Scientia Horticulturae 125 (2010) 248–255

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Characterization of a new fungal antagonist of *Phytophthora capsici*

L. Robles-Yerena[a,1], R.A. Rodríguez-Villarreal[a,1], M.A. Ortega-Amaro[d,2], S. Fraire-Velázquez[b,3], J. Simpson[c,4], R. Rodríguez-Guerra[a,1], J.F. Jiménez-Bremont[d,*]

[a] Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Campo Experimental General Terán, km. 31.0 Carr, Montemorelos-China, Apdo. Postal No. 3, General Terán, Nuevo León, Mexico
[b] Universidad Autónoma de Zacatecas, Unidad Académica de Biología Experimental, Guadalupe, Zacatecas, México C.P. 98600, Mexico
[c] Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Unidad Irapuato, Apdo. Postal 629, Irapuato, Gto., México C.P. 36500, Mexico
[d] Instituto Potosino de Investigación Científica y Tecnológica, A.C. Camino a la Presa de San José 2055, Lomas 4ª sección, San Luis Potosí, San Luis Potosi, México C.P. 78216, Mexico

ARTICLE INFO

Article history:
Received 10 July 2009
Received in revised form 15 January 2010
Accepted 31 March 2010

Keywords:
Capsicum annuum
Biocontrol
Phytophthora capsici
Root rot

ABSTRACT

The capacity of many microorganisms for antagonism towards pathogens is unknown and previously uncharacterized species may be potentially useful in this respect. This work presents the evaluation of the capacity of a previously uncharacterized Ascomycete, denominated here as UA to protect chili pepper (*Capsicum annuum*) against *Phytophthora capsici*, and other soil-borne plant pathogens and establishes the role of UA as a biocontrol agent. Inoculation of UA 2 days before the inoculation of *P. capsici* led to 77.8% survival of pepper plants. Simultaneous confrontation of both microorganisms *in vitro* led to 53.1% growth inhibition of *P. capsici*, while the inoculation of *P. capsici* 3 days after the inoculation with UA improved growth inhibition up to 73%. Simultaneous confrontation *in vitro* of UA with *Fusarium oxysporum* and *Fusarium solani* led to 41.2 and 50% growth inhibition, respectively, but had no effect on *Rhizoctonia solani* or a binucleate *Rhizoctonia* isolate. Moreover, formation of zoosporangia and the germination of zoospores were completely inhibited by exposure to undiluted filter sterilized filtrate. UA produces septate mycelia, but could not be classified in detail due to a lack of spores or reproductive structures. However, sequencing of Internal Transcribed Spacer 1, 2 and the 5.8S genes indicated that this fungus is a member of the Ascomycetes.

1. Introduction

Soil-borne plant pathogens such as *Fusarium oxysporum*, *Fusarium solani* and *Rhizoctonia solani* in association with wilt caused by *Phytophthora capsici* are recurrent problems in the chili pepper production regions of Mexico (Velásquez-Valle et al., 2001; González-Chavira et al., 2002). The latter species is the most devastating of the soil-borne pathogens which attack pepper and is capable of infecting both subterranean and aerial tissues of the plant (Ristaino and Johnston, 1999) and also causes serious losses in other horticultural crops throughout the world (Hausbeck and Lamour, 2004; Lee et al., 2001; Tamietti and Valentino, 2001).

Due to a lack of resistant cultivars, control of soil-borne pathogens of pepper is mainly aimed at *P. capsici* and involves numerous applications of fungicide both before and after transplanting in the field (Hwan and Kim, 1995). Reports have also indicated that Phytophthora shows resistance to these products (Lamour and Hausbeck, 2000; Parra and Ristaino, 2001). It is unlikely that products designed to eradicate Phytophthora will be effective against other soil-borne pathogens of pepper such as *Fusarium* spp. and *R. solani* (Ascomycetes and Basidiomycetes, respectively) and little is known about products which may be specific for these and other soil-borne pathogen species.

The need to develop more efficient, sustainable, safe and environmentally friendly production systems has led to the development of alternative strategies for the control of pathogens which attack diverse species of crop plants. To combat wilt caused by *P. capsici* in pepper, cultural practices based on the biology and ecology of the pathogen have been suggested (Ristaino and Johnston, 1999). One of these strategies is the use of biocontrol agents in order to reduce the pathogen population resistant to fungicides and the number of applications of fungicides (Shen et al., 2002; Sang et al., 2008).

