Characterization of *Tomato chlorotic spot virus* from hydroponic grown lettuce in Brazil

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Abstract: Lettuce cv. Verônica from hydroponic crops showed virus-like symptoms in the regions of Campinas and Sumaré, São Paulo State, Brazil. Affected lettuce samples were used for electron microscope observations, biological, serological and molecular tests. Pleomorphic enveloped particles were always detected in those samples and experimentally inoculated host plants reacted with tospoviruses-induced symptoms, including lettuce. Some differences were observed in *Gomphrena globosa* plants that reacted with local lesions and systemic mosaic. Two isolates of *Tomato chlorotic spot* virus were identified by DAS-ELISA and by RT-PCR. The sequencing and alignment of the RT-PCR amplified fragments have indicated a higher homology degree with the TCSV sequences stored in GenBank.. This is the first report concerning losses due to a *Tospovirus* on commercial hydroponic lettuce crops in Brazil. Further epidemiological studies for better understanding into *Tospovirus* spreading over hydroponic crops are needed, since TSWV is reported to disseminate through the nutritive solution.

Key words: *Tospovirus, Serology, RT-PCR, Sequencing*

Introduction

Lettuce (*Lactuca sativa*) is an Asian vegetable introduced into Brazil through the Portuguese. Nowadays, it is the most appreciated vegetable in the country where its cultivation reaches a high level of technology, comprising green house, hydroponic and organic cultivation (Filgueira, 2000). Hydroponics is an alternative growing method that allows a relatively high number of plants per area at short time intervals. Although a reduction of disease incidence was thought to be expected on hydroponic lettuce, the presence of virus-like diseases has also been noted in several growing regions in São Paulo State. Lettuce diseases constitute a great problem, since they usually affect leaves, and viruses are especially harmful to commercial lettuce.

In Brazil, the first report of *Tospovirus* in lettuce was made by Costa and Forster (1938), and later by Chagas (1970). At that time, this disease was known as *Tomato spotted wilt virus* (Ie, 1970) and sporadically found on lettuce. But important outbreaks started to be reported from 1986 (Moraes *et al*., 1986). Currently, the genus *Tospovirus* in the family *Bunyaviridae* (De Ávila, 1993) is widely spread in Brazil, where the following are reported: *Tomato spotted wilt virus* (TSWV), *Tomato chlorotic spot virus* (TCSV), *Groundnut ringspot virus* (GRSV), *Chrysanthemum stem necrosis virus* (CSNV), *Zucchini lethal chlorotic virus* (ZLCV), and *Iris yellow spot virus* (IYSV) (Resende *et al*., 1996).

The identification of species in the genus *Tospovirus* is made through host range, serology, and according to divergence in aminoacids of coat protein (N Protein) (Pozzer *et al*., 1996). Such species infect plants in 92 botanical families (Peters, 1998), causing significant losses on several vegetable crops. Infection of lettuce occurs most frequently in summer, leading to losses from 30% to 100% (Moraes *et al*., 1986). In São Paulo State, TCSV and GRSV are predominantly transmitted by *Frankliniella schultzei* (Pavan *et al*., 1993). On lettuce under conventional cultivation these diseases were recently described without inducing significant losses (Colariccio *et al*., 2001; Chaves *et al*., 2001). The present work deals with serological identification and molecular characterization of two *Tospovirus* isolates from hydroponic lettuce.

Material and Methods

Virus samples and host. Virus isolates were obtained from hydroponic lettuce cv. Veronica, collected in the municipalities of Campinas and Sumaré, São Paulo State, Brazil. Plants showed dwarfing, virus-like symptoms characterized by mosaic, necrosis, chlorotic and necrotic ringspot on the leaves (Fig. 1).

Fig. 1. Lettuce (*Lactuca sativa*), from hydroponic crop, Campinas, Brazil, showing dwarfing, mosaic, chlorotic and necrotic ringspots and necrosis caused by *Tomato chlorotic spot virus* (TCSV)

Biological tests. Naturally diseased lettuce leaves were ground in cold (ca. 4º C) 0.5% sodium sulphite and the inoculum rubbed on previously carborundum-dusted leaves of the healthy indicator host plants *Chenopodium amaranticolor*, *Lactuca sativa*, *Lycopersicon esculentum*, *Nicotiana glutinosa*, *N. tabacum* ("Samsun NN" "Turkish" and "White Burley") and *Petunia hybrida*.

