

# Increase of Tospoviral Diversity in Brazil with the Identification of Two New Tospovirus Species, One from Chrysanthemum and One from Zucchini

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## ABSTRACT

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During a survey conducted in several different regions of Brazil, two unique tospoviruses were isolated and characterized, one from chrysanthemum and the other from zucchini. The chrysanthemum virus displayed a broad host range, whereas the virus from zucchini was restricted mainly to the family *Cucurbitaceae*. Double-antibody sandwich-enzyme-linked immunosorbent assay and western immunoblot analyses demonstrated that both viruses were serologically distinct from all reported tospovirus species including the recently proposed peanut yellow spot virus and iris yellow spot virus (IYSV) species. The nucleotide sequences of the nucleocapsid

(N) genes of both viruses contain 780 nucleotides encoding for deduced proteins of 260 amino acids. The N proteins of these two viruses displayed amino acid sequence similarities with the previously described tospovirus species ranging from 20 to 75%, but they were more closely related to each other (80%). Based on the biological and molecular features, these viruses are proposed as two new tospovirus species, designated chrysanthemum stem necrosis virus (CSNV) and zucchini lethal chlorosis virus (ZLCV). With the identification of CSNV and ZLCV, in addition to tomato spotted wilt virus, groundnut ring spot virus, tomato chlorotic spot virus, and IYSV, Brazil harbors the broadest spectrum of tospovirus species reported.

*Additional keywords:* *Bunyaviridae*, multiple sequence alignments, phylogeny tree, tospovirus epidemiology.

Diseases caused by tospoviruses continue to cause significant crop losses worldwide (11). As a result of the economic interest, the number of new tospovirus species identified, in addition to the type species tomato spotted wilt virus (TSWV) of the genus *Tospovirus*, has drastically increased during the last decade.

Although the presence of TSWV was initially reported in the 1940s on various crops in Brazil, three additional species, tomato chlorotic spot virus (TCSV) (8), groundnut ring spot virus (GRSV) (8), and iris yellow spot virus (IYSV) (21), were identified in Brazil only during the last decade. More recently, two unique tospovirus isolates were reported, one from chrysanthemum and another from zucchini plants (10,19,22).

In Brazil, chrysanthemum is largely cultivated in greenhouses under controlled light and water conditions. During 1995, growers in Atibaia County (São Paulo State, Brazil) produced 1.43 million chrysanthemum bundles and 2.943 million pot plants. In 1994, a disease with symptoms typical of a virus infection was found in commercial fields of chrysanthemum in Atibaia County. Symptoms consisted of necrotic lesions surrounded by yellow spots on leaves, followed by necrosis on stems, peduncles, and floral receptacles. The agent was found to be a distinct tospovirus (10,17). Since the first outbreak of the disease, this virus has been detected in different areas in Brazil, also infecting tomato (18).

A new tospovirus was also observed in experimental fields of zucchini (*Cucubita pepo* L. cv. Caserta) in São Paulo State that was causing a severe disease in infected plants (25). The occurrence of a systemic infection in the family *Cucurbitaceae* by a tospovirus in Brazil had previously been reported (14). However, at that time, the virus did not cause significant damage to the crops and was regarded as of minor importance. In addition, field misidentification of this virus as watermelon mosaic virus and the lack of available suitable antiserum masked the occurrence of this virus disease for a long time. Currently, large areas cultivated with zucchini are found infected with this virus, with plants showing symptoms of stunting and high yield losses of marketable fruits. During the last 2 years, this virus has spread from São Paulo State into the central areas of Brazil, thereby increasing in economic importance (20).

In this report, we present findings based on the biology, serology, and nucleocapsid (N) protein gene sequences of the two viruses. We propose that they represent new tospovirus species, designated chrysanthemum stem necrosis virus (CSNV) and zucchini lethal chlorosis virus (ZLCV). The identification of these two new tospovirus species in addition to the currently recognized species demonstrate the large distribution and diversity of the genus *Tospovirus* within this geographical area.

## MATERIALS AND METHODS

**Tospovirus isolates and host range.** From a previous survey conducted in Brazil (19,25), two serologically distinct tospovirus isolates from chrysanthemum (Chry1) and zucchini (BR-09) were selected. The original isolates were maintained in a greenhouse on *Nicotiana benthamiana*, *N. rustica*, or *Datura stramonium* through mechanical inoculation using 0.01 M phosphate buffer, pH 7.0, containing 0.1% sodium sulfite.

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The sequences presented in this paper are accessible in GenBank under AF-067068 for the CSNV N gene and AF-067069 for the ZLCV N gene.

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The host range of isolates Chry1 and BR-09 was determined by mechanical inoculation onto several plant species using extracts from infected *N. benthamiana* in the same phosphate inoculation buffer. The symptoms were monitored weekly, and plants were checked for the virus infection by double-antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) using polyclonal antiserum directed against the N protein.

**Thrips transmission.** The preliminary transmission test was done using three thrips species, *Frankliniella occidentalis*, *F. schultzei*, and *Thrips tabaci*. The first instar larvae (8 h old) were placed on CSNV-infected *D. stramonium* or ZLCV-infected cucumber plants for acquisition. After the 16-h acquisition period, the larvae were transferred to healthy plants and reared until the emergence of adults. Thrips were then inoculated to the leaf disks of petunia or cucumber. The transmissions were confirmed by appearance of local lesions or by ELISA using polyclonal antibodies of each virus.

