DEVELOPMENT OF AN IC-RT-PCR ASSAY FOR THE DETECTION OF *EGGPLANT MOTTLED DWARF VIRUS* AND PARTIAL CHARACTERIZATION OF ISOLATES FROM VARIOUS HOSTS IN GREECE

N.I. Katis[1], E.K. Chatzivassiliou[1,2], C.M. Clay[3], V.I. Maliogka[1], P. Pappi[1], K. Efthimiou[1], C.I. Dovas[4] and A.D. Avgelis[5]

[1] *Aristotle University of Thessaloniki, School of Agriculture, Plant Pathology Laboratory, 54124 Thessaloniki, Greece*
[2] *Agricultural University of Athens, Department of Crop Science, Plant Pathology Laboratory, Iera Odos 75, Votanikos, Greece*
[3] *Horticulture Research International, Wellesbourne, Warwick CV35 9EF, UK*
[4] *Aristotle University of Thessaloniki, School of Veterinary Medicine, Laboratory of Microbiology and Infectious Diseases, 54124 Thessaloniki, Greece*
[5] *Plant Protection Institute, National Agricultural Research Foundation, 71110, Heraklion, Greece*

SUMMARY

In a 10-year survey several *Eggplant mottled dwarf virus* (EMDV) isolates were collected from diverse hosts from all over Greece. In this study, an IC-RT-PCR assay was developed for the detection of the virus. Degenerate primers were designed on conserved regions of the glycoprotein (G) gene of various plant nucleorhabdoviruses. Based on the initial genetic information obtained, EMDV-specific primers were subsequently designed. The method successfully amplified a 284 bp product from all (76) virus samples originating from tobacco, cucumber, eggplant, *Pittosporum tobira* and *Capparis spinosa* (caper). Sequence analysis and Western blot were done for further characterization of representative isolates. Two isolates from tobacco (EMDV-Tob) and cucumber (EMDV-Cu) were also subjected to electron microscopy and host range studies. Sequence analysis of the partial G gene confirmed the classification of EMDV within nucleorhabdoviruses. Genetic comparisons among the EMDV isolates tested indicated low levels of variation with the caper isolate, which is reported for the first time in Greece, being the most deviating isolate. This is the first molecular assay developed for the detection of EMDV and also the first sequence information obtained of its genome.

Key words: EMDV, IC-RT-PCR detection, cytopathology, electron microscopy, Western blot.

INTRODUCTION

Eggplant mottled dwarf virus (EMDV) (genus *Nucleorhabdovirus*, family *Rhabdoviridae)* (Fauquet *et al*., 2005) is known in the Mediterranean region since 1969 (Martelli, 1969). It has been isolated from cultivated, ornamental and wild plant species in north Africa, southern Europe and the Middle East causing characteristic vein clearing, leaf crinkling and curling (El Maatoui *et al*., 1985; Martelli and Hamadi, 1986; Lockhart, 1987; Martelli and Cherif, 1987; Danesh *et al*., 1989; Roggero *et al*., 1995; Polverari *et al*., 1996; Ciuffo *et al*., 1999; Kostova *et al*., 2001; Aramburu *et al*., 2006; Mavric *et al*., 2006; De Stradis *et al*., 2008). EMDV is transmitted in nature by leafhoppers. To date *Anaceratogallia laevis* and *A. ribauti* were identified as vectors of the virus in France (Della Giustina *et al*., 2000) and *Agallia vorobjevi* Dlab transmitted EMDV in experimental trials carried out in Iran (Babaie and Izadpanah, 2003).

In Greece, EMDV was first detected in *Hibiscus rosa-sinensis* (Plavsic *et al*., 1984) and several years later in tomato, eggplant and *Pittosporum tobira* (Kyriakopoulou *et al*., 1994). Afterwards, typical EMDV symptoms were recorded in eggplant crops in Argolida, Lakonia, Messinia, Thessaloniki and Komotini (N.I. Katis, unpublished results). In 1995, severe stunting with shortened internodes, leaf curling and crinkling, chlorotic to yellow mottling, occasionally accompanied by deep green vein banding were observed in tobacco crops of cv. Myrodata (an oriental variety) in Agrinio and of cv. Virginia in Kilkis. Cucumber hybrids exhibiting symptoms resembling cucumber toad skin disease (Neshev and Lecoq, 1996) were also observed in glasshouses at Preveza and Thessaloniki. Preliminary results indicated the presence of EMDV in both crops (Katis *et al*., 2000). From 1997 to 2000 the virus was also detected in several tobacco-growing areas of the country (Chatzivassiliou *et al*., 2004). EMDV affected plants were mainly seen in the outer rows of the fields or close to the glasshouse openings and their incidence never exceeded 0.2% (Katis *et al*., 2000; Chatzivassiliou *et al*., 2004). However, in 2008 in an eggplant crop in Thessaloniki 20-25% of the plants were infected.

EMDV detection has been based on electron microscopy and DAS-ELISA tests (Martelli and Castellano, 1970; Martelli and Hamadi, 1986; Castellano and

Corresponding author: N. Katis
Fax: +302310998854
E-mail: katis@agro.auth.gr

Martelli, 1987; Martelli and Cherif, 1987; Polverari *et al*., 1996; Kostova *et al*., 2001; Mavric *et al*., 2006; Aramburu *et al*., 2006) while molecular assays have not been developed. EMDV is known to form enveloped, bacilliform or bullet shaped virions and by analogy to other nucleorhabdoviruses is supposed to have a non-segmented and single-stranded negative-sense RNA genome, encoding at least five structural proteins: the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the RNA-dependent RNA polymerase (L) in the order 3' N-P-M-G-L 5' (Fauquet *et al*., 2005). However, sequence data from the virus are still lacking.

