Black spot caused by *Pseudocercospora opuntiae* in cactus pear productive systems of Jalisco, Mexico

María Judith Ochoa[1, 2], Luis Ángel Rivera[3], Ramón Ignacio Arteaga-Garibay[4], Marcos Daniel Martínez-Peña[4], Javier Ireta[3], Liberato Portillo[2*]

[1]Facultad de Agronomía, Universidad Nacional de Santiago del Estero, Argentina. Av. Belgrano 1912, CP 4200 Santiago del Estero, Argentina.
[2]Departamento de Botánica y Zoología, Universidad de Guadalajara. A. Post. 1-139, Zapopan, Jalisco 45101, México.
[3]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. Rancho Las Cruces, Tepatitlán de Morelos, Jalisco 47600, Mexico.
[4] Centro Nacional de Recursos Genéticos-INIFAP, Rancho Las Cruces, Tepatitlán de Morelos, Jalisco 47600, México.

*Corresponding Author: liberatoportillo@hotmail.com

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ABSTRACT

Black spot is an important fungal disease widely spread in different cactus pear production systems in Mexico. In Jalisco, the disease was detected in the 1990's; nowadays almost 100% of plantations are damaged by it. The objective of this paper was to study the morphological variability, pathogenicity and virulence of the causal agent in cactus pear production systems, for fruit and vegetable (nopalitos) crops, in Jalisco, Mexico. *Pseudocercospora opuntiae* was isolated and characterized morphologically and molecularly from cladodes collected in cactus pear production systems of Zapopan and Ojuelos showing advanced symptoms of the disease. *Pseudocercospora opuntiae* exhibited high growth rates and conidia development in malt extract at 2% in 16/8 h light/darkness at 26°C. Pathogenicity and virulence were tested in healthy cladodes under field and greenhouse conditions, as well as on individual cladodes, *in vitro* young explants and *Phaseolus vulgaris* inoculated with the fungus. *Pseudocercospora opuntiae* was able to infect under all established conditions, the first symptoms appeared 120 days after inoculation. This is the first report of isolation, identification, morphological and molecular characterization, and pathogenicity of the causal agent of cactus pear black spot in Jalisco, Mexico.

***Keywords*:** *Opuntia*; plant disease; pathogenicity tests; nopalitos.

INTRODUCTION

Opuntia species are native plants of several environments in the Americas, from arid zones at sea level to high elevation arid zones of Andean regions of South America. They are in tropical regions of Mexico, Central and South America and the Caribbean (Anderson, 2001), where temperatures are always above 5ºC and in areas of Canada may reach as low as -40ºC (Nobel, 2011). For this reason, these plants can be a valuable genetic resource for very diverse ecological zones (Nobel and Bobich, 2002).

According to Inglese (2010), in Mexico *Opuntia* is cultivated in 100,000 ha for fruit production and in more than 1'000,000 ha for forage production. The highest producing states are:

Distrito Federal with 4,159 ha; Morelos 1,745 ha; Estado de Mexico 785 ha (Quezada-Salinas *et al*. 2006) and Jalisco 2,400 ha for fruit and 1,500 ha for nopalitos, with Zapopan and Ojuelos being the primary producing municipalities (SIAP, 2013).

Production level in many cactus pear (*Opuntia ficus-indica* (L.) Mill.) plantation systems are low due to scarce economic and social conditions, germplasm quality, dissimilar environments and high incidence of plagues and diseases. Diverse diseases have been associated with decreasing cactus pear production; among the most important ones are "mal del oro", caused by *Alternaria alternata* (Fr.) Keissl. (Granata and Sidoti, 1997), a type of soft rotting related to the bacterium *Dickeya* (Fusickovsky, 2002).

Another recently reported disease is "black spot"; its causal agent was identified in central Mexico as *Pseudocercospora opuntiae* Ayala-Escobar, U. Braun, & Crous (Quezada-Salinas *et al.* 2006; Ayala-Escobar *et al.* 2006). However, the causal agent of black spot has also been suggested to be other genera (Flores *et al*. 2013). Likewise, the infection, colonization and sporulation process of the disease remain to be studied (Quezada-Salinas *et al*. 2013). This fungal pathogen has a great impact on those plantations without proper sanitary management, presenting plant losses from 70–100%, as black spot is a disease causing severe reduction of the photosynthetic area and eventually the death of cladodes (Ochoa, 2013). In the state of Jalisco this disease was detected in 1990, and since then its incidence has increased dramatically, but there is very little information about morphology, mechanisms of pathogenicity, and virulence of the causal agent of black spot. This condition has motivated the aim of the present work to isolate and characterize this fungus in different cactus pear productive systems in Jalisco, Mexico and to confirm it is the same causal agent previously described.

