Characterization of a phytoplasma affecting sunflower crops in Argentina

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ABSTRACT

- Phytoplasmas are endocellular bacteria plant pathogens that are classified into the class *Mollicutes* and cause numerous diseases in different plant species. They exclusively inhabit phloem sieve elements of plants and are transmitted by phloem-feeding insects. Phytoplasmas are characterized by a small genome and by lack of a cell wall. Sunflower plants (*Helianthus annuus* L.) with symptoms of reduced and yellow leaves, shortened internodes, abnormal branches and capitulum with different degrees of deformation: phyllody and virescence, both ray florets and tubular flowers transformed into smaller head, were observed during the 2010/2011 season in Pedro Luro, Buenos Aires province, Argentina. The objective of this work was to identify and characterize this pathogen.
- Ultrathin sectioned phloem vessels of symptomatic and healthy plants were observed by electron microscopy. Total DNA was extracted from healthy and symptomatic plants and analyzed by PCR using universal primers (P1/P7; R16F2n/R16R2) for phytoplasmas.
- PCR using phytoplasmas universal primers amplifies a 1.8 kb and 1.2 kb fragment respectively, including the 16S rDNA gene the 16S-23S spacer region and the 5' end of the 23S rDNA gene. PCR amplification fragments of the expected size were obtained in diseased plants when P1/P7 and R16F2n/R16R2 primers were used. Ultrathin sections of leaf midrib from symptomatic plants revealed abundant pleomorphic bodies in the phloem tissue, by transmission electron microscopy observations. For the analysis of the restriction fragment length polymorphisms (RFLP) patterns, R16F2n/R16R2 PCR products were digested with the restriction enzymes *Hha*I, *Alu*I and *TRU1*L (*Mse*I). P1/P7 PCR fragments were cloned into pGEM-T vector and competent *Escherichia coli* strain JM109 cells were transformed. The obtained sequences were assembled using SeqMan program (Lasergene) and manual adjustment was done when necessary. Almost full 16S rRNA gene and 16S–23S spacer region nucleotide sequences were aligned using the *ClustalW* option of the Meg-Align programme. The resultant sequence of 1806 nucleotide was analyzed using BLAST (NCBI) and aligned with other related phytoplasma sequences. A phylogenetic tree was constructed using the neighbour-joining method, with MEGA4 software, comparing several '*Candidatus* Phytoplasma', related sequences and *Acholeplasma palmae* as the out-group, to root the tree. Bootstrapping was performed 1000 times to estimate the support of the inferred clades. The results from PCR-RFLP of the 16S rDNA, according to the profile obtained with restriction enzymes, showed the affiliation of the sunflower phytoplasma (SunPhy) to the 16SrIII (x-disease group), subgroup J. The 16Sr DNA sequence from SunPhy showed the highest identity (99%) with member of 16SrIII. The phylogenetic tree included the sunflower phytoplasma into the 16SrIII (x-disease) group and showed a closer relationship to 16SrIII subgroup B and J phytoplasmas, already identified in Argentina and Brazil, than other x-disease group sequences.
- These results extend the knowledge of phytoplasmas belonging to x-disease group, demonstrating its wide geographical distribution and numerous hosts. This is the first report of a phytoplasma infecting sunflower plants in South America.
- The study of natural reservoirs and vectors are important subjects to understand this phytoplasma disease.

Key words: phytoplasma; 16SrDNA – RFLP; x-disease

INTRODUCTION

Phytoplasmas comprises a group of phytopathogenic bacteria in the class *Mollicutes*, which cause numerous diseases and losses in many crops, ornamentals, fruit trees and also affect wild plants with worldwide distribution (Lee et al, 2000). The "coconut lethal yellowing" is a disease caused by phytoplasmas that affect coconut (*Cocos nucifera* L.) production dramatically in Central America and the Caribbean area, causing significant economic losses in just a few decades, killing over 80% of plants. Another example of disease caused for phytoplasmas is the "corn stunt" produced by the MBS (Maize Bushy Stunt) found all over America, which causes great losses in corn production in the region. Phytoplasma diseases have been observed also in crops such as sugarcane (*Saccharum officinarum* L.), stone and seed fruit (*Prunus* spp., *Malus* spp.), grapevine (*Vitis vinifera*), citrus (*Citrus* spp.), potato (*Solanum tuberosum* L.), among others (Lee et al, 2000).

