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Cauliflower stunt associated with a phytoplasma of subgroup 16SrIII-J and the spatial pattern of disease

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Abstract Since 2000, a disease has occurred with high levels of incidence in crops of cauliflower grown in the green belt area of the city of São Paulo, Brazil. The symptoms are characterized by stunting, malformation of the inflorescence, reddening leaves, and vascular necrosis, suggesting infection by phytoplasma. These symptoms are similar to those described in *Brassicas* species affected by the aster yellows (16SrI) group of phytoplasma. In the present study, a phytoplasma from the 16SrIII-J subgroup was identified in cauliflower plants based on actual and virtual RFLP patterns and phylogenetic analysis, and was distinct from the phytoplasmas frequently associated with aster yellows disease in *Brassicas*. Pathogenicity assays using dodder confirmed that the identified phytoplasma is the agent of the observed disease, which is here designated as cauliflower stunt. Consequently, this species of *Brassica* may be recognized as a new host for subgroup 16SrIII-J, which has frequently been found in diverse species cultivated in Brazil. The spatial pattern of diseased plants was determined in ten cauliflower plots of 300 to 728 plants each. All plants in these plots were evaluated by visual assessments, assigned as diseased or healthy and mapped. The dispersion index and Taylor's power law were determined for various quadrat sizes and the results showed that the diseased plants were distributed in a random pattern in fields with a low disease incidence and in an aggregated pattern in fields with a disease incidence greater than 25 %. According to an isopath area analysis, diseased plants were predominantly present in the field borders, suggesting that the pathogen is possibly introduced by vector(s) from the external area.

Keywords Yellows · *Brassica oleracea* · Epidemiology

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Introduction

Cauliflower (*Brassica oleracea* var. *botrytis*) is an economically relevant vegetable crop in Brazil. The state of São Paulo is the leader in cauliflower production, with a cultivated area of over 2,295 ha and a yield of approximately 29,262 t. In commercial fields located close to the green belt area of the city of São Paulo, a disease has been observed, characterized by stunted plants, reduced size, malformation of the inflorescence, reddening leaves, and vessel necrosis (Fig. 1). In some cases, small heads arise at the base of stem. The symptoms suggest that this disorder could be associated with phytoplasma infection. This anomaly has been observed since 2000, and its occurrence has intensified in more recent years. The incidence levels

Fig. 1 Cauliflower stunt: **a** - sprout proliferation; **b** - stunted plant with malformation of the inflorescence and light reddening of leaves; **c** - necrosis of the vessel region; **d** - healthy plant

are variable with season of year, variety and the use of insecticides in early crop stages. In some fields, the frequency of symptomatic plants has reached values up to 90 %. Damage to the crops results in low yield and low quality of the final product, thereby reducing their market value. Sometimes growers leave these damaged areas or replace the cauliflower fields with other vegetables, such as eggplant, because the disease has become a limiting factor for cauliflower production.

Phytoplasmas associated with distinct species belonging to the genus *Brassica* have been reported. In Italy during the early 1980s, phytoplasmas were seen by electronic microscopy within the phloem of broccoli and cauliflower plants that exhibited inflorescence malformation, including virescence and phyllody (Bertaccini et al. 1983). Representatives of the 16SrI (aster yellows) group have been identified in association with diseases occurring in distinct species of *Brassica.* An aster yellows phytoplasma was detected in broccoli plants grown in areas of South Italy (Marcone and Ragozzino 1995). Also in Italy, a phytoplasma found in the tissue of diseased cabbage was characterized as belonging to the subgroup 16SrI-B (Bertaccini et al. 1998). In winter oilseed rape plants (*Brassica napus* var. *arvensis*) grown in several areas in the Czech Republic, a phytoplasma of the subgroup 16SrI-B was identified in association with symptoms of stunting, reddening leaves and extensive malformation of floral parts (Bertaccini et al. 1998). In cabbage collected in several regions of Hungary, the presence of a phytoplasma of the aster yellows group was found in plants with a head-forming anomaly characterized by leaves growing outward from the apex (Fodor et al. 1999). During an aster yellows epidemic in southwestern Texas, USA, a phytoplasma of the 16SrI-B subgroup was present in cabbage plants with purple discolouration in leaf veins and at the edges of leaves, and which had a sprout proliferation at the base of the stem (Lee et al. 2003). In a field survey of vegetable crops in Serbia, a 16SrI-B phytoplasma was found in broccoli with symptoms of phyllody and flower proliferation (Duduk et al. 2007). In canola and oilseed rape crops located in Canada, phytoplasmas of subgroups 16SrI-A and 16SrI-B were identified in plants showing typical aster yellows disease symptoms. Although representatives of the aster yellows group have been associated with *Brassica* diseases, a phytoplasma belonging to the 16SrVI group was found in naturally infected cabbage cultivated in Iran, in plants that exhibited small leaves, yellowing, stunting, opening of the head, and proliferation of sprouts at the base of stem (Salehi et al. 2007).

