Isolation and identification of fungi from leaves infected with false mildew on safflower crops in the Yaqui Valley, Mexico

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ORIGINAL

Aislamiento e identificación de hongos de las hojas infectadas con la falsa cenicilla en cultivos de cártamo en el Valle del Yaqui, México

Resumen. La falsa cenicilla es una enfermedad que afecta seriamente los cultivos de cártamo en el Valle del Yaqui, México, y es causada por la infección de un hongo perteneciente al género *Ramularia*. En el presente estudio, un hongo aislado de hojas contaminadas fue cultivado bajo diferentes condiciones de crecimiento con la finalidad de estudiar su desarrollo micelial y producción de esporas, determinándose que el medio sólido de *Septoria tritici*, 18 ºC de incubación y fotoperiodos de 12 h luz-oscuridad, fueron las condiciones más adecuadas para el desarrollo del hongo. Este aislamiento fue identificado morfológicamente como *Ramularia cercosporelloides*, pero genómicamente como *Cercosporella acroptili*, por lo que no se puede aún concluir que especie causa esta enfermedad. Adicionalmente, en la periferia de las infecciones estudiadas se detectó la presencia de *Alternaria tenuissima* y *Cladosporium cladosporioides*.

Palabras clave: *Ramularia cercosporelloides*, *Carthamus tinctorius*, *Cercosporella acroptili*, hongos aislados, Sonora

Abstract. False mildew is a serious disease of safflower crops in the Yaqui Valley, Mexico, and is caused by infection with a fungus belonging to the genus *Ramularia*. In the present study, a fungus isolated from leaf lesions was grown under different growth conditions in order to study its mycelial growth and spore production, determining that the solid medium of *Septoria tritici* at 18 °C of incubation and photoperiod of 12 h light-dark, were the most suitable conditions for the fungal development. The isolated was morphologically identified as *Ramularia cercosporelloides*, but genomically as *Cercosporella acroptili*, therefore cannot be concluded which species causes this disease. Also, in the periphery of false mildew were found the presence of *Alternaria tenuissima* and *Cladosporium cladoporioides*.

Keywords: *Ramularia cercosporelloides*, *Carthamus tinctorius*, *Cercosporella acroptili* isolated fungi, Sonora

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Introduction

Safflower (*Carthamus tinctorius* L.) is an alternative agricultural crop suitable for regions with low water availability during the winter season in Mexico or when other crops are damaged by low temperatures. In our country, safflower is a crop mainly used for edible oil (CESAVESON, 2004; Silveira-Gramont *et al*., 2009). Sonora is one of the States with the largest production of safflower, contributing with about 40% of the national production. During the autumn-winter 2000-2001 crop cycles at the Yaqui and Mayo Valleys, a false mildew appeared in safflower crop, disease unknown in the region until that time. The disease-causing agent was identified as *Ramularia* spp. Studies conducted by local boards of plant health found a 100% disease incidence in

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the crop, which generated 36.4% of yield reduction during this cycle (CESAVESON, 2004). For the 2004-2005 crop cycles, the disease was present immediately after the emergence of the plant, six weeks before compared to previous cycles (CESAVESON, 2006). In 2006, the fungus was isolated from safflower leaves infected with false mildew which was morphologically identified as *Ramularia cercosporelliodes*, and its pathogenicity was determined by *in vivo* assays and confirmed using the Koch's postulates (Huerta-Espino *et al*., 2006). In 2007 disease-resistant varieties, lines 04-787 and 04-0765 (Muñoz-Valenzuela *et al*., 2007) and seeds genetic improvement produced highly tolerant safflower varieties were introduced (Montoya-Coronado *et al.,* 2008); but later, it was found that 78% of the sown acreage presented an infection rate from 51 to 100%. Montoya-Coronado *et al*. (2008) reported that *R. carthami* was the causative agent of false mildew, based only morphological identification. Therefore, considering the importance of the problems caused by false mildew in safflower in Mexico, the scarce scientific reports generated to date, the absence of reports with DNA for sequence *R. cercosporelloides* and the problems associated to isolation by conventional methods, the objectives of this research work were: 1) to isolate the fungus from false mildew affected safflower leaves at the Yaqui Valley, 2) to obtain their DNA sequences and, 3) to establish *in vitro* conditions for their optimal growth.

