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Short Notes

Red valerian (*Centranthus ruber* L.): wild host of *Cucumber mosaic virus* in uncultivated areas of Campania region (Southern Italy)

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Summary. In spring 2009–2010, red valerian plants (*Centranthus ruber* L.) showing stunting, mild leaf vein mosaic, reduction in size of leaves and flowers and occasional deformation and reduction of the number of leaves, were observed in Campania (Southern Italy). The presence of *Cucumber mosaic virus* (CMV) in the diseased plants was demonstrated by the reactions of test plants, electron microscopy, serology and RT-PCR. On the basis of highest identities and closest phylogenetic relationships, CMV from *C. ruber* was most closely related to CMV subgroup I A. Koch's postulates were fulfilled by back-inoculating healthy *C. ruber* seedlings.

Key words: weed, subgroups, phylogenetic analysis.

Introduction

Cucumber mosaic virus (CMV) is one of the most destructive and widespread plant viruses that can infect a broad range of annual, biennal, perennial crops and weeds. Many weeds may act as alternative hosts for CMV, and for this reason they are important in virus epidemiology and control. The virus is transmitted in nature by more than 75 species of aphids in a non-persistent manner, and is also seed-borne in different host plants.

CMV is the type member of the genus *Cucumovirus* in the family of *Bromoviridae*, and has isometric virions of approximately 30 nm diameter. The genome of CMV is divided into three positive single-stranded RNA molecules. RNA1 and RNA2 are encapsidated separately, while RNA3 is encapsidated with a subgenomic RNA (RNA4), encoded by the 3′-half of RNA3 (Palukaitis *et al*., 1992) and involved in encapsidation (Suzuki *et al*., 1991). Some CMV strains are also associated with satellites RNAs (satRNA), which may modify the symptoms induced by the virus in host plants (Suzuki *et al*., 1991; Palukaitis *et al*., 1992; Gallitelli, 2000; García-Arenal *et al*., 2000).

CMV exists as a variety of strains which have been divided into two subgroups, I and II, on the basis of nucleic acid hybridization and serology of the capsid protein (Palukaitis *et al*., 1992). CMV subgroup I has been further subdivided into IA and IB on the basis of phylogenetic analyses (Gallitelli, 2000; Yu *et al*., 2004).

Centranthus ruber (L.) DC (syn. *Valeriana rubra* L.), family Valerianaceae, is a plant native of Mediterranean area, that develops wild in many places due to the ability to adapt to poor soils.

In spring 2009–2010, the 2% of red valerian plants showing stunting, mild vein mosaic and marked reduction in size of leaves and flowers (Figures 1 and 2), and occasional deformation and reduction of the number of leaves, were observed on the volcanic soils of Vesuvius in Campania region (Southern Italy), 3–4 kilometres away from cropped fields.

In order to identify the virus responsible of these symptoms, leaves from twenty symptomatic and ten asymptomatic plants of *C. ruber* were collected in the above mentioned area within an area of 1 km^2. Sub-

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Figure 1. Vein clearing and mosaic on the leaves of Centranthus ruber naturally infected with Cucumber mosaic virus (CMV).

sequently, five out of the twenty symptomatic samples were ground in cold 0.01 M potassium phosphate buffer pH 7–7.2 and mechanically inoculated onto three test plants of each of 14 test species, previously dusted with carborundum powder. The test species were: Chenopodiaceae (*Chenopodium quinoa* Willd., *C. amaranticolor* Coste et Reyn, *C. murale* L.), Cucurbitaceae (*Cucumis sativus* L., *C. melo* L., *Cucurbita pepo L.*), Lamiaceae (*Ocimum basilicum* L.), Leguminosae (*Vigna sinensis* Endl.), Solanaceae (*Nicotiana tabacum* L. "Samsun", *N. glutinosa* L., *N. rustica* L., *N. occidentalis* H.-M. Wheeler, *N. benthamiana* L., *Lycopersicon esculentum* L.). Plants were kept in a greenhouse (20–22°C) and observed daily for symptom expression for 4 weeks.

