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# Present status of some virus diseases affecting legume crops in Tunisia, and partial characterization of *Chickpea chlorotic stunt virus*

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**Summary.** Field surveys were conducted in Tunisia during the 2005–2006, 2006–2007 and 2009–2010 growing seasons to identify viruses which produce yellowing, reddening and/or stunting symptoms of chickpea, faba bean and pea crops. Tissue blot immunoassay (TBIA) results showed that *Chickpea chlorotic stunt virus* (CpCSV) was the most common virus, followed by *Faba bean necrotic yellows virus*, *Bean leafroll virus* and *Beet western yellows virus*. The coat protein (CP) gene nucleotide sequence of seven CpCSV isolates collected from different regions of Tunisia was compared with sequences of five other isolates in the NCBI database. A homology tree of the CP nucleotide sequences was prepared and CpCSV isolates were grouped into two clusters. The first group contained two Tunisian CpCSV chickpea isolates collected from Bizerte and Kef; sequenced regions showed a high nucleotiode homology (95%) to that of the Ethiopian and Sudanese CpCSV isolates. The second group included five Tunisian isolates: two from chickpea, two from pea and one from faba bean, which showed a high homology (96%) when compared with the Moroccan, Egyptian and Syrian CpCSV isolates.

**Key words**: coat protein sequence, TBIA, RT-PCR.

## Introduction

Faba bean (*Vicia faba* L.), chickpea (*Cicer arietinum* L.) and pea (*Pisum sativum* L.) are the most important legume crops in Tunisia and play significant roles in the farming system. These crops are affected by a large range of fungal and viral diseases that can cause economic losses under disease favourable conditions. Around 12 viruses have been identified in Tunisia to affect faba bean (Makkouk *et al*., 1988; Najar *et al*., 2000, 2003), and three viruses have been reported on chickpea (Najar *et al*., 2000; Kumari *et al.,* 2010). Recently, *Chickpea chlorotic stunt virus* (CpCSV, genus *Polerovirus*, family *Luteoviridae*) has been reported to affect chickpea in Ethiopia (Abraham *et al*., 2006), Eritrea (Kumari *et al.,* 2008), Syria (Assad *et al*., 2009), Iran (Bananej *et al*., 2010), Morocco, Egypt and Sudan (Abraham *et al.,* 2009), stimulating investigations about its occurrence in Tunisia.

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## Materials and methods

### Field surveys

Field inspections and sample collection were conducted during April 2006, 2007 and 2010, when the plants in crops were at flowering/pod setting stages. The surveys covered northeastern regions (Bizerte, Menzel Bourguiba, Cap Bon [Menzel Bouzalfa and Menzel Temime]) and northwestern regions (Kef, Jendouba, Beja and Bousalem). A total of 90 samples from nine fields (seven chickpea, two faba bean) were collected in 2006, 728 samples

from 25 fields (20 chickpea, five faba bean) were collected in 2007, and 281 samples from 22 fields (ten chickpea, six faba bean, six pea) were collected in 2010. Plants showing symptoms suggestive of virus infection (yellowing, reddening and stunting) were collected.

**Serological assays**

All collected samples were tested for the presence of viruses using the tissue-blot immunoassay (TBIA) (Makkouk and Kumari, 1996) against a battery of polyclonal and monoclonal antibodies (MAbs). ICARDA's Virology Laboratory provided rabbit polyclonal antisera for the following four viruses: *Chickpea chlorotic dwarf virus* (CpCDV, genus *Mastrevirus*, family *Geminiviridae*) (Kumari *et al.,* 2006), *Broad bean wilt virus* (BBWV, genus *Fabavirus,* family *Comoviridae*), *Alfalfa mosaic virus* (AMV, genus *Alfamovirus,* family *Bromoviridae*) and *Broad bean mottle virus* (BBMV, genus *Bromovirus,* family *Bromoviridae*).

