

Short communication. First report of *Eggplant mottled dwarf virus* in China rose in southern Spain

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Abstract

Eggplant mottled dwarf virus (EMDV, genus *Nucleorhabdovirus*, family *Rhabdoviridae*) is transmitted in nature by leafhoppers and its natural host range includes vegetable crops (eggplant, tomato, potato, pepper), ornamentals (pittosporum, honeysuckle, pelargonium) and wild plants (caper, *Solanum nigrum*). The prevalence of infections is generally very low. EMDV has been demonstrated to be the causal agent of a vein yellowing disease of China rose (*Hibiscus rosa-sinensis*) in southern Italy. In this work, four locations from Málaga and Granada provinces (southern Spain) were surveyed in 2011 to study the prevalence of EMDV infections in China rose by serological and molecular methods. Overall, EMDV was detected in 77.3% of the samples (33 out of 45 samples tested). Mechanical transmission tests and immunoelectron microscopy confirmed the presence of EMDV. The possible causes of such a high and unexpected prevalence are discussed. The use of molecular hybridization with an EMDV specific riboprobe is proposed for early screening of vegetative propagated China rose plants to avoid dissemination of infected material.

Additional key words: ELISA; EMDV; *Hibiscus rosa-sinensis*; molecular probe; *Nucleorhabdovirus*.

Eggplant mottled dwarf virus (EMDV) is a member of the genus *Nucleorhabdovirus* in the family *Rhabdoviridae* (order *Mononegavirales*) (Tordo *et al.*, 2005). The EMDV genome is composed of a linear, single stranded and negative-sense molecule of RNA, contained in enveloped bacilliform particles of 220-232 × 66-72 nm (Martelli *et al.*, 2011). The virus is transmitted in nature by the leafhoppers *Anaceratogallia laevis*, *A. ribauti* and *Agallia vorobjevi* (Della Giustina *et al.*, 2000; Babaie & Izadpanah, 2003), but the mode of transmission is still unclear. Natural host range of EMDV comprises crops (eggplant, tomato, potato, pepper, tobacco), ornamentals (pittosporum, China rose, honeysuckle, pelargonium), and wild plant species (caper, *Solanum nigrum* and *S. sodomaeum*) (Roggero *et al.*, 1995; Martelli *et al.*, 2011). The prevalence of infections is generally very low (1-5%),

with some exceptions recorded in Algeria and Jordan where moderate infections of about 20% have been reported on eggplant (Martelli & Hamadi, 1986; Al-Musa & Lockhart, 1990). More recently, EMDV was demonstrated to be the causal agent of vein yellowing disease in China rose (*Hibiscus rosa-sinensis* L.) plants growing in southern Italy and in imported plantlets (De Stradis *et al.*, 2008). Vein yellowing symptoms similar to those observed in Italy were noticed for the last few years in China rose plants growing in public gardens in southern Spain (Fig. 1). During summer 2011, three locations from Málaga province (Rincón de la Victoria, Torre del Mar and Caleta de Vélez) and one from Granada province (La Herradura), were surveyed to study the prevalence, distribution and association of EMDV infections with vein yellowing disease in *H. rosa-sinensis* by serological and molecular methods.

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Abbreviations used: DAS-ELISA (double-antibody sandwich enzyme-linked immunosorbent assay); EMDV (*Eggplant mottled dwarf virus*).



Figure 1. Vein yellowing associated with EMDV infection in a China rose leaf from a plant grown in a public garden of Torre del Mar (Málaga province, southern Spain).

Forty-five leaf samples were collected from China rose plants in Rincón de la Victoria (2 symptomatic, 3 asymptomatic), Torre del Mar (11 symptomatic, 3 asymptomatic), Caleta de Vélez (15 symptomatic) and La Herradura (5 symptomatic, 6 asymptomatic). All symptomatic plants were approximately 10-15 years old and showed symptoms mainly on leaves (*i.e.* vein yellowing), although deformed flowers showing pale colouring were sometimes associated to leaf symptoms. Samples were stored at 4°C and processed within one week from sampling.

An EMDV isolate (named Hirs-1), from one of the infected China rose plants collected in Caleta de Vélez during this work, was selected for host-range studies and to obtain a specific amplicon to use as specific probe for EMDV molecular detection.