There are only a few epidemiological studies on phytoplasma in annual crop pathosystems, and all of them focus on the spatial pattern of diseased plants (Beanland et al. 2005; Hollingsworth et al. 2008;

Madden et al. 1995). The description of the temporal dynamics of diseases associated with phytoplasmas in annual crops is very difficult, probably due to the length of the latent period of the pathogen in the insect vector and the incubation period in the plant host (Weintraub and Beanland 2006). In the genus Brassica, the first symptoms of a disease associated with phytoplasmas usually appear in the middle of the crop season. Consequently, a short period of time is available for epidemiological measurements. The spatial distribution of diseased plants can be related to the dispersal mechanisms of the pathogen and to the sources of inoculum. Understanding the spatial pattern of diseased plants can assist in the establishment of sampling schemes and in the decision-making process for disease management.

The present study was conducted in order to demonstrate the association between phytoplasma and a disease occurring in cauliflower plants, to identify and to phylogenetically analyze the detected phytoplasma, and to characterize the spatial pattern of diseased plants.

Materials and methods

Plant samples, DNA extraction and PCR amplification Sixty-day-old plants were collected from fields close to the green belt area of the city of São Paulo in the state of São Paulo, Brazil. A total of 37 cauliflower plants were sampled, corresponding to 20 symptomatic and 17 symptomless plants. Nucleic acid for use as template in PCR reactions was extracted from inflorescence and leaf vein tissue, using the Dneasy Plant Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions.

Nested PCR primed by the universal primer pair P1/Tint (Deng and Hiruki 1991; Smart et al. 1996) followed by R16F2n/16R2 (Gundersen and Lee 1996) was used for the detection of phytoplasma. The phytoplasma detected from each sample of cauliflower was considered to be an isolate. Identification was also conducted with nested PCR assays by using the P1/Tint primer pair to amplify 16S rDNA, while the primer pair R16(III)F2/R16(III)R1 (Lee et al. 1994) was used to specifically detect phytoplasmas of the 16SrIII group. DNA from chayote witches' broom phytoplasma, a member of the 16SrIII group, subgroup J, was used as the positive control for both kinds of PCR (Montano et al. 2000). DNA extracted from healthy cauliflower plants, grown from seeds in a greenhouse, was used as negative control.

PCR reactions with final volume of 25 μl were performed using 0.5 μl of each primer (20 pmol/μl), 2.0 μl of deoxynucleotide triphosphates (2.5 mM each NTP), 0.13 μl of Taq polymerase (5 U/μl), 2.5 μl of PCR buffer (for 10 ml: 6.75 ml water; 1.0 ml Tris–HCl-1 M; 0.15 ml $MgCl_2$–1 M; 2.0 ml KCl–2.5 M; 0.1 ml 1 % gelatin [Sigma]), and 19.9 μl of water. The thermocycler conditions for reactions primed by the pair P1/Tint were 35 cycles of denaturing at 94 °C for 1 min, annealing at 56 °C for 1 min and primer extension at 72 °C for 2 min. The second PCR reaction was 35 cycles of denaturing at 94 °C for 1 min (2 min for the first cycle), annealing at 50 °C for 2 min and primer extension at 72 °C for 3 min (7 min in the final cycle). PCR products were analyzed by electrophoresis in agarose gel (1 %), stained with Sybr Safe (Invitrogen), and DNA bands were visualized using an UV transilluminator. The DNA fragment size standard was a 1 kb ladder (Life Technologies).

Restriction Fragment Length Polymorphism (RFLP) analysis—actual RFLP RFLP analyses were carried out with all isolates detected from symptomatic and symptomless plants using the restriction enzymes *Alu*I, *Hpa*II, *Kpn*I, and *Mse*I. Since all isolates were identified as belonging to group 16SrIII, further analyses were performed with the enzymes *Bsh*1236, *Hha*I, *Mbo*I, and *Rsa*I for four isolates from each group of symptomatic and asymptomatic plants, selected at random, in order to identify subgroups within the group 16SrIII.

PCR products of 1.2 kb amplified from the reaction primed by R16F2n/R2 were digested separately with restriction enzymes, *Alu*I, *Rsa*I, *Kpn*I, *Hpa*II, *Mse*I, *Hha*I, *Mbo*I and *Bst*UI, according to the manufacturer's instructions. The resulting digests were separated by electrophoresis on a 5 % nondenaturing polyacrylamide gel, stained with Sybr Safe (Invitrogen) and visualized under a UV transilluminator. The DNA fragment size standard was the ΦX174RF*Hae*III ladder (Biolabs). The restriction patterns from the phytoplasma found in cauliflower samples were compared with previously published patterns of phytoplasmas representative of distinct RFLP groups (Lee et al. 1998; Montano et al. 2000).

Cloning, sequencing, and phylogenetic analysis Two isolates were selected for sequencing of the DNA fragment corresponding to the 16S rRNA gene. DNA fragments amplified by the R16F2n/R2 primer pair were cloned in *Escherichia coli*, DH5α strain, using the pGEM T Easy Vector System I (Promega), according to the manufacturer's instructions. Sequences of cloned DNA fragments found in the strains detected in cauliflower were aligned, then compared among themselves and with the sequences of phytoplasmas classified in various groups and 16SrIII subgroups available in GenBank (Table 1), using the Sequencer 3.0 DNA program software (Gene Codes Corporation Arbor, MI, USA).

