OnLine Journal of Biological Sciences 9 (3): 62-66, 2009
ISSN 1608-4217

Detection of Phytoplasma on Indian Fig (*Opuntia ficus-indica* Mill) in Mexico Central Region

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Abstract: Problem statement: The Indian fig (a species of prickly pear cactus), has been known as Nopal, comprises an approximate area of 100,000 ha, in plantations used for human consumption. "Pyramids" Indian fig area located in the northeastern State of Mexico has been an important Indian-fig area in the country, with 15810 ha, where a phytoplasma has been consistently present in symptomatic plant. **Approach:** An unknown symptomatology in the Indian fig (prickly pear cactus) (*Opuntia ficus-indica* Mill) was analyzed through grafting and a nested-PCR reaction and graft on healthy plants grown in a greenhouse. **Results:** The symptoms found, deforming, buds proliferation, thickening and heart-shaping in cladodes, with arrested plant growth and deep yellowing of cladodes, were all attributed to the presence of a phytoplasma given the amplification of a 1200 pb fragment of the 16S rRNA gene using primers R16 F2/R2 and R16F2n/R2 and 80% of phytoplasma transmission efficiency of successful grafts. **Conclusion:** Although the symptoms observed did not completely match those described for this organism in the region, a 1200 pb fragment was amplified and PCR products restriction analysis leading us to assume that the phytoplasma corresponds to subgroup 16SrII, previously reported for other crops in others world regions.

Key words: Indian fig, phytoplasma, Mexico State, graft, PCR detection

INTRODUCTION

The Indian fig (a species of prickly pear cactus), also known as Nopal, nochtli or nopalli and "tuna" in Cuba (*Opuntia ficus-indica* Mill) is endemic of America, with 258 known species, 57 of which are distributed in Mexico. This cactus comprises an approximate area of 100 000 ha, in plantations used for human consumption[13]. The gourmet vegetable variety of Nopal treasured throughout Mexico, where Nopal *Opuntia ficus-indica* has been a staple food for the past 12,000 years. Also the nopal has been used in Mexico as forage, the nopal-vegetable system, provides about 240,000 tonnes of fresh salad food all over the year and is based on the use of approximately 3 million tons of cattle manure[19].

Specifically, the "Pyramids" Indian fig area located in the northeastern State of Mexico, with over 808 km^2, is home to some 212,029 inhabitants and is the most important Indian-fig area in the country, with 15,810 ha of prickle-pear cactus, 428 ha of "nopalito" cactus and 1350 ha of "xoconostle" cactus[23].

The *Opuntia* species, to which not much attention has been paid and which in many countries, such as Cuba, is not commercially exploited, represents an alternative crop species in areas with impoverished soils, useful in regions with water scarcity that restrain traditional crops.

This species has been used as forage for cattle in dry months, but is also very important as a vegetable for human consumption. It undergoes industrial processing to produce jelly, juice, pectin, fructose, cosmetics and colorants, also has medicinal properties. It represents a growing source of economic income, since it is exported to the US, Canada, Europe and Japan, with about 4000 tons per season[23].

The phytosanitary issues related to Indian fig cultivation include soft rots caused by "Mancha de Ojo" fungi (*Alternaria* spp.), prickly-pear black spot (*Colletotrichum gloesporoides* and *Pseudocercospora*

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sp.), bacteria like the one causing soft rot (*Erwinia carotovora*) and the recently reported cladode coining (male cactus), likely caused by a phytoplasma, as well as bud proliferation caused by a spiroplasma[15,22].

Phytoplasma associated diseases are spread worldwide. To date, some phytoplasmas have been reported related to cactus plants, including *Cactus witches broom phytoplasma* in bean (*Faba phyllody phytoplasma*), *Candidatus aurantifolia phytoplasma* and phytoplasma diseases on cactus pear in California[5-7,11]. This set of phytosanitary issues, among others, leads to significant production drops, of up to 50%, resulting in drastic economic losses for producers[10]. This investigation addresses a phytosanitary issue yet unknown in Saint Martin Pyramids (State of Mexico) through testing and standardization of a DNA purification protocol and molecular analysis by PCR focused on this species, providing a specialized technical-scientific service through the collaboration between CETAS/UCF (Cuba) and Gisena (Mexico), INBIOTECA (Mexico).