EMDV-infected samples were collected during the last 10 years from diverse hosts from all over Greece. Subsequently, an immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) assay was developed for the detection of the virus using primers that amplify a part of the G gene. Representative isolates were further studied using different approaches while initial sequence information on the EMDV genome was obtained for the first time.

MATERIALS AND METHODS

Surveys for EMDV incidence. In order to collect virus isolates and evaluate its presence, surveys were conducted in commercial eggplant, cucumber and tobacco crops from all over Greece, for more than 10 years. EMDV isolates were also obtained from *P. tobira* and *Capparis spinosa* (caper). Samples showing typical symptoms of EMDV infection were tested through mechanical inoculation onto different indicator plants. Leaf extracts were prepared in phosphate buffer (pH 7.0) containing 0.1% sodium sulfite (Na_2SO_3), then mechanically inoculated onto carborundum-dusted leaves of *Nicotiana tabacum* cv. Samsun, *N. glutinosa* and *N. rustica*. A positive response for EMDV presence was the development of chlorotic local lesions on the inoculated leaves 1 week post inoculation (p.i.), followed by systemic vein clearing and crinkling (*N. tabacum*) two to three weeks later.

Development of IC-RT-PCR assay. A pair of degenerate primers (FinDeg1: 5´-ACACCIRTHGGICCWTG GYTRCC-3´, FinDeg2: 5´-CSGCCATCKGRGGGAAC CA-3´) targeting conserved regions of the glycoprotein (G) gene was designed after a multiple alignment of the homologous gene sequence from various plant nucleorhabdoviruses available in GenBank (accession Nos AB011257, AY674964, AY618418). The primers were initially used in IC-RT-PCR for the amplification of the homologous genomic region of EMDV-Egg (DSMZ) isolate (Table 1) using the IC protocol described by Candresse *et al.* (1995). First an RT assay (20 µl) was conducted by adding 1.4 mM random hexamers and 12.7 µl DEPC-treated water to the IC-treated tubes and incubating these for 2 min at 80°C, followed by cooling in ice for 2 min. The rest of the RT mixture, consisting of 2 units MMLV, 0.5 mM dNTPs, 1X buffer, 4 mM DDT and 13 units RNASEOUT (Invitrogen, The Netherlands) was added to each tube and the assay took place as follows: 18°C for 20 min, 37°C for 40 min, 42°C for 15 min and 95°C for 5 min. Three µl of the cDNA were further used in a PCR (20 µl) containing F511 Optimised Dynazyme^{TM} Buffer (Finnzymes, Finland), 0.2 mM of each dNTP, 5% DMSO, 2 units Dynazyme II^{TM} DNA polymerase (Finnzymes, Finland) and 1 µM of each primer. PCR cycling parameters were 95°C for 5 min, followed by 5 cycles of 95°C for 30 sec, 45°C for 30 sec and 72°C for 30 sec and another set of 35 cycles of 95°C for 30 sec, 52°C for 30 sec, 48°C for 30 sec and 72°C for 30 sec.

After cloning and sequencing, the viral origin of the purified PCR product was confirmed and based on the obtained sequence a new virus-specific primer pair (EMDVGup: 5'-GATCTGAGGGAACCATTTTGAG C-3', EMDVGdo: 5'-TCCCTTTATCTTACTGTGC GAAC-3') was designed. An IC-RT-PCR assay was performed in a final volume of 20 µl including the new primers at a concentration of 0.2 µM and also F511, Optimised Dynazyme^{TM} Buffer, 0.25 mM of each dNTP, 5.0 mM DTT, 5% DMSO, 12 units RNASEOUT (Invitrogen, The Netherlands), 0.8 units Superscript^{TM} II RNase H^{-} Reverse Transcriptase (RT) (Invitrogen, The Netherlands), 0.8 units AMV RT (Finnzymes, Finland) and 1 unit Dynazyme II^{TM} DNA polymerase (Finnzymes, Fin-

Table 1. EMDV isolates from Greece (Gr) and Germany (DE) used for the preliminary evaluation of the IC-RT-PCR assay and for further sequences analysis of the partial glycoprotein (G) gene, and accession numbers of the sequences determined in the course of this study

Isolate	Host	Origin	Accession number
EMDV-Cu	*Cucumis sativus*	Thessaloniki (Gr)	AM922317
EMDV-Tob	*N. tabacum*	Kilkis (Gr)	AM922318
EMDV-Egg	*S. melongena*	Lakonia (Gr)	AM922319
EMDV-Pit	*P. tobira*	Thessaloniki (Gr)	AM922320
EMDV-Cap	*Capparis spinosa*	Rhodes island (Gr)	AM922321
EMDV-Tom (DSMZ)	*S. lycopersicum*	DSMZ (DE)	-
EMDV-Egg (DSMZ)	*S. melongena*	DSMZ (DE)	-

land). The cycling conditions were as follows: one cycle at 50°C for 40 min, 52°C for 20 min followed by 94°C for 5 min; 35 cycles segmented in steps: (i) 30 sec at 95°C; (ii) 15 sec at 59°C; (iii) 15 sec at 56°C and (iv) 30 sec at 72°C, followed by a final extension step at 72°C for 2 min. Selected samples originating from various plant species (Table 1), which putatively carried an EMDV infection based on the reaction of the inoculated test plants, were used for the evaluation of the assay. Subsequently, all samples collected during the survey were further analysed using this IC-RT-PCR protocol.

Selected EMDV isolates, that had been assayed by IC-RT-PCR, were further studied with sequence analysis and Western blot. The tobacco (EMDV-Tob) and cucumber (EMDV-Cu) isolates were also subjected to electron microscopy observations, and their experimental host range was recorded.