Materials and methods

In June 2010, leaves samples of the aerial part of the plant with more than three false mildew lesions, were randomly collected from safflower crops variety S-518 extemporaneously sown. Samples were collected at 50 m from the Dr. Norman E. Borlaug St, km 12, Yaqui Valley, at Cajeme, Sonora. Subsequently, they were placed in hermetically sealed bags and transported to the Laboratory of Microbiology and Mycotoxins of the Food Research and Graduate Department of the University of Sonora, where they were stored at 4 °C until further use.

Observation of leaves infected

False mildew lesions in safflower plants were identified according to the description of Huerta-Espino *et al*. (2006) throughout observations of leaves under a stereoscope (CARL ZEISS 475002-9902) (16 and 40X magnification) and optical microscope (Leica, Model DME) (100X magnification). False mildew infected leaves were separated and used for fungal isolation.

Fungal isolation

The fungus was sensitive to chemical disinfection of leaves, making difficult the spores removal from the leaf surface, therefore, four isolation techniques were evaluated:

(i) Extraction of spores with a drop of either water or Tween 20 solution: Leaves with false mildew lesions were randomly selected and a drop of either distilled water or sterile Tween 20 was added over the spot lesion. Spores suspension was removed with a sterile Pasteur pipette, deposited on the culture media, and scattered over the surface of the plate with a sterile glass rod. The plates were stored in an incubator (PRECISION, Thermo Scientific, USA) and developed colonies were observed after 96 and 168 h to temperatures evaluated.

(ii) Extraction of spores from lesions found on leaves: Leaves with more than three false mildew lesions, were randomly selected, washed with sterile water, immersed in sodium hypochlorite solution 0.5% ($v\ v^{-1}$) for 2 min, and the excess of disinfectant was removed with sterile distilled water. Disinfected leaves were placed on Petri dishes containing 20 mL of base agar and incubated at different

temperatures. After 48 h, the mycelium developed on the false mildew lesions was observed with the stereoscope (40x) and the characteristic colonies of *Ramularia* genera were identified, according to the description of Huerta-Espino *et al.* (2006). Subsequently, spores were scrapped off the plate using a glass rod, suspended in 2 mL Tween 20 (0.01%, $v.v^{-1}$) In total, 5 spore suspensions were obtained and, from every suspension, 50 µL aliquots were taken and placed on Petri dishes containing 20 mL of culture medium. They were distributed on the surface of the medium with a sterile glass rod and the plates were incubated at different temperatures evaluated until the appearance of fungal colonies.

(iii) Extraction of spores from lesions found on non-disinfected leaves: Leaves with more than three false mildew lesions were randomly selected and placed on Petri dishes containing 20 mL of agar base. After 48 h, the characteristic colonies of *Ramularia* genera were identified according to the description of Huerta-Espino *et al*. (2006). Later, 5 mL of Tween 20 (0.01% $v\ v^{-1}$), were poured onto the plate and spores were scrapped off the plate using a glass rod. The 5 mL water volume containing the spore suspension was distributed in Eppendorf vials (1 mL each) until further use. From each suspension, 50 µL aliquots were taken and entirely used to inoculate the culture media. Afterwards, they were distributed over the surface of the culture medium with a sterile glass rod and incubated to observe the development of fungal colonies in center and periphery of spot leaf.

(iv) Inoculation with fragments of diseased leaves: Leaves with more than three false mildew lesions were randomly selected and leaf fragments with disease lesions were cut in 1x1 cm pieces. Leaves pieces were washed with sterile water and immersed in sodium hypochlorite solution 0.5% ($v\ v^{-1}$) for 2 min, followed by a sterile distilled water washing. Subsequently, leaves fragments were placed on Petri dishes containing 20 mL of culture medium and incubated at the temperatures previously mentioned, until mycelium developed and subjected to observation. The colonies with the highest incidence of growth were re-cultured in selected media at different temperatures and times of exposure to light evaluated, until obtaining an isolated fungal growth for identification.