MATERIALS AND METHODS

Cladodes with different symptoms of the disease were gathered during the humid and dry seasons of 2012 and 2013 in cactus pear commercial plantations of Ojuelos (Lat. N 21º 43' 41.8", 2,280 masl) and Zapopan (Lat. 20º 78' 33", 1,420 masl), in Jalisco, Mexico, designated for fruit and vegetable (nopalito) production, respectively. Portions of cladodes (0.5 cm^2) were dissected from symptomatic tissues and the stomata cavity. They were disinfected with 1.5% sodium hypochlorite (NaOCl) for 1 min, rinsed three times with distilled sterile water, dried on paper towels, and established on water-agar (WA) (30 g/L of agar in distilled water). From this material, compatible pseudostromata and conidia of *Pseudocercospora* type were isolated and identified following Quezada-Salinas *et al*. (2006) and Ayala-Escobar *et al*. (2006). Samples and media were incubated under natural conditions.

Morphological characterization

In order to find the best conditions for morphological development and conidia production, the fungus was cultivated from 5 mm mycelium discs in Petri dishes containing *Opuntia* agar (OPA) (40 g cactus pear cladodes boiled for 10 min and then blended with 20 g agar, 1 L distilled water); potato dextrose agar (PDA) (200 g potatoes, 20 g de dextrose, 20 g agar, 1 L distilled water) (Gams *et al.*, 1998); WA medium; malt extract (MEA) (15 g malt, 20 g agar, 1 L distilled water) (Gams *et al*. 1998); and V8® juice suspension 30%. 10 Petri dishes for each medium were incubated 16/8 h light/darkness under near-ultraviolet lights at 27°C.

Evaluations were carried out after 30 and 60 days (d) by means of direct observations of colony diameter, color and texture, conidia production and mycelium growth. An analysis of variance and a multiple comparisons using a Tukey test were applied. Statgraphics Centurion XVI 2010 (version 16.1.15, StatPoint Technologies, Inc) was used to perform statistical analyses.

Morphological characterization of conidia was carried out with an Olympus® mod. CX 31 microscope with analysis of image Pro-bonus® software, size (length and width) and septa number for conidia were recorded. For every mounted sample, information from 30 isolated conidia was taken. Conidia were differentiated from conidiophores by color, size and abscission scar form, which was olive-green color and thin; its identification was done following Quezada-Salinas *et al.* (2006) and Ayala-Escobar *et al*. (2006).

Pathogenicity tests

Pathogenicity and virulence of the isolations were tested by means of inoculating pre-cut cladodes, young *in vitro* explants (in the laboratory), and cladodes of approximately six months of age in field and greenhouse conditions. In order to test pathogenicity in other host plants, *Phaseolus vulgaris* L. (Saparrat *et al.* 2009) was inoculated with a concentration of 8 x 10^{-3} mL^{-1} conidia, generated in MEA with a photoperiod of 16/8 h light/darkness at 27°C, using a phosphate buffer for conidia adherence. Pre-cut cladodes were inoculated with 200 µL with the same conidia concentration, once infected they were placed in chambers with 85% humidity. *In vitro* inoculation of young explants was carried out by injecting 20 µL of conidia suspension into stem tissue. *In vitro* explants were tested on WA with 25% of nutritive salts of MS (Murashige and Skoog, 1962) with 4 g L^{-1} activated charcoal. Twelve plants in field conditions of approximately six months of age were inoculated by manual aspersion and during the first 10 d were covered with plastic bags to increase relative humidity. Symptoms were evaluated every week by direct observation and following Quezada-Salinas *et al.* (2006) and Ayala-Escobar *et al*. (2006).

To evaluate pathogenicity activation and virulence of *Pseudocercospora* isolated from *Opuntia ficus-indica* cladodes, infection tests in *Phaseolus vulgaris* plants were performed, as this plant is susceptible to *Pseudocercospora griseola* (*Sacc.*) (Saparrat *et al.* 2009). Twelve plants of 20 d of development were used to test *Pseudocercospora* pathogenicity. Plant inoculation was carried out with colonies of 60 d of development with abundant conidia production on WA, MEA and OPA media. For *Phaseolus vulgaris* inoculation, 50 µL of conidia suspension were placed on two leaves. Controls were sprayed with sterile buffer; plants were placed into a micro tunnel, which was used as a humid chamber.

