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POTEXVIRUS DIVERSITY IN CACTACEAE FROM SÃO PAULO STATE IN BRAZIL

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SUMMARY

Northwestern Brazil is a major center of diversity for cactaceous plants, most of which are grown as ornamentals. *Cactus virus X* (CVX), the only virus reported so far from Brazilian cactaceae, is widely spread. In this study we have characterized viruses of *Opuntia tuna* (OT), *Hylocereus undatus* (HU) and *Schlumbergera truncata* (ST) from the State of São Paulo, showing chlorotic spots and mosaic symptoms. Mechanically inoculated *Gomphrena globosa* reacted with local lesions, except when OT extracts were used as inoculum, whereas *Chenopodium amaranticolor* showed erratic systemic symptoms following inoculations from HU and ST. Transmission electron microscopy showed flexuous particles *ca.* 550 nm in length and bundles of virus particles in leaf cells from naturally and experimentally infected plants. Virus particles reacted faintly with an antiserum to CVX. Nucleotide sequences of the RNA dependent RNA polymerase gene obtained from cloned RT-PCR products, revealed the occurrence of Zygocactus virus X (ZyVX) in OT, mixed infection by ZyVX and Schlumbergera virus X (SchVX) in HU, and mixed infection by ZyVX, SchVX and Opuntia virus X (OpVX) in ST. This is the first report of ZyVX, SchVX and OpVX in Brazil.

Key words: *Flexiviridae*, cactuses, ornamentals, sequencing, phylogeny analysis.

INTRODUCTION

Members of the family *Cactaceae*, *Caryophyllales* and core eudicots (APG, 2003), have spiny succulent stems, differentiated shoots, usually succulent and differently shaped (cylindrical, conical, globose or flattened, often ridged or jointed), photosynthetic leaves (usually few and non persistent), short shoots (areoles) producing a spine or a spine cluster and, often, irritating hairs (Judd *et al.*, 1999). These plants are typical of deserts and other arid habitats, can be epiphytic in tropical forests, and are distributed mainly in North and South America. Although fruits of several species are edible, nearly all genera are grown as ornamentals (Judd *et al.*, 1999; Liou *et al.*, 2004).

Even though Northwestern Brazil is the major center of diversity for cactaceae, many species have been disseminated and vegetatively propagated as ornamentals, thus posing the risk of introduction of new viruses. Moreover, *Hylocereus undatus* has been used as a rootstock for other cactus species, favoring virus dispersal. It is necessary to stress that potexviruses, as well as virus species from other genera in the family *Flexiviridae* that do not have a known vector, rely exclusively on graft-transmission for their survival and dissemination (Martelli *et al.*, 2007).

Several viruses have been found in cacti (Brandes and Wetter, 1959; Sammons and Chessin, 1961; Bercks, 1971; Giri and Chessin, 1975; Chessin, 2002; Min *et al.*, 2006). Among these, *Cactus virus X* (CVX) is the most frequent and the only virus recorded from Brazil so far (Aragão *et al.*, 1993). The family *Flexiviridae* comprises viruses belonging to eight genera, including the genus *Potexvirus* (Fauquet *et al.*, 2005). Potexviruses have a monopartite, positive-strand RNA genome with five open reading frames (ORFs), capped at the 5' end and ending with a poly(A) tail at the 3' terminus (Adams *et al.*, 2004). Moreover, viruses that invade parenchymatous tissues and multiply therein, especially members of the genera *Potexvirus*, *Carlavirus*, *Allexivirus*, and *Trichovirus*, are more readily transmissible by mechanical means than viruses which are phloem-restricted in the natural hosts (Martelli *et al.*, 2007).

It is worth mentioning that, in addition to a definitive (CVX) and a tentative species, i.e. Zygocactus symptomless virus (Fauquet *et al.*, 2005), three new viruses isolated from cacti, named Opuntia virus X (OpVX), Schlumbergera virus X (SchVX) and Zygocactus virus X (ZyVX) have been proposed as members of the genus *Potexvirus* (Koenig *et al.*, 2004).