Phytoplasmas replicate within both insect and plant hosts. In plants, they inhabit nutrient-rich phloem tissues and cause growth disorders, leaf and floral alterations, and abnormal proliferation (Hoshi et al, 2009). Insects capable of transmitting phytoplasmas comprise members of the order Hemiptera, including leafhoppers, planthoppers and psyllids (Lee et al, 2000; Weintraub and Beanland, 2006). Phytoplasmas are cell wall-free prokaryotes, which has the smallest genome size among the bacteria (Christensen et al, 2005). The inability to culture phytoplasmas *in vitro* has hindered their characterization at the molecular level and thus cannot be characterised by the traditional techniques used for bacterial classification. Analyses of full genomic sequences of four phytoplasmas recently determined, indicate that these plant pathogens have lost many metabolic genes, suggesting that reductive evolution is a consequence of being an intracellular parasite (Hoshi et al, 2009). The classification of phytoplasmas is based on the analysis of restriction patterns (PCR-RFLP) and nucleotide sequences of highly conserved genomic regions, such as the ribosomal RNA, ribosomal proteins and Tu elongation factor genes. By means of the analysis of restriction patterns of the 16S rRNA gene, phytoplasma "16Sr" groups have been established. In general this classification coincides with that resulting from the phylogenetic analysis of the genes sequences mentioned above (Lee et al, 1998; Zhao et al, 2010).

During the last years, phytoplasmas from different 16Sr groups have been detected and characterized in South America. In Argentina they have been identified in economically important crops such as garlic (*Allium sativum* L.), alfalfa (*Medicago sativa* L.), strawberry (*Fragaria* x *anannassa* Duch), china-tree (*Melia azedarach* L.) and maize (*Zea mays* L.), causing damage and variable losses (Galdeano et al, 2004; Torres et al, 2004b; Conci et al, 2005; Arneodo et al, 2007; Fernández et al, 2008). These pathogens were also detected affecting ornamentals, weeds, and native plants (Torres et al, 2004; Meneguzzi et al, 2008; Guzmán com. pers).

During 2010/2011 season in Pedro Luro, Province of Buenos Aires, Argentina, sunflower plants (*Helianthus annuus* L.) developed severe symptoms of reduced and yellow leaves, shortened internodes, abnormal branches and capitulum with different degrees of deformation: phyllody and virescence, both ray florets and tubular flowers transformed into smaller head (Fig. 1a, b and c). The objective of the present work was to determine the causal agent of the observed symptomatology, identify and characterize this pathogen.

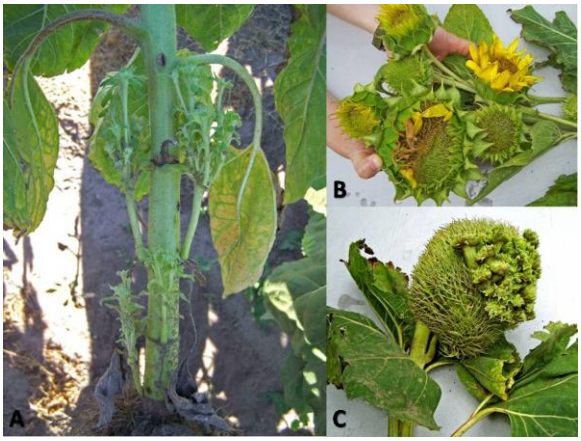

Fig. 1. Plant showing shortened internodes and abnormal branches (A). Plant capitulum with different degrees of deformation, phyllody and virescence (B). Capitulum with capitulums, phyllody, virescence and flowers transformed in smaller capitulums (C).