Molecular characterization

The isolated *Pseudocercospora* strain was subcultured on PDA agar at 28 °C for 72 h. Obtaining the total DNA for genetic identification was done as per Wallace (1987) for DNA extraction technique modified with phenol-isoamyl alcohol with proteinase K and RNase. The pellet was air-dried, and the DNA was resuspended in 100 µL of DNase-free water. The purified DNA sample was stored at -20 °C until use.

DNA samples were amplified using the primer forward ITS1 5'-CTT GGT CAT TTA GAG GAA GAT A-3' and ITS4 5'-TCC TCC GCT TAT TGA TAT-3' (White *et al.* 1990; Redecker

2000; Unoura *et al*. 2011). PCR products were purified using EZ-10 Spin Column PCR Products Purification Kit BS363 (BioBasic Inc., Carlsbad, CA, USA), according to manufacturer's instructions. The product was directly sequenced on an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems, Ontario, Canada) using the forward primer. Ambiguous and incorrectly called bases were manually corrected using BioEdit software, version 2.01 (Technelysium Pty Ltd) and Seaview software version 4.3.3 (Gouy *et al*. 2010). Sequences were then searched using the NCBI BLAST algorithm and GenBank database (http://www.ncbi.nih.gov); those from the top BLAST hits were downloaded for further phylogenetic comparison.

A multiple sequence alignment was performed using the program Clustal X, version 2.0, and the resulting alignment was edited using SeaView (Galtier *et al*. 1996). A phylogenetic tree was constructed based on the sequence distances using Maximum Parsimony algorithm. The phylogenetic analyses were performed using Mega 6 (Tamura *et al*. 2007). Stability or accuracy of the inferred topology was assessed via a bootstrap analysis of 1,000 pseudoreplicates.

RESULTS AND DISCUSSION

Morphological characterization

In an advanced condition of the disease, symptoms found in cactus pear commercial plantations of Ojuelos and Zapopan infected with black spot presented a black subcircular necrotic spot, having low incidence in summer and higher incidence in autumn and the beginning of winter. Coincidence of maximum rainfall periods and relative humidity is an ideal situation for conidia penetration across stomata, according to Ávila *et al*. (2004) for *Pseudocercospora* in olive plantations. The incubation cycle of this pathogen was very slow, with the first symptoms appearing 90 d after plants became infected, which agrees with Quezada-Salinas *et al.* (2006) and Ayala-Escobar *et al.* (2006).

Symptoms of fungal infection appear quickly once *Pseudocercospora* is established: in 8 days qualification goes from a 1 to a 7 in a heptadecimal scale (Figures 1A-G). Injuries initiate with cuticle discoloration in a light green circular shape spot with a small brown point at the center (Figure 1A), which become transparent and oily in appearance (Figure 1B) and an increase in the size of the central brown color, presenting a yellow margin (Figure 1C). Later, the cladode tissue presents a light brown color within the circular spot (Figure 1D), which changes to a dark brown color, presenting collapsed tissue (Figure 1E).

Affected tissues turned black and formations of conidiophores and conidia emerged in small gray protuberances (Figure 1F). Finally, the affected zone changed to a black color and sank across the cladode (Figure 1G). Injuries reached 3–4 cm diameter at the end of the infection cycle. Different symptoms can appear simultaneously in the same cladode.

Figure 1. Symptom progression caused by *Pseudocercospora opuntiae* in cactus pear after 90 d of infection. A) symptom 1, B) symptom 2, C) symptom 3, D) symptom 4, E) symptom 5, F) symptom 6, and G) symptom 7.

Conidiophores and conidia were obtained from *Pseudocercospora* isolations in WA medium after 60 d under natural conditions of light and temperature, which formed colonies of about 1 cm diameter and olive-green color mycelium.

Pseudostromata immersed across olive color stomata of 45-150 µm diameter were located in cactus pear infected tissue (Figuras 2A-C). Conidiophores were dark olive in color of subcylindrical fascicles, without branches (16.47 µm), and obclavate to cylindrical conidia with conical truncated base, obtuse ends, from 3 to 8 septa, and a thin olive color abscission scar (0.5-1.0 µm).

