ORIGINAL RESEARCH PAPER

Microbiology

KEY WORDS: oomycete, *Phytophthora*, pathogenicity.

ISOLATION AND CHARACTERIZATION OF AN OOMYCETE PATHOGENIC IN *CAPSICUM CHINENSE*

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ABSTRACT

The phytopathogenic oomycetes have the capacity to germinate or remain latent until the environment is adequate for establishment and proliferation. These oomycetes are capable of infecting a wide variety of hosts and have been associated with significant economic losses. Large-scale outbreaks are largely due to lack of efficient phytosanitary management, and absence of precise identification of pathogenic microorganism. Correct identification of a causative agent can be challenging because different oomycete pathogens can cause similar symptoms in different hosts while the same pathogen can cause different symptoms in the same host under different conditions. A pathogenic microorganism from the rhizosphere of *Adenium obessum* was isolated and characterized. The strain FR1 was isolated using the growth points technique, and grown in selective culture medium. The isolated strain was morphologically characterized, and also was found to be pathogenic to *Capsicum chinense.* The strain was identified as an oomycete of the genus *Phytophthora*.

INTRODUCTION

Oomycetes are microorganisms belonging to the Straminipila (Cooke et al., 2000), but are often confused with members of the Fungi due to their filamentous growth habit. They can be distinguished from true fungi through biochemical characteristics such as a cell wall composed mainly of 1.3- -glucans, 1.6- -glucans and 1.4- -glucans, rather than chitin (Latijnhouwers et al., 2003). They also differ from the fungi in their synthesis of ergosterol, a compound required for the plasma membrane, which oomycetes acquire from the host (Gaulin et al., 2008). For this reason, application of fungicides that inhibit ergosterol synthesis is ineffective against oomycetes in intensive agricultural systems (Ghannoum and Rice, 1999). Some oomycete species are considered highly phytopathogenic, such as the genera *Phytophthora* and *Pythium*. These have been devastating crops since at least 1840 (Goodwin et al., 1994), and continue to nowadays in different crops around the world (Ristaino, 2002; Fry and Goodwin, 2011; Migliorini et al., 2015; Yang et al., 2017)(Ristaino, 2002; Fry and Goodwin, 2011; Migliorini et al., 2015; Yang et al., 2017).

Fungicide management is not always effective, because pathogenic members of this clade differ in their response; for example, growth in *Pythium* was inhibited in the presence of hymexazol, but the same compound exerted no effect on growth in a *Phytophthora cinnamoni* strain (Tsa and Guy, 1977), and different strains of *Phytophthora* sp. respond different to metalaxyl (Cooke et al., 2003). Effective phytosanitary management for oomycete control requires identification and classification of the target microorganisms before initiating a management plan. Identification to the species level is critical when studying oomycetes, be it to meet research goals or for rapid and accurate diagnosis during pathogen outbreaks. Our objective is to contribute to the knowledge about Phytophthora through the study of its structure and pathogenic capacity

MATERIALS AND METHODS

Microorganism Isolation

The microorganism strain was collected from rhizosphere of ornamental plant *Adenium obessum* var. Lila grown at a nurse (coordinate: 20.64541-89.464140) in Mexico. A selection was made of *A. obessum* seedlings in germination trays that presented severe wilting at stem base. The selected seedlings and the associated potting mixture were removed from the trays. In a sterile environment, the seedlings were separated from the potting mixture and the roots were removed and washed with sterile water. They were then sterilized by rinsing with 2% sodium hypochlorite for 3 mins, followed by three rinses with sterile water, one with 70% ethanol for 3 mins, and a further three rinses with sterile water. The sterilized roots were placed on sterile absorbent paper and fragmented into 1 cm^2 pieces. Each piece was placed in a Petri dish containing 25 ml selective medium (20% clarified V8 juice, 15 g agar + 100 mg L^{-1} pentachlorobenzene (PCNB) and 250 g ml^{-1} ampicillin).

The potting mixture removed from the seedlings was placed in sterile culture boxes (Magenta™), saturated with sterile water and a habanero chili (*Capsicum chinense*) fruit. Root and potting

mixture samples were incubated at room temperature in darkness and checked daily for mycelium growth, them the mycelia hypha tips were transferred to Petri dishes containing selective medium and maintained at room temperature in darkness until growth reached 20% of the box surface. Again, the hypha tips were removed and transferred into new selective culture medium. The mycelium remaining in the box was used for microscope observations. Of the isolates obtained, strain FR1 was chosen because of its rapid growth and capacity in medium with PCNB.

Macroscopic examination

Colony morphology was determined by placing agar blocks (1 cm x 1 cm) into the center of a dish plate containing V8 or PDA agar, allowing it to grow for one week in darkness at 25 °C, and describing colony growth pattern of FR1 (Ann and Ko, 1990; Jung et al., 2011; Parker, 2018). The identification of the genus was based on the observation of morphological characteristics, such as type of mycelium, shape and size of sporangia, size and ornamentation of oogonio (Ho, 1981; Hardham, 2005).