Sequence analysis. The 284 bp DNA amplicons obtained from the five Greek isolates tested (Table 1) were gel-purified using a matrix gel extraction system (Marligen Bioscience, USA) and directly sequenced using 'EMDVGup' and 'EMDVGdo' oligonucleotide primers. The sequences were compared, after clipping the primer-binding regions, against the homologous genomic regions of other rhabdoviruses already deposited in the GenBank using the basic local alignment search tool (BLAST). Nucleotide and amino acid identities among the sequences of the EMDV isolates were determined, after proper alignment with Clustal-X (Thompson *et al*., 1997), using GeneDoc (http://www.psc.edu/biomed/genedoc).

Western blot analysis. Extracts of infected leaves were denatured in loading buffer (2×) (100 mM Tris-

Table 2. Test plant reaction to infection by EMDV-Tob and EMDV-Cu isolates.

Test plants	Symptoms			
	EMDV-Tob		EMDV-Cu	
	Local	Systemic	Local	Systemic
Amaranthaceae				
Celosia cristata	nt	nt	ChL	----
Gomphrena globosa	RL	----	RL	----
Chenopodiaceae				
Chenopodium amaranticolor	nt	nt	----	----
C. quinoa	ChL	----	ChL	----
Cucurbitaceae				
Cucumis sativus cv. Pepinex	nt	nt	ChL	VC
C. sativus cv. Knossos	----	----	ChL	----
C. melo	----	----	----	----
Cucurbita pepo	----	----	----	----
Citrullus lanatus	----	----	----	----
Luffa acutangula	nt	nt	----	VC, Cr
Solanaceae				
Capsicum annuum cv. Rupia	ChL	VC	----	----
Datura stramonium	ChL	----	ChL	----
Solanum lycopersicum cv. Dombo	ChL	VC	nt	nt
Nicotiana benthamiana	ChL	VC	----	VC, Cr
N. cavicola	nt	nt	ChL	VC, VN
N. clevelandii	ChL	VC	----	VC, Cr
N. glutinosa	ChL	VC	ChL	----
N. occidentalis	nt	nt	ChL	VC, VN
N. paniculata	nt	nt	ChL	VC, VN
N. rotundifolia	nt	nt	ChL	VC, VN
N. rustica	ChL	VC	ChL	VC, Cr
N. sylvestris	----	----	nt	nt
N. tabacum cv. Samsun	ChL	VC, Cr	ChL	----
cv. White Burley	nt	nt	ChL	----
cv. Xanthi	nt	nt	ChL	----
Petunia hybrida	ChL, NL	VC, Cr	----	----
Physalis floridana	----	NL, VC	NL	VC, VN
Solanum melongena	----	VC	ChL	VC, VN

ChL=chlorotic lesions, VC=vein clearing, VN=vein necrosis, NL=necrotic lesions, Cr=crinkling, RL=red lesions, --- = no local or systemic reaction, nt= not tested.

HCl pH 6.8, 2% β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) for 5 min at 100°C. The samples were analysed on 12% SDS-polyacrylamide vertical slab gels (Bio-rad mini gel system) according to Laemmli (1970). For immunodetection, proteins were transferred electrophoretically from SDS-polyacrylamide gels to PVDF membranes (Westran Clear Signal, Whatman) at 50 mA for 2.5 h. The membrane was blocked with 2% bovine serum albumin (BSA) and was probed with a EMDV-specific polyclonal rabbit antiserum (supplied by Dr S. Winter, Braunschweig) diluted 1:4000 in PBS-Tween/2% BSA for 3 h. The electroblot was then treated for labelling with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5000) for 1 h and the antigen-conjugate reaction was visualised by adding Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Electron microscopy. *Negative staining***.** Virus particles were visualised by grinding a small portion (9 mm^2) of a *N. tabacum*- or *Cucumis sativus*-infected leaf in 20 µl of 0.05% (w/v) bacitracin plus 50 µl of 2% (w/v) methylamine tungstate pH 7.0. The homogenate was transferred onto carbon-filmed EM grids for 30 sec, the excess removed using filter paper and then air dried.

*Decoration tests***.** Virus particles were first attached to carbon-faced collodion EM grids using an immunosorbent electron microscopy (ISEM) protocol, followed by decoration (Derrick, 1973; Milne and Luisoni, 1977) with a polyclonal EMDV antiserum (AS-0136, DSMZ). Grids were floated on 1/1000 EMDV antiserum diluted in 0.05 M phosphate buffer pH 7.5 for 45 min. The virus was trapped by transferring for 20 min the grids onto drops of infected leaf material homogenised in the same buffer. After rinsing with buffer, the grids were transferred onto 1/10 or 1/50 EMDV antiserum (diluted in the same buffer) for 30 min. After rinsing with ultrapure water (Elga maxima), the grids were stained using a saturated aqueous solution of uranyl acetate (UA). EMDV-infected tomato leaf material (EMDV-Tom, DSMZ, Table 1) was used as a positive control.

Ultrathin sectioning. Small pieces of an infected tobacco leaf were fixed for 1 h in 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2, rinsed with the same buffer, then postfixed for 2 h in 1% (w/v) osmium tetroxide in the same buffer. After rinsing the samples were dehydrated with graded ethanol , infiltrated with LR White resin (London Resin Company UK.) for 3 days and polymerised for 20 h at 60°C. Ultrathin sections were cut with a Reichert Ultracut E microtome using a Diatome diamond knife and stained with aqueous uranyl acetate for 10 min and lead citrate for 3 min at 20°C. Grids were examined under a JEOL 100 CX II electron microscope at 80 kV accelerating voltage and photographed on Kodak 4489 cut film.

Virus particle sizing. Measurements were made using projected images. Magnification was calibrated with a diffraction grating replica (2160 lines per mm).

