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IDENTIFICATION AND *IN VITRO* CONTROL OF THE CAUSAL AGENT OF WILT IN ONION

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Onion seedlings with wilt symptoms were collected from the state of Guerrero, Mexico, from which *Fusarium oxysporum* f. sp. *cepae* was isolated and identified, based on the colony and conidia structure´s morphological characteristics. A pathogenicity test was conducted, inoculating healthy onion seedlings with the isolated pathogen, while the control seedlings were sprayed with sterile distilled water only. Control seedlings remained healthy while inoculated ones developed wilt symptoms seven days after inoculation, similar to those observed in the production area. *F. oxysporum* f. sp. *cepae* was re-isolated and the genomic DNA extraction, PCR, and sequencing test were carried out. Based on morphological identification and pathogenicity tests, it was determined that *F. oxysporum* f. sp. *cepae* was the causal agent of wilt in onion, although the molecular identification was only carried out on a genus level. Additionally, different biocontrol products were evaluated *in vitro* including fungicides and phyto-extracts in order to determine the inhibition of the pathogen. The products used were as follows: *Trichoderma asperellum* strain obtained at CSAEGro (100%), *T. harzianum* (100%), prozycar (100%), cupravit (100%) manzate (100%), busan (100%), Q-2000 (60%), neem extract (51%) and garlic extract (27%).
Keywords: *Allium cepa*, diagnosis, *F. oxysporum* f. sp. *cepae*, biocontrol, fungicides, phyto-extracts

INTRODUCTION

Onion is the fifth most important vegetable crop grown in Mexico, which is among the top 10 major producing countries (SIAP, 2013). In regions where this *Amaryllidaceae* is grown there are frequent incidences of fungal diseases that cause root, neck and bulb rot in onion, at different phenological stages from transplanting to harvest (Agrios, 2005). In the conventional and intensive production systems, fungicides are mainly used to control soil borne fungal diseases such as blight and root rot caused by *Fusarium oxysporum* f. sp. *cepae* (Agrios, 2005); because the chemical control has the advantage of being effective in the short term, and can treat diseases safely without much labor; they are economical when used at recommended doses, and producers can obtain higher yields and quality products; therefore, increasing their economic situation (Mendoza, 1990; Hafeez *et al*., 2016). However, in recent years several controversies have been generated because the excessive use of fungicides creating food safety risk; which generates side effects such as pollution and environmental damage and even creates resistance of pathogen strains (Gisi and Sierotzki, 2008). The need to find safe alternatives to increase agricultural productivity, has stimulated the advance search towards other control strategies of fungal diseases that do not involve environmental and other health risk. There are a group of fungi and bacteria that have antagonistic effects against pathogenic soil inhabitant, this action can be exploited as a form of biological control against plant diseases. Among these beneficial microorganisms, the fungus *Trichoderma* is found (Vargas *et al.*, 2012.); which have been extensively studied; are found naturally in a number of agricultural soils; are the most used antagonists worldwide because of its ubiquity, versatility, and adaptability, ability to be isolated, cultured, and easy to be manipulated. *Trichoderma* spp. are found in the rhizosphere and protects the plant roots, they also colonize flowers, seeds, leaves and reduce damage caused by diseases in a wide range of crops, especially onion; moreover, it is inexpensive and keeps the pathogen within the natural balance, promoting various beneficial activities in the agroecosystem. However, among its disadvantages, it is noted that this fungus exerts a slow control, does not completely eliminates the disease and its results are unpredictable (De França *et al*., 2014). On the other hand, there is an increase in the use of active antifungal