Electron microscopy. Infected lettuce leaf extracts were negatively stained with 2% uranyl acetate and observed under a Philips EM 208 electron microscope.

Serology. The identification of the *Tospovirus* species infecting lettuce samples was performed by DAS-ELISA (Converse and Martin, 1991), using polyclonal antibodies against coat protein from TSWV, TCSV, GRSV and CSNV. Absorbance (A405 nm) evaluations were made after addition of the substratum (*p*-nitrophenilphosphate), using a *Microplate reader 3550-UV* (Bio-Rad). The results were analysed by the relation between the readings (mean of 3 readings) of the infected samples and the readings of the healthy ones (I/H).

RT-PCR

Extraction of 1µg of viral RNA was performed according to Chomczynski and Sacchi (1987) from 1g of infected lettuce leaves, using primers named BR60 (5´ AGAGCAATSGTGTCA 3´), designed to align with the 3`untranslated region of the S RNA from nucleotide 1 to 15 (complementary sense), and BR65 (5`ATCAAG CCTTCTGAAAGTTCAT 3`), designed to align with the coat protein gene (N), from nucleotide 433 to 453 (viral sense) according to Eiras *et al*. (2001). Samples were then placed in a PTC-100 MJ-Research thermocycler and the reagents of the Taq Polymerase kit (recombinant Gibco BRL) added according to the manufacturer´s instructions. After an initial heating at 94° C for 5 min, the amplification was reached by 30 cycles of 94º C for 1 min followed by 48º C for 1 min and 72° C for 1 min , and by a final heating at 72° C for the extention. The amplified DNA fragments were visualized on a 1% agarose ethydium bromide gel stained in a UV transslluminator (Sambrook *et al*., 1998)

Cloning and sequencing

Amplified products via RT-PCR were purified from the agarose gel by the Concert Rapid Gel Extraction System (Life Technologies) kit, cloned into the pGEM-T vector (Promega) and used for transforming competent *E. coli* cells (DH5-α). Procedures were made according to Sambrook *et al*.(1989) to suppliers directions. Amplified products were sequenced by the terminal chain reaction technique (Sanger *et al*., 1977), using the automatic ABI 377 sequencer and the ABI Prism Big Dye Terminator Cycle

Sequencing Ready Reaction kit – Ampli Taq DNA Polymerase, FS (Perkin Elmer) according to the manufacturer´s instructions. The alignment of the obtained sequences was performed with the Sequencer 3.1 program (Gene Codes Corporation) and comparisons with sequences from GenBank were performed through the BLAST program of the National Center for Biotechnology Information – NCBI.

Results and Discussion

Inoculated indicator host plants such as *P. hybrida* and *C. amaranticolor* reacted with local necrotic rings. Systemic mosaic, crinkle of younger leaves and necrosis were noted on *L. esculentum*, *N. debneyi*, *N. glutinosa* and *N. tabacum* while mosaic, necrosis and stunting occurred on lettuce (Table 1).

Electron microscope observations showed consistent presence, in extracts of infected lettuce leaves, of enveloped, rounded and pleomorphic particles, 80-100 nm in diameter, comparable to *Tospovirus* particles.

By means of DAS-ELISA, using the antisera to the main tospoviruses occurring in Brazil, TCSV was identified in the lettuce samples. Tests were considered positive when absorbance values from infected plant extracts were threefold higher than those from healthy plant ones, corresponding to 0.850 and 0.960 for samples from Campinas and Sumaré, respectively. No reaction was obtained with the remaining antisera.

It is worth mentioning that TCSV has been serological detected in dual infection with GRSV in hydroponic lettuce in the municipalities of Amparo and Itatiba, São Paulo State (Colariccio *et al*., 1998). Recent surveys indicated that TCSV has also been prevalent in different vegetable growing areas in São Paulo State and that GRSV predominates on lettuce in the sub-middle São Francisco Valley river, Pernambuco State (De Ávila *et al*., 1996). Both TCSV and GRSV, which are efficiently transmitted by the thrips species *F. occidentalis* and *F.schultzei* (Wijkamp *et al*., 1995; Borbon and Garcia, 1996), are prevalent in tropical and subtropical