The aim of our study was to identify viruses in *Opuntia tuna* and *Schlumbergera truncata* showing chlorotic spots and *Hylocereus undatus* with mosaic symptoms

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Table 1. Identity percentage at the nucleotide level of partial RdRp sequences from species and isolates of the genus *Potexvirus*[(a)]

	OT	HU1	HU2	HU3	ST1	ST2	ST3	ST4	ST5
AltMV	62.8	62.3	62.1	63.1	64.9	63.1	62.9	64.9	63.9
BaMV	57.6	57.4	59.1	60.3	60.2	58.1	57.7	59.2	57.9
CalVX	62.8	62.1	60.6	63.2	62.8	60.8	62.3	63.5	61.5
ClYMV	64.7	65.1	65.3	64.9	64.6	63.9	65.2	64.0	64.8
CsCMV	64.4	65.0	64.7	65.4	65.4	65.6	64.7	65.5	65.2
CymMV	58.7	59.8	61.2	59.5	62.5	61.0	59.3	58.6	59.2
FoMV	60.3	60.5	58.8	60.3	63.1	58.9	60.3	62.2	59.5
HdRSV	66.5	66.5	64.7	67.6	64.0	64.2	67.0	65.1	65.9
HVX	64.0	64.6	61.7	65.4	65.0	61.8	64.6	64.8	62.0
LVX	61.0	61.5	60.0	61.0	59.5	60.9	61.2	60.4	61.2
MaMV	54.5	55.2	55.0	54.9	57.5	57.4	54.9	55.7	56.6
MinVX	61.2	62.0	58.8	60.9	62.0	57.8	62.0	62.2	59.4
NMV	57.5	59.1	58.6	59.4	60.8	58.9	58.0	57.2	58.7
NVX	61.3	60.6	61.2	61.3	65.4	61.2	61.7	63.9	61.2
PAMV	59.2	59.5	60.1	59.8	62.5	61.2	59.5	66.3	60.3
PapMV	64.0	64.5	64.8	64.9	65.4	64.6	64.5	63.9	64.0
PepMV	59.1	60.0	61.4	59.1	61.3	62.0	58.8	64.5	59.1
PlAMV	65.5	65.2	62.8	64.9	64.6	63.2	65.6	64.0	64.5
PVX	63.7	63.9	62.5	64.0	65.7	61.7	64.0	64.3	62.8
ScVX	58.5	57.6	60.9	58.4	61.7	60.6	58.6	63.5	60.7
SMYEV	62.7	63.1	60.3	62.4	64.2	60.4	63.0	62.9	60.5
TVX	65.0	66.0	62.8	66.3	63.9	63.1	65.4	59.4	64.8
WClMV	59.6	60.2	60.8	60.2	59.8	61.0	60.1	59.2	60.2
Cactaceae viruses									
CVX	75.3	75.8	75.0	76.7	74.4	74.8	75.8	76.4	75.6
OpVX	73.8	74.7	71.8	73.6	**96.6**	72.2	74.1	**85.8**	71.8
SchVX	76.1	76.2	**94.1**	75.3	70.7	**87.8**	76.4	72.7	**81.8**
ZyVX	**95.2**	**97.1**	76.8	**95.8**	74.1	76.8	**95.7**	**85.0**	**84.9**
OT	–	**95.8**	76.0	**94.7**	73.8	76.3	**99.1**	**85.6**	**85.7**
HU1	–	–	75.9	**96.4**	74.1	77.0	**96.3**	**84.7**	**85.2**
HU2	–	–	–	75.8	71.0	**86.7**	76.1	72.2	**81.5**
HU3	–	–	–	–	73.8	76.5	**95.5**	**84.4**	**84.6**
ST1	–	–	–	–	–	73.0	74.5	**87.6**	72.7
ST2	–	–	–	–	–	–	76.8	74.2	**90.0**
ST3	–	–	–	–	–	–	–	**86.6**	**86.6**
ST4	–	–	–	–	–	–	–	–	**83.9**
ST5	–	–	–	–	–	–	–	–	–

(a) See Fig. 3 for GenBank accession numbers.