The culture media used for propagation of mycelia and spores were V-8 medium (15 g agar, 3 g $CaCO_3$, 200 mL juice of 8 vegetables, and graduated to 1000 mL with H_2O), *Septoria tritici* medium (ST) (1000 mL H_2O, 18 g agar, 4 g sucrose, 4 g yeast extract, 4 g malt extract) (Huerta-Espino *et al*., 2006), potato and dextrose agar media (PDA Bioxon), and Czapeck Bioxon medium (BD Bioxon). Cultures and re-cultures of the inocula were incubated at different temperatures (18, 20, 25, and 30 °C) and times of exposure to light (exposure cycles of 12h light-dark and 24 h dark). From these experiments, the best fungal growing conditions were selected for further propagation and cultivation trials. The spores developed in each culture media were suspended or re-cultivated, according to the isolation technique used.

After determining the most suitable growth conditions *in vitro*, the isolation was inoculated in 20 mL of culture medium contained in a 125 mL Erlenmeyer flask and incubated for 7 days at 18 °C. Then, 10 mL of sterile water was added and stirred for 5 min using a disinfected magnetic bar. An aliquot of 25 µL of the spore suspension was taken and deposited on a slide for observation. The observations were made using an optical microscope and 10 to 20 images of the spores were randomly taken with the software Image-Pro Plus v. 6.3 X (Media Cybernetics, Inc., USA, 1993-2008) at magnifications of 100, 400, and 1000x. The length and width of 25 randomly selected spores were measured. The experiments were performed in triplicate and measurements obtained from the three trials were averaged, with a total of 75 data.

Fungal identification

The descriptions for morphological identification of the database Nomenclature and Species Banks were employed to identify *Ramularia* isolated species (Robert *et al*., 2005).

DNA sequence analyses

Samples of isolated fungi were sent to the Center for Genomic Biotechnology of the National Polytechnic Institute (Reynosa, Tamaulipas, Mexico) for genomic identification. Genomic DNA was extracted from 240 h-old mycelia cultures grown in Potato Dextrose Broth (PDB). DNA extracted (1 mL) was placed into a 2 mL microcentrifuge tube and spun for 2 min at 12,000 rpm. The supernatant was aspirated and discarded. Then, 100 µL of PrepMan ®Ultra Sample Preparation Reagent were added. The sample was shaked for 30 s using a Vortex, heated for 10 min at 100 °C, and cooled down to room temperature for 2 min. Then, the sample was centrifuged at 12,000 rpm for 2 min and 50 µL of the supernatant were transferred into a new microcentrifuge tube. The supernatant was ready for Polymerase Chain Reaction (PCR) (Protocol PrepMan®Ultra, Applied Biosystems, USA). Purified DNA was used to obtain the sequence. The primers ITS1/ITS4 and NL1/NL4 were used to amplify both, the fungal DNA region segment D2, which belongs to a large rDNA sub-unit LSU, and the DNA 26S segment. The sequencing conditions included a denaturation process carried out at 94°C during 5 min, followed by a cycle at 94°C during 30 s, and an annealing step at 58°C (for ITS) and at 63°C (for 26S), both during 30 s. An extension was carried out at 72°C for 1 min for a total of 36 cycles and a final extension at 72°C and 4°C for 7 and 5 min, respectively. The genomic DNA sequence was finally obtained using a fluorescent Big Dye 3.1 (Applied Biosystems). The PCR products were sequenced using a Sequencing Applied Biosystems Mod. 3130 and generated sequences were aligned with BLAST algorithm (GenBank) of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast).

Microcultures

The microculture technique reported by Tuite (1969) was employed with the following modifications: 1 cm^2 agar plugs were obtained from sterile solid medium and placed onto a slide. The slide was placed into a humidified chamber, consisting of a 9 mm-diameter Petri dish which bottom had been previously covered with gray paper and moistened with 5 mL of sterile distilled water, to prevent dehydration of the culture medium. Each agar plug was inoculated with a straight handle loop in both the center and the ends, and covered with a coverslip. The chamber was incubated under previously selected conditions and observed until the appearance of the fungal colony.

Results

Observation of infected leaves

Fungal colonies and spores characteristic of the *Ramularia* genera were observed on the surface of false mildew infected safflower leaves (Figure 1). Disease lesions on safflower leaves were similar to those reported by Huerta-Espino *et al*. (2006). In summary, they were observed as brown circular or angular-irregular shaped necrotic lesions on the leaves, (2-20 mm in diameter), and sometimes, it a diffuse yellowish halo and fungus growth (resembling the whitish mold) was observed. Next, ovoid, cylindrical (20-45 x 3.5-10 µm) septated (0 to3 septum), solitary conidia or formed in short chains at scar sites on conidiophores, were observed.