**Antiserum production and serological studies.** Antiserum was prepared against the N protein from purified nucleocapsid preparations (8). Antisera raised in rabbits were further purified by ammonium sulfate precipitation, eluted over a DEAE-Sephacel column (Sigma Chemical Co., St. Louis) and conjugated to alkaline phosphatase (1 mg/ml). Isolates Chry1 and BR-09 were compared in DAS-ELISA and dot blot (2,7) with other tospovirus species, TSWV, TCSV, GRSV, Impatiens necrotic spot virus (INSV), watermelon silver mottle virus (WSMV), and the recently reported IYSV from Brazil (IYSV<sub>BR</sub>) (21), using polyclonal antibodies against N proteins directed to the respective viruses. Purified nucleocapsid protein extracts (100 ng) were used as antigen sources, and extracts of healthy plants were included as a control. The concentration of the N protein was determined by a BioRad protein assay (BioRad Laboratories, Hercules, CA) according to the manufacturer's procedures.

**Isolation of nucleocapsid RNA.** Nucleocapsids were purified as described by De Ávila et al. (5), with minor modifications. Pellets obtained after centrifugation over a 30% sucrose cushion were resuspended in 400 µl of RNA extraction buffer (0.2 M NaAc and 10 mM EDTA; pH 6.0) overnight at 4°C. Viral RNA was extracted from nucleocapsid preparations after adding sodium dodecyl sulfate (SDS) to a final concentration of 1% (wt/vol), followed by phenol and phenol/chloroform (1:1 vol/vol) extractions. RNA was precipitated for 30 min at -70°C after the addition of 1/10 (vol/vol) of 3 M NH<sub>4</sub>Ac and 2.5 (vol/vol) of 96% ethanol, pelleted, and subsequently washed with 70% ethanol. The RNA was dissolved in diethylpyrocarbonate-treated water and analyzed by electrophoresis on a 1.2% agarose gel.

**Polymerase chain reaction (PCR) amplification and cloning.** For both isolates, the 3'-terminal regions of the small (S) RNA comprising the N protein genes and the noncoding regions were amplified in two steps using reverse transcription PCR (RT-PCR) (24). In the first step, the primers BR-060 (5' CATGGATCCTG-

CAGAGCAATTGTGTCA 3'), complementary to the first 15 nucleotides of the 3' end of the S RNA containing a *Bam*HI site (underlined), and BR-066 (5' CATGGATCCTGTAATGTTCCATAGCA 3'), which is identical to a conserved tospoviral S RNA sequence at nucleotide position 2,058 to 2,974 of the TSWV S RNA strand, were used for RT-PCR analyses on nucleocapsid RNA. Amplification resulted in a fragment of 850 to 900 base pairs (bp) for CSNV and ZLCV, respectively. Based on the sequence of this fragment obtained from both species, a new primer, CZ1 (5' CCCGGATCCG-

TABLE 1. Experimental host range as determined for chrysanthemum stem necrosis virus (CSNV) and zucchini lethal chlorosis virus (ZLCV)

Host	CSNV		ZLCV	
	Local	Systemic	Local	Systemic
<i>Amaranthaceae</i>				
<i>Gomphrena globosa</i>	CS, NS <sup>a</sup>	...	CL	...
<i>Balsaminaceae</i>				
<i>Impatiens</i> spp.	NS	...	...	...
<i>Chenopodiaceae</i>				
<i>Beta vulgaris</i>	NS	...	...	...
<i>Chenopodium quinoa</i>	NS	...	...	...
<i>Chenopodium amaranticolor</i>	NS	...	...	...
<i>Compositae</i>				
<i>Helianthus annuus</i>	...	...	...	...
<i>Zinnia elegans</i>	CL	Mo	...	...
<i>Cucurbitaceae</i>				
<i>Citrullus lanatus</i>	CL	...	...	VC, M, LD, PD
<i>Cucumis sativus</i>	CL	...	...	VC, M
<i>Cucurbita melo</i>	CL	...	...	M
<i>Cucurbita pepo</i>	CL	M	...	M, LD, PD, GR
<i>Convolvulaceae</i>				
<i>Ipomoea setosa</i>	...	...	...	...
<i>Leguminosae</i>				
<i>Phaseolus vulgaris</i>	CL	VC	...	...
<i>Pisum sativum</i>	NR	Mo, W	...	...
<i>Vigna unguiculata</i>	CL	Mo, LD	...	...
<i>Portulacaceae</i>				
<i>Portulaca oleracea</i>	...	...	...	...
<i>Solanaceae</i>				
<i>Datura stramonium</i>	NS, NR	VC, M, E	...	...
<i>Lycopersicon esculentum</i>	NS, NR	CS, VN	...	...
<i>Nicotiana benthamiana</i>	CS	VC, M, LD	CL	M, GR
<i>Nicotiana glutinosa</i>	NS	...	...	...
<i>Nicotiana occidentalis</i>	CS	VC, M	CL	...
<i>Nicotiana rustica</i>	CS	VC, M	...	...
<i>Nicotiana tabacum</i> TNN	NR	VC	...	...
<i>Nicandra physalodes</i>	CS	VC, M	...	...
<i>Physalis occidentalis</i>	NS	M, TN	...	...
<i>Capsicum chinense</i> PI 159236	NS	...	...	...
<i>Petunia hybrida</i>	NS	...	...	...