MATERIALS AND METHODS

Fresh leaf midribs (0.5 g) of healthy and six symptomatic sunflower plants were ground in liquid nitrogen and total DNA was extracted with CTAB (hexadecyltrimethil-ammonium bromide) following the Doyle and Doyle protocol (1990). DNA concentration was determined spectrophotometrically (Sambrook et al, 1989) and estimated by 1% agarose gel electrophoresis stained with ethidium bromide.

Ultrathin sectioned phloem vessels of symptomatic and healthy plants were observed by electron microscopy. Small pieces of tissues (2 x 3 mm) collected from leaf midribs were fixed in glutaraldehyde 2.5%, paraformaldehyde 2.5%, postfixed in osmium tetroxide 1% and contrasted in uranyl acetate 0.5%. The samples were dehydrated in acetone and embedded in Spurr low viscosity resin. Ultrathin sections were performed with a diamond knife and contrasted with lead citrate and uranyl acetate 2%. The sections were observed on a transmission electron microscope (EM) (Jeol JEM 1220).

Two pairs of universal primers were used in direct PCR assays, P1/P7 primers (Schneider et al, 1995) to amplify a 1.8 kb fragment including the 16S rRNA gene, the 16S–23S spacer region and the 5´ end of the 23S rRNA gene. The parameters used were: 35 cycles of 1 min denaturation at 95 ºC (3 min for the first cycle), 30 sec annealing at 55 ºC and 2 min polymerization at 72 ºC. Second pair of primers used were R16F2/R16R2 that amplify a 1.2 kb fragment corresponding to 16S rRNA gene (Lee et al, 1993), the cycling conditions were: 35 cycles of 1 min denaturation at 94 ºC, annealing for 2 min at 54 ºC and primer extension for 2 min at 72 ºC. DNA from healthy plants and without DNA templates were included in all cases as negative controls. PCR-RFLP analysis was carried out using R16F2/R16R2 PCR products digested with the restriction enzymes *Hha*I, *Alu*I and *TRU1*L (*Mse*I) (New England Biolabs). Restriction fragments were resolved in 1.5% agarose (Biodynamics) + 0.5% agarose Metaphor® (BioWittaker Molecular Applications) gel, buffered in TBE, stained with ethidium bromide and visualized by UV light. The group and subgroup classification was done by comparison with the published data (Lee et al, 1998). In order to sequence the 16S DNAr amplification of sunflower phylody phytoplasma (SunPhy), the P1/P7 PCR amplification was ligated to pGEM-T vector (Promega) according to manufacturer's instructions and competent *Escherichia coli* strain JM109 cells were transformed. The sequences obtained were assembled using the SeqMan program (Lasergene software, dnastar ver. 5, 2001), and manual adjustment was done when necessary. Almost full 16S rRNA gene and 16S–23S spacer region nucleotide sequences were aligned using the ClustalW (Meg-Align) with other related sequences to determine the similarity percentage. A phylogenetic tree was constructed using the neighbour-joining method, with MEGA4 software, comparing several '*Candidatus* Phytoplasma', related sequences and *Acholeplasma palmae* as the out-group. Bootstrapping was performed 1000 times to estimate the support of the inferred clades.

RESULTS

These results are the first report of a phytoplasma that infects sunflower plant in Argentina. The presence of abundant pleomorphic bodies within phloem cells has been established by EM observation of ultrathin sections of leaf midrib in symptomatic plants (Fig 2) and was not observed in healthy ones.