Host range studies. The experimental host range (Table 2) of two virus isolates from field tobacco (EMDV-Tob) and cucumber (EMDV-Cu) was determined following mechanical inoculation of six plants of each tested species in three repetitions. Inoculated hosts were kept in an insect-proof glasshouse for three to five weeks for symptom development. Plants not showing symptoms were back inoculated onto *N. tabacum* cv. Samsun, or *C. sativus* cv. Pepinex for EMDV-Tob and EMDV-Cu, respectively.

RESULTS

Surveys for EMDV incidence. EMDV was identified in several Greek prefectures (Fig. 1). The virus was found in eggplant in Argolida (5 infected samples), Lakonia (12), Messinia (2), Rodopi (2) and Thessaloniki (10); tobacco in Aetoloakarnania (2), Fokida (2), Karditsa (5), Kilkis (2), Pieria (1), Rodopi (2), Trikala (2) and Xanthi (1); cucumber in Messinia (1), Preveza (2) and Thessaloniki (2); *P. tobira* in Rhodes island (9) and Thessaloniki (13) and caper in Rhodes island (1).

IC-RT-PCR detection and sequence analysis. A band of 376 bp with no further unspecific products, was initially amplified from the G gene of the EMDV-Egg (DSMZ) isolate using the degenerate primers FinDeg1 and FinDeg2 (not shown).

The EMDV-specific primers that were further designed based on the obtained sequence successfully amplified a 284 bp G gene fragment from all samples assumed to be positive for EMDV, based on test plant reactions (Fig. 2). Additional experiments performed using total RNA extracts from various EMDV isolates gave also satisfactory results, comparable to those of IC-RT-PCR (not shown). BLAST analysis confirmed the viral origin of the sequences, which were deposited in the EMBL-EBI under the accession numbers reported in Table 1. The sequences showed highest amino acid (aa) identity (43%) with the homologous genomic region of the nucleorhabdoviruses *Potato yellow dwarf virus* (PYDV, accession No. ADE45273) followed by *Rice yellow stunt virus* (34%) (RYSV, AB011257) and *Iranian maize mosaic virus* (31%) (IMMV, DQ186554). Pairwise comparisons of the partial G gene sequences from all EMDV isolates displayed a divergence that ranged from 86% to 97% at the nucleotide (nt) level and 95% to 100% at the aa level (Fig. 3). The sequence of the caper isolate was the most divergent at the nt level.

Western blot analysis. A main band of 55-60 kDa, recognized by the EMDV-specific polyclonal antiserum, was

Fig. 1. Map of Greece showing the areas surveyed for the detection of EMDV.

consistently detected in all preparations from EMDV-infected plants (tobacco, cucumber, eggplant, *P. tobira*, *C. spinosa*) (Fig. 4). Comparative analysis of the size of this protein with those of other closely related nucleorhabdoviruses [PYDV, RYSV, IMMV and *Taro vein chlorosis virus* (TVCV)] suggested that it probably corresponds to the nucleocapsid protein. Other less intense lower and higher molecular weight bands, putatively corresponding to the remaining structural proteins encoded by rhabdoviral genome, were also detected in most of the analysed samples. No comparable bands were obtained from preparations of healthy plant tissues.

Electron microscopy. Negatively stained preparations from tobacco and cucumber leaf material yielded mostly bullet-shaped and rarely bacilliform rhabdovirus-like particles (Figs 5a,b). Particles which had retained the outer membrane (Fig. 5a) measured *ca.* 98 nm in width, whereas particles with no apparent outer membrane and mostly bullet-shaped (i.e. naked nucleocapsids) measured *ca* 81 nm in width. Some preparations yielded bullet-shaped particles (Fig. 5b) with one rounded and one truncated end, whose central core was penetrated by the stain. These particles were shorter and often tapered.

In IEM tests, particles from both cucumber and tobacco leaves were clearly decorated by the EMDV antiserum (Fig. 5c) at both 1/50 and 1/10 dilutions. The intensity of decoration was apparently equivalent to that shown by the positive control (genuine EMDV against homologous antiserum).

In ultrathin sections paracrystalline arrays of rhabdovirus-like particles were seen around the nuclei, accumulating in the perinuclear space between the inner and

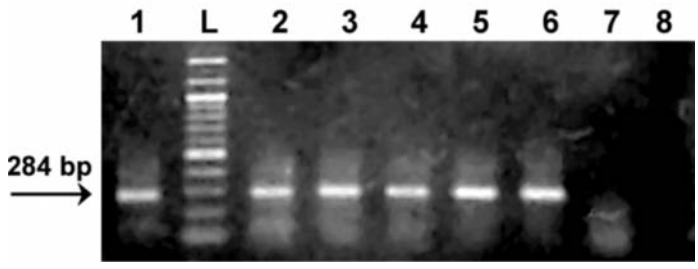

Fig. 2. Gel electrophoresis of IC-RT-PCR products amplified from different viral isolates using primers EMDVGup and EMDVGdo. Lane 1, isolate EMDV-Egg (DSMZ); lane 2, EMDV-Tob; lane 3, EMDV-Cu; lane 4, EMDV-Egg; lane 5, EMDV-Cap; lane 6, EMDV-Pit; lane 7, healthy tobacco; lane 8, healthy cucumber; lane L, 100 bp DNA ladder.

```
EMDV-Tob TGCGAACAAGAACATGTTCATGATAAGGAATGTAGATACAGGAGACACTAATTGGTGGAATCCTGTATACTCTTATTTTGATAAGAGTGA 90
EMDV-Pit ..................................................................C....G..................... 90
EMDV-Cu  ..............................................C..............C.....................A..... 90
EMDV-Egg ........G.....................................T..C..................................C........ 90
EMDV-Cap .......................T.....CA..........G.....C...........C..............C..C..C.....C.. 90

EMDV-Tob TATGTGCAACATCAGCAGCATCAATCCCTACAAGCAGATGATGGACCAAGGGAGCCCCATCAAGTTCAATTTTTGGAGAGGAGAAGCAGT 180
EMDV-Pit ..................................................................T.............................. 180
EMDV-Cu  ..................................................................T.....A.................G........ 180
EMDV-Egg ..................................................A..T...........C...........G........ 180
EMDV-Cap C.............................................T...........T..T..A..T..C..C........T..G..... 180

EMDV-Tob ACTCTACCCTCCATATACAGGACCACTTCAATGGACTCCCAGAGCCAACCCCAACTCCATTCGCA                        245
EMDV-Pit ..............C........C.....................................                        245
EMDV-Cu  .......................C.....................................                        245
EMDV-Egg .......................C.........................T......T.......                        245
EMDV-Cap T..T..T.....G..C........CT.G..G.....C...........T.....T..........                        245
```