<sup>a</sup> CL = chlorotic lesion, CS = chlorotic spot, E = epinasty, GR = growth reduction, LD = leaf deformation, M = mosaic, Mo = mottling, NR = necrotic rings, NS = necrotic spots, PD = plant death, TN = top necrosis, VC = vein clearing, VN = vein necrosis, W = wilting, and ... = no symptoms.



Fig. 1. Symptoms on A, chrysanthemum infected with chrysanthemum stem necrosis virus (CSNV) and B, zucchini infected with zucchini lethal chlorosis virus (ZLCV).

GGAAAGTTTGCAGTGT 3'), identical to a highly conserved region (nucleotide position 723 to 740 of the CSNV N gene) present in the sequence of both isolates, was designed. In the second step, a 500-bp fragment was amplified using primers CZ1 and UHP (supplied by I. Cortés), the latter having the potential to anneal to the intergenic regions of both medium (M) and S RNA of any tospovirus. First-strand cDNA synthesis was carried out using avian myeloblastosis virus-reverse transcriptase (Pharmacia Biotechnology Inc., Uppsala, Sweden). Amplification conditions were selected (24). Amplified PCR fragments were cloned in the pGEM-T vector (Promega Corp., Madison, WI) or digested with *Bam*HI, gel-purified, and subsequently cloned into the *Bam*HI site of pBS/KS<sup>+</sup> (Stratagene Inc., La Jolla, CA). Recombinant clones from at least three independent RT-PCR reactions were sequenced by the chain termination method (26) in an automatic ABI PRISM 377 DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Sequences were compiled and analyzed using algorithms of the Genetic Computer Group (GCG) package from the University of Wisconsin (9). GenBank searches were performed using BLAST (1). PileUp data from GCG were further analyzed by using the PAUP 3.1.1. package (Illinois Natural History Survey, Champaign, IL). A phylogenetic tree was constructed based on 100 replicates using midpoint rooting.

## RESULTS

**Host range and symptoms.** The isolate Chry1, designated CSNV in this study, caused typical tospovirus symptoms in several hosts tested, inducing necrotic chlorotic local lesions and concentric rings on the inoculated leaves, followed by vein necrosis, mosaic or mottling, and leaf deformation on full-grown leaves. Mechanical inoculation of CSNV on the original host reproduced identical symptoms to the naturally infected chrysanthemum field (Fig. 1A). Severe symptoms on the inoculated hosts were observed within 2 weeks postinfection, eventually leading to the death of most plants. The experimental host range of CSNV was broad, infecting 19 different hosts in seven botanical families (Table 1). In

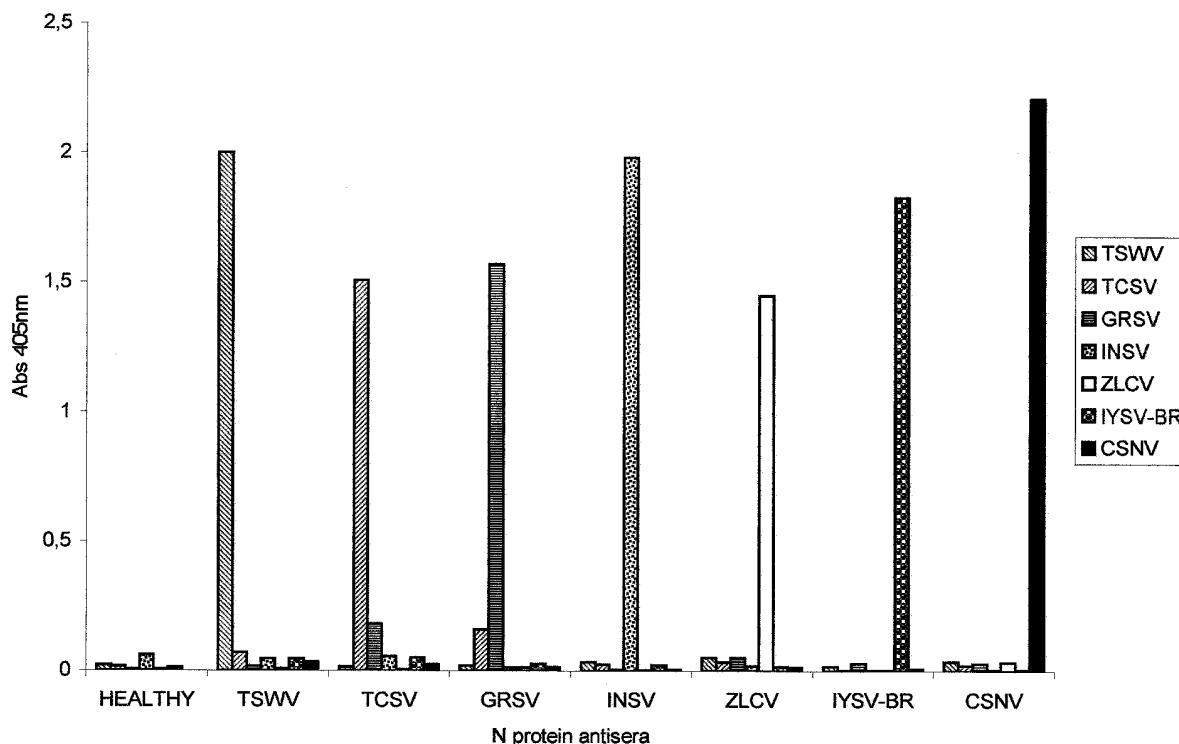
most cases, the symptoms observed were similar to those caused by TSWV.