AltMV= *Alternanthera mosaic virus*; BaMV= *Bamboo mosaic virus* CalVX= Caladium virus X; ClYMV= *Clover yellow mosaic virus*; CsCMV= *Cassava common mosaic virus*; CVX= *Cactus virus X*; CymMV= *Cymbidium mosaic virus*; FoMV= *Foxtail mosaic virus*; HdRSV= *Hydrangea ringspot virus*; HVX= *Hosta virus X*; LVX= *Lily virus X*; MaMV = Malva mosaic virus; MinVX= Mint virus X; NMV= *Narcissus mosaic virus*; NVX= *Nerine virus X*; OpVX= Opuntia virus X; PAMV= *Potato aucuba mosaic virus*; PapMV= *Papaya mosaic virus*; PepMV= *Pepino mosaic virus*; PlAMV= *Plantago asiatica mosaic virus*; PVX= *Potato virus X*; SchVX= Schlumbergera virus X; ScaVX= Scallion virus X; SMYEV= *Strawberry mild yellow edge virus*; TVX= *Tulip virus X*; WClMV= *White clover mosaic virus*; ZyVX= Zygocactus virus X. Potexvirus isolates from: *Hylocereus undatus* (HU), *Opuntia tuna* (OT), *Schlumbergera truncata* (ST)

(Fig. 1) from the Brazilian State of São Paulo and to characterize the genetic diversity of virus isolates in the RNA dependent RNA polymerase (RdRp) gene.

MATERIALS AND METHODS

Virus sources and mechanical transmission. Epidermal tissues from stems of *O. tuna*, *H. undatus* and *S. truncata* were ground in 0.01 M phosphate buffer pH 7.0 supplemented with 0.1% sodium sulfide and mechanically inoculated to species of *Amaranthaceae, Chenopodiaceae* and *Solanaceae.*

Electron microscopy. Virus particles from stem tissue extracts of the three cactus species and from leaves of experimentally infected herbaceous hosts were visualized by transmission electron microscopy after negative staining with 2% uranyl acetate. Naturally infected tissues were processed for cytological studies according to the methods described by Martelli and Russo (1984). Decoration tests were done with an antiserum to CVX