principles in which various plants containing antimicrobial properties, helps with crop protection and increases plant quality and production; these have the advantage of being less toxic and more readily degradable; such as garlic, neem and cinnamon, whose effects are attributed to the presence of a group of secondary metabolites in different parts of plants that gives them a natural protection (Kadam *et al*., 2014.); they are applied as foliar extracts sprayed or incorporated in the rhizosphere of the plant, to counteract the damage caused by soil pathogens such as *Fusarium oxysporum* f. sp. *cepae*. However, the antifungal activity depends on the type of extract and evaluation time (Villa *et al*., 2015). The alternative control with biological, organic and chemical products must be assessed in each host-pathogen interaction, to integrate the best control strategy to help reduce the damages and economic losses caused by this disease. Thus, the objectives of this research were: i) identify the pathogen associated with onion wilt based on morphology and pathogenicity test, ii) evaluate *in vitro* biological, chemical and organic fungicides on the pathogen associated with onions wilt.

MATERIALS AND METHODS

***Isolation*:** In the growing season of spring-summer 2013, in the community of Ixcateopan, Guerrero, Mexico, plant samples with symptoms of yellowing, wilting, leaf necrosis and root rot were collected in a commercial onion plot where the purple onion variety Cojumatlan was cultivated. The samples were placed in paper bags, transported to the Plant Pathology Laboratory of Professional Studies at the Center of the Agricultural College of the State of Guerrero (CEP-CSAEGro), to realize the phytosanitary diagnosis. The collected plant tissue was washed with tap water and cut into small pieces of about a centimeter (the sick and healthy parts) and were disinfected with 2% sodium hypochlorite for 2 min, rinsed with sterile water, dried with sterile blotting paper, placing five pieces in Petri dishes with potato dextrose agar (PDA) culture medium. The Petri dishes were incubated at 25°C in darkness. After 72 hours the development of the colonies was observed, the most frequent strains were transferred to new Petri dish with PDA culture medium for identification.

***Morphological identification*:** A portion of the medium with mycelium of the fungus grown on PDA culture medium was placed in a test tube with 10 mL of sterile distilled water, in order to obtain monosporic culture. Then a series of dilutions were performed to obtain 5 orders of dilution. From the last dilution a sample was deposited in Petri dish with PDA culture medium, distributing evenly and incubating at 25 °C for 24 hr. After fungal growth the mycelium was re-isolated in PDA culture medium for another five days. Shape, color of the colony and type of conidia of the fungus involved in the onion wilt damage was used in order to carry out the identification using keys established by Watanabe (2002) and Leslie and Sumerell (2006).

***Pathogenicity tests*:** Once purified, the isolation was grown on PDA culture medium in order to increase inoculum. After 10 days of growth, suspensions were prepared in sterile distilled water and were adjusted to a concentration of 6×10^3 conidia mL^{-1}. Twelve one month seedlings were used, of which eight were inoculated with the pathogen by spraying 2.5 mL (pre calibration) of the suspension using a manual Truper® sprayer; four control seedlings were used applying only sterile distilled water. The inoculated seedlings were placed in Styrofoam trays 60 × 40 cm, disinfested with 70% ethanol. Incubated at 26°C and 80% relative humidity, the symptoms were recorded daily for eight days. When the symptoms of the fungus appeared in advance growth in the damaged tissues, 10 samples (0.5 cm^2) were taken and disinfected with 2% sodium hypochlorite for 2 min, then washed three times with sterile distilled water and placed in PDA culture medium. The morphological characteristics of the colonies and re-isolated fungi were compared with the original strains to verify Koch's postulates and the whole procedure was performed twice.

***Molecular identification*:** DNA extraction was performed from 50 to 100 mg of mycelium, using the DNeasy^{MR} kit, following the manufacturer's protocol. The procedure was repeated for four samples of the fungus. Universal PCR reactions were performed to fungi with primers ITS-1fu 5'-tccgtaggtgaacctgcgg-3' and ITS-4 5'-tcctccgcttattgatatgc-3' (White *et al.,* 1990); which amplify an internal intergenic spacer (ITS) and generated a product of approximately 500 and 900 base pairs (bp). This phase was conducted using the methodology described by Diaz *et al*. (2015). The PCR-amplified fragments were directly sequenced and the results were compared with sequences available in the gene bank (GenBank) of the National Center for Biotechnology Information (NCBI) at the National Institute of Health (NIH) USA.