In contrast, isolate BR-09, designated ZLCV in this study, displayed a more restricted experimental host range. ZLCV induced systemic chlorotic symptoms, necrosis of basal leaves, malformation (narrow and curled leaf blades), and reduction in plant growth. This isolate induced only local lesions on *N. occidentalis*, but systemically infected *Gomphrena globosa*, *N. benthamiana*, and several other cucurbitaceae species (Table 1). Zucchini plants infected with this virus showed severe mosaic, leaf distortion, stunting (Fig. 1B), and often death of the plant, as originally observed under field conditions.

**Thrips transmission.** Many local lesions were observed when CSNV was inoculated by *F. occidentalis* and *F. schultzei*, but not by *T. tabaci*. The transmission of CSNV was confirmed by two *Frankliniella* species. The transmission of ZLCV was evaluated by ELISA; however, no transmission was found by any of the three thrips tested. Further studies on identification of vector species of ZLCV are ongoing.

**Serological comparison of CSNV and ZLCV to other tospoviruses.** The serological relationship of CSNV and ZLCV was evaluated by DAS-ELISA and dot blot using polyclonal antibodies directed against the N proteins of TSWV, TCSV, GRSV, INSV, IYSV, and WSMV. No positive reaction was observed for CSNV and ZLCV with antibodies of other tospoviruses applied (Fig. 2), nor did they cross-react with each other. The results strongly support CSNV and ZLCV as representatives of serologically distinct tospovirus species. Western immunoblot analysis confirmed the results obtained by ELISA, although a slight cross-reaction of CSNV with antibodies against TSWV, TCSV, and GRSV was observed (data not shown).

**Molecular cloning, sequence determination, and analysis of CSNV and ZLCV N protein genes.** Analysis of the genomic RNAs of CSNV and ZLCV revealed the presence of three RNA segments with sizes corresponding to those of RNAs of TSWV (data not shown). Using primer BR-060 in combination with BR-066 and primer CZ1 in combination with UHP, two overlapping



**Fig. 2.** Serological reactions among six tospoviruses showing amount of relatedness using polyclonal antisera against their respective nucleocapsid (N) proteins and infected plant extracts as an antigen source.

fragments comprising the 5' and 3' ends of the N gene including a part of the intergenic region of the S RNA were successfully amplified for CSNV and ZLCV by RT-PCR. After verifying the S RNA-specificity on northern blot hybridization containing viral RNA, the amplified fragments were purified, cloned into pGEM-T or pBS/KS<sup>+</sup>, and sequenced. The open reading frame (ORF) representing the N gene of CSNV contained 780 nucleotides. It started with an AUG codon at nucleotide position 153 (numbered from the 5' terminal end) and terminated with a UAA stop codon at position 933 (Fig. 3), thereby potentially coding for a protein of 260 amino acid residues with a predicted  $M_r$  of about 29 kDa. The ORF in the ZLCV N gene also encoded a deduced protein of 260 amino acids and a predicted  $M_r$  of 29 kDa, starting from nucleotide

position 242 and terminating at nucleotide position 1,022 (Fig. 4). The 3' untranslated region (UTR) of the S RNA of ZLCV was revealed to be much longer (220 bases) than that of all the other tospovirus species (approximately 130 bp). This was not a cloning artifact, since several independent RT-PCR amplifications using different primers combinations and subsequent sequence analysis of the fragments obtained always revealed the same longer 3' UTR sequence (data not shown). For both ZLCV and CSNV, the predicted gene product corresponded in size to the N protein determined from SDS polyacrylamide gels (data not shown). A search in the EMBL protein database revealed sequence similarity of the deduced amino acid sequences of CSNV and ZLCV clones to the N proteins of reported tospovirus species, thereby confirming the