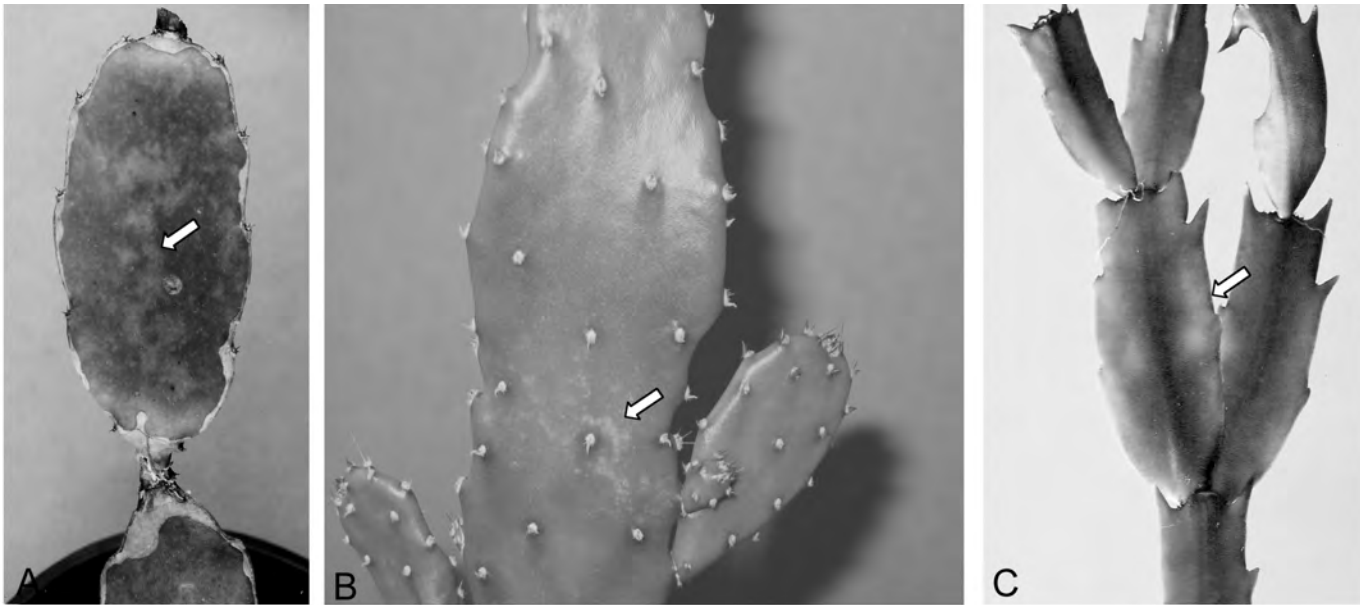

Fig. 1. Symptoms (arrows) shown by *Hylocereus undatus* (A), *Opuntia tuna* (B) and *Schlumbergera truncata* (C) from São Paulo State, Brazil.

diluted at 1:50 in 0.1 M phosphate buffer pH 7.0.

RNA extraction and RT-PCR. Total RNA was extracted from epidermal tissues of the three infected cactus species using Trizol LS reagent according to the manufacturer's instructions (Gibco BRL, USA). Reverse transcription and PCR amplification were performed using the primers potex 1 (CAY CAR CAR GCX AAR GAY SA) and potex 2 (TCD GTR TTD GCR TCR AAD GT) and the conditions described by Gibbs *et al.* (1998).

cDNA cloning and sequencing. After purification of the amplified products from each cactus using the Concert Rapid Gel Extraction Kit (Gibco BRL, USA) as suggested by the manufacturer, amplicons were cloned into the pGEM-T vector (Promega, USA). Twelve independent clones from each viral gene product were sequenced by the ABI Prism, using the Big Dye Terminator System (PE Applied Biosystems, USA).

Phylogenetic analyses. The nucleotide sequences were submitted to BLAST (Altschul *et al.*, 1990), aligned with the corresponding sequences of 19 potexvirus species and the carlavirus *Potato virus M* (PVM) according to Koonin and Dolja (1993) and automatically translated using the software SeqPup v. 0.6f (Gilbert, 1995) for Macintosh. The percentage of identity at the nucleotide level between sequences was determined by PAUP v. 4.0b10 for Macintosh. Maximum parsimony analysis was made with nucleotide and amino acid sequences using PAUP v. 4.0b10 with heuristic search and equal weighting (Swofford, 2002). The stepwise addition algorithm was also used and bootstrap values were determined using the branch-and-bound method (Thompson, 1987). Neighbor-joining (NJ) analysis with nucleotide sequences from the clones of Brazilian CVX, OpVX, SchVX, ZyVX, and *Potato virus X* (PVX) was done with PAUP v. 4.0b10, after estimating the nucleotide substitution and gamma distribution with the software Modeltest v. 3.06 (Posada and Crandal, 1998). Sequences used in alignments and phylogenetic analysis were from the viruses listed in Fig. 3.

RESULTS

Mechanical transmission and host range. In general, the symptoms caused by viruses isolated from *H. undatus* (HU), *O. tuna* (OT) and *S. truncata* (ST) appeared 15 days post-inoculation. *Gomphrena globosa* showed local lesions when inoculated with OT extracts and *Chenopodium amaranticolor* had erratic systemic symptoms following inoculation with extracts from HU and ST. All virus isolates induced chlorotic local lesions in *C. murale* and local and systemic symptoms in *C. quinoa. Nicotiana glutinosa* reacted with vein banding, mosaic and deformation of the leaves following inoculation with HU extracts. In contrast, none of the viruses caused symptoms in *N. benthamiana*, *N. clevelandii*, *N. debneyi*, *N. megalosiphon*, *N. tabacum* cv. White Burley and *Datura stramonium.*

Electron microscopy. Flexuous particles *ca.* 550 nm in length were observed in dips from all naturally infected cacti and experimentally infected *C. amaranticolor* plants (Fig. 2A). Similar particles were also seen in dips

from *N. glutinosa* leaves experimentally infected with HU. The cytoplasm of cells of thin-sectioned stem tissue of the three cacti contained virus particles arranged in bundles (Fig. 2C) or in large disorderly accumulations (Fig. 2B, C,D). No visible cytopathic differences could be observed in the cells infected by each of the three viral three isolates. All isolates were weakly decorated by an antiserum to CVX.

