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SHORT COMMUNICATION

OCCURRENCE OF *CUCURBIT YELLOW STUNTING DISORDER VIRUS*
AND *CUCUMBER VEIN YELLOWING VIRUS* IN TUNISIAS. Yakoubi¹, C. Desbiez², H. Fakhfakh¹, C. Wipf-Scheibel², M. Marrakchi¹ and H. Lecoq²¹Laboratoire de Génétique Moléculaire, Immunologie et Biotechnologie, Faculté des Sciences de Tunis, Campus Universitaire, 2092 El Manar, Tunis, Tunisia²INRA, Station de Pathologie Végétale, Domaine Saint Maurice, B.P. 94, 84143 Montfavet cedex, France

SUMMARY

During surveys carried out in June and October 2005 and August 2006 for assessing the occurrence of cucurbit-infecting viruses in Tunisia, samples of squash, melon and snake cucumber showing virus-like symptoms were collected from the major cucurbit-growing areas. Besides mosaic-inducing viruses, DAS-ELISA and RT-PCR tests showed the presence of yellows-inducing viruses: *Cucurbit aphid-borne yellows virus* (CABYV, *Polerovirus*), *Cucurbit yellow stunting disorder virus* (CYSDV, *Crinivirus*) and *Cucumber vein yellowing virus* (CVYV, *Ipomovirus*). While CABYV, a virus previously reported from Tunisia, was detected throughout the country, CYSDV and CVYV, reported here for the first time, were found only in the Sahel and the Southern part of the country. The identification of CYSDV and CVYV was confirmed by immunosorbent electron microscopy. Phylogenetic analysis done on parts of the CP gene sequence showed that Tunisian CYSDV isolates have high sequence identity (99-100%) with isolates that have recently emerged in the Middle East, Southern Europe and the United States. In contrast, CVYV isolates from Tunisia are more divergent (96.6%) from Middle East isolates and are only distantly related (94.5-95%) to isolates which recently emerged in Spain, Portugal and France.

Key words: BPYV, CABYV, crinivirus, CVYV, CYSDV, ipomovirus, melon, squash, whitefly.

Viral diseases are a worldwide problem of cucurbit crops and a major limiting factor for production (Blancard *et al.*, 1994, Zitter *et al.*, 1996). In Tunisia, cultural practices, environmental conditions and the lack of awareness of some growers about viral diseases are some of the factors that result in a high incidence of viral diseases.

It is particularly important to identify the causal agents of virus diseases for implementing effective and

durable control measures. Several virus diseases causing mosaic symptoms were previously reported in Tunisia including *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV), *Papaya ringspot virus* type Watermelon (PRSV-W), *Zucchini yellow fleck virus* (ZYFV), *Squash mosaic virus* (SqMV) and *Cucumber mosaic virus* (CMV) (Cherif and Ezzaier, 1987). We undertook surveys in some of the major cucurbit-producing areas in Tunisia in order to update the relative importance of the different viruses infecting cucurbit crops. Indeed, yellowing diseases are becoming increasingly important in Tunisia, as in many areas of the world (Wisler *et al.*, 1998). *Cucurbit aphid-borne yellows virus* (CABYV, Genus *Polerovirus*, Family *Luteoviridae*) was recently reported as causing yellowing symptoms in cucurbits in Tunisia (Mnari Hattab *et al.*, 2005).

One hundred and eighteen samples of infected cucurbit plants [snake cucumber (*Cucumis melo* var *flexuosus*), zucchini squash (*Cucurbita pepo*), squash (*Cucurbita moschata*) and melon (*Cucumis melo*)] showing virus-like symptoms were collected from the main cucurbit growing regions of Tunisia: Bizerte and Cap Bon (Northern Tunisia), Monastir (Sahel region) and Degache (Southern Tunisia) at the end of the growing season in June and October 2005 and August 2006, when symptoms are most apparent.