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1      AGAGCAATTGTGTCAATTTTATTCAAAAACCTCAGCACTCAGTAAGATATATATCACATTA
61     GGACAATCAAAGAGCGACTGCGGAATACTCTGCACGACTTGTAACCCTAAACCAAATTC
121    ATTGAAAGCAAACCTAAACTCTATTTAATCATC  ATG TCT AAA GTT AAG CTT ACA
                                           M  S  K  V  K  L  T
174    AAG GAA AAC ATT GTT GCT TTG CTG ACA CAA GCT GGT GAA GTT GAA TTT
      K  E  N  I  V  A  L  L  T  Q  A  G  E  V  E  F
222    GAG GAA GAA CAG AAC CAA ATT GCA TTC AAC TTC CAA AGT TTC TGC AAT
      E  E  E  Q  N  Q  I  A  F  N  F  Q  S  F  C  N
270    GAC AAC CTG GAT CAG ATC AAA AAC ATG AAC TTA ATA TCA TGT TTG ACA
      D  N  L  D  Q  I  K  N  M  N  L  I  S  C  L  T
318    TTT CTT AAG AAT CGT CAA AGC ATA ATG AAA GTT GTA AAA CAA AGT GAT
      F  L  K  N  R  Q  S  I  M  K  V  V  K  Q  S  D
366    TTC ACT TTT GGC AAA ATC ACT ACC AAA AAG AAT TCT GAT AGG ATT GGA
      F  T  F  G  K  I  T  T  K  K  N  S  D  R  I  G
414    CCA AAT GAC ATG ACT TTC AGG AGA TTG GAT AGC TTG ATA AGA GTC AAG
      P  N  D  M  T  F  R  R  L  D  S  L  I  R  V  K
462    CTT ATT GGA AGA ACT AAG AGT GAT GAA GAT CTA AAC ACT ATC AAA TCA
      L  I  G  R  T  K  S  D  E  D  L  N  T  I  K  S
510    AAG ATT GCT TCT CAC CCT CTA GTT CAG GCA TAC GGG CTT AGC TTG AAT
      K  I  A  S  H  P  L  V  Q  A  Y  G  L  S  L  N
558    GAT GCA AAG TCT GTT AGA TTG GCT ATA ATG CTT GGA GGT AGT CTC CCT
      D  A  K  S  V  R  L  A  I  M  L  G  G  S  L  P
606    CTC ATT GCT TCA GTT GAG AGC TTC GAA ATG ATT AGC GTT GTA CTA GCT
      L  I  A  S  V  E  S  F  E  M  I  S  V  V  L  A
654    ATC TAC CAG GAT TCA AAG CAC AAA GAG CTT GGA ATA GAT GAA AAG AAA
      I  Y  Q  D  S  K  H  K  E  L  G  I  D  E  K  K
702    TAT GAT ACT AAA GAA GCT TTA GGG AAA GTT TGC ACT GTT TTA AAG AGC
      Y  D  T  K  E  A  L  G  K  V  C  T  V  L  K  S
750    AAA GGA TTT GCT ATT GAC GAA CAA CAG ATG GAA AAA GGA AAA GAA TAT
      K  G  F  A  I  D  E  Q  Q  M  E  K  G  K  E  Y
798    GCA AAT ATT CTC AAG GCT TGT GAC CCA AGA ATG AAA GGA GCC ATT GCT
      A  N  I  L  K  A  C  D  P  R  M  K  G  A  I  A
846    ATG GAA CAT TAC AGT GAT TCT CTC AAC AAG TTC TAT GAG ATG TTT GGA
      M  E  H  Y  S  D  S  L  N  K  F  Y  E  M  F  G
894    GTT CAA AAG GGA TCT AAA CTT ATT CCT AAA GAT CTT GTT TAA AGCAAAC
      V  Q  K  G  S  K  L  I  P  K  D  L  V  *
943    TATTTAAAATAGCTTATATATATATAAGTCTAAAGTTTGTAAAGGTGTGTTAAAGATTTA
1003   AAGTGTGTCTAAAGTGTGTAAAGTAATATTTATGTTTGTATTAGTTAAA

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**Fig. 3.** Complete nucleotide sequence of the chrysanthemum stem necrosis virus (CSNV) nucleocapsid (N) gene. The deduced amino acid sequence is shown below the nucleotide sequence. The ATG start codon is in bold and underlined, and the asterisk indicates the stop codon.

identity as the N protein. Amino acid sequence identity of the CSNV N protein to those of other *Tospovirus* species varied between 25 to 75%, whereas for ZLCV N protein ranged between 20 to 75%. For both viruses, the highest similarity was observed with TSWV, TCSV, and GRSV and the lowest with groundnut bud necrosis virus (GBNV), WSMV, watermelon bud necrosis virus (WBNV), peanut yellow spot virus (PYSV), and IYSV (Table 2). Comparison of the ZLCV N protein against that of CSNV showed 80% identity.

## DISCUSSION

Eight tospovirus species have been established to date based on serology, host range, and sequence data of the nucleocapsid protein gene (3,6,12,13,15,23,27,28,30). In this study, two new tospovirus species, CSNV and ZLCV, are proposed. The serological comparison clearly showed that these two isolates were distinct from other tospoviruses. Cloning and subsequent sequence analysis of the N protein gene indeed revealed that, based on the amino acid