RT-PCR and sequence analyses. Products of 648 bp in size were obtained by RT-PCR using total RNA extracts from HU, OT and ST as template. DNA amplicons corresponded to a fragment of the RdRp gene. Sequences of virus isolates from OT (OT1), HU (HU1, HU2, HU3) and ST (ST1, ST2, ST3, ST4 and ST5) were similar to those of potexvirus species with nucleotide identity ranging from 54.5 to 97.1%, as shown by analysis using the PAUP program. Nucleotide identity with CVX sequences ranged from 74.4 to 76.7%. Viral sequences determined in this study were deposited in GenBank under the accession numbers EU676009 (OT), EU670720-EU670722 (HU1-HU3) and EU676004-EU676008 (ST1-ST5). The clones corresponding to the RdRp gene from OT were identical to one another. Very high nucleotide

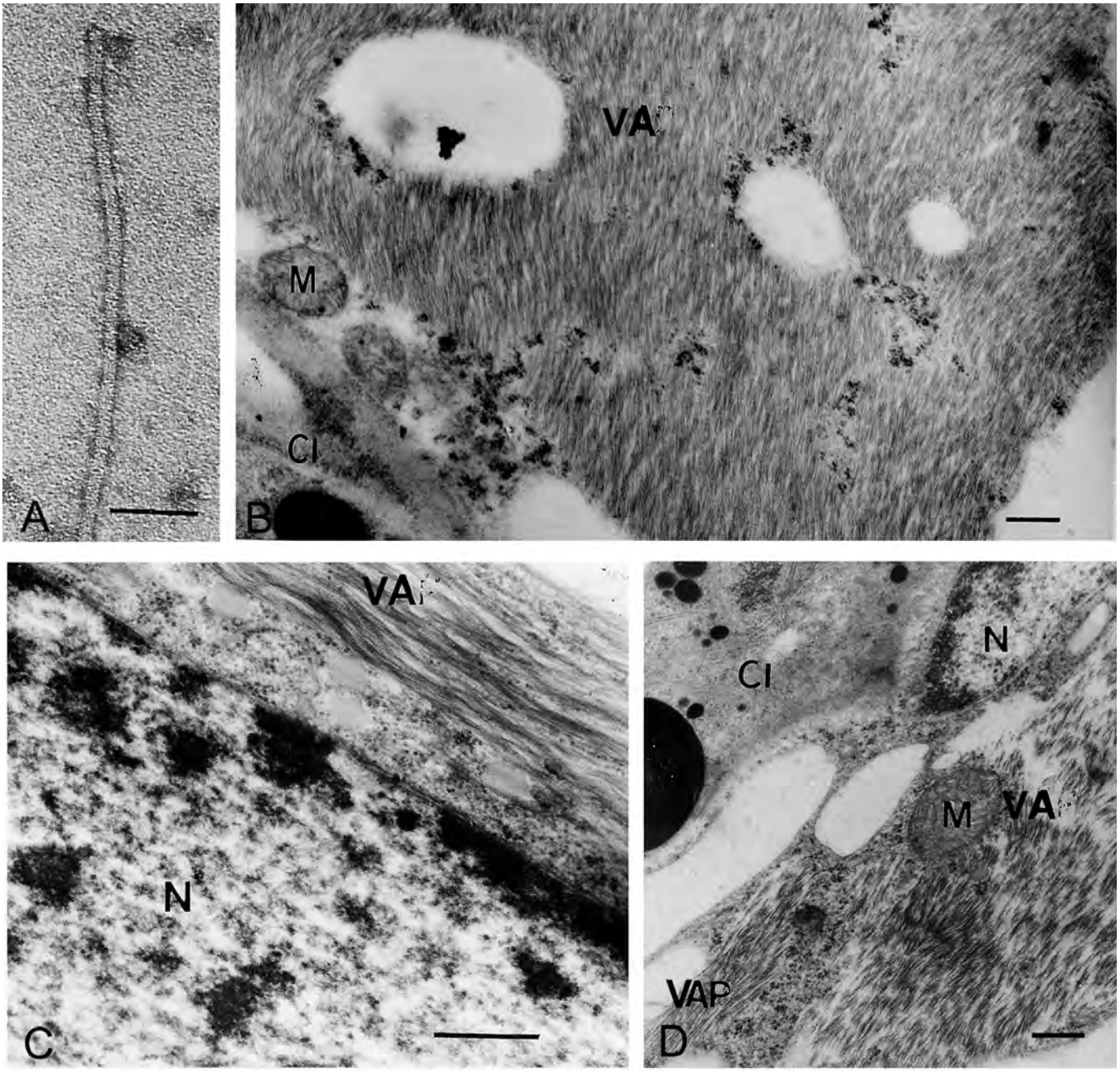

Fig. 2. Negatively stained flexuous particles from *Schlumbergera truncata* stems (A). Bar =100 nm. Ultra-thin sections of stem tissues of *Hylocereus undatus* (B), *Schlumbergera truncata* (C) and *Opuntia tuna* (D), showing cytoplasmic aggregates of virus particles (VA, VAP). Cl = chloroplast, M = mitochondrion, N = nucleus. Bars = 500 nm

identities were found between clones from OT and ZyVX (95.2%), HU1 and ZyVX (97.1%), HU2 and SchVX (94.1%), ST1 and OpVX (96.6%), ST2 and SchVX (87.8%) and ST3 and ZyVX (95.7%) (Table 1).

Phylogenetic analyses. The phylogenetic tree constructed under maximum parsimony conditions showed that RdRp sequences of potexviruses from cactaceae and relative clones formed a monophyletic group supported by 100% bootstrap (Fig. 3). In particular, the tree comprised two major clades formed by OT, ST3, HU3, HU1, ZyVX and ST5, ST2, HU2, SchVX, plus a smaller clade formed by ST4, ST1 and OpVX. However, whitin this clade, clone ST4 was separate from ST1 and OpVX (Fig. 3).