MATERIALS AND METHODS

In a field trip to Indian fig (*Opuntia ficus-indica* Mill) producing areas in May 2006 (Fig. 1), apparently diseased plants were observed, which were collected and taken to Gisena (Integrated Phytosanitary Services Group, ENA, SA de CV), located in Texcoco, State of Mexico, for analysis. Studies were conducted on symptomatology in collected specimens; additionally, diseased buds were grafted onto 10 healthy plants grown in a greenhouse.

Collected specimens (10) displayed serious deforming, excessive budding, rounded and long cladodes and atypical yellowing. Young buds and cladodes were sampled from both diseased and healthy specimens, from which DNA was purified and two extraction methods were compared: (a) DNAzol Plan Kit and (b) the extraction method used by[12] with CTAB (Cetyl trimethylammonium bromide). Afterwards, DNA quality was checked in a 0.8% agarose gel.

A nested PCR (Polymerase chain reaction) was done with DNA, using the universal primers of gene 16rDNA, F2/R2+[16] and R16F2n/R2 with modifications. A brief explanation of the steps involved follows: The sequence used in this step for amplification in the thermocycler was: 2 min at 94°C, (1 min at 94°C, 1 min at 56°C and 1 min at 72°C, 30 cycles) and 4 min at 72°C ending with 10°C ∞. Amplified fragments were observed in a 0.8% agarose gel in 1 × TAE stained with etidium bromide (0.5 µg) and observed in a UV transluminator.

RESULTS

Description of symptoms associated to phytoplasma in the field: 1 ha of Nopal production area was random tested in Saint Martin Pyramids (State of Mexico) to found disease disorder responsible for fig wilt disease (Fig. 1). The symptoms were observed in around 15% of plant examined. In total of 10 cladodes samples were collected. After 60 days the phytoplasma transmission to healthy *Opuntia* plants, we found that transmission efficiency occurred in 80% of successful grafts.

A number of symptoms were observed in specimens collected in the field, including yellowing in old plantations, colorless of cladode epidermis, cladode deforming and curly. Also, cladodes may appear yellow and thickened without losing their typical ear-like shape or, alternatively, produce heart-shaped cladodes (Fig. 2). Rounded shapes may be found in couples and, in some cases, cladodes displayed atypical buds.

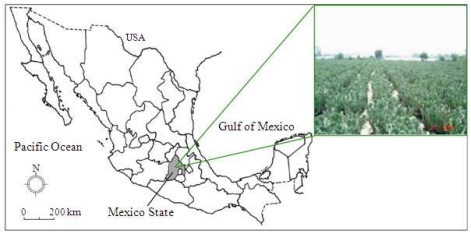

Fig. 1: Mexico map, showing the central region. The specific county the "Pyramids" Indian fig area located in the northeastern State of Mexico, with over 808 km^2

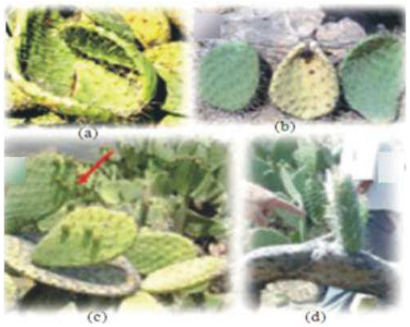

Fig. 2: Symptoms found in field were yellowing in old plantations; (a): De-coloring of cladode epidermis, cladode deforming; (b): Cladodes may appear yellow and thickened without losing their typical ear-like shape or, alternatively, produce heart-shaped cladodes: (c): And cladodes displayed atypical proliferation buds; (d): Cladodes displaying a heart shape, with buds, or thickened as a finger