Mechanical sap inoculations revealed successful transmission of the virus from red valerian to all the inoculated test plants of each species, except *O. basilicum*. Virus induced chlorotic-necrotic local lesions developed on *C. quinoa*, *C. murale*, C. *amaranticolor* and *V. sinensis*, 7–10 days post inoculation (dpi). The

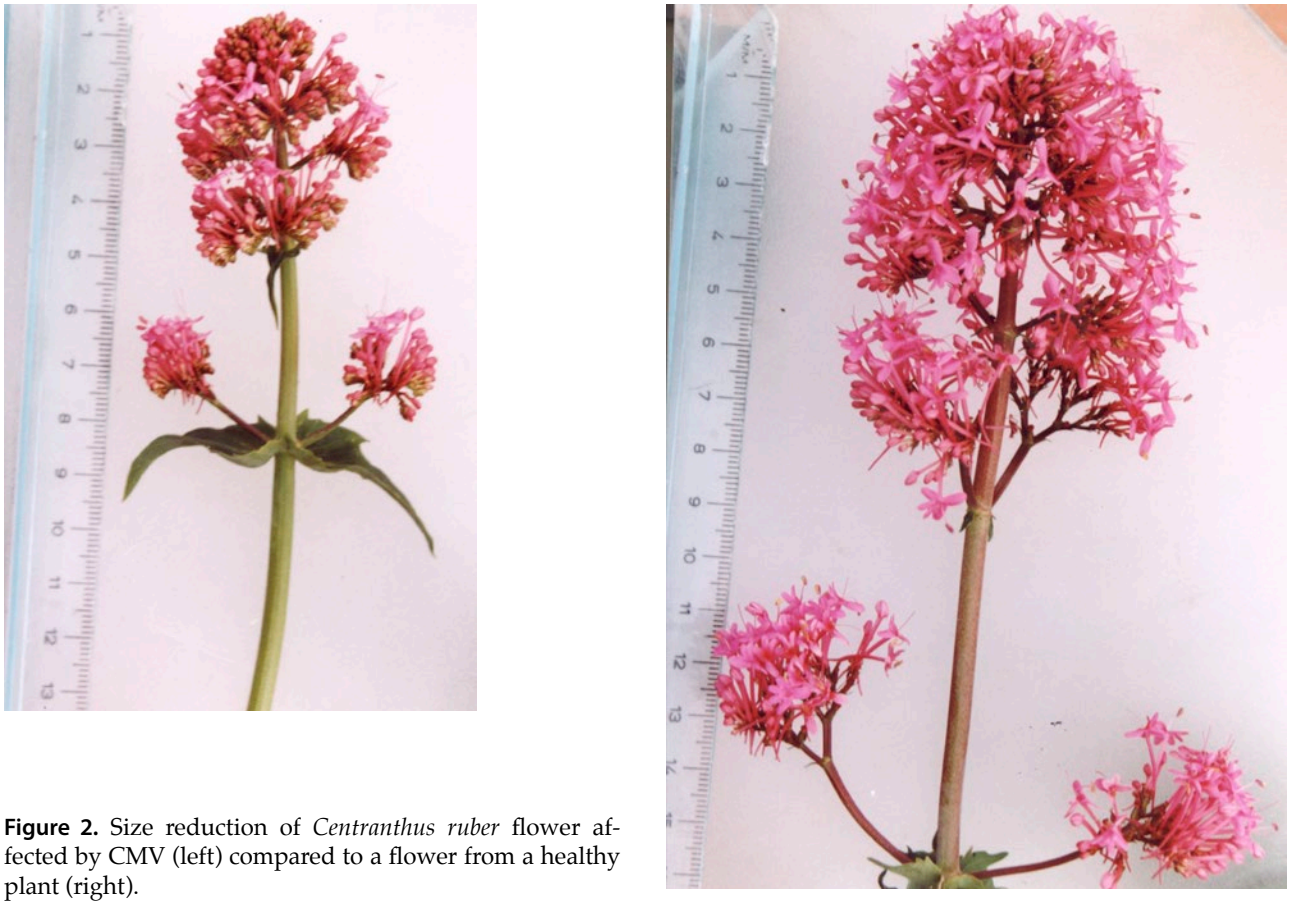

Figure 2. Size reduction of *Centranthus ruber* flower affected by CMV (left) compared to a flower from a healthy plant (right).

virus also induced systemic mosaic on *N. tabacum*, *N. benthamiana*, *N. occidentalis*, *N. glutinosa*, *N. rustica* and *L. esculentum*, 14–16 dpi. During spring (20–25 dpi), *L. esculentum* inoculated plants also exhibited fern leaf symptoms. *Cucumis sativus* , *C. melo* and *C. pepo* showed chlorotic mottle and malformations on younger leaves. The host range and symptoms induced on indicator plants were typical of those induced by CMV.

The indications given by biological assays were confirmed by the electron microscopy observations of leaf dips of symptomatic *C. ruber* plants, mounted in 1% aqueous uranyl acetate and observed with a Philips EM 208 electron microscope. These observations revealed the consistent presence of isometric particles approximately 30 nm in diameter, typical of *Cucumovirus* particles.

Subsequently, the thirty *C. ruber* samples, twenty symptomatic and ten asymptomatic, were analysed by DAS-ELISA, using commercially produced antiserum (Loewe-Phytodiagnostica Biochemical, Sauerlach, Germany), against CMV, according to manufacturer's instructions. Only the symptomatic plants were positive to CMV, confirming the association of CMV with the diseased plants and indicating that the virus is probably not latent in *C. ruber*.