A variety of organisms have been described as biocontrol agents against soil-borne plant pathogens including populations of the same pathogen species (Silva and Bettiol, 2005). Organisms which have been studied as biocontrol agents for *P. capsici* include *Burkholderia cepacia* (Ezziyyani et al., 2004), *Pseudomonas*

* Corresponding author. Tel.: +52 444 8342000; fax: +52 444 8342010.
E-mail addresses: jbremont@ipicyt.edu.mx, jbremont@yahoo.com (J.F. Jiménez-Bremont).
[1] Tel.: +52 8262670539.
[2] Tel.: +52 444 8342000.
[3] Tel.: +52 4928993420.
[4] Tel.: +52 4626239667.

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doi:10.1016/j.scienta.2010.03.016

spp. (Virgen-Calleros et al., 1997), *Glomus intraradices* (Zheng et al., 2005), *Bacillus* spp. (Guillén-Cruz et al., 2006) and *Trichoderma* spp. (Sid Ahmed et al., 1999). However, considering the biodiversity of microorganisms it is probable that other species of microorganisms associated or not with pepper roots will show activity against soil-borne pathogens which attack this crop. An example is the recently discovered fungus *Muscodor albus* which produces wide spectrum volatile antimicrobial compounds (Strobel et al., 2001; Ezra et al., 2004).

Although three species of bacteria have been evaluated for their combined efficiency as biocontrol agents against fungi associated with wilt in pepper (Guillén-Cruz et al., 2006), it is important to consider the specificity of action and interaction of these agents (Huang, 1992; Camprubi et al., 1995; Girlanda et al., 2001; Whipps, 2001). This complex situation implies the necessity to develop new strategies based on the utilization of diverse biocontrol agents that could not be defeated by the pathogen, and remains unaffected among the biocontrol agents.

With the aim of identifying fungi associated with wilt in pepper, samples were obtained from pepper crops grown in Guanajuato State, one of the main pepper producing regions of Mexico. In this study, various isolates of a septate non-sporulating fungus as well as a reduced number of Zygomycetes were obtained from roots of pepper plants cultured on fields where Fusarium, Rhizoctonia, Alternaria and *P. capsici* pathogens were previously isolated. One isolate of the septate fungus and two of the Zygomycetes were screened in order to establish their potential as biocontrol agents against root pathogens of pepper. Only the septate fungus was capable of protecting pepper plants against *P. capsici*. This fungus is most probably a non-sporulating, unclassified Ascomycete (UA). We describe the activity of this UA as a new biocontrol agent for root pathogens of pepper. In particular, the effects of this UA fungi and its filtrate on growth inhibition of *P. capsici* were evaluated, as well as the protection of pepper seedlings.

2. Materials and methods

2.1. Fungal strains and characterization

Three isolates of a previously uncharacterized Ascomycete (UA) associated with pepper roots in Guanajuato State, Mexico (GPUA1, 2, 3) were obtained by hyphal tip culture in 2005. After determining that GPUA1 was able to protect pepper seedlings against *P. capsici* damage (as described below), the other two isolates associated to pepper roots (GPUA2, 3) and one obtained from common bean (*Phaseolus vulgaris* L.) roots in Puebla State (PBUA1) Mexico, were included in the study. Characterization of growth rate and morphology was determined by visual observation and measurement of colony size daily on Spezieller Nahrstoffarmer agar (SNA), potato dextrose agar (PDA) and water-agar (WA) medium at 26 °C. Additionally, samples of mycelia of fungi grown on PDA were observed on an Olympus BX60 microscope and images were captured using Image Pro Plus, Version 2. GPUA1, 2, 3 and PBUA1 isolates were similar in growth rate and characterized by the ability of producing a pink coloration on SNA medium (Nelson et al., 1983).