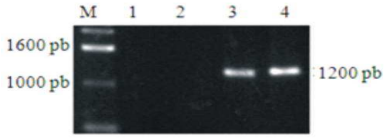

Fig. 3: Electroforesis of agarose gel 0.8% in TAE 1X showing the nested-PCR results. The fragments 1,200 bp correspond to phytoplasma 16S rRNA gene; (Lane 1): Negative control; (Lane 2): Health plant; (Lanes 3 and 4): Disease plants with symptoms

ADN extraction and analysis through nested PCR: During Indian fig ADN extraction using both methods (DNAzol Plan Kit and CTAB extraction), optimal purification was achieved when samples were taken from young leaves. However, low yields (50-100 ng mL^{-1}) occurred when old leaves were used. ADN quality was confirmed through spectrophotometry, followed by PCR analyses with those samples that yielded favorable readings, such as 3 and 4 and exceptionally others where extracted DNA was not clearly differentiated, like 1 and 2, for comparison purposes (Fig. 3).

The presence of phytoplasmas was confirmed using nested-PCR with primers R16 mF2/R1 and R16F2n/R2. Last, in several samples analyzed through PCR from young buds and cladodes, the presence of an amplified 1200 pb fragment was confirmed (Fig. 3). This amplified fragment matches the sequence of rDNA gene 16S used universally for phytoplasmas, hence confirming the presence of this pathogen in analyzed samples from the Pirámides Indian fig region located in northeastern State of Mexico.

For characterization, sequence of the amplified DNA was restriction analysis with the enzymes *Tru*9I. The *Tru*9I digestion pattern of the amplified product using nested-PCR with primers R16mF2/R1 and R16F2n/R2 detected in *O. ficus-indica* was identical to theorethical digestion pattern obtained from Candidatus Phytoplasma aurantifolia (subgroup 16SrII-B) (Genbank Accession No. MOU15442)[26], Papaya bunchy-top disease (subgroup 16SrII) (Genbank Accession No. DQ286948)[4] and soybean phyllody phytoplasma (subgroup 16SrII-C) (Genbank Accession No. EF186816)[20].

DISCUSSION

Cacti are perceived as apparently strong plants due to their morphology, so that this is likely one reason that many diseases are widespread in the genera *Echinopsis*, *Opuntia* and *Cerus*, where grafting is frequently practiced.

Common symptoms observed in *Cactaceae* infested with phytoplasmas seem to disrupt the metabolism of growth regulators, which stimulates the production and promotes the growth of axillary buds. Consequently, plants grow rapidly under a metabolic unbalance which may be lethal. We observe symptoms of excessive stem and shoot proliferation similar a case to reported in *Opuntia monacantha*[11] This microorganism causes a disease that, in the early stages, is relative benign but later may kill the host, depending on the species and the environmental conditions.

Seemingly, some cacti species are immune, asymptomatic, or displaying only minor or masked symptoms. The underlying reason remains unknown. The most commonly reported symptoms or damage include: (a) Deformity and/or abnormal shape (stunted), with growth arrest; (b) Excessive bifurcations or dichotomies, with new buds in each areole, even to the plant's upper portion; (c) Epidermis colorless (manly yellow to off-white); (d) Necrosis and/or deformation in flowers and buds.

Most phytoplasmas reported in subtropical and tropical Australia have been assigned to the 16SrII group; however, members of group 16SrXII are associated to some economically important diseases including *Strawberry lethal yellows*, *Papaya dieback* and *Grapevine yellows*[2,3,25]. These phytoplasmas have been diagnosed through PCR with primers specific for the 16S rRNA gene and cause abnormal growth, severe cladode proliferation and lack of flowers, fruits and spine production, lacking the presence of viral particles under the electronic microscope[24].