Koch's postulates were fulfilled by back inoculating *C. quinoa*, *V. sinensis*, *C. pepo* and *N. glutinosa* sap to healthy *C. ruber* seedlings (three plants for each inoculation). Inoculated plants reacted showing stunting, mosaic and reduction in leaf size. In the case of *C. quinoa* and *V. sinensis*, back inoculation was performed after three monolesion passages. The presence of CMV in the back-inoculated symptomatic *C. ruber* plants was confirmed by DAS-ELISA.

In order to verify the transmissibility of CMV by *C. ruber* seeds, 165 plants derived from seed from two diseased plants were tested by DAS-ELISA, with negative results, suggesting that CMV is not seed-transmitted in this host.

For RT-PCR, total nucleic acids were extracted from the leaves (100 mg) of three symptomatic and one asymptomatic *C. ruber* plants using the silica capture method (Foissac *et al*. (2000). An aliquot (2 μL) was used as template for reverse transcription with the enzyme Superscript III (Invitrogen, Carlsbad, CA, USA), following manufacturer's instructions. Incubation mixtures for PCR amplifications contained 2 μL of cDNA, 1.5 mM $MgCl_2$, 200 μM each dNTP, 400 nM for each primer and 2.5 U of *Taq* DNA polymerase (Sigma-Aldrich, St Louis, MI, USA). Primers used were CMV3F1 and CMV3R (Davino *et al*., 2005), specific for a conserved region of CMV coat protein gene (CP), and primers M1 and M2, specific for a region coding for CMV movement protein (MP) (Lin *et al*., 2004). All reagents were combined and maintained at 95°C for 3 min. Thirty-eight cycles of PCR were performed by heating at 95°C for 30 sec, 50°C (primers CMV3F1 and CMV3R) or 55°C (M1 and M2) for 1 min, and 72°C for 1 min, followed by final period at 72°C for 10 min. As the positive control, a CMV isolate from the authors' collection was used. PCR amplicons of the expected size (436 bp and 842 bp, respectively) were purified using Promega purification kit (Wizard® SV gel and PCR Clean-UpSystem, Madison, WI, USA) and directly sequenced (BMR Genomics, Padova, Italy) in both directions using the specific primers. The sequences obtained were analysed using the BLASTn program with default parameters (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al*., 1990).

Using primer pairs for the CP and MP regions of CMV genome, the expected size amplicons of 436 bp and 842 bp, respectively, were amplified from the symptomatic samples genome. However, no amplification was obtained from the asymptomatic plant of *C. ruber* used as the negative control, which previously resulted negative to CMV by ELISA. The analyses of the movement protein sequences of the three *C. ruber* isolates, revealed a 100% identity to each other and 99% with CMV sequences published in the GenBank database (http://blast.ncbi.nlm.nih.gov/). This sequence was submitted to GenBank database under the accession number JF951999.

For phylogenetic analysis, the coat protein sequences of the CMV isolated from red valerian were compared to the corresponding sequences of 20 CMV species retrieved from GenBank database entries: seven belonging to subgroup IA (Accession numbers D10538, U22821, AJ511990, D10539, D00386, D12499, AJ276481), six to subgroup IB (Accession numbers AB008777, AB042294, U20219, D28780, Y16926) and seven to subgroup II (Accession numbers AF063610, AJ304398, AF198103, L15336, AJ304403, AF127976, M21464).

The coat protein sequences obtained in this study and corresponding to the isolates previously cited, had 100% identity to each other (GenBank accession N° JF802035) and showed an average identity of 96,