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1      AGAGCAATTGTGTCAATTTTACTCAAAAACCAAACTCAGCCAGATATTTCTCCACATA
61     TTGCAC TTGCGAAGCGACTGCAAAGCCCTTAACACAGCTTGTTACACCCTAAACCAAGCTC
121    GATACAACCATACTGCATCTCTACAGTTGCTACGATTTTTTACAGTTACTATAGTGATTAC
181    ATCTGTTTTTAAATATACCATATCTTTCCAGTTGTTACATTCTCTACAGTTCTTAAATCAA
241    T   ATG TCA AAA GTC AAG CTT ACG AAG GAG AAC ATT GTT GCT TTG CTG
        M   S   K   V   K   L   T   K   E   N   I   V   A   L   L
287    ACT CAA GCA ACA GAA GTG GAA TTT GAA GAA GAA CAA AAT CAA ACT GCT
        T   Q   A   T   E   V   E   F   E   E   E   Q   N   Q   T   A
335    TTC AAC TTT AAA ACT TCC TAT GAG GGA AAT CTC AAG CTG ATC AAG AAC
        F   N   F   K   T   S   Y   E   G   N   L   K   L   I   K   N
383    ATG AGC ATC ACA TCG TGT TTG ACG TTC CTG AAA AAC CGC CAG AGC ATT
        M   S   I   T   S   C   L   T   F   L   K   N   R   Q   S   I
431    ATG AAG GTT GTC AAA CAA AGT GAT TTT AAT TTT GGC AAA GTC ACT ATT
        M   K   V   V   K   Q   S   D   F   N   F   G   K   V   T   I
479    AAA AAA GTT TCT GAC AAA ATT GGA CCT AAT GAT ATG AcT TTT AGG AGA
        K   K   V   S   D   K   I   G   P   N   D   M   T   F   R   R
527    CTG GAC AGC ATG ATA AGA GTG AAG CCG ATT GAA GCC ACT GCA AAT GAT
        L   D   S   M   I   R   V   K   P   I   E   A   T   A   N   D
575    GAA AAC TTA TCT GCT ATT AGA TCA AAA ATT GCT TCA CAT CCT CTG GTT
        E   N   L   S   A   I   R   S   K   I   A   S   H   P   L   V
623    CAG GCT TAT GGG TTG AGT TTA ACC AAT GCC AAA TCA GTT CGC CTT GCA
        Q   A   Y   G   L   S   L   T   N   A   K   S   V   R   L   A
671    ATC ATG TTA GGA GGT AGC ATA CCT CTT ATT GCT TCT GTT GAC AGT TTT
        I   M   L   G   G   S   I   P   L   I   A   S   V   D   S   F
719    GAG ATG ATC AGT GTT GTT TTA GCT ATA TAT CAA GAT TCA AAA CAT AAA
        E   M   I   S   V   V   L   A   I   Y   Q   D   S   K   H   K
767    GAG CTA GGA ATT GAC CTG AAG AAA TAT GAT ACT ACT GAA GCC CTT GGG
        E   L   G   I   D   L   K   K   Y   D   T   T   E   A   L   G
815    AAA GTT TGC TCT GTT CTC AGA AGC AAA GGC TTT GAC ATT GAT GAT GCT
        K   V   C   S   V   L   R   S   K   G   F   D   I   D   D   A
863    CAG ATG GAA AAA GGA AAG GAA TAT GCA AAT ATC. CTC AAA GCA TGT GAT
        Q   M   E   K   G   K   E   Y   A   N   I   L   K   A   C   D
911    CCA AGG TTG AAA GGA AGT GTT GCT ATG GAA CAT TAC AGT GAA ACT CTC
        P   R   L   K   G   S   V   A   M   E   H   Y   S   E   T   L
959    AAC AAG TTC TAC AAC ATG TTC GGA GTC AAG AAG GAG GAG AAA CAT GTT
        N   K   F   Y   N   M   F   G   V   K   K   E   E   K   H   V
1007   CCT AAA GGT GTT GCA TAA AAAC TTTTAAATGTCTTTATAAGTAATCCAAGCTA
        P   K   G   V   A   *
1060   TGTGTGTTGTGCTAAATCAAAAGAAATAAAAACAAAAATATGTGTGTTGTATATCTATGT
1121   AAGTGTTAATGTCTCTGTGTGCTTAAATCAAAATATAATTAAGTTTGTTTTAACTTTCTGT
1181   GTGAATTTAAGTTTCATAATAAGATTCAAAAAACAAAAACAAAAACAAAA

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**Fig. 4.** Complete nucleotide sequence of the zucchini lethal chlorosis virus (ZLCV) nucleocapsid (N) gene. The deduced amino acid sequence is shown below the nucleotide sequence. The ATG start codon is in bold and underlined, and the asterisk indicates the stop codon.

sequence comparisons with other tospoviral N genes, two new species were involved. CSNV and ZLCV thus would be representatives of two new serogroups, each representing a new tospovirus species.

Although the serogroup concept has been widely used as one of the first parameters toward establishment of new tospovirus species, it sometimes is confusing and conflicting. Whereas newly identified tospoviruses can be classified as a new species based on their N protein gene sequence data, they also can be classified with other tospovirus species within one serogroup based on their serological relatedness. Moreover, whereas GBNV, WBNV, and WSMV all represent a distinct species and due to their serological cross-reactions have been classified within one serogroup (IV), this rule has been applied differently for the tospovirus species TSWV, TCSV, and GRSV. The latter three species all are serologically related but, in contrast to the members of serogroup IV, classified within two different serogroups, I and II, respectively (6,7). To avoid this confusion for the future and avoid the existence of two different classification tools, we recommend use of the species concept for taxonomic purposes.

A phylogenetic tree based on the amino acid sequences of the N protein genes shows that CSNV and ZLCV belong to a cluster

formed by TSWV, TCSV, GRSV, and INSV (Fig. 5). This is supported by the multiple amino acid sequence alignment of the N protein genes that shows ranges of identical or highly conserved amino acids present in the aforementioned viruses, but absent or diverging from more distantly related viruses, e.g., GBNV, WSMV, WBNV, and PYSV (data not shown). However, the clustering of CSNV and ZLCV with TSWV, GRSV, and TCSV, all of them occurring in Brazil, showed highly conserved amino acid domains (Fig. 6) and may represent an adaptation to its ecological niche.