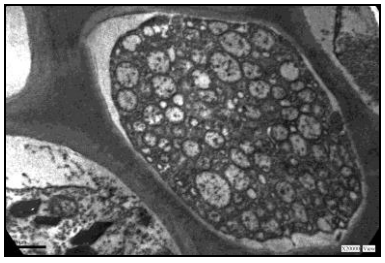

Fig. 2. Transmission electron micrograph showing abundant pleomorphic bodies (white arrow) of phytoplasma in phloem cells of sunflower plants.

DNA amplification and RFLP analysis

PCR products of 1.8 kb and 1.2 kb were obtained from symptomatic plant DNA using universal primers P1/P7 and R16F2/R16R2 respectively, there was no amplification in healthy plants DNA and negative control (Fig. 3).

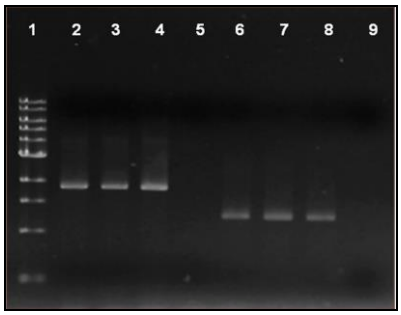

Fig. 3. PCR products obtained with universal primers P1/P7 (wells 2 to 5) and R16F2/R16R2 (wells 6 to 9) only three of the six samples are shown)
1- DNA Ladder 1 Kb (NEB)
2 and 6 - plant Nº 1
3 and 7 - plant Nº 2
4 and 8 - plant Nº 3
5 and 9 - healthy plant

The detected phytoplasma was classified on the basis of RFLP analysis, according to the classification scheme previously established by Lee et al, 1998. The profile obtained was compared with members of 16SrIII subgroups J and B, showing the affiliation of the SunPhy to the 16SrIII (x-disease group), subgroup J. All the analyzed samples produced the same restriction patterns with *Hha*I, *Alu*I and *TRU1*L (*Mse*I) enzymes (Fig. 4).

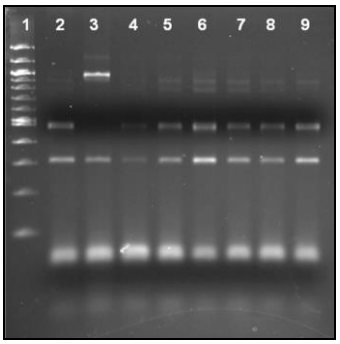

Fig. 4 RFLP analysis of 1.2 kb PCR product (R16F2/R16R2 primers) of SunPhy digested with *Hha*I
1- 1 Kb DNA Ladder (NEB)
2- X-disease subgroup J (control)
3- X-disease subgroup B (control)
4 to 9- Sunflower symptomatic plants

Phylogenetic analysis

The 1806 bp of 16Sr DNA sequence from SunPhy showed the highest identity (99%) with members of 16SrIII. The phylogenetical analysis, using a complete sequence of 16Sr rDNA, clustered SunPhy with high confidence values (100) into the x-disease phytoplasma group (16Sr III), forming a particular cluster with *Garlic decline phytoplasma* (16Sr III-J) and *Chayote witches'-broom phytoplasma* (16Sr III-J), already identified in Argentina and Brazil respectively (Fig. 5).