(A)

```
EMDV-Tob ANKNMFMIRNVDTGDTNWWNPVYSYFDKSDMCNISSINPYKQMMDQGSPIKFNFWRGEAVLYPPYTGPLQWTPRANPNSIR 81
EMDV-Pit ......................C..................................................... 81
EMDV-Cu  ..................................................................... 81
EMDV-Egg ..R.............................................R.............................L.... 81
EMDV-Cap ..........I..................................................................... 81
```

(B)

Fig. 3. Multiple nucleotide (A) and amino acid (B) alignment of the partial G gene from different EMDV isolates. Nucleotide sequences are presented in (+) sense DNA.

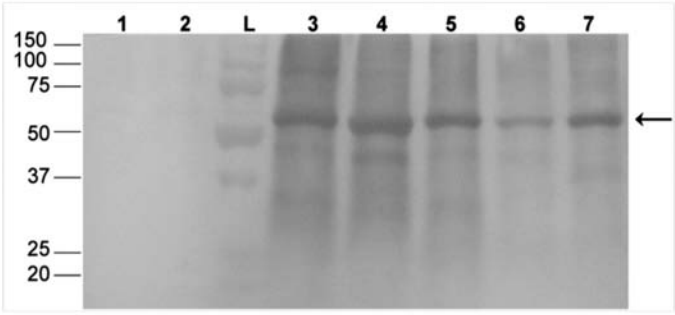

Fig. 4. Western blot analysis for the detection of EMDV isolates. Lane 1, healthy tobacco; lane 2, healthy cucumber; lane 3, EMDV-Tob; lane 4, EMDV-Cu; Lane 5, EMDV-Egg (DSMZ); lane 6, EMDV-Cap; lane 7, EMDV-Pit; lane L, Bio-Rad dual color prestained precision plus protein standard. Numbers on the left are molecular mass values in kDa.

outer lamellae of the nuclear membrane (Fig. 6a). Some particles were budding from the inner membrane (Fig. 6b). In transverse section (TS) virus particles revealed the internal ring-like structure reported for EMDV (Russo and Martelli, 1973) and the presence of outer membrane projections (Fig. 6b). Particles in thin section measured *ca.* 72 nm (mean value from 80 measurements) in diameter and up to 232 nm in length.

Hollow tubular or ring-like structures with a similar diameter to that of the virus particles were also seen in cisternae of the endoplasmic reticulum (Fig. 6a). Nuclei appeared large and the nucleoplasm was pale and uniformly granular; other organelles such as chloroplasts did not seem to be adversely affected (Fig. 6a).

Host range studies. The host range of EMDV-Tob was similar to that reported by Martelli and Rana, (1970) and especially to that of an EMDV strain from tobacco in Italy (Polverari *et al.,* 1996). However, the host range of EMDV-Cu was slightly different from that of the strain isolated from cucumber in Italy (Roggero *et al.*, 1995). In most experiments local lesions appeared 10 days post inoculation, whereas systemic infections were observed one month later. Interestingly, the cucumber isolate could infect locally various *N. tabacum* cultivars tested with no systemic spread (Table 2).

DISCUSSION

EMDV occurs in Greece as well as throughout the Mediterranean region. Representative infected samples were collected from a range of crops and areas of the country and characterized. The survey, carried out for more than 10 years, showed that EMDV infects various hosts belonging to different plant families, but it displays a rather limited spread. Due to its constantly low incidence it could be considered as one of the minor components of the viral pathosystem of tobacco, cucumber and eggplant crops (Papavasiliou *et al.*, 2001;