A MAb used to detect *Faba bean necrotic yellows virus* (FBNYV, genus *Nanovirus*, family *Nanoviridae*) (3-2E9) was provided by A. Franz (Franz *et al.,* 1996), and a broad-spectrum legume-luteovirus MAb (5G4) was provided by L. Katul (Katul, 1992), BBA, Braunschweig, Germany. To identify the individual luteoviruses infecting legume crops in the investigated areas, samples that gave positive reactions with the broad-spectrum MAb 5G4 were further tested against the following specific luteovirus MAbs: *Beet western yellows virus* (BWYV, genus *Polerovirus*, family *Luteoviridae*) (A5977; from Agdia, Elkhart, IN, USA), *Bean leafroll virus* (BLRV, genus *Luteovirus*, family *Luteoviridae*) (4B10; Katul, 1992), *Soybean dwarf virus* (SbDV, genus *Luteovirus*, family *Luteoviridae*) (PVAS-650; from American Type Culture Collection, ATCC, Rockville, Maryland,USA), a mixture of three MAbs (1-1G5, 1-3H4 and 1-4B12) produced against an Ethiopian isolate of CpCSV (CpCSV-Eth), and a mixture of three MAbs (5-2B8, 5-3D5 and 5-5B8) produced against a Syrian isolate of CpCSV (CpCSV-Sy) (Abraham *et al.,* 2006*,* 2009).

**RNA extraction and RT-PCR assays**

The following seven samples that reacted with CpCSV MAb were used in this study. Six of them reacted with CpCSV-Sy: three chickpea isolates (TuC35-06 from Cap Bon, TuC84-06 from Bizerte and TuC215-201 from Beja), one faba bean isolate (TuV258-10 from Kef) and two pea isolates (TuP163-10 and TuP166-10 from Cap Bon). One chickpea sample (TuC311-07 from Kef) reacted with CpCSV-Eth. In addition, a Syrian CpCSV isolate (SV1-03) (Abraham *et al*., 2009) was used as a control.

Total RNA was extracted from infected plants using the RNeasy Plant Mini Kit from Qiagen (Cat. No. 74904; Hilden, Germany) following the methods described by Mackenzie *et al*. (1997). A one-step RT-PCR reaction was carried out by One-step RT-PCR Kit from Invitrogen Corporation, Carstland, CA, USA (Cat. No.12574-01) using the CpCSV-specific primer pairs (CpCSV-F: 5-TAGGCGTACTGTTCAGCGGG-3 and CpCSV-R: 5-TCCTTTGTCCATTCGAGGTGA-3) (Kumari *et al.,* 2008).

Amplification was performed in an automated thermal cycler (Applied Biosystems Gene) programmed for the following thermo-cycling conditions: 48ºC for 48 min for cDNA synthesis, 5 min at 94ºC for reverse transcriptase inactivation and initial denaturation, followed by 35 cycles of 30 s at 94ºC, 1 min at 55ºC and 2 min at 72ºC, and a final extention for 10 min at 72ºC.

PCR 15 µl products were analysed by electrophoresis on agarose gel (1%) in TBE buffer (90 mM Tris borate, 2 mM EDTA) at 120 V for 45 min. The amplified DNA fragments were stained with ethidium bromide (0.5 µg $mL^{-1}$) and visualized under a UV transilluminator. The size of fragments was determined using 1 kb DNA molecular weight ladder (IX, Cat.N. 11 449 460 001, Boehringer Mannhein, Mannhein, Germany).

**Sequence analysis**

The PCR amplification products of the seven Tunisian isolates were purified using Qiaquick® PCR Purification Kit 50 from Qiagen (Cat. No. 28104) (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplicons were sequenced by using the ABI Prism® 3100 Genetic Analyser BigDye® Terminator Sequencing standard. A search for homology with coat protein genes deposited in the GenBank was carried out with the BLAST program (Altshull *et al*., 1990). The sequence obtained was employed to further study homology among the CpCSV isolates. The homol-

ogy tree was performed using DNAMAN Sequence Analysis Software package (Lynnon Biosoft, Quebec, Canada).

The following are the GeneBank Accession Numbers of isolates from other countries used for nucleotide sequence comparison: CpCSV-Syria (EU541270 from faba bean), CpCSV-Egypt (EU541269 from faba bean), CpCSV-Morocco (EU541267 from faba bean), CpCSV-Sudan (EU541263 from chickpea), CpCSV-Ethiopia (EU541257 from faba bean); BLRV-USA (AF441393); BWYV-France (AF167482); and *SbDV*-Japan (AB038147)

## Results

### Field observations

The most commonly observed symptoms suggestive of virus infection in chickpea, faba bean and pea crops were stunting and yellowing, chlorosis, reddening of the leaves and tip wilting. Based on symptoms observed the virus disease incidence in the fields was estimated to range from 0.5 to 30% during 2006 and 2010 growing seasons and from 0.5 to 50% during the 2007 growing season. The greater virus disease incidence was recorded in several chickpea fields in Cap-Bon and Kef regions.