Table 1 Sequences of 16S rDNA used in this study and the respective GenBank accession numbers

Classification	Disease	GenBank accession no.
16SrIII-J	Cauliflower stunt (CfS)	HM237045
16SrI-B	Maize bushy stunt (MBS)	AY265208
16SrII-A	Peanut witches'-broom (PnWB)	L33765
16SrIII-A	Canadian peach X-disease (CX)	L33733
16SrIII-B	Clover yellow edge (CYE)	AF175304
16SrIII-C	Pecan bunch (PB1)	FJ376626
16SrIII-D	Goldenrod yellows (GR1)	FJ376627
16SrIII-E	Spiraea stunt (SP1)	AF190228
16SrIII-F	Milkweed yellows (MW1)	AF510724
16SrIII-G	Walnut witches'-broom (WWB)	AF190226
16SrIII-H	Poinsettia branch-inducing (PoiBI)	AF190223
16SrIII-I	Virginia grapevine yellows (VGY)	AF060875
16SrIII-J	Chayote witches'-broom (ChWB)	AF147706
16SrIII-K	Strawberry leafy fruit (SLF)	AF274876
16SrIII-L	Poisettia exuberant flower-inducing (EF-MM)	EU169138
16SrIII-M	Montana potato purple top (PPT-MT117-1)	FJ226074
16SrIII-N	Alaska potato purple top (PPT-AK6)	FJ376629
16SrIII-P	Dandelion virescence (DanVir)	AF370119
16SrIII-R	Cirsium white leaf (CirWL)	AF373105
16SrIII-S	Western peach X-disease (WX)	L04682
16SrIII-U	Eggplant giant calix (EB02-Br06)	HM589213
16SrIV-A	Coconut lethal yellowing (LYJ-C8)	AF498307
16SrV-A	Elm yellows (EY1)	AY197655
16SrVI-A	Clover proliferation (CP)	AY390261
16SrVII-B	Erigeron witches'-broom (EriWB)	AY034608
16SrVIII-A	Loofah witches'-broom (LFWB)	AF353090
16SrIX-A	Pigeon pea witches'-broom (PPWB)	AF248957
16SrX-A	Apple proliferation (AP)	AJ542541
16SrXI-A	Rice yellow dwarf (RYD)	D12581
16SrXII-B	Australian grapevine yellows (AUSGY)	L76865
16SrXIII-C	Chinaberry yellows (CbY)	DQ444264
16SXIV	Bermuda grass white leaf (BGWL)	AF248961
16SrXV-A	Hibiscus witches'-broom (HibWB)	AF147708
16SrXVI	Sugar cane yellow leaf syndrome (SCYLS)	AY725228
16SrXVII-A	Papaya phytoplasma (PAY)	AY725234
16SrXVIII-A	American potato purple top wilt (APPTW)	DQ174122
Acholeplasma laidlawii		M23932

A phylogenetic tree was constructed from the sequence present in a cauliflower isolate (CfS) and sequences from several phytoplasmas (Table 1), using the Molecular Evolutionary Genetic Analysis (MEGA), software, version 4 (Tamura et al. 2007), with the Neighbour -Joining method. Bootstrapping was performed 1,000 times, and *Acholeplasma laidlawii* served as the outgroup.

Computer-simulated RFLP analysis—virtual RFLP Computer-simulated RFLP analysis was performed on sequences of the 1,246-bp genomic fragment of an isolate from cauliflower (CfS) and chayote witches' broom phytoplasma (ChWB, GenBank accession number AF147706). The trimmed and aligned sequences were exported to the pDRAW32 program (AcaClone Software) for computer-simulated restriction digestion and virtual gel plotting (Wei et al. 2007). Each aligned DNA fragment was digested *in silico* with the 17 restriction enzymes previously indicated for phytoplasma classification, and a virtual 3.0 % agarose gel electrophoresis image was plotted and captured as a PDF file (Wei et al. 2007). The virtual RFLP profiles obtained for strains representative of the phytoplasma found in cauliflower were compared with those generated by the chayote phytoplasma.

Transmission by dodder Two symptomatic cauliflower plants were collected in the field and kept in pots under greenhouse conditions. The presence of phytoplasma was confirmed by nested PCR (primers P1/Tint and R16F2n/16R2) and identification by actual RFLP analysis. Dodder (*Cuscuta subinclusa*) was grown on both cauliflower plants. From each one, dodder was established on three periwinkle plants (*Catharanthus roseus*) grown from seeds. Transmission was evaluated based on symptoms exhibited by the periwinkle plants and detection of phytoplasmas by nested PCR.