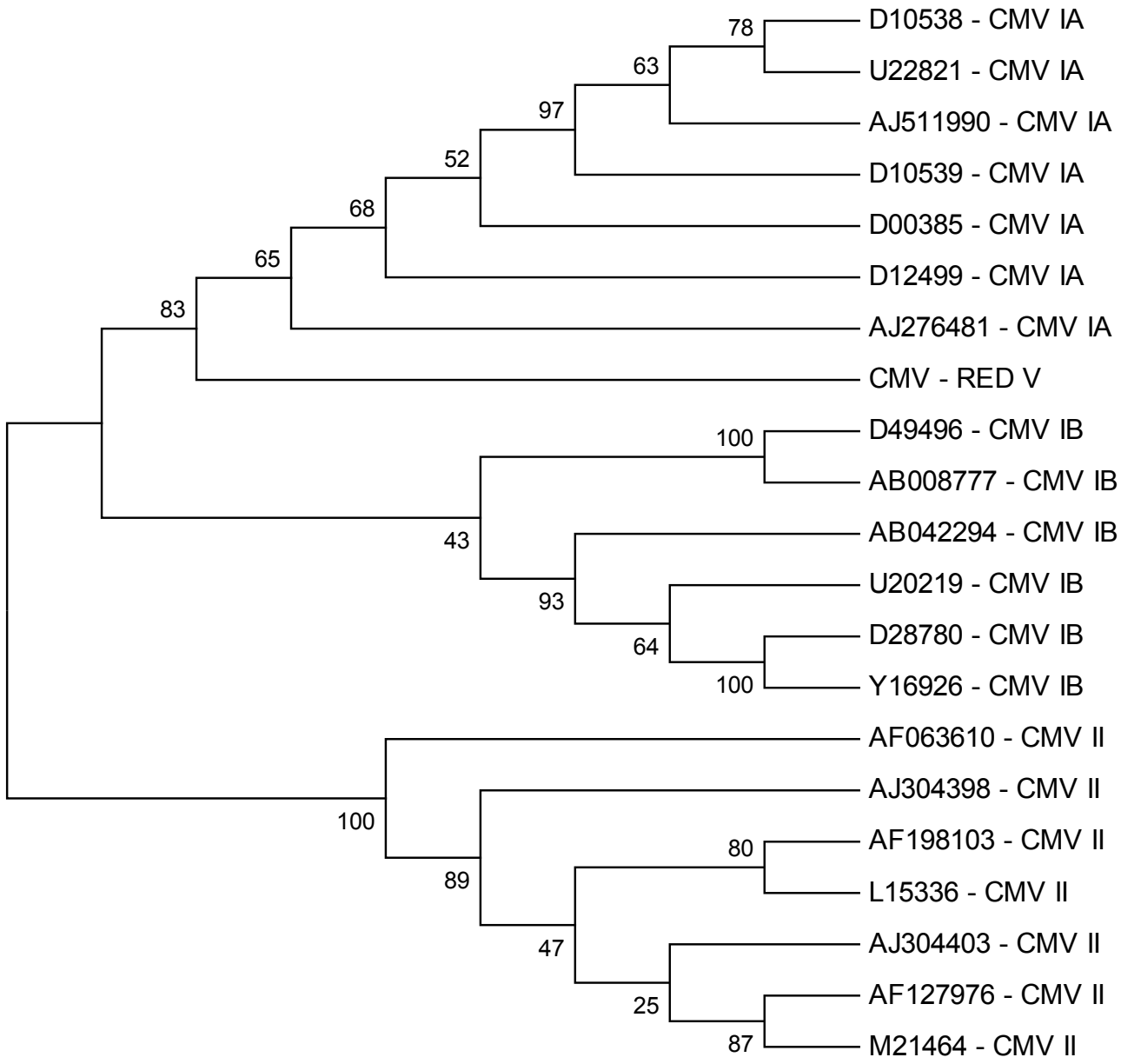

Figure 3. Unrooted phylogenetic tree calculated from a conserved region of the coat protein gene (CP) of CMV species belonging to the three subgroups IA, IB and II. CMV-RED V is the sequence obtained by the authors.

92 and 81% with those of CMV strains belonging to subgroups IA, IB and II, respectively. A phylogenetic tree was inferred using the program MEGA v. 3.1 (Kumar *et al.*, 2004) with the neighbour-joining method, and the statistical reliability of the branches was evaluated by performing bootstrap analysis (1,000 replicas). The results showed that CMV isolated from *C. ruber* formed a monophyletic group supported by 100% bootstrap and appeared closely related to AJ276481 (subgroup IA) (nucleotide identity of 96%) (Figure 3). More distant relationships were observed for subgroups IB and II. In particular, the tree comprised three major clades formed by the CMV isolates belonging to the three subgroups.

The studies at the biological, serological and molecular level show that the *C. ruber* disease is caused by an isolate of CMV. Based on the analysis of coat protein gene sequence, this isolate belongs to subgroup IA.

A previous study by Crescenzi *et al*. (1993) in Campania investigated CMV strains, and the role played by so-called arable weeds (within a crop or in areas adjacent to crop plants) in epidemiology of the virus. They showed the occurrence of CMV with greater frequency of strains of subgroup I (formerly WT strains) in 20 different species of weeds, demonstrating an important role of these weeds as survival hosts for this virus. The present study also indicates that red valerian plants may play a role in the epidemiology of CMV, allowing the virus to overwinter during the unfavourable season and be reintroduced by aphids on agricultural crops during the growing season.

Although *C. ruber* is listed as natural host of CMV by some authors (Klinkowski and Uscdraweit, 1968; Horvath, 1980), to our knowledge this is first description of CMV symptoms on this host in nature.

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