***Phase I: Biological control in vitro, cellophane test*:** Metabolites of native *Trichoderma asperellum* strain CEP-CSAEGro, same as was provided by the Plant Pathology area of CEP-CSAEGro, and a commercial strain *T. harzianum* obtained from the NatuControl product was evaluated (Dennis and Webster, 1971). In order to use and activate the strain the plate dilution method was used (Michel *et al*., 2009; Singleton *et al.*, 1992). The antagonist fungi in Petri dishes with PDA medium and cellophane was processed, two days after the biocontrol agent was also processed and the cellophane was removed and in that same Petri dishes the phytopathogenic fungus was placed (Michel *et al*., 2014). The diameter of the radial growth was measured until the control Petri dishes were filled (6 days).Three treatments were evaluated: T1 = control (phytopathogenic fungus only); T2 = *T. asperellum* native strain (CEP-CSAEGro) and T3 = *T. harzianum* commercial strain (NatuControl). A completely

randomized design was used with four replications. The variables evaluated were: a) mycelial growth of the phytopathogenic fungus (PF) measured in cm, b) percent of inhibition was calculated with the equation: % inhibition = [(D1-D2) / D1 × 100] (Arzate *et al*., 2006); where: D1 = diameter of the colony of PF growing in Petri dishes with PDA culture medium and D2 = diameter of fungal colony growing in Petri dishes with PF in PDA culture medium, where *Trichoderma* spp. was previously grown on the cellophane, releasing enzymes and metabolites onto the PDA culture medium. The data obtained from the evaluation of the variables was submitted in an analysis of variance and multiple comparison test of means using the Tukey´s Honest Significance Difference (HSD) method with a significance level of 5%. All statistical analyses were performed using the Statistical Analysis System (SAS, 2014) software.

***Phase II: Chemical and organic control*:** In this phase the chemical fungicide treatments with carbendazim (Prozycar), copper oxychloride + mancozeb (Cupravit mix), mancozeb (Manzate) Tiocianomitiltio (Busan), iodine (Q-2000), and the organic fungicides treatments such as *azadireachtin* (neem extract) and *Allium sativum* (garlic extract) plus a control without fungicides were implemented. The eight treatments were distributed under a completely randomized design with four replications; 32 experimental units (Petri dishes) were generated. Fungicides were deposited at the bottom of each Petri dish, then added 15 mL of PDA culture medium in liquid form; gently stirring until dissolved and allowed to solidify at room temperature. In the center of the Petri dish PF was placed, then the colony diameter was measured daily for six days. The variables were mycelial growth and the percentage inhibition. The data obtained from the evaluation of the variables was also submitted in an analysis of variance and multiple comparison test of means using the Tukey´s Honest Significant Difference (HSD) method with a significance level of 5%. All statistical analyses were performed using the Statistical Analysis System (SAS, 2014) software.

RESULTS AND DISCUSSION

***Isolation*:** An isolate was obtained from the colonies that were grown in PDA culture medium which resulted as the causal agent of onion wilt, it was also observed that wilt symptoms were more frequent when high humidity in the atmosphere, soil and high rainfall occurred, interspersed with periods of drought.

***Morphological identification*:** The colonies of the isolated strains from inoculated seedlings showed cottony white mycelial growth with a slight purplish brown coloration; similar to the original isolate (Fig. 1A). This pathogen showed