Spatial pattern analysis The spatial pattern of cauliflower stunt was determined in ten plots from a farm located in São Paulo State, 23° 30′ 06″ S and 47° 27′ 29″ W, Brazil. The plots had similar conditions of management and were surrounded by forests. The incidence of disease was assessed by visual inspection of the plants for the symptoms of stunting, malformation of the inflorescence and necrosis of conductive vessels. The location of each symptomatic plant was recorded on maps. Plots 1 to 3 were planted with hybrid Barcelona and assessed in September 2007, plots 4 to 7 were planted with hybrid Cindy and assessed in March 2008, and plots 8 to 10 were planted with hybrid Sarah and assessed in April 2008. The number of plants in each plot ranged from 300 to 728. The analysis of distribution of diseased plants in each plot was performed on quadrats of four (2 by 2), nine (3 by 3) and 16 (4 by 4) plants.

The dispersion index (D), a function of the observed variance (V_{obs}) and the binomial variance (V_{bin}), was estimated for each area and its significance was tested by the chi-square test (Gottwald et al. 1996). A value of D statistically equal to 1 indicates a random distribution of symptomatic plants in a given area. A value of D greater than 1 indicates aggregation (Gottwald et al. 1996).

The binary form of Taylor's power law relates the observed variance (V_{obs}) and the expected binomial variance (V_{bin}). In this case, $\log(V_{obs}) = \log(A) + b\log(V_{bin})$, where $\log(A)$ and b are parameters. Linear regressions were performed for all plots in each quadrant size by the least squares method. A random spatial distribution of symptomatic plants is inferred when $b=1$ and $\log(A)=0$. There is a constant level of aggregation for all incidence values when $b=1$ and $A>1$. When $b>1$, the degree of aggregation varies according to the disease incidence. The equality of parameters b and $\log(A)$ was tested by the t-test, using the parameter estimate and its standard error (Madden et al. 1995).

Isopath areas for each plot were calculated with Statistica 6.0 software (Stasoft) using the standardized least square weighted by distance. The matrix of the non-transformed proportion of diseased plants in each quadrat was used. For each plot, the number of isopath areas previously chosen was the same. However, the levels of each isopath area were arbitrarily chosen to highlight possible differences.

Results

The presence of phytoplasma was detected in 16 out of 20 symptomatic plants by the amplification of approximately 1.2-kb DNA fragments using nested PCR primed by the P1/Tint-16F2n/R2 primer pair. Amplification products were also obtained from DNA extracted from 10 out of 17 symptomless plants, indicating

infection by phytoplasma. Further PCR analysis of positive cauliflower samples using group-specific primer pairs yielded a 0.8-kb product, indicating that the phytoplasma detected in both symptomatic and asymptomatic plants was a member of 16SrIII group.

The collective RFLP patterns generated by the restriction enzymes *Alu*I, *Hpa*II, *Kpn*I, and *Mse*I revealed that all cauliflower isolates were indistinguishable from each other and were identical to the patterns produced by the Chayote witches' broom phytoplasma, representative of the subgroup 16SrIII-J. Further RFLP analysis performed with the eight selected isolates revealed indistinguishable restriction patterns for the endonucleases *Bsh*1236, *Hha*I, *Mbo*I, and *Rsa*I, which were identical to those produced for the reference phytoplasma. All restriction enzymes characterized the phytoplasma found in the cauliflower as a member of group 16SrIII, but *Hha*I and *Bst*UI were the key enzymes that allowed classification of this phytoplasma in the 16SrIII-J subgroup.

Two out of the eight isolates identified by actual RFLP were chosen for sequencing. Three clones of each isolate were sequenced, and a majority consensus sequence was selected for each isolate. Because the results of sequencing showed that the majority of the consensus sequences were identical, one of them was selected to represent the phytoplasma found in cauliflower. This 1246-bp sequence was designated CfS and deposited in GenBank under the accession number HM237045.

Virtual RFLP patterns generated by the phytoplasma detected in cauliflower were identical to those produced by chayote phytoplasma for all 17 restriction enzymes used for *in silico* digestion (Fig. 2).

Analysis of the nucleotide sequences of 16S rDNA demonstrated 99 % similarity between the sequence of cauliflower phytoplasma and the sequence from chayote witches' broom phytoplasma (GenBank: AF147706) belonging to the 16SrIII-J subgroup. The similarity coefficient (*F*) based on virtual RFLP patterns produced by 17 enzymes was calculated as described elsewhere (Lee et al. 1998). The results revealed that the *F* value was equal to 1.0 when the DNA sequence of the phytoplasma found in association with the diseased cauliflower was compared to the nucleotide sequence of representative of the 16SrIII-J subgroup.

Phylogenetic analysis of phytoplasmas from 20 groups, 11 subgroups of the 16SrIII group, and the isolate representing the phytoplasma found in cauliflower generated the tree shown in Fig. 3. The arrangement of branches, supported by bootstrapping values, revealed that the cauliflower phytoplasma is closed related to other members of the 16SrIII group and specifically affiliated with the phytoplasma of 16SrIII-J subgroup, in agreement with previous results of PCR assays and RFLP analysis.