Microscopic Examination

Oomycete morphology was described using an ligth microscope. The strain was growed on V8 culture medium for 30 days in darkness to promote formation of vegetative structures such as mycelium and sporangia, and reproductive structures (Safaiefarahani et al., 2015). Samples were taken from the mycelium and placed on a slide where a drop of 20% methyl blue was added to dye the structures. After staining, the mycelia were observed with a microscope (Nikon, Eclipse E200), and the oomycete classified based on reported taxonomic keys (Gallegly and Hong, 2008).

Scanning electron microscopy

For observations of the structure of the strain FR1 in an SEM, it was necessary to perform with *C. chinense* seed root as support, to avoid losing the sample during tissue fixation. *C. chinense* seeds were disinfected as previously reported (Bojórquez-Quintal et al., 2014). Six disinfected seeds were placed in petri dishes containing MS medium (Sigma-aldrich) for 7 days in darkness, then seeds were infected using mycelium plugs (0.7 mm diameter). After 7 days, the infected seedlings were fixed in FAA solution (10% formaldehyde, 5% acetic acid, 50% ethanol, v/v/v) for 2 h. After fixation the tissue samples were prepared by dehydrating as previously reported (Enkerli et al., 1997; Pathan et al., 2009; Mutalib, 2014). Samples were dried to critical-point in a dryer (Tousimis Samdri-795®, Maryland, USA), exchanging ethanol with liquid CO_2 and later with gaseous CO_2. Finally, the samples were mounted on adhesive carbon film and dusted with gold (21 nm particles) (Denton Vacuum Desk II®). Prepared samples were viewed in a scanning electron microscope (JEOL model JSM-6360LV®).

Pathogenicity test in C. chinense

Pathogenicity test for seed, the seeds were disinfected and placed in petri dishes with wet absorbent paper, next to them mycelium plugs were placed. After 7 days, the number of germinated seeds and the presence of mycelium were observed. The control treatment was performed in a similar way, but with sterile culture medium plug.

Developed seedlings (45 days) were used to test pathogenicity. Disinfected seedlings were reached by dipping only the root in 80% ethanol for 5 min, followed by three rinses with sterile distilled water, one with 30% sodium hypochlorite for 15 min, and four more with sterile distilled water. After disinfection the seedlings were placed in a hydroponic system in liquid MS culture medium. The medium was inoculated by adding segments of V8 culture medium containing mycelia. After 24 h, the roots were observed using an optical microscope. Pathogenicity of the oomycete in fruit was assessed by placing a mycelium plug, or one with agar medium without mycelium, next to the fruit under sterile conditions in a phytotron at 25 °C and 80% relative humidity for seven days.

RESULTS AND DISCUSSION

Our current primary research focus is microorganisms which attack plant roots and exhibit resistance to common antibiotics and fungicides. To this end we collected samples of the rhizosphere and roots of *A. obessum* seedlings presenting symptoms of root rot. Of the strains isolated from both inoculums, only those were selected that grew in V8 culture medium with PNCB and ampicillin, and had the ability to infect fresh *C. chinense* fruit. Characterization was done of strain FR1 since it was deemed a probable oomycete.

Colony morphology in V8 culture medium included ill-defined petaloid structures that had neither chrysanthemum-like nor coralloid shape (Fig. 1a), similar to those reported for *Phytophthora cactorum*, *P. palmivora*, *P. cinnamomi* and *P. capsici* (Eggers et al., 2012; Hudler, 2013; Widmer, 2014). In contrast, PDA culture medium produced a uniform fluffy morphology (Fig. 1b) similar to the species *Phytophthora cambivora* (Tian and Babadoost, 2004; Vannini and Vettraino, 2011).

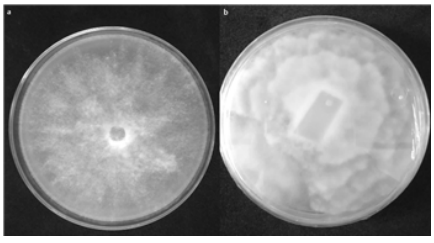

Figure 1. Growth pattern of FR1 strain in V8 (a) and PDA (b) culture media.

Microscopic structure characterization was done by staining with methylene blue and observing samples with an optical microscope and SEM. The culture characterized had been grown for twenty days in darkness to promote formation of structures such as chlamydospores, hyphal swellings and sexual reproduction structures such as antheridia and oogonia (González et al., 2017). Observed structures included: coenocytic mycelium, branched and irregular hyphae (Fig. 2a), as well as hyphal swellings (Fig 2b). Scanning electron microscope (SEM) microphotographs of FR1 showed obpyriform and rounded sporangia, with a single apical papilla.