A binucleate *Rhizoctonia* isolate (Gto17S2) previously reported as a biocontrol agent against pepper root pathogens, a *R. solani* isolate (Zac9-P3) pathogenic on pepper (Laguna-Estrada et al., 2005), a monozoospore isolate (PCT-17) (Hernández, 2007) of *P. capsici* kindly provided by Dr. S. Fernandez-Pavia (IIAF/UMSNH, Mexico) and monoconidial pathogenic isolates of *F. oxysporum* (CH1P4-1) and *F. solani* (CH1P4-1) obtained from pepper roots in Guanajuato State were also used in the analysis (Laguna-Estrada et al., 2005). The UA, Rhizoctonia and *P. capsici* strains were maintained on acidified PDA medium (González-Chavira et al., 2004), whereas Fusarium species were maintained on SNA medium. For long term storage, isolates were preserved at −80 °C in the above broth media with the addition of 50% glycerol.

2.2. Co-inoculation of pepper seedlings

The ability of GPUA1 to protect pepper seedlings against *P. capsici*, *F. oxysporum*, *F. solani* and *R. solani* was tested. Seeds of a *C. annuum* "guajillo" landrace were surface sterilized with 1% NaClO solution for 1 min, washed twice in sterile distilled water and dried on sterile filter paper. Seeds were sown in groups of 20–25 in a Petri dish containing 2% WA in a growth chamber at 25 °C and a 12 h photoperiod. After 4 days seeds were transferred in groups of three to fresh WA and left to germinate and develop roots (approximately 8 days later). These plants were then inoculated at the base of the hypocotyls with a fragment (2 mm^2) of a 10 days old colony of GPUA1. Two days later plants were inoculated at the same site with fragments of 5 days old colonies of either *P. capsici* or *R. solani* or with 5 μl of a suspension containing 1×10^6 spore ml^{-1} of *F. oxysporum* or *F. solani*. Plants inoculated individually with each of the pathogens or with GPUA1 and the uninoculated plants were used as controls. Each set of inoculations was repeated 3 times. Treatments were evaluated 8 days after inoculation of the pathogens using a plant reaction scale as disease index from 1 to 5 where 1 = no visible symptoms, 2 = 1–10% root rot, 3 = 11–25% root rot, 4 = 26–50% root rot, 5 = >50% root rot or plant dead. The data were analyzed by random ANOVA and the averages compared.

2.3. Confrontation of UA strains with P. capsici

UA strains and the *P. capsici* strain were either inoculated simultaneously at a distance of 3 cm on Petri dishes containing PDA or the UA strain was inoculated 3 days before inoculation of *P. capsici* also at a distance of 3 cm, all cultures were incubated at 26 °C. Controls were individual inoculations of each UA strain or *P. capsici*. All confrontations were carried out in triplicate. Growth of *P. capsici* was evaluated 8 days after inoculation by measuring colony radius from the original point of inoculation in the direction of the UA strain. Confrontations between GPUA1 and the Fusarium and Rhizoctonia isolates were carried out as described above for the simultaneous inoculations.

2.4. Evaluation of GPUA1 filtrates

GPUA1 was inoculated into 150 ml of PDB (potato dextrose broth) in a 250 ml flask and grown with agitation of 150 rpm at 26 °C for 16 days. The liquid medium was then filtered through 3 layers of sterilized Whatman no. 1 filter paper, sterilized by autoclave (for 15 min at 121 °C and 1.2 kg cm^{-2}) and undiluted or diluted to 25, 50 and 75%. The different concentrations of filtrate were used to replace water in the preparation of PDA plates. Alternatively, the filtrate was filter sterilized using 0.22 μm millipore filters and diluted to 50% before preparing PDA medium as above. *P. capsici* was inoculated in the centre of Petri dishes containing PDA prepared with the four concentrations of autoclaved filtrate and with the filter sterilized sample. The Fusarium and Rhizoctonia isolates were only inoculated on PDA prepared using the filter sterilized sample. Uninoculated PDA plates served as controls for contamination by mycelium of GPUA1. All treatments were carried out in triplicate and incubated at 26 °C for 5 days when colony diameter was measured. After 5 days of incubation fragments of *P. capsici* grown on undiluted autoclaved filtrate were removed and inoculated on normal PDA in order to determine viability.

2.5. Inhibition of zoosporangia and germination of zoospores

Undiluted filter sterilized media were inoculated with fragments of a 6 days old *P. capsici* culture in order to determine