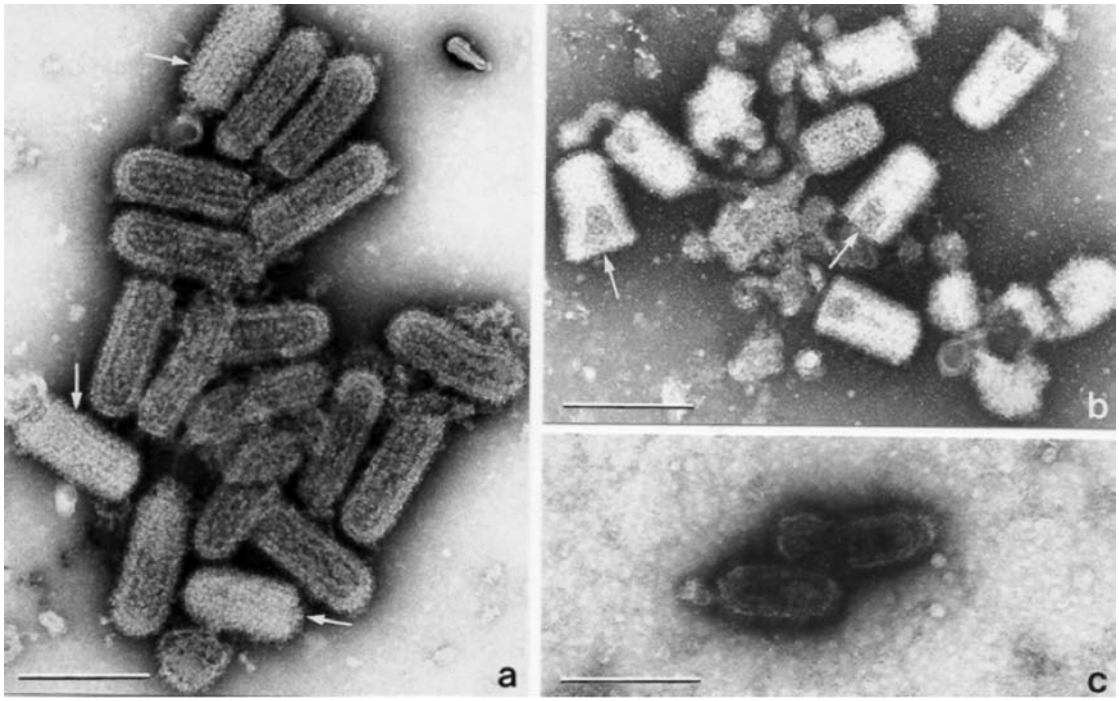

Fig. 5. a. Bacilliform and bullet-shaped rhabdovirus-like particles from *Nicotiana tabacum* leaf negatively stained with methylamine tungstate. Some particles (arrows) show the enveloping membrane. Bar = 200 nm. b. Bullet-shaped rhabdovirus-like particles stained with methylamine tungstate; stain has penetrated the core of the virus particles (arrows). Bar = 200 nm. c. Bacilliform rhabdovirus-like particles decorated by EMDV antiserum diluted 1:10 and stained with uranyl acetate. Bar = 200 nm.

Chatzivassiliou *et al*., 2004). Nevertheless early season infections can result in the development of severe symptoms and high yield losses in individually affected plants.

EMDV is not seed-transmitted (El Maataoui *et al.,* 1985) and therefore its spread depends primarily on the presence of alternative virus sources. Consequently, primary infections in susceptible crops depend on the presence and activity of insect vectors. EMDV is known to be transmitted by the leafhoppers *Anaceratogallia laevis*, *A. ribauti* (Della Giustina *et al*., 2000) or *Agallia vorobjevi* (Babaie and Izadpanah, 2003). According to Babaie and Izadpanah (2003) the virus is transmitted in a persistent manner, thus migrant vectors should colonize infected plants in order to feed long enough to acquire and transmit the virus. In almost all crops surveyed during this study, EMDV affected plants appeared in the peripheral rows and near the openings of the glasshouses, but no secondary spread was observed. A similar spread pattern was also recorded in tobacco crops in Italy (Polverari *et al*., 1996). Based on the observed disease distribution it seems most likely that the vectors do not thrive on the surveyed crops, which therefore do not represent sources of inoculum for further virus spread. The question is whether the vector has to feed for a long time on a plant to transmit the virus. Since EMDV can be transmitted by mechanical inoculation, the vector could infect epidermis and parenchyma cells. So the virus might be transmitted with a low efficiency after short probes, infecting also non-preferred hosts of a vector. It is not known whether the three identified EMDV vectors or any other vector is implicated in EMDV epidemiology in Greece. Our attempts to transmit EMDV by common aphid species or whiteflies gave negative results as previously reported (Martelli and Russo, 1973; El Maatoui *et al*., 1985; Al Musa and Lockhard, 1990; Babaie and Izadpanah, 2003), indicating that they are most probably not involved in the virus spread. While the vector status of EMDV in Greece remains unknown its high incidence recorded in an eggplant crop in 2008 indicates that its spread, at least under certain conditions, is a rather complicated phenomenon. Occasionally high disease incidence has also been reported in Iran (Babaie and Izadpanah, 2003).

In order to identify EMDV sources and the vector involved, as well as for a better understanding of its epidemiology, a reliable detection method is necessary. So far virus detection has been based on electron microscopy and DAS-ELISA (Martelli and Castellano, 1970; Martelli and Hamadi, 1986; Castellano and Martelli, 1987; Martelli and Cherif, 1987; Polverari *et al*., 1996; Kostova *et al*., 2001; Mavric *et al*., 2006; Aramburu *et al*., 2006). In this study the development