All samples were tested for the presence of ZYMV, WMV, CMV, PRSV, SqMV, CABYV and *Cucumber vein yellowing virus* (CVYV) using the double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with specific polyclonal antibodies prepared at INRA-Montfavet. Tests for *Cucurbit yellow stunting disorder virus* (CYSDV) and *Beet pseudo-yellows virus* (BPYV) were conducted by RT-PCR using specific primers: CYSDV-CP-5' (5'-ATGGCGAGTTCGAGTGAGAA-3') and CYSDV-CP-3' (5'-TCAATTACCACAGCCACTG-3') (Desbiez *et al.*, 2003) and BPYV-CP-5' (5'-CTGACATATGGGAGATAATGATGATGG-3') and BPYV-CP-3' (5'-CTGACTCGAGTCAGTTTCCATAA-GAAGC-3') that we defined according to GenBank sequence AB085612.

The DAS-ELISA tests showed that a large proportion (72%, 85/118) of the samples collected reacted positively with a CABYV antiserum. However, in some

cases, CABYV was not detected in plants with typical yellowing symptoms (Fig. 1), suggesting the presence of other viruses. CYSDV was detected by RT-PCR in 12.7% (15/118) of the samples in single or in mixed infection with CABYV or CYSDV, whereas CVYV was found in 6.8% (8/118) of the samples in single or in mixed infection with CABYV, CYSDV or ZYMV. CYSDV was detected in melon and snake cucumber plants while CVYV was found in zucchini squash and melon plants. BPYV was not detected in the samples analysed. CABYV was found in samples from all over the country, while CYSDV and CVYV were detected only in samples from the Sahel (Monastir) and Southern (Degache) regions. CYSDV and CVYV which are common in some countries of the Mediterranean basin are reported here for the first time in Tunisia.

CYSDV (Genus *Crinivirus*, family *Closteroviridae*) is transmitted in a semi-persistent mode by *Bemisia tabaci*. It induces severe yellowing symptoms that begin as an interveinal mottle and intensify as leaves age (Abou-Jawdah *et al.*, 2000) (Fig. 1). Particles are flexible rods 750-800 nm long (Liu *et al.*, 2000). The genome consists of two molecules of single stranded RNA of positive polarity designated RNA1 and RNA2 (Célix *et al.*, 1996). CYSDV was first reported by Hassan and Duffus (1991) in the United Arab Emirates and it has spread to many countries around the Mediterranean basin. It has been reported in Spain (Celix *et al.*, 1996), Lebanon (Abou-Jawdah *et al.*, 2000), Portugal (Louro *et al.*, 2000), Morocco (Desbiez *et al.*, 2000) and more recently in France (Desbiez *et al.*, 2003). CYSDV has been reported for the first time in Northern America in Texas in 1999 (Kao *et al.*, 2000) and is further spreading causing severe outbreaks in 2006 in California, Arizona and Northern Mexico (Brown *et al.*, 2007, Kuo *et al.*, 2007).

CVYV (Genus *Ipomovirus*, family *Potyviridae*) is the causal agent of a severe disease of cucurbits, characterized by vein clearing followed by leaf chlorosis and of-



Fig. 1. Typical yellowing symptoms caused by CYSDV on naturally infected snake cucumber in Monastir region.

ten associated with severe yellowing of older leaves. Virions are filamentous particles and the genome is a single stranded, positive-sense, RNA molecule (Lecoq *et al.*, 2000). CVYV is readily transmitted mechanically and by the whitefly *Bemisia tabaci* in a semi-persistent manner (Mansour and Al-Musa, 1993). CVYV was originally identified in Israel (Cohen and Nitzany, 1960) and later in the eastern Mediterranean basin (Al-Musa *et al.*, 1985; Yilmaz *et al.*, 1989) and more recently has been reported in Spain (Cuadrado *et al.*, 2001), Portugal (Louro *et al.*, 2004) and France (Lecoq *et al.*, 2007).

The Tunisian isolates of CYSDV and CVYV were partially characterized using serological and molecular methods. Crude extracts from infected snake cucumber and zucchini leaves found singly infected by CYSDV or CVYV respectively, were observed with a Philips CM 10 electron microscope using pyroxilin and carbon-coated grids and 1% ammonium molybdate, pH 7.0, as