The transmission of phytoplasma from cauliflower to periwinkle was observed approximately 30 days after the first contact between periwinkle plants and dodder. Initial symptoms were characterized by foliar chlorosis,

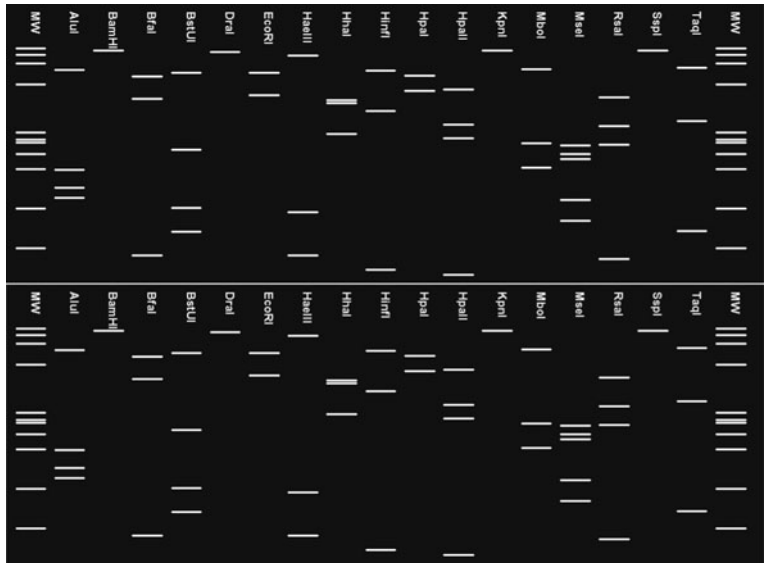

Fig. 2 Virtual RFLP patterns from the *in silico* digestion of the 16S rRNA gene. F2nR2 fragments from cauliflower stunt phytoplasma (CfS) (*top*) and Chayote witches'-broom (ChWB) (*bottom*), used as a reference for the 16SrIII-J subgroup. Simulated digestions were performed with 17 endonucleases: *Alu*I, *BamH*I, *Bfa*I, *Bst*UI, (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. MW=PhiX174DNA-*Hae*III marker

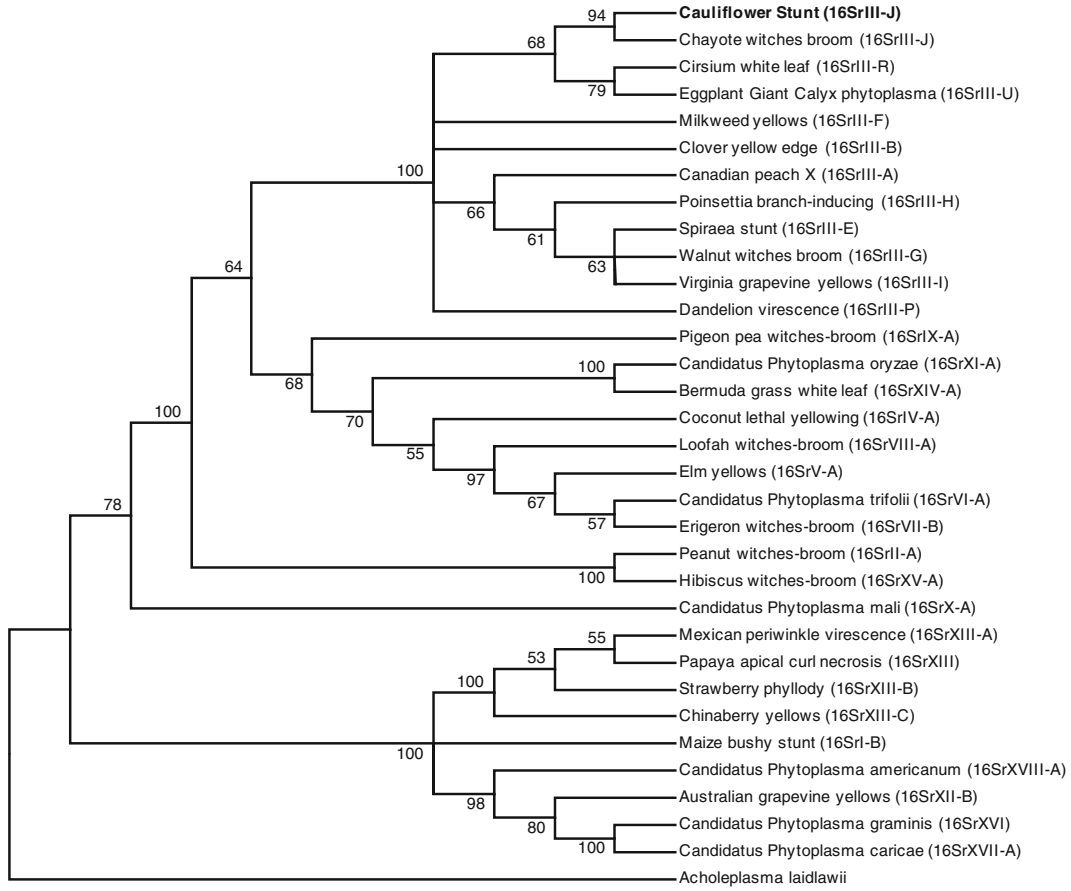

Fig. 3 Phylogenetic tree based on the sequence of 16S rDNA from the phytoplasma associated with cauliflower stunt (CfS, this paper), phytoplasmas representative of 20 groups, phytoplasmas belonging to 11 subgroups of group 16SrIII, and *Acholeplasma laidlawii* as the outgroup. The numbers on the branches represent bootstrap values for 1,000 replicates

followed by shoot proliferation and generalized stunting. PCR assays consistently demonstrated the presence of phytoplasma associated with diseased tissues, and molecular identification confirmed that the phytoplasma detected in periwinkle was identical to those found in cauliflower plants.