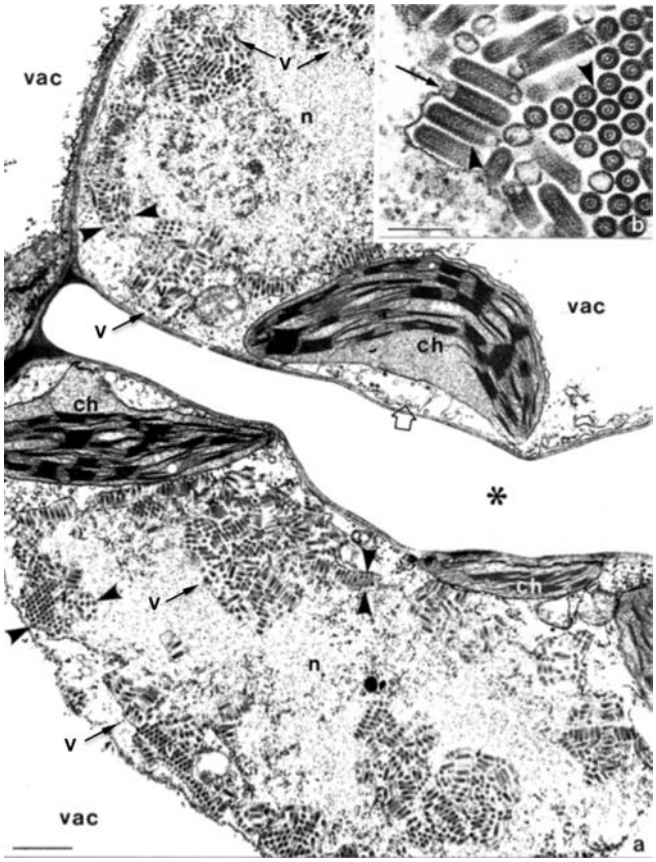

Fig. 6. a. Parts of two thin-sectioned *Nicotiana tabacum* mesophyll cells, containing accumulations of rhabdovirus-like particles (v) accumulating between the inner and outer membrane (arrowheads) of the nuclei (n). Chloroplasts (ch), vacuoles (vac) and the intercellular space (*) can also be seen. Tubular or ring-like structures can be seen within endoplasmic reticulum cisternae (open arrow). Bar = 1 µm. Inset. A bullet-shaped rhabdovirus-like particle budding from the inner nuclear membrane (arrow) into the perinuclear space. Membrane projections of the rhabdovirus-like particles can be seen (arrowheads). Bar = 200 nm. n = nucleus, v = virus, ch = chloroplast, vac = vacuole.

of an IC-RT-PCR assay is reported for the first time. The successful use of this procedure for the detection of a number of virus isolates confirmed its reliability. The immunocapture step was included in both the generic and specific RT-PCR assays so as to increase the specificity of the reaction and alleviate the need for laborious RNA extraction. However, RT-PCR without the immunocapture step yielded also satisfactory results. This assay will certainly benefit further EMDV epidemiological studies.

A range of different approaches, including Western blot analysis, electron microscopy and mechanical inoculations were also undertaken in order to further characterize the Greek EMDV isolates from various crops. All infected plants that were tested with Western blot reacted with the homologous antiserum revealing similar banding patterns. Negative staining preparations with methylamine tungstate of the Greek isolates EMDV-Tob and EMDV-Cu showed the presence of mainly bullet-shaped particles with a width ranging from 81 (nucleocapsid) to 98 nm (whole particles). Previously reported values for EMDV virion size were 50-250×70-95 nm for bullet-shaped and 300-320×95 nm for bacilliform particles in negatively stained preparations (Martelli and Castellano, 1970; Martelli and Hamadi, 1986; Castellano and Martelli, 1987; Martelli and Cherif, 1987; Polverari *et al*., 1996). There can be significant differences between the stain used and how recently the microscope magnification was calibrated that suggest caution in interpreting differences in reported sizes. Furthermore, the length of bullet-shaped particles should be treated with particular caution, as it is generally recognized that such particles represent incomplete virions. Francki *et al.* (1985) stated that more consistent measurements could have probably been obtained from thin-sections. Particles in thin sections prepared herein measured 232×72 nm. Previously reported values for EMDV bacilliform particles in thin-section are 220×65-66 nm (Martelli and Castellano, 1970; Martelli and Hamadi, 1986; Martelli and Cherif, 1987). Finally rhabdovirus-like particles were found accumulating close to the nuclear membrane which, along with the rest of the cytopathological observations made in this study are typical of those reported for other EMDV strains (Martelli and Castellano, 1970; Martelli and Hamadi, 1986; Castellano and Martelli, 1987; Martelli and Cherif, 1987; Polverari *et al*., 1996).

In addition, the host range of the Greek isolates EMDV-Tob and EMDV-Cu that were tested herein is similar to that previously reported (Martelli and Russo, 1973), although some differences were observed. The available data cannot explain why the EMDV-Cu isolate did not systemically infect tobacco. This phenomenon might be due to either a low virus concentration and\or the induction of a defense response from the host plant. However, differences in the pathogenicity and host range of EMDV-Cu compared to EMDV-Tob, which infects tobacco, might also be important.

In this study, initial EMDV sequence information was obtained for the first time. Sequence analysis of the partial G gene showed that EMDV shares the highest amino acid sequence similarities (31-43%) with other nucleorhabdoviruses (PYDV, RYSV, IMMV), thus confirming its previous classification in this virus group based on EM observations (Martelli and Castellano, 1970; Martelli and Hamadi, 1986; Castellano and Martelli, 1987; Martelli and Cherif, 1987; Polverari *et al*., 1996). Genetic comparisons among the various EMDV isolates we studied indicate low levels of variation. The caper isolate, which is reported for the first time in Greece, is the most distantly related. Further sequencing of the EMDV genome is desirable as it could provide useful information for determining the extent

of molecular variability within the species, which may reflect on pathogenicity, and for evaluating the evolutionary relationships of the virus with other plant and animal rhabdoviruses.

ACKNOWLEDGEMENTS

We thank Dr. Stephan Winter (DSMZ-Deutche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweing, Germany) for the generous gift of EMDV-infected plant material (eggplant and tomato isolates) and the EMDV-specific antiserum and Dr. L. Tomassoli (Rome, Italy) for providing the caper isolate from Rhodes.

REFERENCES

Al-Musa A.M., Lockhart B., 1990. Occurrence of *Eggplant mottled dwarf virus* in Jordan. *Journal of Phytopathology* **128**: 283-287.

Aramburu J., Galipienso L., Tornos T., Matas M., 2006. First report of *Eggplant mottled dwarf virus* in mainland Spain. *Plant Pathology* **55**: 565.

Babaie Gh., Izadpanah K., 2003. Vector transmission of *Eggplant mottled dwarf virus* in Iran. *Journal of Phytopathology* **151**: 679-682.

Candresse T., Lanneau M., Revers F., Macquaire G., German S., Dunez J., Grasseau N., Malinovsky T., 1995. An immunocapture PCR assay adapted to the detection and the analysis of the molecular variability of *Apple chlorotic leaf spot virus*. *Acta Horticulturae* **386**: 136-147.