Furthermore, in the periphery of lesions, spores and mycelium of *Alternaria* and *Cladosporium* were observed; consequently, the isolation of pure conidia from the leaf spot was difficult. Independently from host and leaf surface,

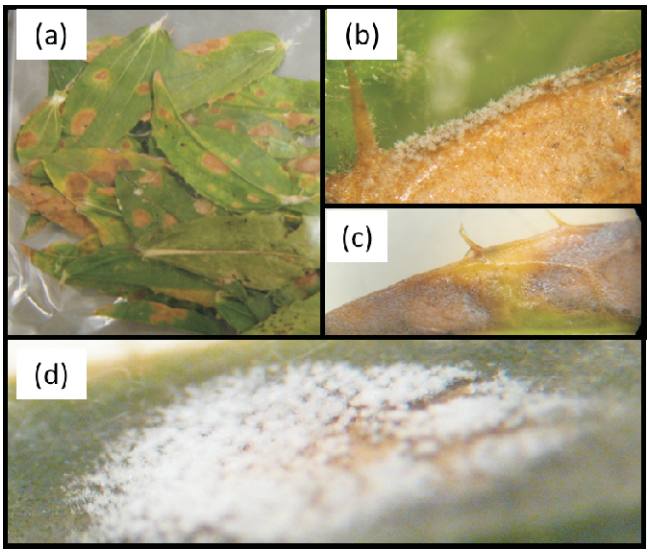

Figure 1. Safflower leaves infected with false mildew (a), *Ramularia* colonies developed on infected leaves at 40x (b), 16x (c) and 100x (d) magnification.

Heuser and Zimmer (2002) found that the removal of spores only from *Ramularia* sp., *Cladosporium* sp., and others spores was difficult.

Fungal isolation

"Extraction of spores from lesions found on leaves" and "extraction of spores from lesions found on non-disinfected leaves" were the most appropriate isolation techniques for the development of *Ramularia, Cladosporium* and *Alternaria* after 48 h of incubation.

ST culture medium at 18 °C, with a photoperiod of 12 h light-dark, was the most suitable culture conditions for the propagation of isolate genomic identified as *C. acroptili*. Areas with white to salmon pink-colored mycelium were found, as well as diffuse pigmentation of colonies obtained with spores cultivated in medium ST and extracted with technique extraction of spores from lesions found on leaves. These colonies were re-cultivated in new ST culture media and incubated under the same conditions, until full adaptation of the fungus to the environment from which a pure culture might be possible to obtain. The isolation and adaptation of colonies from ST culture medium was obtained until the fifth re-cultivation (Figure 3).

Table 1 . Qualitative characteristics of *Ramularia* mycelium and spores observed in different culturing conditions after 168 h of incubation, starting from an isolation developed at 18 °C and 12 h light/12 h dark

Culture medium	Incubation temperature	Mycelia	Spores
	(ºC)		
V-8	25	++	+++
	18	++	+++
ST	25	++	++
	18	++	+++
PDA	25	+	SE
	18	+++	+
Czapeck	25	++	SE
	18	+	+

+ Low development, + + half-developed, + + + abundant development
SE = without spores

With respect to culture conditions, V-8 and ST media were suitable for the production of spores. It was observed that, the ST media had the highest density of mycelium on the surface and production of spores after 7 days of incubation (25 °C or 18 °C, and photoperiod of 12 h light-dark) when spores inoculated on culture media ST and V-8 came from a suspension in sterile water (Table 1). Therefore, the culture conditions selected for the propagation of isolation were: ST medium, 18 °C and a photoperiod of 12 h. Under these conditions, the spores generated had the following dimensions in size, 17.21 ± 5.63 in length and 7.54 ± 1.40 µm wide.

Fungal identification and DNA sequence

The fungal genus isolated from the central part of the false mildew was morphologically identified as *Ramularia* and the fungi from the periphery of lesions were identified as *Cladosporium* and *Alternaria*. According to the database reported by Robert *et al*. (2005), the presumptive species were

Ramularia cercosporelloides, *Cladosporium cladosporioides,* and *Alternaria tenuissima*.