The disease incidence ranged from 4 % to 42 %. The binomial index of dispersion (D) showed an aggregated pattern of diseased plants for the majority of the plots with a quadrat size of 4 by 4 (Table 2), especially for incidence values higher than 25 %. The D values were usually higher for the largest quadrat sizes.

Estimates of b values were statistically different from 1, and $\log(A)$ values were statistically higher than zero ($P<0.05$), which indicated a significant pattern of aggregation of symptomatic plants within 3 by 3 and 4 by 4 quadrat sizes (Table 3). Values of b greater than 1 also indicated that the degree of aggregation was a function of disease incidence. The relationship between $\log(V_{obs})$ and $\log(V_{bin})$ was significant for 3 by 3 and 4 by 4 quadrat sizes, where most of the points were located above the binomial line (Fig. 4).

Each level of grey in the isopath areas represents a range of disease incidence (Fig. 5). Disease outbreaks on the edge of the plots indicate that the disease was probably introduced from outside the plot and advanced toward the center of the areas. Foci also arose in the center of the plots as the incidence of disease increased, as occurred in plots 3, 4 and 5.

Discussion

The initial diagnosis, based on symptoms present in cauliflower plants, was subsequently confirmed by the amplification of 1.2-kb DNA fragments from 80 % of

Table 2 Disease incidence (*p*) and dispersion index (*D*) analysis of cauliflower stunt based on symptomatic plants

Plots	*p*	*D*[a]		
		2×2	3×3	4×4
1	0.0590	1.1952	1.1439	1.0895
2	0.0460	1.0130	0.9524	0.9002
3	0.0675	1.1239	1.3553	1.2843
4	0.1700	1.0612	0.9806	1.4653
5	0.3695	1.1598	1.5979*	1.9913*
6	0.3377	1.1385	1.1848	1.5499*
7	0.3805	1.1919*	1.3943*	1.4438*
8	0.2602	1.2076*	1.3456*	1.8722*
9	0.3057	1.3089*	1.5449*	1.6510*
10	0.4273	1.4099*	1.7380*	1.9164*

[a] *D* values for indicated quadrat size by plot and assessment date for cauliflower plots with symptomatic plants. Values presented for each assessment date are *D* (=observed variance/binomial variance). Significances (*) were calculated with chi-square distribution. Values of *D* not significantly from 1 indicate that the pattern of symptomatic plots is random. Values of *D* significantly higher than 1 ($P<0.05$) indicate aggregated pattern of symptomatic plants

the symptomatic plants, indicating that a phytoplasma was consistently associated with the disease, which was here designated cauliflower stunt (CfS). The detection of phytoplasma in symptomatic plants of periwinkle, used in pathogenicity assays performed with dodder, confirmed that a phytoplasma was present in affected cauliflower plants. The cauliflower plants exhibited symptoms characterized by stunting, a reduction in size, malformation of the inflorescence, foliar edges with reddening, and dark discolouration of the vessel region (Fig. 1). Sometimes small sprouts were found in the base of the stem. These symptoms were more intense when the plants were infected in the early stages of growth.

Table 3 Log(*A*) and *b* parameters for Taylor's power law and R^2 for each quadrat

Quadrats	*b*	Log(*A*)	R^2
2×2	1.07	0.18[a]	0.98
3×3	1.18[a]	0.44[a]	0.96
4×4	1.33[a]	0.86[a]	0.98

[a] Values significantly different from 1 for *b* and zero for log(*A*), by *t*-test, at 5 % probability

The detection of phytoplasma in symptomless cauliflower plants provides evidence that the incidence of disease could be underestimated when evaluated solely by the observation of symptomatic plants present in the field. Symptomless plants harbouring phytoplasmas were reported for other species of the *Brassica* genus, including cabbage, broccoli, turnip (Wang and Hiruki 2001), and canola (*B. rapa*) and oilseed rape (*B. napus*) (Olivier et al. 2010). The detection of phytoplasmas in tissues of symptomless plants may be attributed to late infections caused by leafhoppers, as demonstrated in previous reports for canola and oilseed rape (Olivier et al. 2010) and lettuce (Zhou et al. 2002). Our findings are in agreement with this theory, because cauliflower crops have a short growing cycle, and plants infected recently would probably not express external symptoms within the growing season. Despite the high sensitivity of the technique used for detection, the phytoplasma was not evidenced in 20 % of symptomatic plants. The crop was grown in a relatively small and homogeneous area, the plants were submitted to the same practices, and were sampled in the same stage of growth pointing that the symptoms were induced by the phytoplasma. The lack of detection of the phythoplasma in symptomatic plants is an event that is not rare and has previously been described for diverse species as carrot, cabbage, onion (Lee et al. 2003) and potato (Santos-Cervantes et al. 2010). Low titres of phytoplasma in the sampled tissues (Wang and Hiruki 2001; Bertaccini and Duduka 2009) and irregular distribution in the host (Marcone 2010) must be considered among the diverse factors probably responsible for the lack of detection by PCR.