Mechanical transmission tests of Hirs-1 were carried out by grinding healthy and diseased China rose leaves with sterile pestle and mortar in the presence of neutral 0.03 M phosphate buffer (Na_2HPO_4), containing 0.2% DIECA (diethyldithiocarbamic acid), 75 mg mL⁻¹ of active charcoal and carborundum 600 mesh. Plants tested were *Nicotiana benthamiana*, *Nicotiana glutinosa*, *Solanum lycopersicum*, *Ocimum basilicum*, *Phaseolus vulgaris* and *Chenopodium amaranticolor*. Inoculated plants were kept for 8 weeks in an insect-proof greenhouse at 22-24°C for symptoms observation. No symptoms were observed when asymptomatic plants were used as inoculum, whereas inoculum from symptomatic China rose were infectious only on *N. glutinosa* and *N. benthamiana* plants.

These plants showed chlorotic local lesions after approx. 10 days from inoculation, whereas stunting, systemic vein clearing and deformed leaves were observed after 3-4 weeks.

Electron microscope observations of leaf dips and ultrathin sections of symptomatic *Nicotiana* spp. and China rose plant (isolate Hirs-1) revealed the consistent presence of rhabdovirus-like particles ca. 230 × 70 nm in size in the analyzed samples, thus confirming the indication given by the biological assay. Virus particles, observed in China rose plant, were clearly decorated by immunoelectron microscopy (Milne & Luisoni, 1977) with a polyclonal antiserum to an Iranian potato isolate of EMDV (Fig. 2), kindly provided by Dr. B.E.L. Lockhart (Lockhart, 1987), confirming the identity of the virus of the Hirs-1 isolate.

Serological detection of EMDV in China rose leaf samples from Torre del Mar, Rincón de la Victoria and La Herradura, was performed by using double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) commercial kit (Loewe Biochemica GmbH, Germany), following manufacturer's instructions. A good correlation was observed between presence of symptoms and serological EMDV detection. In particular, EMDV was serologically detected in 11 symptomatic and in 2 asymptomatic samples from Torre del Mar (14 samples in total), in 3 out of 5 symptomatic samples from La Herradura (11 samples in

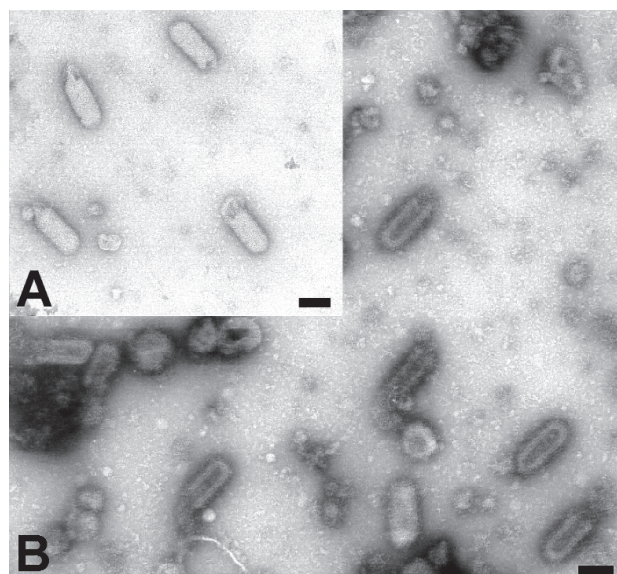


Figure 2. Rhabdovirus-like particles observed in China rose symptomatic leaf dip (A) and virus particles from the same sample decorated with the EMDV antiserum (B). Bars = 100 nm.

total) and in two symptomatic samples from Rincón de la Victoria (5 samples in total).