Host plants	Symptoms on host plants			
	TCSV – Campinas Isolate		TCSV – Sumaré Isolate	
	LOCAL	SISTEMIC	LOCAL	SISTEMIC
Gomphrena globosa	NL	WM	NL	WM
Impatiens sp	NR	VC, NR, LD	NR	VC, NR, LD
Chenopodium quinoa	NP	-	NP	-
C. amaranticolor	NP	-	NP	-
Emilia sonchifolia	NL	M, W	NL	M, W
Cucurbita pepo	-	-	-	-
Datura stramonium	NR	VN, LD, SN	NR	VN, LD, SN
Lycopersicon esculentum	NR	M, B, SN	NR	M, B, SN
Nicotiana glutinosa	NL	M, LD	NL	M, LD
N. tabacum cv. White burley	NR	LP, M, LD	NR	LP, M, LD
Petunia hybrida	NL	-	NL	-

Bronzing: B; Leaf deformation: LD; Line Patterns: LP; Mosaic: M; Necrotic lesions: NL; Necrotic points: NP; Stem necrosis: SN; Vein clearing: VC; Vein necrosis: VN; Wilting: W; Whiten mosaic: WM

Table 1 – Symptoms induced on several hosts by Tomato chlorotic spot virus isolates from hydroponic lettuce

regions (Wijkamp *et al*., 1995) and are reported only from Brazil, South Africa (De Ávila *et al*., 1993) and Argentina (Dewey *et al*., 1996).

Through RT-PCR, using the oligonucleotides BR60 and BR65, DNA fragments with 442 bp were amplified (Fig. 2). No amplified product was obtained from healthy plants. The two oligonucleotides align with S RNA in the coat protein gene (N gene) and permit amplification of at least 5 different *Tospovirus* species, ie TSWV, TCSV, GRSV, INSV and CSNV (Eiras *et al.,* 2001). The sequencing and alignment of the RT-PCR amplified fragments indicated a higher degree of homology with the sequences of TCSV stored in GenBank (Fig. 3; Table 2).

Molecular studies in Brazil have identified and characterized different *Tospovirus* species (Bezerra *et al.*, 1999; Pozzer *et al*., 1999), and these species constitute a limiting factor on vegetable crop production, mainly on *Asteraceae* and *Solanaceae* (Colariccio *et al*., 2001; Lima *et al*., 2000). In the present work 2 TCSV isolates were characterized from hydroponic lettuce in Campinas and Sumaré regions where the field culture covers areas from 25 to 200 ha and from 5 to 25 ha, respectively (www.cati.sp.gov.br). TCSV is the main *Tospovirus* in São Paulo State where it is harmful to different crops especially to vegetables (Nagata *et al*., 1995; Colariccio *et al*., 2001).

The identification and characterization of TCSV on hydroponic growing lettuce may be important to future breeding programs. However, data concerning the behaviour of different lettuce cultivars towards *Tospovirus* species are not consistent so far. The breeding of cultivars with a good resistance level to TCSV is the best control strategy, since thrips control is not efficient. Originally grown in Brazil during autumn and winter, lettuce had its cycle extended by breeders who developed cultivars for spring and summer (Filgueira, 2000). Consequently, lettuce crops remain exposed the whole year to vectors and *Tospovirus* sources. Thus, eradication of weed hosts close to hydroponic lettuce associated with other cultural practices should be undertaken to prevent attack by *Tospovirus*. Further epidemiological studies for better understanding of *Tospovirus* spread into hydroponic crops are needed, since TSWV has been reported to disseminate through the nutritive solution (Paludan, 1985).

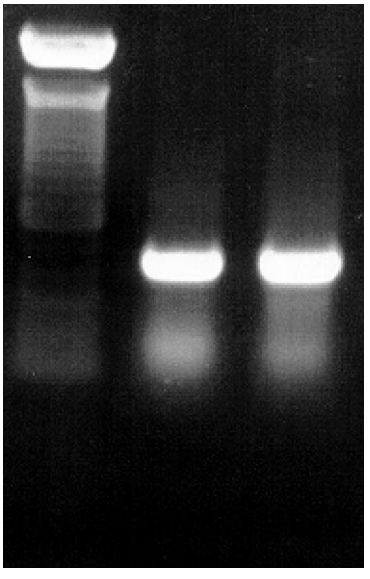

Fig. 2. Results of RT-PCR for total RNAs samples, amplified with primers BR60 and BR65. Lane 1 correspond to 100 bp DNA ladder (Gibco BRL), lanes 2 and 3 correspond to Campinas and Sumaré TCSV isolates, respectively.