### Serological tests

Laboratory (TBIA) results of 90, 728 and 281 symptomatic plant samples collected respectively during 2006, 2007 and 2010 growing seasons showed that 51 (56.7%), 414 (56.9%) and 128 (45.5%) samples reacted positively with 5G4 MAb. Whereas, FBNYV was detected only in four (4.4%), 88 (12.1%) and six (2.1%) samples collected during 2006, 2007 and 2010, respectively (Table 1). When samples that reacted positively with 5G4 MAb were further tested using specific luteovirus MAbs, three luteoviruses (BLRV, BWYV and CpCSV) were identified (Table 1). There were an additional 22 faba bean (collected during 2007), one chickpea (from 2010) and one pea samples, which reacted with the broad spectrum MAb 5G4 but did not react with any of the specific MAbs used (Table 1).

The majority of the 5G4 positive samples collected from chickpea, faba bean and pea fields during 2006 and 2010 growing seasons reacted with CpCSV MAbs. However, in 2007, in faba bean fields, CpCSV and BWYV incidence was approximately equal.

Most of the CpCSV positive samples reacted with CpCSV-Sy MAb except one chickpea sample collected during April 2006, one faba bean and six chickpea samples collected during April 2007, which reacted with CpCSV-Eth MAb (Table 2).

### RT-PCR assays

All seven Tunisian (three chickpea, one faba bean and two pea) samples and one chickpea sample that respectively reacted serologically with CpCSV-Sy and CpCSV-Eth as well as the reference CpCSV isolate (SV1-03) generated amplicons of the expected size (413 bp) using CpCSV-specific primers.

### Sequence analysis

The coat protein (CP) gene nucleotide sequence analysis divided the CpCSV Tunisian isolates in two groups. The first group contained two Tunisian CpCSV isolates from chickpea (TuC84-06 from Bizerte [GeneBank Accession No. HQ324136] and TuC311-07 from Kef [GeneBank Accession No. HQ324135]) which showed high homology (95%) to that of the Ethiopian (EU41257) and Sudanese (EU 541263) CpCSV isolates. The second group included five Tunisian isolates: two collected from chickpea (TuC35-06 [GeneBank Accession No. HQ.324137], TuC215-2010 [GeneBank Accession No. HQ199307]), two from pea (TuP163-2010 [GeneBank Accession No. HQ199308] and TuP166-2010 [GeneBank Accession No. HQ199309]), and one from faba bean (TuV 258-2010 [GeneBank Accession No. HQ199310]), and showed a high sequence homology (96%) with the Moroccan (EU541267), Egyptian (EU541269) and Syrian (EU541270) isolates. However, all isolates were clearly distinct (Fig. 1) from other luteoviruses such as BWYV, BLRV and SbDV (less than 70% homology).

## Discussion

The field surveys conducted in northeastern and northwestern regions of Tunisia showed that luteoviruses and FBNYV were the main viruses causing yellowing, chlorosis and reddening on faba bean, chickpea and pea crops. These viruses

have been reported earlier on faba bean and chickpea (Najar *et al.,* 2000), whereas this is the first report of FBNYV affecting chickpea in Tunisia.

Further serological testing of samples that reacted with 5G4 MAb allowed the identification of three luetoviruses (BWYV, BLRV and CpCSV). CpCSV, a proposed new member of the genus *Polerovirus,* was found in this survey to be the prevalent virus causing yellowing and stunting in chickpea fields.