Castellano M.A., Martelli G.P., 1987. Tomato vein yellowing in Italy, a disease caused by *Eggplant mottled dwarf virus*. *Phytopathologia Mediterranea* **26**: 46-50.

Chatzivassiliou E.K., Efthimiou K., Drossos E., Papadopoulou A., Poimenidis G., Katis N.I., 2004. A survey of tobacco viruses in tobacco crops and native flora in Greece. *European Journal of Plant Pathology* **110**: 1011-1023.

Danesh D., Lockhart B.E.L., 1989. *Eggplant mottled dwarf virus* in potato in Iran. *Plant Disease* **73**: 856-858.

De Stradis A., Parrella G., Vovlas C., Ragozzino A., 2008. Vein-yellowing of *Hibiscus rosa-sinensis* caused by *Eggplant mottled dwarf virus* in southern Italy. *Journal of Plant Pathology* **90**: 359-361.

Della Giustina W., Javoy M., Bansept P., Morel E., Balasse H., Goussard N., Passard C., 2000. Les cicadelles du genre *Anaceratagallia* vectrice du virus responsable de la maladie de la peau de crapaud du concombre. *PHM-Revue Horticole* **420**: 40-43.

Derrick K.S., 1973. Quantitative assay for plant viruses using serologically specific electron microscopy. *Virology* **56**: 652-653.

El Maataoui M., Lockhart B.E.L., Lesemann D.E., 1985. Biological, serological, and cytopathological properties of tomato vein yellowing virus occurring in tomato in Morocco. *Phytopathology* **75**: 109-115.

Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A., 2005. Virus taxonomy. 8th Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, CA, USA.

Francki R.I.B., Milne R.G., Hatta T., 1985. Plant Rhabdoviridae. In: Francki R.I.B., Milne R.G., Hatta T., (eds). Atlas of Plant Viruses, Vol. 1, pp. 73-100. CRC Press, Boca Raton, FL, USA.

Katis N.I., Chatzivassiliou E.K., Avgelis A.D., Manoussopoulos I., Lecoq H., 2000. Occurrence of *Eggplant mottled dwarf nucleorhabdovirus* (EMDV) in tobacco and cucumber in Greece. *Phytopathologia Mediterranea* **39**: 318.

Kostova D., Masenga V., Milne R.G., Lisa V., 2001. First report of *Eggplant mottled dwarf virus* in cucumber and pepper in Bulgaria. *Plant Pathology* **50**: 804.

Kyriakopoulou P.E., Rana G.L., De Stradis A., 1994. Tomato vein yellowing in tomato in Greece. *Proceedings of the 7th Panhellenic Phytopathological Congress*, *Athens 1994*: 3.

Laemmli U.K., 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lockhart B.E.L., 1987. Evidence for identity of plant rhabdoviruses causing vein-yellowing disease of tomato and *Hibiscus rosa-sinensis*. *Plant Disease* **71**: 731-733.

Martelli G.P., 1969. Bacilliform particles associated with mottled dwarf of eggplant (*Solanum melongena* L.). *Journal of General Virology* **5**: 319-320.

Martelli G.P., Castellano M.A., 1970. Electron microscopy of *Eggplant mottled dwarf virus*. *Phytopathologia Mediterranea* **9**: 39-49.

Martelli G.P., Russo M., 1973. *Eggplant mottled dwarf virus*. *CMI/AAB Descriptions of Plant Viruses* **115**.

Martelli G.P., Hamadi A., 1986. Occurrence of *Eggplant mottled dwarf virus* in Algeria. *Plant Pathology* **35**: 595-597.

Martelli G.P., Cherif C., 1987. *Eggplant mottled dwarf virus* associated with vein yellowing of honeysuckle. *Journal of Phytopathology* **119**: 32-41.

Martin D.K., 1983. Studies on the main weed host and aphid vector of *Lettuce necrotic yellows virus* in South Australia. In: Plumb R.T., Thresh J.M. (eds). Plant Virus Epidemiology, pp. 211. Blackwell Scientific Publications, Oxford, UK.

Mavric I., Tušek Žnidarič M., Viršček Marn M., Dolničar P., Mehle N., Lesemann D-E., Ravnikar M., 2006. First report of *Eggplant mottled dwarf virus* in potato and tomato in Slovenia. *Plant Pathology* **55**: 566.

Milne R.G., Luisoni E., 1977. Rapid electron microscopy of virus preparations. *Methods in Virology* **6**: 265-281.

Neshev G., Lecoq H., 1996. Cucumber toad skin disease, a new virus disease of cucumber in Bulgaria. *Rastitelna Zashtita* **9**: 25.

Papavasiliou C., Hatziloukas E., Dovas C.I., Efthimiou K., Avgelis A.D., Katis N.I., 2001. Incidence of insect-borne cucurbit viruses in Greece. *Phytopathologia Mediterranea* **40**: 195.

Plavsic B., Eric Z., Milicic D., 1984. Rhabdovirus-like particles associated with vein yellowing of *Hibiscus rosa-sinensis* L. *Phytopathologia Mediterranea* **23**: 49-51.

Polverari A., Castellano M.A., Marte M., 1996. The natural occurrence of Eggplant mottled dwarf rhabdovirus in tobacco in Italy. *Journal of Phytopathology* **144**: 25-27.

Roggero P., Milne R.G., Masenga V., Ogliara P., 1995. First report of Eggplant mottled dwarf rhabdovirus in cucumber and pepper. *Plant Disease* **79**: 321.

Russo M., Martelli G.P., 1973. A study of the structure of *Eggplant mottled dwarf virus*, *Virology* **52**: 39-48.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G., 1997. The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876-4882.

Received September 30, 2010
Accepted January 31, 2011