At the nucleotide level, sequence data comparisons revealed that the leader sequence of the N protein of ZLCV is the longest observed among tospoviruses, 220 nucleotides compared with an average length of 130 nucleotides (Fig. 4). This result was confirmed even by using different primer combinations to amplify this region and by the sequencing of its clones (data not shown). Sequence analysis did not reveal any specific sequence motif or secondary structure related to this region. The implication of this long leader sequence remains to be elucidated.

With the identification of CSNV and ZLCV, in addition to TSWV, GRSV, TCSV, and the recently identified IYSV<sub>BR</sub> (closely related to the Asian species), Brazil harbors the broadest spectrum

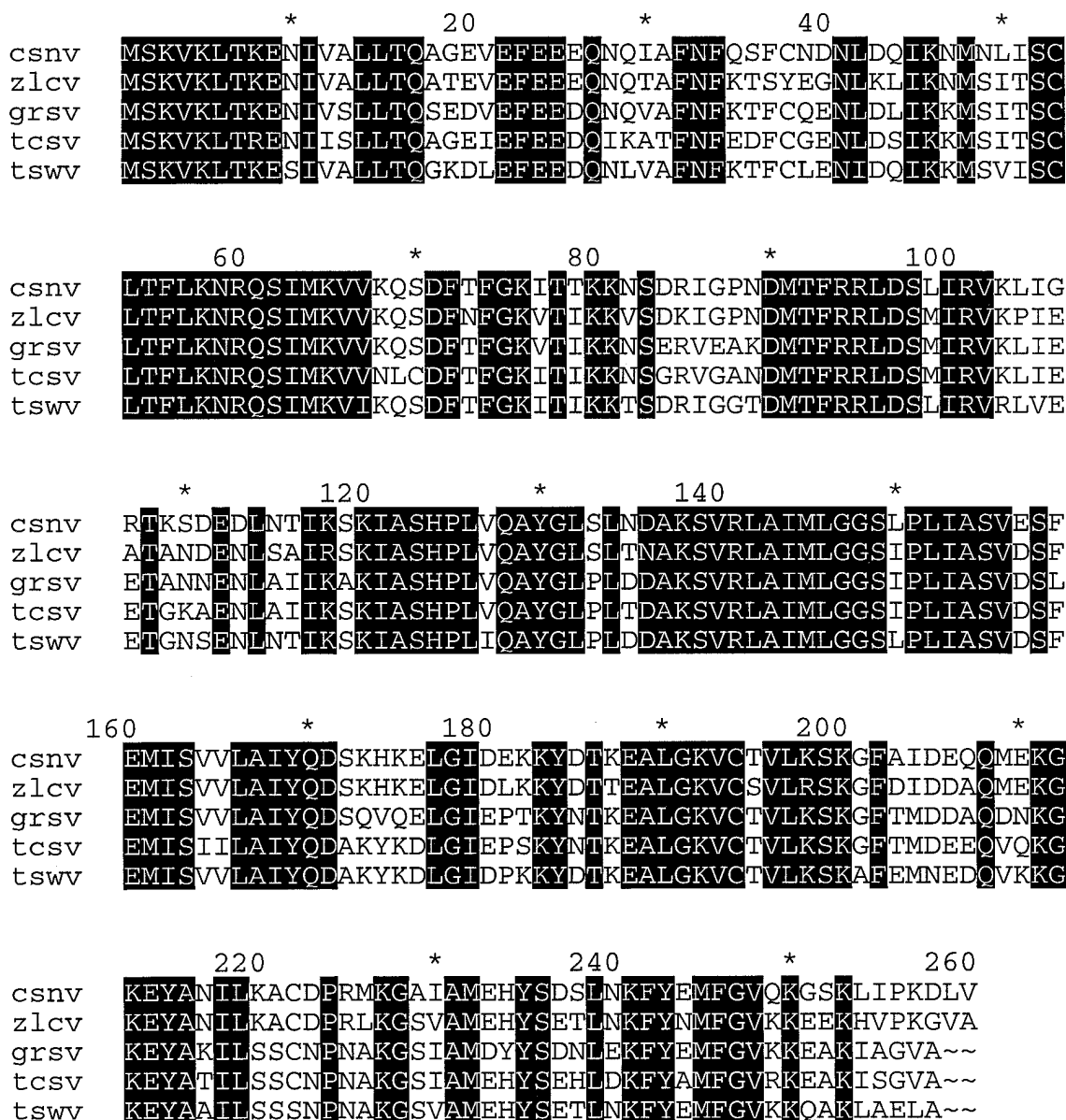


Fig. 5. Phylogenetic tree of tospovirus species showing the evolutionary relationships based on the nucleocapsid (N) protein sequence. The tree was constructed with the computer program PAUP 3.1.1., using PileUp data obtained from GCG as input based on 100 replicates using midpoint rooting.

of tospovirus species reported. The presence of IYSV<sub>BR</sub> only in onion fields in Brazil could be explained by the introduction of the crop to the country and its adaptation (21). Other tospovirus species prevalent in Asia (INSV, GBNV, WSMV, and WBNV) have not yet been reported in Brazil.