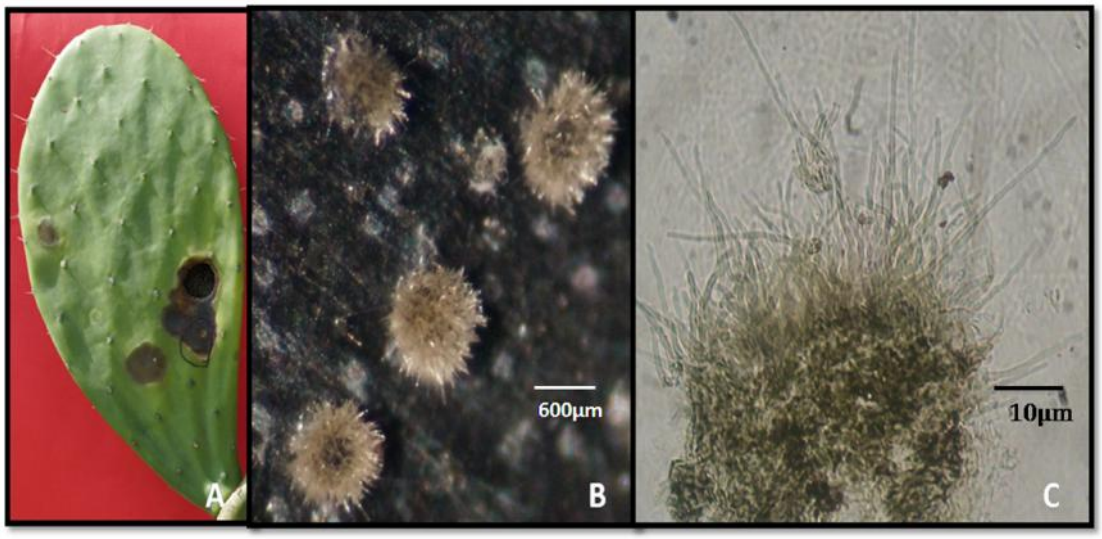

Figure 2. Symptoms of black spot in circular shape found in cactus pear plantations in Ojuelos and Zapopan, Jalisco, Mexico. A) cladode with circular dark brown spots with tissue necrosis, B) pseudostromata type with conidiophores in fascicles, C) pseudostromata with conidiophores and conidia (lateral view).

For conidia formation, the *Pseudocercospora* strain was transferred to MEA medium where a large number of conidia were generated after 30 d under 16/8 h light/darkness and 27°C. In this medium, olive-green inner mycelia developed with conidia of 20-89 µm length and 2.5-6.0 µm width on average, from three to eight septa with a size of 16.43 µm (Figures 3A-F). These morphological characteristics agree with those reported by Beiharz (1994) for *Pseudocercospora* and also match morphological analyses for *P. opuntiae* (Ayala-Escobar *et al*. 2006; Quezada-Salinas *et al.* 2006).

After 30 and 60 d of inoculation in all media, diameter, color and texture of the colony, as well as conidia production and mycelium growth were evaluated. After 60 d *Pseudocercospora* presented different morphologies in all media (Table 1), producing abundant pseudostromata, conidiophores and conidia. Highest conidia production was in MEA medium with 16/8 h light/dark and at 27°C. These results differ from those reported by Ayala-Escobar *et al*. (2006), who found the best *Pseudocercospora* development in OPA media. On the other hand, the use of near ultraviolet wavelength light was not necessary for its sporulation, as cited by Barrera (2011).

Table 1. Morphological characteristics of *Pseudocercospora opuntiae* colonies after 30 and 60 d of inoculation in all media.

Medium	Colony diameter (cm) after 30 d	Colony diameter (cm) after 60 d	Color	Mycelium	Conidia
MEA	2.16±0.091 c	4.18±.16b	brown/ olive-green	Aerial	Presented at 30 d
V8®	2.63±.07 a	6.8±.15c	dark green/whitish	Aerial	Presented at 60 d
NA	1.46±.16 d	1.86±.15d	dark green/whitish	Aerial	No presented
WA	2.27±.07 b	4.48±.2a	Olive-green	Immersed	Presented at 30 d

Means followed by the same letter are not significantly different (p = 0.05) using Tukey test.