For EMDV molecular detection, a digoxigenin riboprobe was prepared from a cDNA fragment of about 400 bp of the polymerase gene, amplified from the EMDV isolate Hrsi-1, by using the primer pair described by Alfaro-Fernández *et al.* (2011). Insert of a recombinant plasmid, pEMES, was sequenced on both strands from two independent bacterial colonies at MWG Biotech (Ebersberg, Germany). Sequences obtained were identical (GenBank Acc. no. HE661590) and showed 99% and 97% identity with two Spanish EMDV isolates from *Pittosporum tobira* (HM636918 and HM636919). The pEMES plasmid was then used to produce an EMDV specific riboprobe and all the China rose samples from Caleta de Vélez were checked for the presence of EMDV in dot blot hybridization tests (15 symptomatic samples). The plasmid was linearized by digestion with appropriate restriction enzyme prior to transcription of plus-sense digoxigenin-labelled RNA probe, adopting methods and protocols previously described (Parrella *et al.*, 2004). Briefly, total nucleic acids extracted from leaf samples were spotted onto nylon membranes (Hybond N⁺) and fixed by exposure to UV light for 30 s prior to hybridization. Controls consisted of pEMES plasmid, used for the probe production, and leaf extracts from healthy China rose. Membrane was prehybridized for 20 min at 70°C in 5 mL of Dig Easy Granules buffer (Roche Diagnostics, Mannheim, Germany) and hybridized at 55°C overnight. Membrane was washed three times with 0.1 X SSC (0.15 M NaCl plus 0.015 M sodium citrate) and 2% SDS at 60°C for 20 min each. Detection was performed with an anti-digoxigenin antibody and the chemoluminescent substrate CDP star (Roche Diagnostics, Mannheim, Germany). ChemiDoc System apparatus and Quantity One software (Bio-Rad Laboratories, Hercules, CA) were used to detect chemoluminescent signals. All symptomatic China rose samples from Caleta de Vélez produced a clear hybridization signal in dot-blot hybridization test, while no signal was observed with healthy plant material (Fig. 3).

In summary, EMDV was detected by DAS-ELISA in 18 out of 30 samples tested and by dot-blot hybridization in 15 out of 15 samples tested. Overall, EMDV was detected in 77.3% of the samples (33 out of 45 samples tested) and 88.8% of positive samples were correlated with vein yellowing symptoms.

In a previous work we have reported sporadic infection caused by *Alfalfa mosaic virus* (AMV) in China rose in

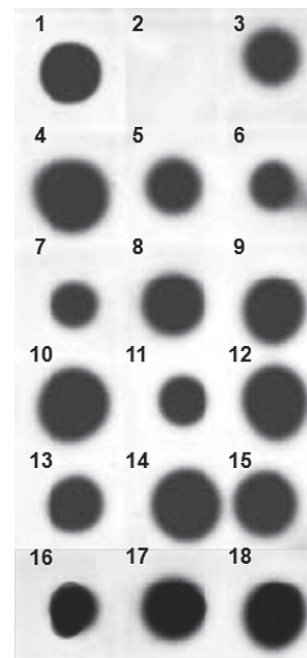


Figure 3. Dot-blot hybridization with a specific EMDV riboprobe. 1, pEMES plasmid used for the probe production; 2, healthy China rose plant; 3, *Nicotiana tabacum* plant mechanically infected by EMDV (isolate Hrsi-1); 4-18, symptomatic China rose plants sampled in Caleta de Vélez (Málaga province, southern Spain).

southern Spain (Parrella *et al.*, 2012), but the findings reported in the present paper demonstrate the widespread occurrence and the high prevalence of EMDV in China rose plants in southern Spain. EMDV has been detected previously in northern Spain, infecting eggplant and cucumber (Aramburu *et al.*, 2006) and more recently in *Pittosporum tobira*, in eastern Spain (Alfaro-Fernández *et al.*, 2011). Nevertheless, to our knowledge EMDV has not been found in southern Spain until now and this report represents also the first finding of the virus in China rose in Spain. Since China rose is an evergreen plant, EMDV infected plants constitute a permanent source of the virus. EMDV has a natural host range including important economic crops as tomato, pepper, eggplant, tobacco, potato, cucumber and muskmelon. Nevertheless, only moderate prevalence of EMDV infections has been reported so far (Martelli & Hamadi, 1986; Al-Musa & Lockhart, 1990). Based on the knowledge of natural transmission of EMDV, the high rate of infection found in the present survey could be only partially explained by vector activity. The suspicion that the China rose plants from the public gardens inspected were already infected by EMDV when they were planted is strong. The situation seems to be quite similar to that reported in Italy where

China rose plants sold in commercial markets were found already infected by EMDV (De Stradis *et al.*, 2008). Moreover, since it was observed that these plant are pruned frequently (usually every two years) it cannot be excluded a role in the EMDV dissemination by pruning tools. The use of high sensitive methods for EMDV detection, as the specific riboprobe developed in the present work, could be useful for early screening of plants before commercialization or transplanting in private or public gardens, avoiding a possible negative impact on vegetable crops of economic importance grown in close areas.

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