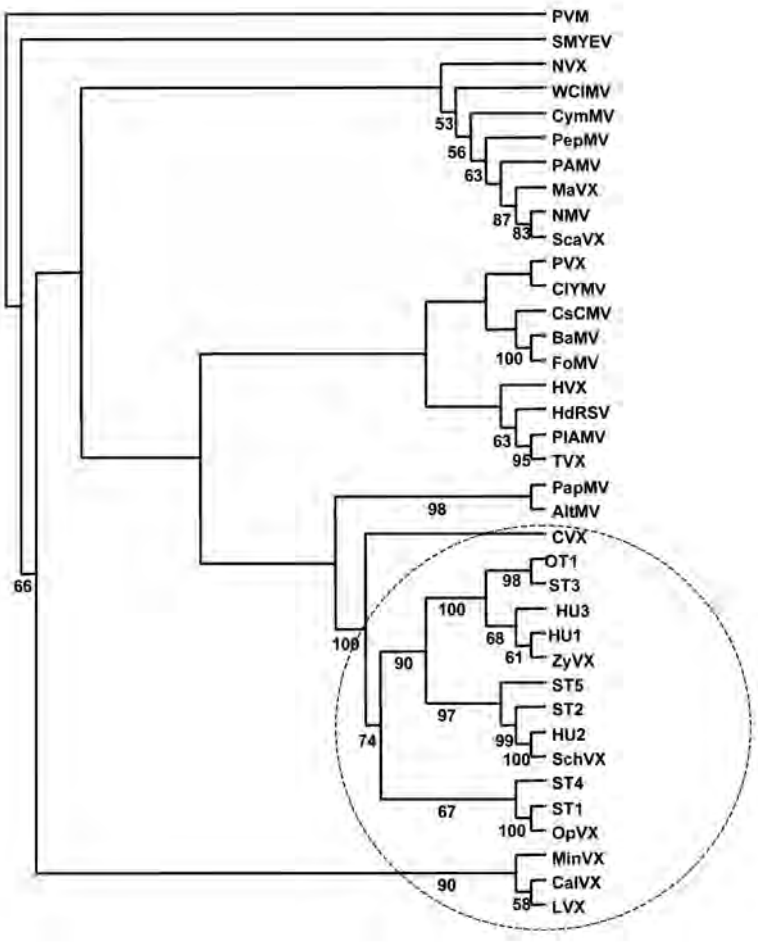

Fig. 3. Maximum parsimony tree contructed with partial RdRp sequences of viruses isolated from *Hylocereus undatus* (HU1, HU2, HU3), *Opuntia tuna* (OT1) and *Schlumbergera truncata* (ST1, ST2, ST3, ST4, ST5) and comparable potexvirus sequences retrieved from GenBank. *Alternanthera mosaic virus* (AltMV, NC00773), *Bamboo mosaic virus* (BaMV, NC001642), *Cactus virus X* (NC002815), Caladium virus X (CalVX, AY727533), *Clover yellow mosaic virus* (ClYMV, NC 001753), *Cassava common mosaic virus* (CsCMV, NC001658), *Cymbidium mosaic virus* (CymMV, AF 016914), *Foxtail mosaic virus* (FoMV, NC001483), *Hosta virus X* (HVX, AJ620114), *Hydrangea ringspot virus* (HdRSV, NC006943), *Lily virus X* (LVX, NC 007192), Malva mosaic virus (MaVX, NC008251), Mint virus X (MVX NC006948), *Narcissus mosaic virus* (NMV, NC001441), *Nerine virus X* (NVX, AB219105), Opuntia Virus X (AY366209), *Papaya mosaic virus* (PapMV, NC001748), *Pepino mosaic virus* (PepMV, AJ308445), *Plantago asiatica mosaic virus* (PlAMV, Z21647), *Potato aucuba mosaic virus* (PAMV, S73580), *Potato virus X* (PVX, AF111193), *Scallion virus X* (ScaVX, NC004300), Schlumbergera virus X (SchVX, AY366207), *Strawberry mild yellow edge virus* (SMYEV, D12517), *Tulip virus X* (TVX, NC004322), *White clover mosaic virus* (WClMV, X16636) and Zygocactus virus X (ZyVX, AY366208). Bootstrap values are indicated near the branches. *Potato virus M* (PVM, NC001361) was used as outgroup.

DISCUSSION

The viruses morphologically similar to potexviruses described in this study are widely spread in different genera of the family *Cactaceae*. Infections have been reported in more than 40 different species within this family (Koenig *et al.*, 2004). In Brazil, among the 28 definitive and 18 tentative species of the *Potexvirus* genus (Fauquet *et al.*, 2005), only the occurrence of CVX, PVX, *Alternanthera mosaic virus* (AltMV), *Bamboo mosaic virus* (BaMV), Caladium virus X (CalVX), *Cassava common mosaic virus* (CsCMV), *Cymbidium mosaic virus* (CymMV), Patchouli virus X (PaVX), and *White clover mosaic virus* (WClMV) has been recorded (Costa and Kitajima, 1972; Chagas *et al.*, 1977; Lin *et al.*, 1977; Mulder *et al.*, 1987; Souto *et al.*, 1991; Meissner *et al.*, 1992; Aragão *et al.*, 1993; Rivas *et al.*, 2005; Duarte *et al.*, 2008).

OpVX, SchVX and ZyVX induced different symptoms on herbaceous hosts, with *C. amaranticolor* showing erratic systemic symptoms when inoculated with HU and ST extracts, thus suggesting the presence of a possible mixture of potexvirus species or strains in the natural hosts. ZyVX from OT did not induce symptoms in *Gomphrena globosa*, as previously reported (Giri and Chessin, 1975).