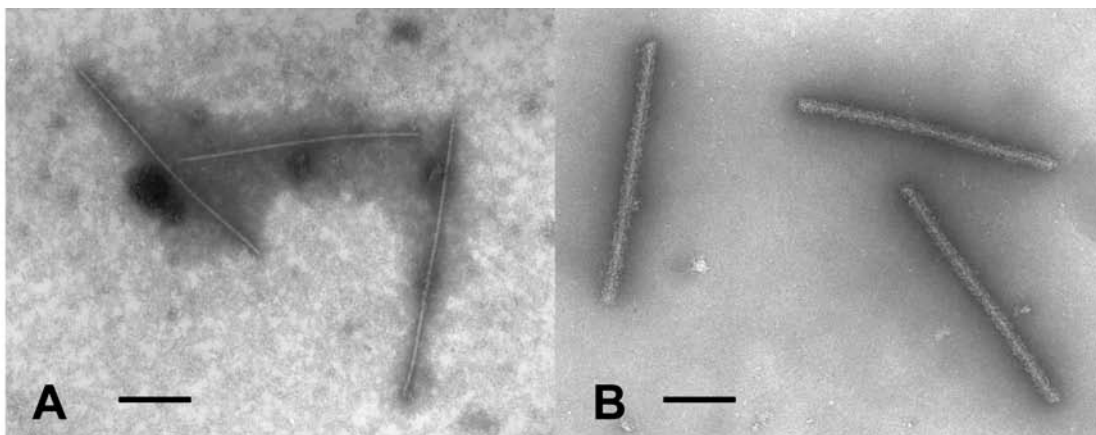


Fig. 2. Particles of the Tunisian *Cucurbit vein yellowing virus* isolate observed after trapping on CVYV-activated grids and negative staining using ammonium molybdate in crude extracts of infected zucchini squash leaves (A), and CVYV particles decorated by a polyclonal antiserum against a Sudanese CVYV isolate at a dilution of 1:5 (B). Scale bars represent 200 nm.

negative stain. Grids were coated with CVYV or CYSDV antisera at a dilution of 1:500. Decoration of virus particles was performed by incubating grids for 20 min at 37°C with antiserum diluted 1:10 (CYSDV) and 1:5 (CVYV) in 0.06 M phosphate buffer pH 7.2.

This assay revealed the presence of few very flexuous filamentous particles in extracts from plants infected by CYSDV and numerous rigid filamentous particles in extracts from plants infected by CVYV. In both cases particles were decorated by their corresponding antisera (data not shown and Fig. 2).

Total RNA was extracted from 50 mg of symptomatic leaves using TRI-reagent (Molecular Research Center Inc., Cincinnati, OH, USA), resuspended in 20 µl of RNase-free H₂O and heated for 5 min at 65°C before testing by RT-PCR. One µl of total RNA was used in an RT-PCR mixture (25 µl) containing 2.5 µl of 10x one-step RT-PCR reaction buffer (100 mM Trizma-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂ and 0.01% gelatin), 0.5 µl of 10 mM dNTP, 2.5 U AMV reverse transcriptase (Promega, Madison, WI, USA), 2.5 U Taq DNA polymerase and 100 ng of each specific primer: CYSDV-CP-5' and CYSDV-CP-3' (Desbiez *et al.*, 2003) or CVYV-CP-5' (5'-GCTTCTGGTTCTCAAGTGGGA-3') and CVYV-CP-3' (5'-GATGCATCAGTTGTCAGATG-3') (Bananej *et al.*, 2006).

This procedure successfully amplified parts of CVYV and CYSDV coat protein genes from total nucleic acids of infected tissue yielding expected-size fragments of 536 bp and 756 bp, respectively. No amplification was ob-

tained from extracts from healthy melon or squash plants.

The amplified fragments of 4 and 5 isolates of CVYV and CYSDV respectively, were sent for direct sequencing to Genome Express (Grenoble, France). The nucleotide sequences of the 4 CVYV isolates were identical, thus only one was deposited in GenBank under number EF538680. Similarly, the 5 CYSDV nucleotide sequences were identical, and one was deposited under number EF538681. The deduced amino acid sequences of CYSDV or CVYV were compared to sequences in the GenBank database using the BLAST and FASTA procedures (Altschul *et al.*, 1997; Pearson and Lipman, 1988). Sequence alignments and a distance matrix were obtained with Clustal W included in DAMBE (Thompson *et al.*, 1994). Sequences of CYSDV and CVYV strains available from databases were added to the analysis: CYSDV strains from France (AY204220), Jordan (DQ903107 and DQ903111), Spain (AF312809 and AJ243000), Iran (AY730779), Mexico (AF312807), Texas (AF312806), Lebanon (AF312803), Turkey (AF312801) and Saudi Arabia (AF312797) and CVYV strains from Israel (AF233429), Portugal (AY424869), Spain (AY578085) and France (EF441272). Neighbour-joining trees were built with MEGA (Molecular Evolutionary Genetics Analysis), version 3.1 (Kumar *et al.*, 2004). Robustness of the inferred evolutionary relationships was assessed by 500 bootstrap replicates.