When the cauliflower phytoplasma and the phytoplasma representative of subgroup 16SrIII-J, used as reference, were compared based on actual and virtual RFLP analysis and value of the coefficient of similarity, it was confirmed that the cauliflower phytoplasma was a member of subgroup 16SrIII-J. In agreement, the branching of phylogenetic tree showed that the phytoplasma found in cauliflower was closely related to phytoplasma of the subgroup 16SrIII-J.

Although phytoplasmas of the 16SrI (aster yellows) group have frequently been found in plants in countries of the Northern hemisphere that

exhibit similar symptoms to those present in Brazil, the results described here revealed the presence of a phytoplasma belonging to the 16SrIII (X-disease) group in naturally infected cauliflower plants. In Brazil, representatives of the 16SrIII group have commonly been characterized in association with several botanical species. Diverse hosts are found among vegetable crops, including tomato (Amaral-Mello et al. 2006), chayote (Montano et al. 2000), eggplant (Amaral-Mello et al. 2011), pumpkin (Montano et al. 2006), and other species such as China tree (Duarte et al. 2009), begonia (Ribeiro et al. 2006), and *Celosia argentea* and *Celosia spicata* (Eckstein et al. 2012). Our results show that cauliflower is a new host for phytoplasma belonging to the 16SrIII group, specifically the 16SrIII-J subgroup. Previous investigations demonstrated that representatives of this subgroup are associated with chayote witches' broom (Montano et al. 2000), pumpkin yellows (Montano et al. 2006), and eggplant (Amaral-Mello et al. 2011). In addition, the results of the present study also revealed that a phytoplasma belonging to the 16SrIII-J group is associated with cauliflower stunt. In South America, phytoplasma of 16SrIII-J group was also found in association with Chinaberry trees in Bolivia (Harrison, et al. 2003) and garlic crops in Argentina (Galdeano et al. 2004). Moreover a phytoplasma associated with coffee crispiness in Colombia was also described as belonging to the same clade (Galvis et al. 2007). These findings suggest a low specificity of the agent in relation to the host species, and that the discovery of possible insect vectors for these pathosystems would be useful to delineate new management strategies for disease control.

The spatial analysis of cauliflower stunt revealed that the dispersion pattern of diseased plants is random when the disease incidence is lower than 25 % and aggregated at higher incidences. The cauliflower plots used in this study were adjacent to forests and had poor weed control. Specific studies to identify the vector of the disease have not been performed, but it is well known that phytoplasmas can be transmitted by sap-sucking insects, such as leafhoppers, planthoppers and psyllids (Weintraub and Beanland 2006). Insects that act as vectors of the agent of cauliflower stunt may arise from outside the plots and shelter in weeds among the rows. Plants that remain outside the plantation are fundamental to the maintenance of the inoculum and the spread of disease caused by phytoplasmas (Weintraub and Beanland 2006). The control of weeds may also contribute to modify the incidence of the disease (Weintraub and Beanland 2006).

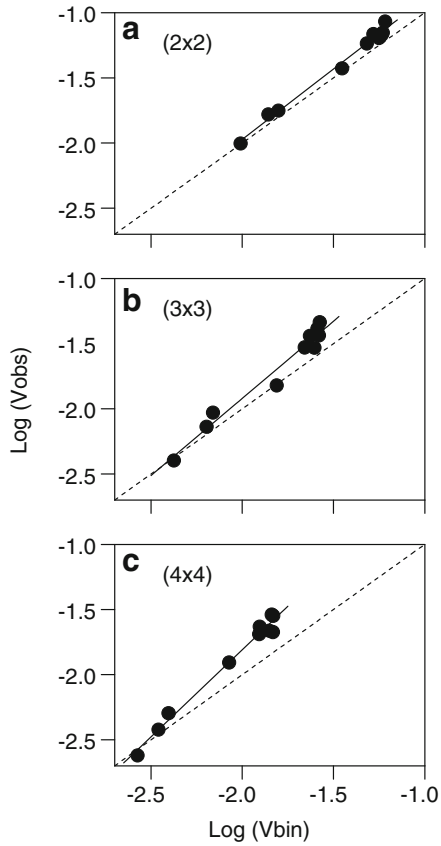

Fig. 4 Relationship between the log of the observed variance (V_{obs}) and the log of the theoretical binomial variance (V_{bin}) of the incidence of cauliflower stunt-diseased plants in ten plots in Brazil for three quadrat sizes. Log (A) is different from zero, and b is different from 1 ($P<0.05$). The *solid line* represents $\log(V_{obs}) = \log(A) + b\log(V_{bin})$ fitted to the data by linear regression. The *dashed line* is the binomial line (A=b=1). Each plot (◦) was assessed at one time. Quadrat size is indicated in each panel

Disease incidences higher than 5.2 % were required to detect aggregation of aster yellows in lettuce associated with phytoplasma (Madden et al. 1995). Beanland et al. (2005) reported for the pathosystem that disease incidences ranged from 3 % to 72 % and that most of the evaluated fields exhibited clustering of disease.