Based on the serological reaction with two CpCSV MAbs (Eth and Sy), most of samples reacted with the CpCSV-Sy MAb and only one faba bean and seven chickpea samples reacted with the CpCSV-Eth MAb. In addition, the comparison of the CP gene nucleotide sequence of Tunisian CpCSV isolates with other CpCSV isolates revealed that two Tunisian isolates (collected from chickpea) clustered with serogroup-I isolates from Sudan and Ethiopia, and five Tunisian isolates (collected from chickpea, faba bean and pea) clustered with serogroup-II isolates from Egypt, Morocco and Syria (Abraham *et al.,* 2009). These results suggested that both CpCSV serogroups are present in Tunisia. In this comparative study, a CpCSV-CP amplicon smaller than that used by Abraham *et*

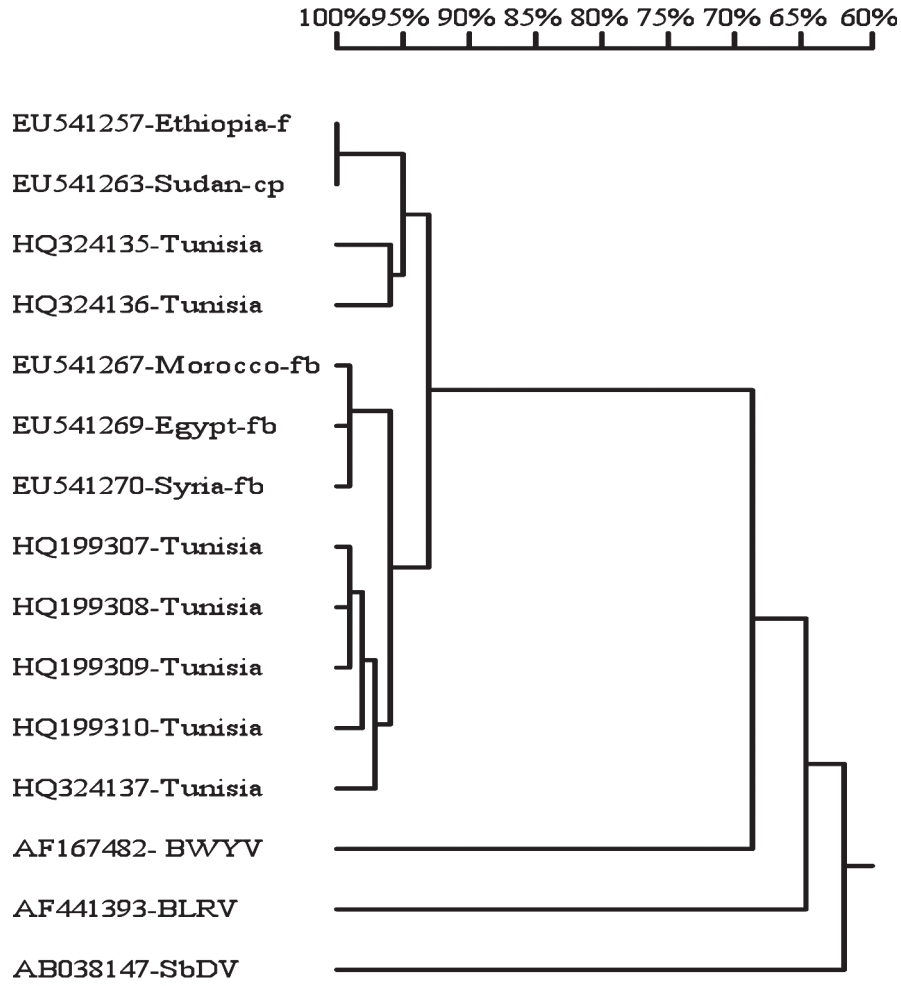


Figure 1. Homology tree deduced from multiple alignments of the CP gene nucleotide sequence of Tunisian, Ethiopian, Sudanese, Egyptian, and Syrian isolates of BLRV, BWYV and CpCSV. The tree was constructed using DNAMAN software with 1000 Bootstrap replicates. Values at nodes indicate significance in bootstrap analysis.

