academicJournals

Vol. 8(8), pp. 724-733, 19 February, 2014
DOI: 10.5897/AJMR2013.6413
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Morphological and molecular characterization of pathogenic isolates of *Fusarium* spp. obtained from gladiolus corms and their sensitivity to *Jatropha curcas* L. oil

Liliana Córdova Albores[1], Silvia Bautista Baños[1], Jorge Martínez Herrera[1], Laura Barrera Necha[1], Mónica Hernández López[1] and Andrés Cruz Hernández[2]

[1]Instituto Politécnico Nacional-Centro de Desarrollo de Productos Bióticos. Carr. Yautepec-Jojutla km. 6, San Isidro, CEPROBI 8, Yautepec, Morelos, C. P. 62731, México.
[2]Universidad Autónoma de Querétaro. Laboratorio de Microbiología, Facultad de Ciencias Naturales-Biología. Av. de las Ciencias s/n, Juriquilla, Querétaro, Delegación Santa Rosa Jauregui. C.P. 76230. México.

Accepted 30 January, 2014

The State of Morelos is the third biggest gladiolus producer in Mexico. However, this ornamental is affected by the disease named corm rot or fusarium yellows, characterized by leaf yellowing, epinasty and wilting, and caused by fungi of the genus *Fusarium*. The first objective was to corroborate the pathogenicity of the 45 isolates obtained. The second objective was to identify and characterize morphologically and molecularly by polymerase chain reaction-internal transcribed spacer (PCR-ITS), the highly pathogenic isolates and comparatively analyze the fungal species involved with the reference strain *Fusarium oxysporum* f. sp. *gladioli* (Fog). The third objective was to quantify the phorbol esters in *Jatropha curcas* oil and evaluate their antifungal potential on mycelial growth and conidial germination of different *Fusarium* species. Eleven isolates were highly significant pathogenic ($P < 0.001$). Three fungal species were identified in basal stems and damaged corms taken from field plants, namely *F. oxysporum*, *Fusarium solani* and *Fusarium proliferatum*. Molecular analyzes corroborated the species identified and their sequences were deposited in the National Center for Biotechnology Information (NCBI) gene bank. The percentage of oil obtained was 61.5 %; the phorbol ester content in the oil was 1.52 mg g^{-1} of 12,13-phorbol myristate. All species identified and the reference strain was sensitive to the 5 mg mL^{-1} oil concentration.

Key words: Corm rot, *Fusarium*, molecular analysis, phorbol esters, *Fusarium* development.

INTRODUCTION

The gladiolus is one of the main crops in the State of Morelos, which ranks as Mexico's third biggest producer of this flowering plant. Corm rot is caused by *Fusarium* spp. and results in losses of 60-80% during storage (SAGARPA, 2006). Members of the genus *Fusarium* are among the most important plant pathogens worldwide. *Fusarium* species are widely distributed in soil and organic substrates and are abundant in cultivated soils in temperate and tropical regions (Booth, 1985). Some species of this genus produce mycotoxins in stored food

*Corresponding author. E-mail: lbarrera@ipn.mx. Tel: 52 7353942020.

and cause disease in animals and humans (Ortoneda et al., 2003). Like many soil-borne fungi, the genus *Fusarium* is amply endowed with means of survival, one of its mechanisms being the ability to rapidly change both its host and its morphology and behavior (Booth, 1985; Alves-Santos et al., 1999; Katan and Di Primo, 1999; Ortoneda et al., 2003). Differentiation of *Fusarium* spp. is based on physiological and morphological characteristics, such as the size and shape of macroconidia, presence or absence of microconidia and chlamydospores, and colony morphology. Subtle differences in a single feature can delineate species. Characterization by molecular techniques using polymerase chain reaction (PCR) to amplify the internal transcribed sequences (ITS) allows identifying organisms that cannot be distinguished morphologically, and they can also help to understand the mechanisms of pathogenic variation and therefore, to develop effective management strategies (Flores-Olivas et al., 1997; Alves-Santos et al., 1999; Baayen, 2000; Haan et al., 2000). Management by synthetic fungicides leads to resistance, environmental pollution, elimination of beneficial entomofauna and operator poisoning. Consequently, there are a large number of reports on the development of alternatives such as the use of essential oils (Pauli and Knobloch, 1987; Pintore et al., 2002; Barrera-Necha and García-Barrera, 2008; Barrera-Necha et al., 2009; Tripathi et al., 2009); however, there are few reports on the use of vegetable oils. *Jatropha curcas* belongs to the family Euforbiaceae. In Mexico, the latex of this plant is used to treat mouth infections and digestive problems caused by certain fungi, and to make biodiesel, which gives it considerable economic importance (Martínez-Herrera, 2006). The seeds contain chemical compounds, among which tannins, saponins and phorbol esters stand out for their possible biological activity. *J. curcas* oil has insecticidal activity (Wink et al., 1997), and it has also been tested as an antimicrobial agent to treat infections, including sexually transmitted ones, in humans (Aiyelaagbe et al. 2007). Based on this, studies have focused on fungal development in *in vitro* tests demonstrating that mycelial growth of several fungi, including *Alternaria alternata*, *Aspergillus flavus*, *A. fumigans*, *A. niger* and *Fusarium chladmydosporum*, is inhibited when grown on seed oil and crude seed extracts at concentrations of 100 and 500 µl (Srivastava et al., 2012). In an attempt to evaluate the fungicidal effect of different plant organs of *J. curcas*, it was found that seeds followed by the fruit pulp and the whole fruit had significant inhibition of the mycelia of the fungus *Colletrotrichum gloeosporioides* isolated from papaya fruits (Rahman et al., 2011). Therefore, it is necessary to carry out studies to evaluate the use of different antifungal compounds to control fungal diseases such as phorbol esters contain in seed oil. The aims of this study were to verify the pathogenicity of the isolates obtained from gladiolus corms in the State of Morelos, to morphologically and molecularly characterize *Fusarium* isolates obtained and evaluate their response to *J. curcas* seed oil.