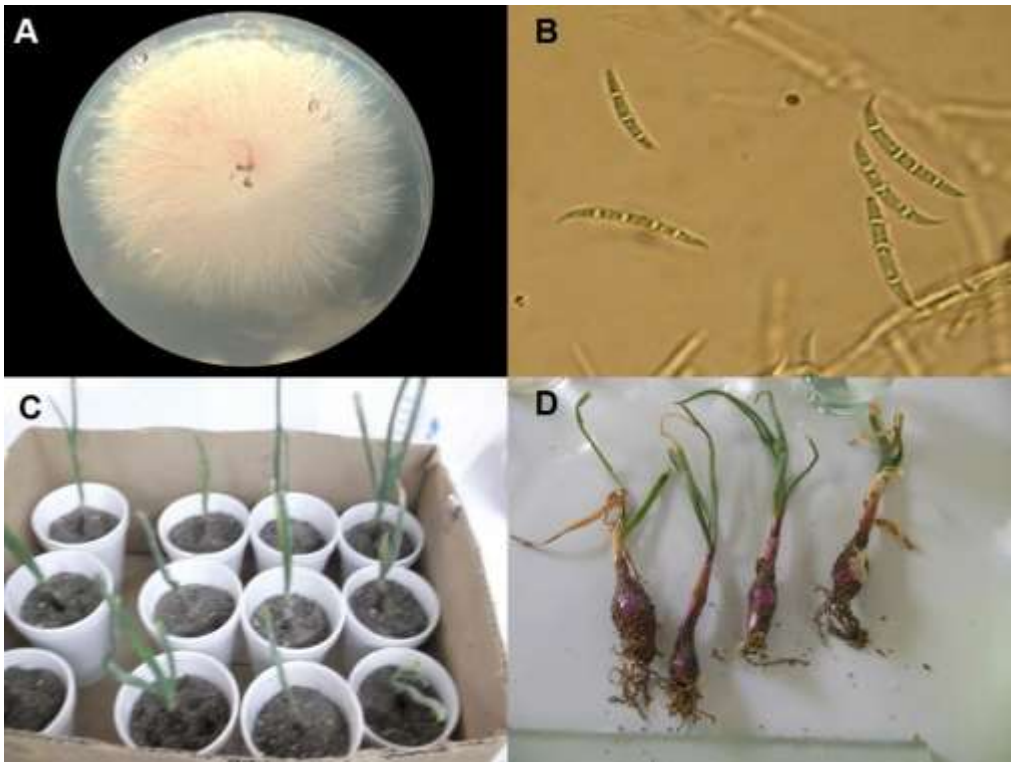

Figure 1. Identification of the pathogen associated with the onion crop. A) *F. oxysporum f. sp. cepae* in PDA; B) Macroconidia of *F. oxysporum f. sp. cepae*; C) seedlings inoculated with *F. oxysporum*; D) Symptoms induced by *F. oxysporum f. sp. cepae* in onion.

microconidia of one to two cells with dimensions 6-15.8 × 1.9-3.7 microns, macroconidia of 3-5 cells, bent towards both ends of 29.1-45 × 2.9-4.7 (Fig. 1B) which correspond to *Fusarium oxysporum.* The morphological characteristics mentioned match those described by various authors such as Booth (1971), Nelson *et al*. (1982), Watanabe (2002) and Leslie and Sumerell (2006).

***Pathogenicity test*:** Seven days after the inoculation with conidia suspension all seedlings showed wilt symptoms (Fig. 1C and 1D). Control seedlings remained free of symptoms. From the symptomatic seedlings, the pathogen was re-isolated and newly identified which coincided with the initially isolated pathogen from the field and the host specificity was identified as *F. oxysporum* f. sp. *cepae*.

***Molecular identification*:** When compared with sequences available in GenBank, 100% similarity of sequence of our investigation were found with *Fusarium* spp. (accession number KF293358.1). Due to high genetic diversity of *Fusarium* and the use of universal fungal primers, causal agent could be identified to genus level only through sequencing. This result corroborates the findings by morphological identification and pathogenicity test.s