Sequence comparisons showed that the CYSDV isolates from Tunisia shared 99-100% nucleotide sequence identity with isolates that recently emerged in the Middle East, Southern Europe (Spain, Portugal and France), the United States and Morocco, suggesting a common origin (Fig. 3). Tunisian CVYV isolates shared only 94.5-96.6% nucleotide sequence identity in the central part of the coat protein with isolates from Spain, the Middle East and France (Fig. 4).

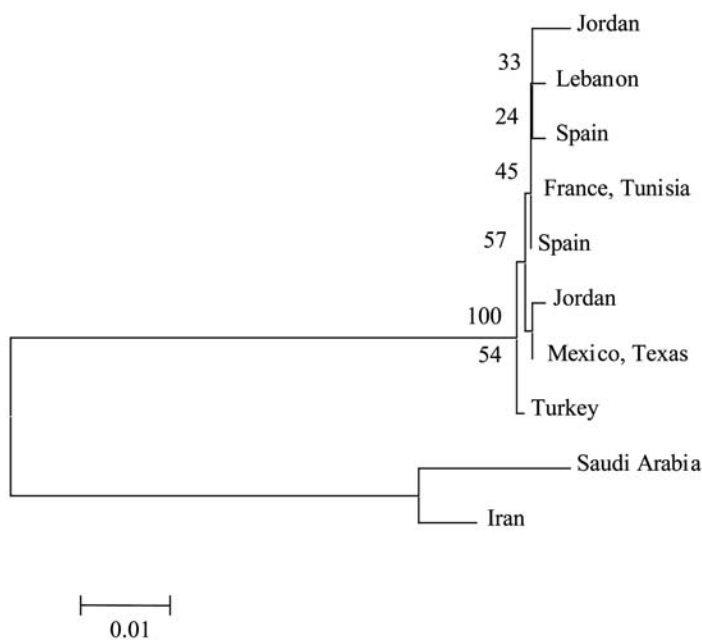


Fig. 3. Phylogenetic analysis of the nucleotide sequence of part of the coat protein gene of CYSDV isolates. The neighbour-joining unrooted tree was established by the MEGA program. Bootstrap analysis was applied using 500 bootstrap replicates. Scale bar represents a genetic distance of 0.01.

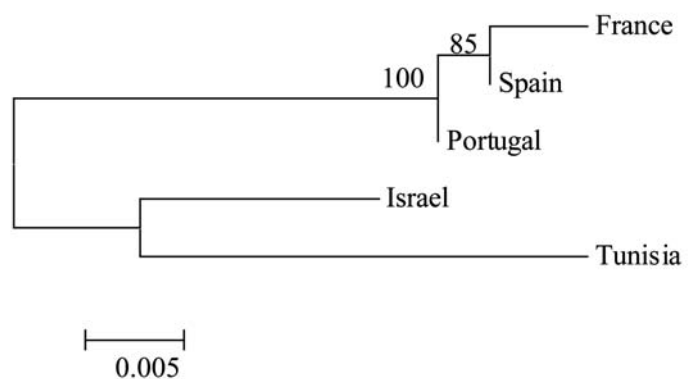


Fig. 4. Phylogenetic analysis of the nucleotide sequence of the core part of the coat protein gene of CVYV isolates. The neighbour-joining unrooted tree was established by the MEGA program. Bootstrap analysis was applied using 500 bootstrap replicates. Scale bar represents a genetic distance of 0.005.

The rapid spread of CYSDV in the Mediterranean Basin and in North America makes it one of the most typical examples of emerging viruses infecting cucurbits (Lecoq *et al.*, 1998, Brown *et al.*, 2007). The situation for CVYV is slightly different. The relatively distant relationships with isolates from the Middle-East and with those emerging in Spain, Portugal and France suggest either a distinct origin for the Tunisian isolates or that CVYV has already evolved on its own for some time in Tunisia.

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