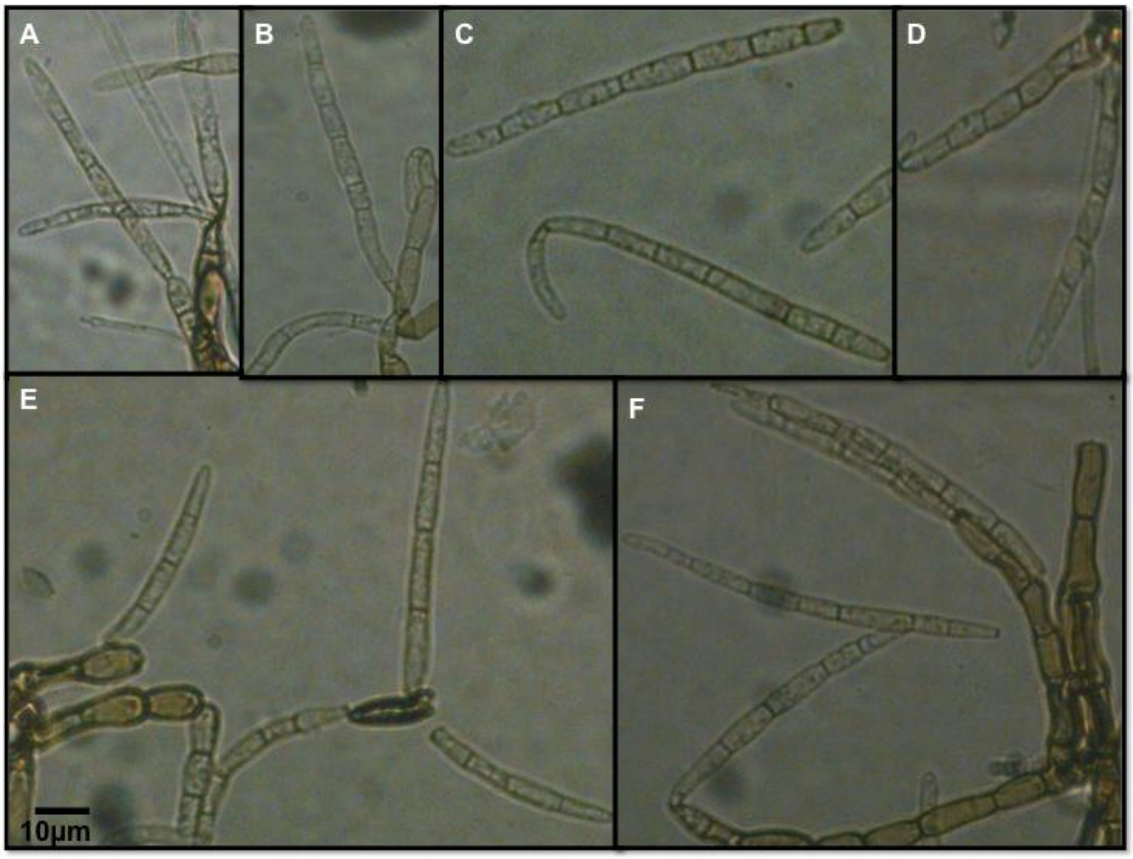

Figure 3. Different sizes of conidia and conidiophores developed in MEA. A), B) and D) brown and olive-green conidia united to branched conidiophores, C), E) and F) solitary cylindrical conidia with a marked thin abscission scar.

Pathogenicity tests

Black spot symptoms in cut cladodes appeared after 30 d of infection with cuticle discoloration. The presence of dark brown spots originated from the infection point and exhibited symptomatic dehydrated tissue. Landa (2012) found that cut cladodes are more vulnerable to *Salmonella typhimurium* in comparison with cladodes collected by removal at the nodes, which indicates that defense mechanisms may diminish when cladodes are cut, allowing the establishment of diseases in less time. Saénz *et al.* (2006) indicated that horticultural products after harvest present a fast senescence process and become more susceptible to microorganism invasion.

Injuries of material grown under field conditions were taken for symptom evaluation. Conidia concentration 8 x 10^{-3} mL^{-1} is ideal to induce black spot, as reported by Quezada-Salinas *et al.* (2006). After 30 d, 20% of field and greenhouse inoculated plants, presented symptom 1 characterized by the appearance of circular to oval spots, which were oily and light green in color. This symptom prevailed in the majority of the plants without an apparent advance up to week 9, where 5% presented symptoms 2 and 3. Symptoms 4, 5 and 6 appeared from week 10 on. Finally, symptom 7 began in 5% of the plants in week 12 and evolved rapidly in week 14 and 15, where 78% of the plants presented collapse of affected tissue. In field and greenhouse conditions, only two of the infected plants presented the advance of the disease up to symptom 2. These results demonstrated (Figure 4) that *Pseudocercospora opuntiae*

has a very long incubation period, which in controlled infection conditions encompasses approximately 109 d, coinciding with those results from Quezada-Salinas *et al.* (2006).