The presumptive fungal species (after morphological identification) were genomically aligned with the nucleotide sequence obtained (Figure 2). *R. cercosporelloides* was 100% identical to *Cercosporella acroptili* (gb|GU214689.1|) by genomic alignment; *C. cladosporioides* (gb|AY213695.1|) and *A. tenuissima* (gb|FJ755240.1|) were confirmed (verified with BLAST algorithm in NCBI, February 2013).

Discussion

The present research work reports the genomic identification of *Cercosporella acroptili* (=*Ramularia acroptili* in MycoBank Database, International Mycological Association, accessed on February 2013) in safflower. Other studies have reported the presence of *Ramularia carthami* Z. as the species isolated from false mildew (Montoya-Coronado *et al*., 2008; Borbon-Garcia *et al*., 2011). However, Huerta-Espino *et al.* (2006), reported that *R. cercosporelloides* was the fungi that caused false mildew in safflower. Nowadays, *R. carthami* is recognized as *Ramularia cynerae* (MycoBank Database, International Mycological Association, accessed on February 2013) but it is not *R. cercosporelloides* (a nom. nov. for *Cercosporella carthami*, see notes in Koike *et al*., 2011).

Ramularia acroptili and *C. acroptili* have been reported in *Acroptilon repens* (Berner *et al*., 2005), whereas *R. cercosporelloides* and *C. carthami* have only been reported in *Carthamus tinctorius* (Kirschner, 2009; Huerta-Espino *et al.*, 2006). At this time, the question was still in the air, what causes false mildew on safflower, *R. carthami, C. acroptili* or *C. carthami*?

The fungal taxonomy is a dynamic and progressive discipline that constantly generates changes in its nomenclature. Traditionally, fungi are classified according to their morphology and sexual status; however, they have also been classified from an ecological and biological panorama (Guarro *et al.,* 1999). Disputes to name species commonly occur, particularly when fungi have the ability to spread using different types of reproduction, which varies with the geographical zone in which the sample was collected, or simply with the time of collection (Shenoy *et al*., 2007). For this reason, there are many synonymous for some species and they are constantly reclassified. Also, the difficulty of the

(a)
GCGGAGGAAAAGAAACCAACAGGGATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCGCAAGCCC
GAGTTGTAATTTGTAGAGGATGCTTCTAGGTAGCGACCGGTCTAAGTTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTA
TGTGACCGGCCCGCACCTTCTACGTAGCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGAGGTAAATTTCTTCT
AAAGCTAAATACCGGCCAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTTAAAAAG
CACGTGAAATTGTTGAAAGGGAAGCGCTCACAACCAGACTTCTGGGCGGTGTTCCGCCGGTCTTCTGACCGGTCTACTCGCCGTCCC
GAGGCCAACATCATCTGGGACCGCCGGATAAGACCTCAGGAATGTAGCTTCCCTCGGGAAGTGTTATAGCCTGTGGTGATGCGGCG
CGTCTCGGGTGAGGTCCGCGCTTCGGCAAGGATGTTGGCGTAATGGTTGTCAGCGGCCCGTCT

(b)
AGACCGATAGCGAACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTCAAACAGCACGTGAAATTGTTG
AAAGGGAAGCGCTTGCAGCCAGACTTGCTTACAGTTGCTCATCCGGGTTTCTACCCGGTGCACTCTTCTGTAGGCAGGCC
AGCATCAGTTTGGGCGGTAGGATAAAGGTCTCTGTCACGTACCTCCTTTCGGGGAGGCCTTATAGGGGAGACGACATACT
ACCAGCCTGGACTGAGGTCCGCGCATCTGCTAGGATGCTGGCGTAATGGCTGTAAGCGGCCCGTCTTGAAACACGGACCA
AG

(c)
TAGACCGATAGCGAACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTTAAAAAGCACGTGAAATTGTT
AAAAGGGAAGGGATTGCAACCAGACTTGCTCGCGGTGTTCCGCCGGTCTTCTGACCGGTCTACTCGCCGCGTTGCAGGCC
AGCATCGTCTGGTGCCGCTGGATAAGACTTGAGGAATGTAGCTCCTTCGGGAGTGTTATAGCCTCTTGTGATGCAGCGAG
CGCCGGGCGAGGTCCGCGCTTCGGCTAGGATGCTGGCGTAATGGTCGTAATCCGCCCGTCTTGAAACACGGACCAA

Figure 2. Nucleotide sequences from the genera isolated from leaves of safflower false mildew: a) *Ramularia*, b) *Alternaria*, c) *Cladosporium*.