Some typical symptoms have been previously described for Indian fig phytoplasmas, for example formation of multiple buds, called "witches broom"[8] following the method by Ahrens and Seemuller[1] and using primers R16m F2/R16m R1 and R16 F2/R2[17]. A 1.2 Kb rDNA fragment was found, which upon sequencing (accession AJ 293216) was aligned, displaying a high similarity to the subgroup 16S rII Fab bean phyllody phytoplasma (99.7%) (Accession No. X23432), a member of subgroup C, group 16Sr and with the "cactus witches-broom" phytoplasma found in Mexico (99.4%) accession AF200718, primers report associated with phytoplasma in Yunna (China)[8].

More recently, Choueiri *et al*.[11] confirmed the presence of phytoplasmas in Lebanon, with infections in *Monacantha opuntia* (Haworth) observed as symptoms evident with the stem showing excessive proliferation, concentrated in three types of symptoms; phytoplasma was detected through nested PCR with

amplification of 881 pb rDNA fragments using primers R16m F2/R1 and R16 F2/R2. This ADN sequence was registered as an accession in GenBank as AY939815. Such sequence appeared as highly similar to several strains (isolates) of the 16SrII phytoplasma group, with the closest similarity with two witches broom phytoplasma isolates found in cacti reported in China[8] and Mexico[18] with (99.8%), registered as Accessions in GenBank (AJ2932216 and AF320575), respectively, as well as in bean (*Faba phyllody phytoplasma*) accession X83432 (99.7%) and *Candidatus elaurantifolia phytoplsma* accession U15442 with 99.3%. This seems to be the first report of a phytoplasma infection belonging to group 16SrII. Similar results were reported by[12] in Australia in a legume, with symptoms described as *Australian luceme yellows* (ALuY), analyzed through electron microscopy and at a molecular level using specific primers for phytoplasmas belonging to region 16S-23S rRNA through PCR and LRLP+[21]. The sequence of amplified fragments matched the group of *Faba bean phyllody phytoplasma*, or subgroup 16SrII[9]. Other symptoms in bean (*Vicia faba* L.) observed in Antequera (Malaga, southern Spain) by Castro S and Romero[9] were attributed to phytoplasmas, following a phylogenetic analysis placed it in a cluster belonging to group 16SrIII along with the member of X-disease phytoplasma.

When ADN was extracted from symptomatic plants as described by Cai *et al*.[8], the authors amplified a sequence of 16S rDNA of specific phytoplasmas through nested PCR, using primers P1/P7, R16f2/r2 or fU5/rU3. Recently, Granata *et al*.[14] showed the aligned sequence corresponding to an Indian fig phytoplasma from plantations in Carlentini (Sicily, Italy) diagnosed through grafting, PCR and AFLP of gene 16S rRNA, a 1525 bp sequence belonging to the 16SrII-C ribosomal group was obtained.

Despite being one of the major Indian fig producers worldwide, Mexico has not conducted in-depth studies on phytoplasma infections and its dissemination across the main cactus areas. The present study reports new phytoplasma symptoms in the Piramides region, which do not totally match those reported to date; however, these are highly important, given that Indian fig represents the main source of income for local inhabitants and the current propagation practice is likely to favor a greater dissemination of this pathogen in the future.

CONCLUSION

The present research found in San Martín de Las Pirámides (State of Mexico) disease disorder responsible for fig wilt disease. The symptoms were observed in around 15% of plant examined. The phytoplasma transmission assays showed a high efficiency in 80 % of successful grafts. The 1200 pb fragment was amplified using nested-PCR with primers R16mF2/R1 and R16F2n/R2 specific to phytoplasma diagnostic. The PCR products restriction analysis leading us to assume that the phytoplasma corresponds to subgroup 16Srll.

ACKNOWLEDGMENT

This research was possible thank to source of financial and collaboration between two Mexican institution (GISENA and INBIOTECA, Universidad Veracruzana) and one Cuban (CETAS, Universidad de Cienfuegos).

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