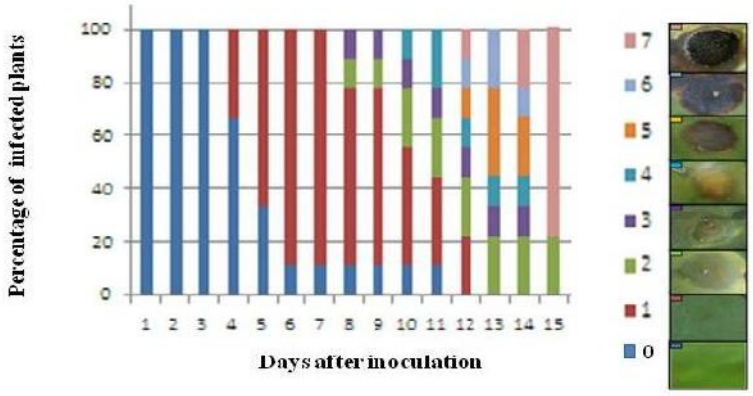

Figure 4. Symptoms progression after inoculation. Scale: 0 healthy plant, 1-7 observed symptoms: 1-2: Initial cuticle discoloration changing to a clear green color with small olive-green points; 3-5: Spots turn dark brown and their diameter increases to 3-4 cm, presenting a yellow margin and sinking of central part additionally; 6-7: The affected part dries up leaving a visible woody tissue, in many occasions it becomes detached leaving orifices that cross the cladode.

Nevertheless, differing from what Quezada-Salinas *et al.* (2006) report, symptom 7, where the injury becomes a black-colored circle, did manifest at the end of the infection cycle. Besides that, symptoms appeared in a similar way and in the same period in field and greenhouse incubation conditions. Injuries observed in infected plants corresponded to circular spots with more intense black color in comparison to those found in commercial plantations. Morphology of injuries under controlled infection conditions depends on the manner in which inoculation is carried out, as was demonstrated by Quezada-Salinas *et al.* (2006).

After 25 d, plants of *Phaseolus vulgaris* presented oily spots of light green color (Figure 5A). Around the spots, chlorotic tissue appeared and spread on the leaf surface (Figure 5B). The advance of symptoms was delimited by the appearance of yellowish tissue with an oily consistency (Figure 5C). Affected tissue suffered total necrosis (Figure 5D) throughout the entire leaf surface (Figure 5E). These symptoms appeared from the inoculation of three culture media (WA, MEA and OPA).

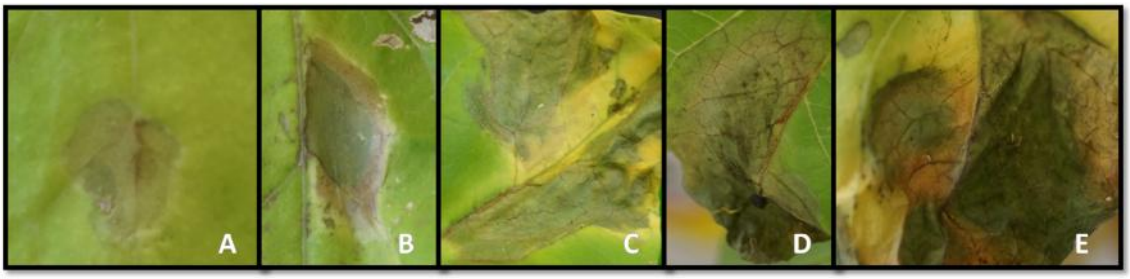

Figure 5. Symptom progression in *Phaseolus vulgaris* caused by *Pseudocercospora opuntiae* isolated from *Opuntia ficus-indica* cladodes.

Symptoms provoked by *Pseudocercospora* in *P. vulgaris* plants are similar to those found in *Opuntia* cladodes but likely due to tissue consistency, injuries were observed with major intensity and in less time. That a *Pseudocercospora* strain isolated from black spot injuries of *Opuntia* cladodes has the ability to infect other species of economic importance, verifies that *P. opuntiae* may not always be host-specific, which indeed represents huge implications in the epidemiology of the disease needing be studied.