Figure 3. Colonies and spores isolated from false mildew in medium ST at 18°C.

morphological identification causes frequent changes in the classification by genera, *Ramularia* and *Cercosporella* for example (Kirschner, 2009). Moreover, Tautz *et al*. (2003) mentioned that one of the disadvantages of the traditional taxonomy systems is the dependence on specialists whose skills are lost upon retirement; other is the difficulty to access the specialized literature.

In the present work, a genomic identification was done. Our isolated strain showed 100% genomic alignment with a nucleotide sequence of a strain CBS120252, isolated from *Acroptilon repens* in Turkey and identified as *R. acroptili* (GenBank accession numbers GU214689) (Crous *et al*., 2009). As a matter of fact, on February 2013, the GenBank accession numbers GU214689, corresponds to *C. acroptili* and there is no accession for *R. cercosporelloides* in the database nucleotide sequence. It is interesting to mention that both strains were isolated from *Astarecea* plants, which confirm the presence of the genera *Ramularia* in *Astarecea*. Berner *et al*. (2005) reported a fungal isolate from *A. repens*, tentatively identified as *R. acroptili,* but finally it was confirmed as *C. acroptili*. In our report, the isolate from *C. tinctorius* was tentatively identified as *R. cercosporelloides* but it was confirmed as *C. acroptili.*

These reports support the necessity for highly specific techniques for the identification of fungi in safflower and also for *Mycosphaerella* anamorphs (telemorph of *Ramularia*). Given the difficulties in the identification of species, Hibbett *et al.* (2007) suggested the use of DNA for identification, especially in isolates that cannot be easily distinguished or when they show low taxonomic differentiation.

For this reason, while further data is not available, we agree with Kirschner (2009) proposal in using several studies for the classification of a species. These may include morphological descriptions by simple light microscopy, ultrastructure's descriptions using scanning electron microscopy, molecular analysis, observations of interactions, and host-parasite infection.

The existing controversy in the names reported for the fungi genera isolated from false mildew in safflower *Ramularia* and *Cercosporella,* is a problem that is illustrated naming this isolate *R. cercosporelloides* as reported by Braun (Kirshner, 2009). However, the aim of the present study does not focus on the discussion of the methods and techniques for fungal identification; but this necessity is evident.

Moreover, our isolates showed slow growth and development in artificial media. However, for further studies, the use of ST medium, 18 °C, and photoperiods of 12 h is recommended. The use of sterile water to suspend the spores reduces the mycelium production time. This might be due to the beginning of the germination of spores in the presence of water or to the competition of the spores by the agglomeration over the culture medium.

On the other hand, with respect to the isolated fungi from the periphery of the spot leaf, the presence of *Alternaria* in safflower has been reported as a recurring contaminant in

the field (Irwin 1976, Mortensen *et al*. 1983) and our studies have recently showed the presence of *A. tenuissima* in safflower seed (Quintana *et al.*, 2011). Also, *Cladosporium* is a fungus phylogenetically related to the *Ramularia* genera (Kirshner 2009), and is described as an opportunistic fungus of secondary contamination in agricultural crops, and their spores are deposited on injuries caused by other phytopathogenic fungi (Crous *et al*. 2007). It is possible that in safflower, *C. cladosporioides* spores are deposited on the surface of leaves with false mildew.

The existence of either antagonistic or synergistic interactions between the fungi isolated in the present study is also possible. These interactions have been reported in other phytopathogenic fungi, for example *C. cladosporioides* and *Sclerotia sclerotionum* in bean (*Phaseolus vulgaris* L.) (Boland and Hunter, 1988). However, this hypothesis was not confirmed.

Conclusion

This first isolation of *C. acroptili*, *A. tenussima,* and *C. cladosporium* from false mildew infected safflower is reported. *C. acroptili* was isolated and genomically identified from false mildew infected safflower leaves planted and grown at the Yaqui Valley, Sonora, Mexico. However, we propose to name our isolation as *R. cercosporelloides,* until sufficient data for its proper identification be obtained.

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