Bioassays and the light decoration of virus particles by the CVX antiserum supported the notion that *O. tuna*, *H. undatus* and *S. truncata* were not infected with this virus. However, infection by other potexvirus species was ascertained as shown by the positive recovery of 12 clones obtained by RT-PCR from each of the cacti under study. Sequences corresponding to the partial RdRp gene contained four (I to IV) of the eight conserved RdRp motifs identified by Koonin and Dolja (1993). High nucleotide identity with cactus viruses was ascertained, except for CVX (74.4 to 76.7%). The virus from OT had the highest identity with ZyVX, viruses from HU with SchVX and ZyVX, and viruses from ST with OpVX, SchVX and ZyVX. This was taken as evidence that mixed infection occurred in natural populations of HU (SchVX and ZyVX) and ST (OpVX, SchVX and ZyVX). In particular, clone ST4 had a nu-

cleotide sequence identity higher than 85.0% with OpVX and ZyVX, and the identity of clone ST5 with SchVX and ZyVX was higher than 81.8%.

Sequences of the virus isolates characterized in this study were within the lower limits of the potexvirus range of diversity (72 and 80% at the nucleotide and amino acid levels, respectively) (Adams *et al.*, 2004; Fauquet *et al.*, 2005). For example, the complete RdRp sequence of CVX had 70.3, 71.0 and 74.4% nucleotide identity with OpVX, SchVX and ZyVX, respectively (data not shown). Koenig *et al.* (2004) proposed that OpVX, SchVX and ZyVX should be regarded as distinct species in the genus *Potexvirus* based on diversity in their coat protein and other genes, as well as in symptomatology.

Because the RdRp is the only protein universally encoded in the nondefective positive-strand RNA viruses, its phylogeny is the principal determinant of the evolutionary framework in this vast virus group (Martelli *et al.*, 2007). Thus, phylogenetic analysis based on RdRp sequences revealed two major clusters (Fig. 3). Viruses from cactaceae characterized in this study fell into a subcluster of the largest of the two clusters which comprised AltMV, BaMV ClYMV, CsCMV, CVX, FoMV, HdRSV, HVX, OpVX, PlAMV, PapMV, PVX, SchVX, TVX and ZyVX. Sherpa *et al.* (2007) also reported that potexviruses can be divided into two groups. The cluster formed by the HU, OT and ST clones, CVX, OpVX, SchVX and ZyVX was strongly supported by a bootstrap value of 100%, in accordance with similar results obtained by Rivas *et al.* (2005) and more recently by Côté *et al.* (2008).

The tree topology constructed under NJ confirmed the monophyly of cactaceae viruses. Pairwise identities between viruses from the cluster formed by OT1, ST3, HU3, HU1 and ZyVX, supported by 100% bootstrap, ranged from 94.7 to 99.1%. This result is within the limits of intra-specific diversity of potexviruses (Adam *et al.*, 2004; Fauquet *et al.*, 2005). It is worth pointing out that clone ST4 did not cluster with either OpVX or ZyVX, although the nucleotide identity between these viruses is 85.8 and 85.0%, respectively. Similar results were obtained when the ST5 sequence was compared with SchVX and ZyVX (81.8 and 84.9%, respectively), although it clustered with SchVX and the clade was supported by 92% bootstrap. Such results suggest that a recombination event between potexviruses took place in *Schlumbergera truncata*. Homologous recombination can play an important role in the genetic diversity of RNA viruses and their evolution (Bruyere *et al.*, 2000).

In conclusion, this study reports for the first time the presence of three different potexviruses other than CVX in single or mixed infection in Brazilian cacti. According to Martelli *et al.* (2007) there is no region of flexiviral genomes that is left untouched by genetic variation. Additionally, the study of the variability and modification of the genetic structure of plant virus populations is an important aspect of plant pathology and may be highly relevant for the development of strategies for controlling virus diseases (García-Arenal *et al.*, 2001).

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