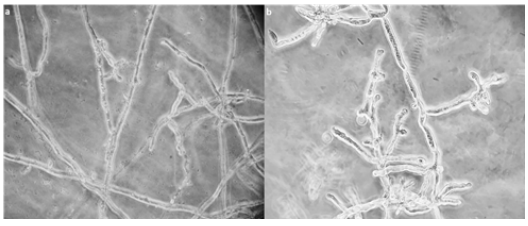

Figure 2. Micrograph of FR1 mycelium strain showing (a) asepted, ramified, irregular hyphae and (b) hyphal swellings.

These coincide with characteristics described for the species *P. capsici* (Fig 3a). The mycelium also exhibited abundant hyphal swellings and chlamydospores (Fig. 3b). Among the sexual structures was amphigynous antheridium. This condition is caused by growth of the initial oogonial through the initial antheridium, which has been reported for *P. capsici* and *P. cinnamomi* (Hemmes and Bartnicki-Garcia, 1975; Hüberli et al., 1997). The formed oospore is considered plerotic (Fig. 4).

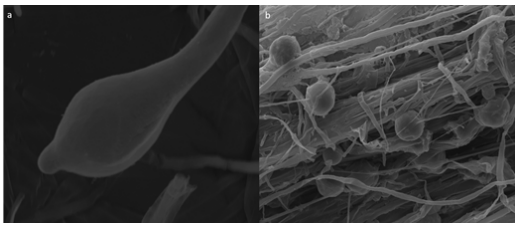

Figure 3. SEM micrograph of (a) sporangia and (b) chlamydospores.

Strain FR1 identification was done using established morphological keys (Gallegly and Hong, 2008). The first classification of FR1 derives from antheridium structure, which exhibited amphigynous antheridium (Fig 4). However, the strain was able to grow at temperatures above 30 °C, the sporangia did not detach easily from the sporangium and it had chlamydospores. Overall, the present characterization suggests that FR1 is a *P. nicotianae* strain.

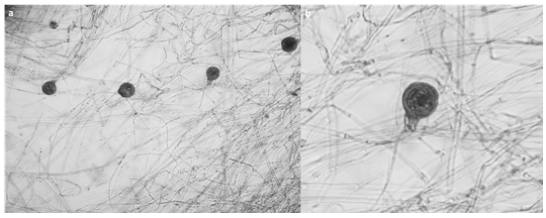

Figure 4. Micrographs FR1 oomycete sexual structures.

Plant inoculation to determine oomycete ability to infect tissue

Considered to be an aggressive pathogen in plants, *P. nicotianae* has the ability to cause infection in different plant tissues. Microorganisms have been described capable of causing disease in horticultural crops, mainly in plants of the *Capsicum* (Allagui and Lepoivre, 2000). To verify the isolated oomycete's infective capacity, *C. chinense* seeds, seedlings and fruit were infected with it. Three days after inoculation of the seeds the oomycete colonized them and apparently prevented radicle emergence. Twenty-four hours after inoculation of the seedling roots, the oomycete had colonized them, causing structural deformations, exhibiting mycelial growth on their surface, and necrosis (Fig. 5).

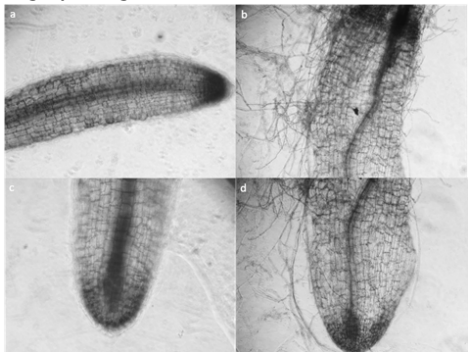

Figure 5. *C. chinense* roots: (a and b) elongation and cellular division sections; (c and d) cellular division sections; (a and c) uninoculated, (b and d) inoculated.

Seven days after inoculation of the fruit with the strain FR1, white mycelium was plainly visible on the surface, confirming its ability to proliferate on *C. chinense* fruit (Fig. 6). Considering the morphological and pathogenic characteristics of the isolated strain it is probable that it corresponds to an oomycete of the *Phytophthora* genus, while its coincidence with the sexual characteristics of *P. nicotianae* suggests it belongs to these species.

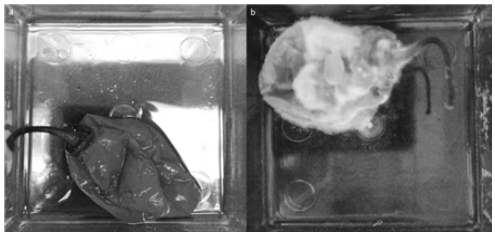

Figure 6. C. *chinense* fruit: (a) sterilized, (b) seven days after inoculation with Fr1.

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