Molecular characterization

A comparison of the sequence obtained in this study with those deposited in GenBank database was performed using DNA sequence alignment in BLAST and a phylogenetic analysis. The nucleotide sequence was identified as *Pseudocercospora opuntiae* BSJ1 (GenBank accession number: KF975410). Identity of sequences was determined based on the highest percentage (a minimum of 97%) of total nucleotide match with sequences from nucleotide database in the GenBank (Rosselló-Mora and Amann, 2001; Morgulis *et al*. 2008), and were corroborated with a phylogenetic analysis (Figure 6).

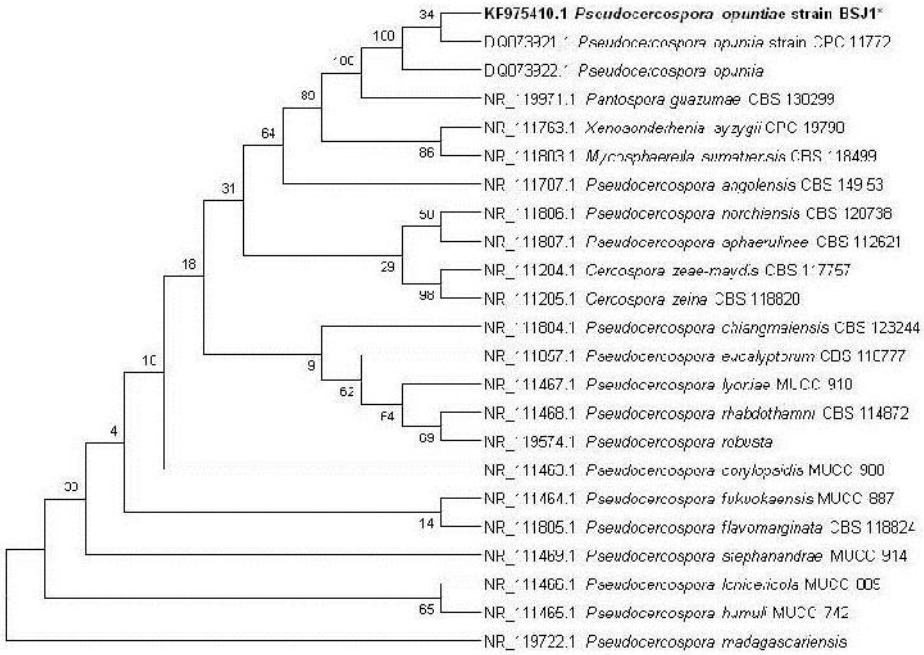

Figure 6. Phylogram based on the ITS 1 and 2 region sequences of *Pseudocercospora* strain identified in this study. The tree was generated using the Maximum Parsimony method. Data set was subjected to 1,000 bootstrap pseudoreplicates. Reference sequences were obtained from GenBank database. Sequence from the isolated strain is indicated by an asterisk. GenBank accession numbers are given in the labels.

CONCLUSIONS

Black spot is a severe disease in several cactus pear producing regions of Mexico, its etiology was described for the center of the country, but other regions need accurate information of the disease in their own enviroment.

In this research, the fungus involved with black spot symptoms in cactus pear cladodes from productive systems (fruit and vegetable) in western Mexico (Ojuelos and Zapopan, Jalisco) was identified. The study is the first verification of the causal agent for Jalisco. Though the isolated fungus shows some variations in morphological characteristics of development, amplification of the ITS region revealed a high level of conformity with taxonomic identification reported by Quezada-Salinas *et al*. (2006).

Pathogenicity of the isolated fungus from cladodes showed the symptoms in pre-cut cladodes, field and greenhouse plants, and *in vitro* explants. Virulence also was shown in *Phaseolus vulgaris* and different symptoms than those reported by Saparrat *et al.* (2009) for *Pseudocercospora griseola* were observed.

The causal agent of cactus pear black spot in the state of Jalisco, in Mexico, corresponds to *Pseudocercospora opuntiae*. Fungal infection route, as well as incidence level (low in summer and high in autumn), will allow us to recommend cultural managment practices which may reduce host inoculum and infection. Knowledge of inoculum sources, the manner in which the fungus infects, infection time, and pathogen response to environmental temperature may aid in the control of this pathogen.

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