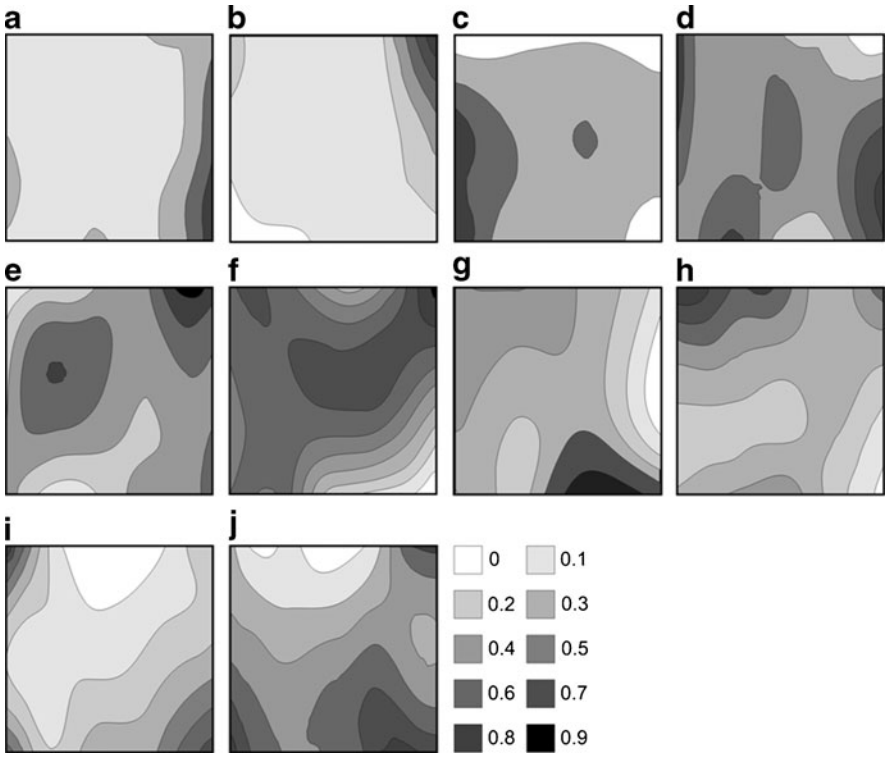

Fig. 5 Isopath area maps of ten cauliflower plots assessed for stunt disease. The colors indicate variations in incidence; i.e., *white* for lower incidence and *black* for higher incidence, in addition to the intermediate colors. The Y-axis represents the crop rows

The slope of Taylor's power law indicates that the spatial pattern of diseases with the vector depends more on the behaviour of the vector than on the characteristics of the pathogen or host (Taylor 1984). The slope obtained for cauliflower stunt varied from 1.07 to 1.33, depending on the quadrant size. Similar ranges of *b* for the same quadrat sizes were determined for other phytoplasma diseases: from 1.14 to 1.26 for coconut lethal yellows (Bonnot et al. 2010) and from 1.08 to 1.18 for lettuce aster yellows (Beanland et al. 2005; Madden et al. 1995). This similarity of *b* values indicates that the spread of these diseases follows the same pattern and is probably shared by the same group of vectors.

In the isopath analysis, it was observed that the foci of disease concentrated at the edge of the plots, indicating that the disease is introduced in the field from external areas. The presence of secondary foci when disease incidence increased indicates that the disease advanced toward the centre of the area, and phytoplasma insect vectors may be sheltered by weeds that grow among rows. In vineyards in North America, vines infected with phytoplasma exhibit aggregation, and the occurrence of diseased plants is related to the transmission by insects that move into the vines from adjacent woods (Beanland et al. 2005).

More studies are needed to identify the insects that act as phytoplasma vectors and to identify the weeds that are natural phytoplasma reservoirs. We suggest that the intensive cauliflower plantation in these areas ensures the simultaneous presence of the phytoplasma, and that the vector and weeds that grow inside and around the plantation area can result in a higher infection pressure.

Cauliflower stunt was confirmed as a disease associated with a 16SrIII-J phytoplasma, which is also associated with other diseases found in important horticultural species, mainly in South America. Epidemiological aspects revealed that diseased cauliflower plants were aggregated, and the pathogen was introduced from outside areas, indicating the presence of vector(s) in this pathosystem. Thus, the discovery of the vector(s) of this specific phytoplasma would improve the management strategies for disease control in cauliflower and other vegetable crops.

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