Although tospoviruses have been known in Brazil since 1938 (4), it is only during the last decade that they have become a major problem for several horticultural and ornamental crops. Currently, the occurrence of tospoviruses in Brazil shows differences in geographical distribution, prevalence, and economic impact for each tospovirus species (18). During the last 5 years, GRSV has widely spread in the northeastern part of Brazil, becoming prevalent in this region and mostly infecting tomato crops. TSWV and TCSV

are widely spread and occur in many different crops, although their incidence has slowly decreased throughout the last couple of years. In contrast, the occurrence of IYSV remains restricted to the northeastern part of the country, where it is a major problem in onion crops at the São Francisco River basin, resulting in yield losses of up to 100%. The new tospovirus species, CSNV and ZLCV, proposed in this work are currently growing in economic importance having rapidly spread within the last 2 years into new areas geographically distinct from where they were initially reported. Due to its broad host range, CSNV is anticipated to become as widespread and important as TSWV, TCSV, and GRSV, which infect many economically important crops. As Brazilian tospoviruses can be transmitted with high efficiency by *F. schultzei* (29), it is expected that this thrips species, native in Latin America (16), also plays an important role in the transmission of these two new tospovirus species. Preliminary studies of transmission of CSNV showed that the virus was transmitted by *F. schultzei* with high efficiency. The identification of vector species of ZLCV is currently ongoing.

The contrasting features concerning the biological characteristics of tospoviruses found in Brazil do not only reflect the adaptation of these viruses to distinct ecological niches, but also their adaptation to distinct hosts and vectors. With respect to the latter, it is interesting that, in addition to thrips *F. occidentalis*, *F. schultzei*, and *T. tabaci*, the presence of *T. palmi* in Brazil has also been reported (T. Nagata, unpublished data). Tospovirus species more prevalent in Asia have not yet been detected, but the presence of *T. palmi* already creates conditions for their occurrence, accentuating the growing economic importance and threat of tospoviruses for Brazilian agriculture, horticulture, and flower industries.

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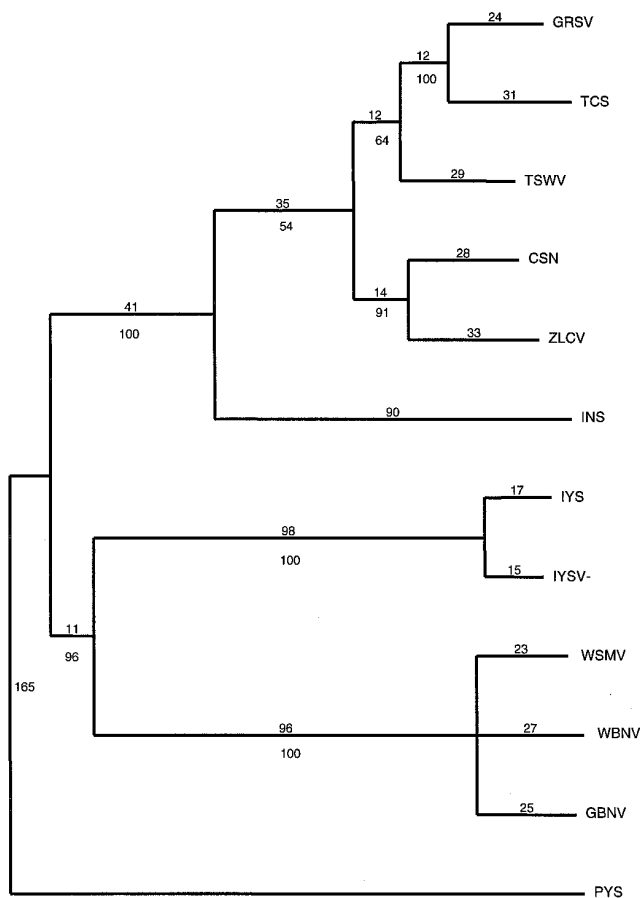


Fig. 6. Multiple amino acid sequence alignment of tospoviral nucleocapsid (N) proteins occurring in Brazil. Identical amino acids are shaded black.

TABLE 2. Amino acid identity between tospoviral nucleocapsid (N) proteins

Species <sup>a</sup>	TSWV	TCSV	GRSV	INSV	GBNV	WSMV	WBNV	IYSV	IYSV <sub>BR</sub>	PYSV	ZLCV	CSNV
TSWV	100	77	78	55	32	33	32	35	35	24	72	75
TCSV		100	81	53	30	31	30	33	32	20	73	72
GRSV			100	54	33	33	32	33	33	24	75	73
INSV				100	31	32	30	31	30	23	52	54
GBNV					100	86	85	43	44	20	32	30
WSMV						100	85	42	43	23	31	25
WBNV							100	43	44	22	30	30
IYSV								100	90	21	32	33
IYSV <sub>BR</sub>									100	21	32	32
PYSV										100	20	25
ZLCV											100	80
CSNV												100

<sup>a</sup> TSWV = tomato spotted wilt virus, TCSV = tomato chlorotic spot virus, GRSV = groundnut ring spot virus, INSV = Impatiens necrotic spot virus, GBNV = groundnut bud necrosis virus, also referred as PBNV = peanut bud necrosis virus, WSMV = watermelon silver mottle virus, WBNV = watermelon bud necrosis virus, IYSV = iris yellow spot virus, IYSV<sub>BR</sub> = IYSV from Brazil (GenBank no. AF067070), PYSV = peanut yellow spot virus, ZLCV = zucchini lethal chlorosis virus (GenBank no. AF067069), and CSNV = chrysanthemum stem necrosis virus (GenBank no. AF067068).

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