***Phase I. Biological control in vitro, cellophane test*:** The mycelial growth of the phytopathogenic fungus showed highly significant evidence (P <0.0001) in all evaluated dates, the native and commercial strains of *Trichoderma* did not allow the growth of the pathogen, which at 144 hours (6 days) reached a diameter of 8.6 cm (Table 1). Therefore, it was determined that the pathogenic fungus grew at a rate of 0.06 cm hour^{-1}. Similarly, the metabolites of *T. asperellum* and *T. harzianum* strains obtained from CSAEGro inhibited the growth of *F. oxysporum* f. sp. *cepae*, the control grew normally in PDA culture medium (Figure 2). In a similar study, Michel *et al*. (2014) used the *T. asperellum* in *F. oxysporum* f. sp. *gladioli* strain and reported 60.6% inhibition, this result is lower than that reported in the present study where the same species *T. asperellum* inhibited 100% to *F. oxysporum* f. sp. *cepae*. Diaz *et al*. (2014) confirmed findings of this investigation where the authors also used *T. asperellum* CSAEGro strains and found that the biocontrol agent retarded the presence of *Rhizoctonia solani* in cucurbits under greenhouse conditions. Meanwhile Sundaramoorthy and Balabaskar (2013) evaluated different species of *Trichoderma* against *F. oxysporum* f. sp. *lycopersici in vitro* and *T. harzianum* was able to inhibit the pathogen in only 53%, this percentage of inhibition is lower than that reported here. El-Katatny and Imam (2012) employed two strains of *T. harzianum* against tomato postharvest pathogens that also included *Fusarium* spp. These authors stated that with the use of *T. harzianum* strains, incidences ranging from 12.5 to 87.5% were obtained i.e. the biocontrol effects of the strains did not suppress the pathogen, therefore the commercial *T. harzianum* strain used in this investigation was effective in controlling the *F. oxysporum* in onion.

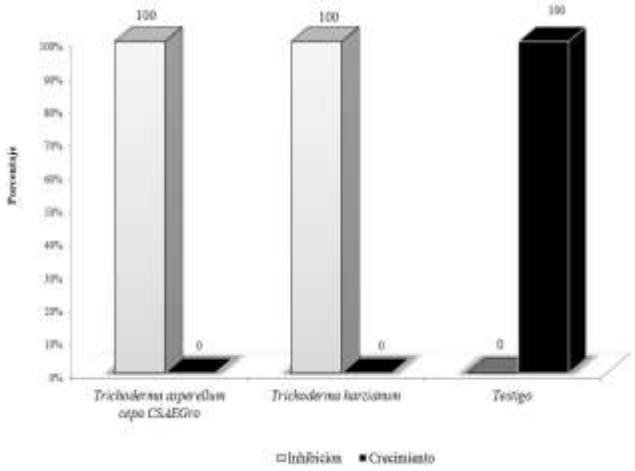

Figure 2. Percentage of inhibition and growth of *F. oxysporum f. sp. cepae,* depending on the treatments from test I.

***Phase II: Chemical and organic control*:** The two characteristics evaluated at this phase showed significant differences in all evaluation dates (P <0.0001). The fungus did not show any signs of development with carbendazim fungicide, copper oxychloride + mancozeb, mancozeb and tiocianomitiltio had a 100% inhibition on pathogen development, the iodine and the extracts were least effective (Table 2 and Fig. 3); the pathogenic fungus mycelium grew at an average rate of 0.27 cm day^{-1}, after 144 h covered the entire PDA culture medium surface of the Petri dish. According to Kadam *et al*. (2014), who worked with *F. oxysporum* f. sp. *gladioli* and used several types of the fungicides used in their investigation, reported the following results: carbendazin (65.33%), copper oxychloride (50.77%), mancozeb (32.00%), these results are lower than those found in the

Table 1. Diameter of the colony of *F. oxysporum f. sp. cepae* in the biological control *in vitro*, using the cellophane test, evaluated at six times.

Treatment	Time (h)					
	24	48	72	96	120	144
Control	1.8 a	3.1 a	4.5 a	5.5 a	6.6 a	8.6 a
Trichoderma sp.	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b
T. harzianum	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b

Mean values in columns with the same letter are not statistically different (Tukey´s HSD, $P \leq 0.05$)

Table 2. Diameter of the colony of *F. oxysporum f. sp. cepae* using chemical and organic products for control, evaluated at six times.