Table 1. Serological test (TBIA) results of legume symptomatic samples collected during April 2006, 2007 and 2010 from different regions of Tunisia.

| Samples collection date | No. of fields surveyed | No. of samples tested | No. of samples which reacted positively with[a] | | | | |
|---|---|---|---|---|---|---|---|
| | | | FBNYV (3-2E9) | 5G4 | BWYV (Agdia, A5977) | BLRV (4B10) | CpCSV (Eth and Sy)[b] |
| Faba bean (*Vicia faba* L.) | | | | | | | |
| April 2006 | 2 | 9 | 1 | 2 | 0 | 0 | 2 |
| April 2007 | 5 | 118 | 26 | 60 | 18 | 6 | 14 |
| April 2010 | 6 | 84 | 3 | 58 | 1 | 0 | 57 |
| Chickpea (*Cicer arietinum* L.) | | | | | | | |
| April 2006 | 7 | 81 | 3 | 49 | 0 | 1 | 48 |
| April 2007 | 20 | 610 | 62 | 354 | 20 | 35 | 299 |
| April 2010 | 10 | 142 | 0 | 66 | 3 | 0 | 62 |
| Pea (*Pisum sativum* L.) | | | | | | | |
| April 2010 | 6 | 55 | 3 | 4 | 0 | 1 | 2 |
| Total | | | | | | | |
| April 2006 | 9 | 90 | 4 | 51 | 0 | 1 | 50 |
| April 2007 | 25 | 728 | 88 | 414 | 38 | 41 | 313 |
| April 2010 | 22 | 281 | 6 | 128 | 4 | 1 | 121 |

[a] All samples were negative to BBWV, AMV, CpCDV, BBMV, SbDV. Virus acronyms used are: FBNYV, *Faba bean necrotic yellows virus*; BWYV, *Beet western yellows virus*; BLRV, *Bean leafroll virus*; CpCSV, *Chickpea chlorotic stunt virus*; BBWV, *Broad bean wilt virus*; AMV, *Alfalfa mosaic virus*; CpCDV, *Chickpea chlorotic dwarf virus*; BBMV, *Broad bean mottle virus*; 5G4, a broad spectrum monoclonal antibody reacting with all legume luteoviruses,

[b] CpCSV-Eth, a mixture of three MAbs (1-1G5, -3H4 and -4B12) produced against an Ethiopian isolate of CpCSV; CpCSV-Sy, a mixture of three MAbs (5-2B8, -3D5, 5B8) produced against a Syrian isolate of CpCSV.

Table 2. Results of TBIA reaction with two CpCSV MAbs of plant samples collected during April 2006, 2007 and 2010 from different regions of Tunisia.

| Samples collection date | No. of samples tested (positive to 5G4) | No. of TBIA positive samples | TBIA reaction with different CpCSV MAbs[a] | |
|---|---|---|---|---|
| | | | CpCSV-Eth | CpCSV -Sy |
| Faba bean | | | | |
| April 2006 | 2 | 2 | -[b] | + |
| April 2007 | 60 | 13 | - | + |
| | | 1 | + | - |
| April 2010 | 58 | 57 | - | + |
| Chickpea | | | | |
| April 2006 | 49 | 47 | - | + |
| | | 1 | + | - |
| April 2007 | 354 | 293 | - | + |
| | | 6 | + | - |
| April 2010 | 66 | 62 | - | + |
| Pea | | | | |
| April 2010 | 4 | 2 | - | + |

[a] CpCSV-Eth, a mixture of three MAbs (1-1G5, -3H4 and -4B12) produced against an Ethiopian isolate of CpCSV; CpCSV-Sy, a mixture of three MAbs (5-2B8, -3D5, 5B8) produced against a Syrian isolate of CpCSV.

[b] +, reacted positively; -, did not react.

*al*. (2009) was employed, It is possible to speculate that using a larger amplicon, more variability could be found. Moreover, 22 faba bean, one chickpea and one pea samples gave a positive reaction with the legume broad spectrum MAb 5G4 but did not react with any of the specific MAbs used. This suggests that further work is needed to clarify the identity of this luteovirus.

CpCSV has been reported to affect many legume crops (e.g. chickpea, faba bean, lentil and pea) as well as some leguminous weeds and four wild non-legume plant species (Abraham *et al*., 2006, 2009; Kumari *et al*., 2008; Assad *et al*., 2009) in several countries. To better understand the epidemiology of CpCSV in Tunisia, more work is needed to identify the host range of the virus, and the efficiency of aphid vectors under Tunisian conditions. In addition, screening chickpea germplasm for resistance to luteoviruses (e.g. BWYV, CpCSV, BLRV) under Tunisian conditions is needed.

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