by more than one ssRNA virus cannot be ruled out. The

patterns of dsRNAs synthesized in tissues infected by typical plant ssRNA viruses do not correspond with the number and sizes of the dsRNAs associated with CCRS disease. Among the known dsRNA viruses from plants, only the phytoeviruses genus, whose type species is wound tumor virus (9), have a genome composed of 12 dsRNAs, although their sizes are different from those observed for the dsRNAs associated with the CCRS disease.

The small, circular RNAs could be either viroids or, alternatively, viroidlike satellite RNAs of the virus generating the dsRNAs. The detection in CCRS symptomatic tissue of at least the most abundant of the two small, circular RNAs in constant association with the 12 dsRNAs is more consistent with a satellite nature. However, viroidlike satellite RNAs have been found so far only associated with members of the sobemo-, luteo-, and nepoviruses (4), and the genome organization and expression of these viruses is not consistent with the pattern of dsRNAs found in tissues affected by the CCRS disease. In order to discriminate between the viroid or satellite nature of the small, circular RNAs from cherry, it is necessary to study in depth their infectious capacity and, particularly, whether they are endowed with autonomous replication or are functionally dependent on a helper virus. Sequencing of

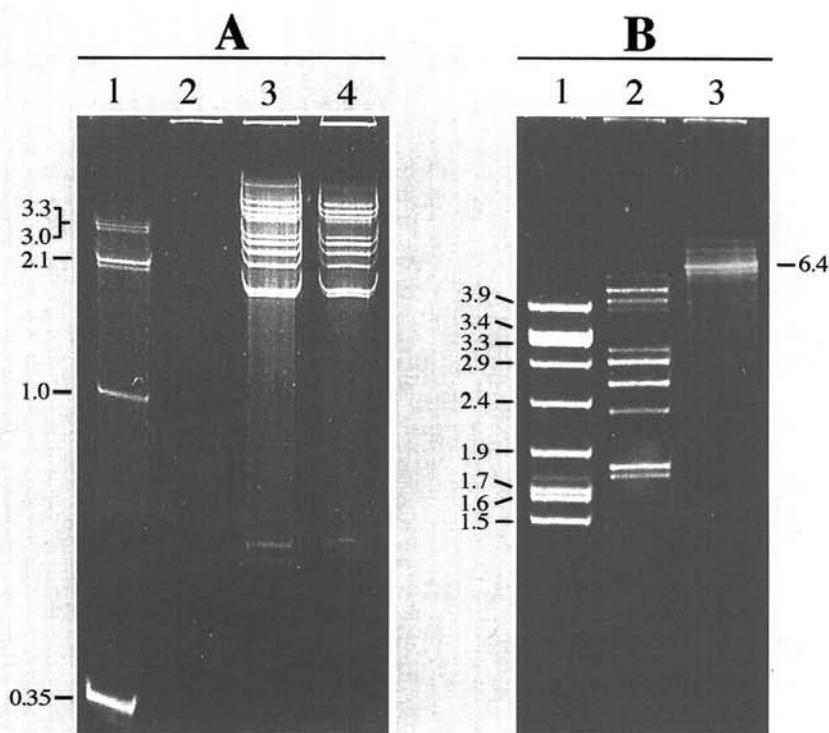


Fig. 3. Analysis by polyacrylamide gel electrophoresis of dsRNAs from cherry. (A) Lane 1, dsRNAs from cucumber mosaic virus (CMV)-infected *Nicotiana tabacum*. Lane 2, extract from an asymptomatic cherry plant. Lanes 3 and 4, extracts from two cherry plants exhibiting the characteristic symptoms of the cherry chlorotic rusty spot disease. (B) Lane 1, dsRNAs from maize rough dwarf virus (MRDV). Lane 2, extract from a cherry plant exhibiting the characteristic symptoms of the cherry chlorotic rusty spot disease. Lane 3, dsRNAs from tobacco mosaic virus (TMV)-infected *N. tabacum*. Numbers on the margins indicate molecular sizes of the markers in kbp. The electrophoresis was overrun in (B) to improve the separation of the largest dsRNAs. (The two smallest ones have migrated out of the gel.)



Fig. 4. Susceptibility to nucleases of the dsRNAs associated with the cherry chlorotic rusty spot disease. Lanes 1 and 2, dsRNAs from cucumber mosaic virus (CMV)-infected *Nicotiana tabacum* after incubation in $0.1\times$ SSC and in the same buffer plus RNase, respectively. Lanes 3 to 5, dsRNAs associated with the cherry chlorotic rusty spot disease after treatment with DNase, RNase in $2\times$ SSC, and RNase in $0.1\times$ SSC. Other details as in the legend to Figure 3.

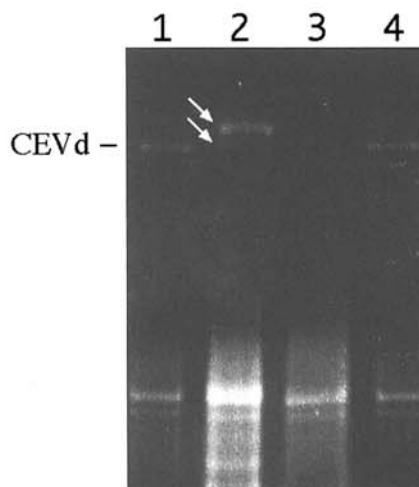


Fig. 5. Analysis by double polyacrylamide gel electrophoresis of nucleic acid extracts from cherry enriched in viroidlike RNAs. The first nondenaturing gel was stained with ethidium bromide, and a segment of 1 cm centered around the position of the viroid standard citrus exocortis viroid (CEVd) was cut and applied to the top of a second denaturing gel, which is the one illustrated. Lanes 1 and 4, partially purified CEVd preparation. (The migration of the circular form is indicated on the left.) Lanes 2 and 3, extracts from a cherry plant displaying the typical symptoms of the cherry chlorotic rusty spot disease and from an asymptomatic control, respectively. Arrows in lane 2 indicate the migrations of the small, circular RNAs. (The less abundant species is barely visible in the photograph.)

the most abundant cherry viroidlike RNA has confirmed that it is a circular RNA of 451 nt with sequence and structural similarities to viroid and viroidlike satellite RNAs which include the presence of hammerhead ribozymes in both polarity strands (F. Di Serio, J. A. Daròs, A. Ragozzino, and R. Flores, *unpublished*).

An important question that still remains unexplained is the source of the primary inoculum of the CCRS disease. From the symptomatic point of view, this disease appears different from the virus and virus-like diseases reported previously in cherry (14). Although fruit alterations have been found associated with other cherry diseases, for example little cherry, the fruit and also the leaf symptoms are clearly different (14). However, there are close similarities between CCRS disease and the Amasya cherry disease described in Turkey (3,5) and recently associated with a series of presumably viral dsRNAs that have not been recovered from healthy leaf tissues (1). The death of the trees some years after the onset of the typical symptoms is a significant feature of the Amasya cherry disease that until now has not been observed in trees affected by CCRS disease. However, this difference in symptom expression could be due to the influence of environmental factors or to the varieties grown in both areas, since local Turkish varieties seem to be particularly sensitive to the Amasya cherry disease (5). On the other hand, an important difference between CCRS and Amasya cherry diseases is the association of at least one small, circular RNA only with CCRS. Further investigations are needed to determine whether these two cherry diseases are induced by the same agent and to define the nature of that agent.

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