Treatment	Time (h)					
	24	48	72	96	120	144
Prozycar	0.00 c	0.00 d	0.00 c	0.00 c	0.00 c	0.00 d
Cupravit	0.00 c	0.00 d	0.00 c	0.00 c	0.00 c	0.00 d
Manzate	0.00 c	0.00 d	0.00 c	0.00 c	0.00 c	0.00 d
Busan	0.00 c	0.00 d	0.00 c	0.00 c	0.00 c	0.00 d
Q-2000	1.68 b	1.98 c	2.39 b	2.63 b	2.83 b	3.15 c
Garlic extract	2.18 ab	2.88 c	3.63 b	3.70 b	3.93 b	4.28 c
Neem extract	3.65 a	4.25 ab	5.68 a	5.68 a	6.10 a	6.33 b
Control	2.98 ab	5.65 a	6.90 a	6.90 a	7.43 a	8.68 a

Mean values in columns with the same letter are not statistically different (Tukey´s HSD, $P \leq 0.05$)

present investigation. Morales (2013) evaluated the tiocianomitiltio on *F. oxysporum in vitro* and reported 100% inhibition, this result coincides with our results. Bokshi *et al*. (2007) in a study conducted in postharvest of *Cucumis melo*, used iodine as one of their treatments against *Fusarium* sp., *Alternaria* sp. and *Rhizopus* sp., to prevent rotting of fruits, and found that hot iodine when used as post-harvest treatment controls most of the rot causing pathogens in storage. Montes (2009) mentioned that allicin from garlic (*Allium sativum* L.) exhibits broad-spectrum antifungal activity. Benkeblia (2004) found that garlic has an antifungal marked inhibitory effect as concentrations are increased against *Penicillium*, *Cyclopium* and *F. oxysporum*. Alkahil (2005), in *Fusarium oxysporum* isolated from tomato, tested plant extracts including garlic (*Allium sativum* L.), the extract was the best with almost 95% of fungicidal activity. Kadam *et al*. (2014) used neem as a fungicide against *F. oxysporum* f. sp. *gladioli* and reported a 35.00% inhibition, the results described for garlic extract overshadowed the findings in this investigation and in the case of neem the inhibition was lower than that obtained against *F. oxysporum* f. sp. *cepae*.

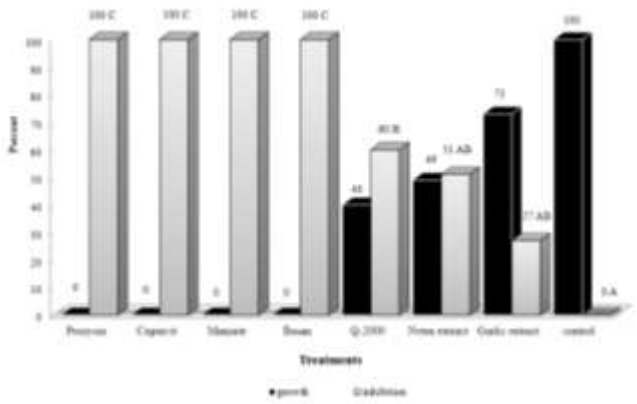

Figure 3. Percentage of inhibition and growth of *F. oxysporum* f. sp. *cepae*, for the treatments in the trial II.

***Conclusions*:** Morphological identification and pathogenicity tests identified *F. oxysporum* f. sp. *cepae* as the causal agent; however, molecularly the identification was only carried out on the genus level. The biocontrol agents *T. asperellum* and *T. harzianum* completely eliminated the pathogen; the carbendazim fungicide copper oxychloride + mancozeb, mancozeb and Tiocianomitiltio obtained 100% fungal growth inhibition, while plant extracts were not as efficient in inhibiting the pathogen.

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