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AGAGCAATTG TGTCAATTTT ATTCAAAAAC ATACTACTCA GCAACACAAA          50
TCATCACATT GCCAGGATAA GTAACGACTG CGGTCTACAG AG(t/c)CGTACTT     100
TCTTACCTTG AATCACATCT CTCGAGAGCG GTCTAGATCT ACACTGCCAA A       151

ATG TCT AAG GTC AAG CTC ACC AGA GAG AAC ATT ATC TCT CTT CTA    196
 M   S   K   V   K   L   T   R   E   N   I   I   S   L   L
ACT CAG GCT GGA GAA ATC GAG TTT GAA GAA GAT CAA AAT CAA GCT    241
 T   Q   A   G   E   I   E   F   E   E   D   Q   N   Q   A
GCA TTC AAC TTC AAG ACT TTT TGC GGA GAA AAT CTT GAT TCA ATC    286
 A   F   N   F   K   T   F   C   G   E   N   L   D   S   I
AAG AAA ATG AGC ATT ACC TCA TGT TTG ACT TTC CTG AAA AAT CGC    331
 K   K   M   S   I   T   S   C   L   T   F   L   K   N   R
CAG AGC ATC ATG AAA GTT GTG AAC CAA AGT GAT TTT ACC TTT GGG    376
 Q   S   I   M   K   V   V   N   Q   S   D   F   T   F   G
AAA ATC AC(a/g) ATC AAA AAG AAT TCT GGA AGG GTT GGA GCT AAT    418
 K   I   T       I   K   K   N   S   G   R   V   G   A   N
GAT ATG ACT TTC AGA AGG CTT GAT                               442
 D   M   T   F   R   R   L   D
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Fig. 3. Nucleotides (above) and deduced aminoacids (below) of the TCSV hydroponic lettuce isolates, from Campinas and Sumaré, amplified by RT-PCR. The primers BR60 and BR65 are underlined. The start codon (ATG) of the coat protein gene is shown in bold at the position 152. The untranslated region is indicated in italic. Differences on nucleotides between the two lettuce isolates, Campinas and Sumaré, are indicated, respectively, in parenthesis. No aminoacids changes were observed between the two isolates. The nucleotides are numbered from the 5' end viral strand.

	TCSV-C[1]	TCSV-S[2]	TCSV-J[3]	TCSV[4]	TCSV[5]	GRSV[6]	TSWV[7]	ZLCV[8]	CSNV
TCSV-C[1]	_	99	99	97	99	84	80	85	84
TCSV-S[2]	100	_	99	98	99	84	80	85	83
TCSV-J[3]	99	99	-	99	96	81	78	74	73
TCSV[4]	98	98	98	-	96	81	79	74	74
TCSV[5]	95	95	95	96	-	82	78	74	74
GRSV[6]	84	84	84	83	87	-	78	75	74
TSWV[7]	78	78	78	77	80	79	-	74	76
ZLCV[8]	73	73	72	72	75	76	74	-	77
CSNV[9]	74	74	74	73	75	74	77	80	-

1. TCSV Campinas isolate (hydroponic); **2.** TCSV Sumaré isolate (hydroponic); **3.** TCSV Solanum gilo isolate (AF413110); **4.** TCSV isolate BR03 (S54325); **5.** TCSV (AF282982, AAG23654.1); **6.** *Groundnut ringspot virus* - GRSV (AF25271, AAF64317.1); **7.** *Tomato spotted wilt virus* - TSWV (AB038341, BAB18308.1); **8.** *Zucchini lethal chlorosis virus* - ZLCV (AF067069, AAF04198.1); **9.** *Chrysanthemum stem necrosis virus* - CSNV (AF067068, AAF04197.1). *The *GenBank* accession numbers are indicated in parenthesis.

Table 2 - Comparison (homology in percentage) among nucleotides sequence (above the diagonal) and translated aminoacids (below the diagonal) of the *Tomato chlorotic spot virus* (TCSV) lettuce isolates coat protein with other *Tospovirus* sequences of the *Genbank**.

Besides cultural practices, it is of pivotal importance that new lettuce lines be introduced into breeding programs targeting *Tospovirus* resistance, since break of resistance to this virus species has been observed. Losses due to Tospovirus have been reported to reach 100% on field lettuce crops (Moraes *et al*., 1986), but there is no information so far on losses affecting hydroponic lettuce.

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