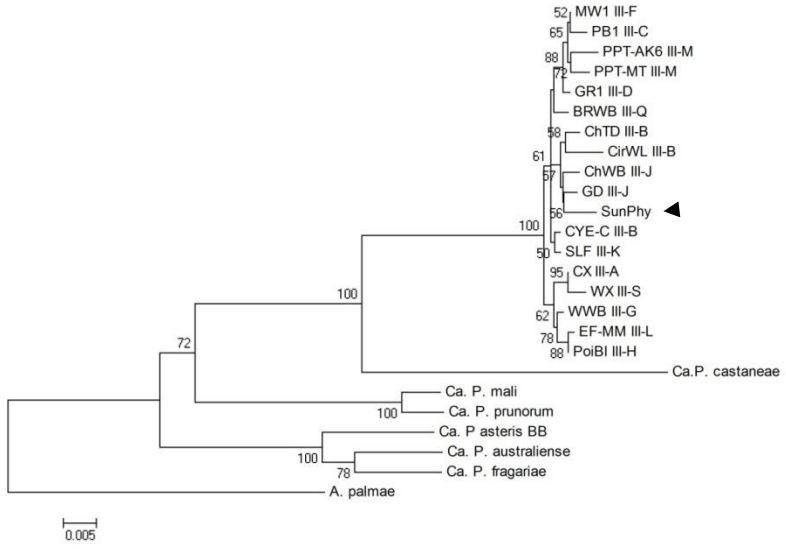

Fig. 5 Phylogenetic tree constructed by neighbour-joining method of 16S rRNA gene sequences from 17 representative isolates of 16Sr III group, six '*Ca*. Phytoplasmas' and *Acholeplasma palmae* as an outgroup. The numbers on the branches are bootstrap (confidence) values. The arrow shows the SunPhy sequence.

DISCUSSION

In this work we identify and characterize a phytoplasma in a new host in Argentina. The sunflower phyllody phytoplasma is associated with the symptomatology descript above. The RFLP analysis of 16Sr DNA SunPhy sequence determined that this patogen belongs to 16SrIII group (x-disease), subgroup J. Phylogenetic analysis of this sequence has shown a closer relationship with phytoplasmas from subgroup J, which is consistent with RFLP analysis. The x-disease group (16SrIII) comprises several subgroups. It is one of the largest and most heterogeneous phytoplasma group, including pathogens of various plant hosts, with symptoms that range from yellowing to witches´ broom and decline around the world (Seemüller et al, 1994; Davis et al, 1998). In South America, phytoplasmas from 16SrIII, subgroup J, has been associated with Chayote witches´-broom (ChWBIII) in Brazil (Montano et al, 2000), Garlic Decline in Argentina (Galdeano et al, 2004) and China tree yellows found in Bolivia (Harrison et al, 2003). In Argentina several diseases have been associated with phytoplasma infections (Gómez et al, 1996; Conci et al, 1998; Galdeano et al, 2004, Torres et al, 2004, 2004b; Conci et al, 2005; Arneodo et al, 2007; Meneguzzi et al, 2008; Fernández et al, 2008) where group x-disease is the most widely represented, just as in Brazil. A very interesting fact to consider is that all x-disease phytoplasmas detected in South America belong only to subgroups B and J and not to other 16SrIII subgroups (Galdeano et al, 2004). Other studies suggest that an evolutionary divergence could have occurred in South American phytoplasmas originated by geographical or ecological separation (Montano et al, 2000, 2001; Barros et al, 2002; Conci et al, 2005) fact that could also be related to the distribution of x-disease, and other 16Sr groups in South America.

The sunflower phytoplasma detected in Argentina is the first report of a phytoplasma from x-disease group affecting *Helianthus annuus* L. worldwide. Argentina is one of the main sunflower exporters in the world and therefore the SunPhy becomes more important. Previous studies have reported the infections caused by phytoplasmas from 16SrII and 16SrVI groups in sunflower in Iran (Tazehkand et al, 2010).

Epidemiological studies on phytoplasmal diseases reported a wide range of disease incidence, which has been related mainly to environmental conditions and the presence of efficient insect vectors. Keeping in mind that there are species of wild *Helianthus* in sunflower producing areas as wild sunflower (*Helianthus annuus* L.) naturalized in the central region or the prairie sunflower (*H. petiolaris* L.) that could act as a alternate host in nature would be very important initiate studies to establish the role played by these species in the disease. In order to increase the knowledge of the impact generated by these diseases and to control through strict quarantine regulations is necessary to study local phytoplasma genomic level, understand the fundamental biological mechanisms, to determine whether there are different phytoplasmas involved in each disease, identify alternative hosts, vectors involved and develop diagnostic systems.

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