MATERIALS AND METHODS

Sampled material

Corms and plants (yellowing of leaves and corms with basal rot) of five varieties ('White foam', 'Red ewe', 'White ewe', 'Yellow' and 'Sancerri') were collected in the field in seven producing municipalities in the State of Morelos. A total of 34 plants showing symptoms of corm rot were collected from August to October 2011 from commercial fields in Cuautla, Yautepec, Ayala, Tlanepantla, Temixco, Yecapixtla and Totolapan.

Isolation of fungi

Soil and roots were removed from gladiolus corms by washing with running water and then corms were immersed in an acaricide solution (AK®20) at a concentration of 15 ml l^{-1} for 5 min and left to dry. They were disinfected with a sodium hypochlorite solution at 5% for 3 min, rinsed with sterile distilled water and placed in humid chambers for 24 h to promote fungal growth. From mycelium developed in the corms, isolates were made on Potato Dextrose Agar (PDA) medium and incubated at room temperature for 7 days. Monosporic cultures were established as described by Leslie and Summerell (2006).

Pathogenicity tests

Forty-five isolates showed differences in the color of the colonies and initially they were selected as different species. Healthy corms of the variety 'White foam', pre-peeled and pre-treated with a contact fungicide (Captan® 2 g L^{-1}), were planted in sterile soil (121°C, 15 lb pressure for 3 h) under greenhouse conditions. Once the approximately 1-cm stem emerged, 5 mL of the isolated spore solution of each isolate to examine were added at a concentration of 1×10^6 spores ml^{-1}. The reference strain used was *Fusarium oxysporum* f. sp. *gladioli* (Fog) already molecularly identified (Garduño-Pizaña et al., 2010) and it was treated in the same way without inoculation. Plants were assessed three weeks after inoculation.

To statistically assess pathogenicity, a damage scale was developed based on the corm rot symptoms observed in the plants, assigning it numerical values: no symptoms (plant and corm) (1); epinasty/stunting (2); yellowing (3); plant did not emerge with root development (4); plant did not emerge (5) (Mendoza, 1977).

Data analysis

A completely randomized design with 5 replicates for each isolate and a control treatment (reference strain, labeled as Fog) were used. Analysis of variance of repeated means and a multiple comparison against the control were performed with the Holm-Sidak method ($P < 0.05$) using the Sigma Stat 3.5 statistical package designed by STATCON© Witzenhausen, Germany.

Morphological characterization

For taxonomic identification of the isolates, the classification methodology of Nelson et al. (1983) and Leslie and Summerell (2006) was used. These authors suggest taking as references the

colony colors on PDA, and the length of the phialides, chlamydospores, microconidia and macroconidia. The length of the structures was measured using ImageTool© software for Windows Version 3.0 Alpha 4 designed by the Center for Health Sciences, University of Texas. Morphological observations and measurements were made with a compound microscope at 40x.

Molecular characterization

DNA extraction from 12 monosporic isolates was performed by the method described by Doyle and Doyle (1990), with modifications. The quality of the DNA obtained was verified by electrophoresis on 1% agarose gel stained with ethidium bromide (0.1 µg μl^{-1}) and observed in a gel documentation system (G Box®; Syngene, England). DNA concentration was calculated by measuring the optical density at 260 nm with the following formula:

$$[DNA] = \frac{O.D._{260} \times 50 \times Dilution}{1000}$$

PCR for ITS primers

The ITS regions were amplified with the primers ITS1 (TCCGTAGGTGAACCTGCGG), ITS2 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) (White et al., 1990; Haan et al., 2000; Abd-Elsalam et al., 2003; Moreno-Velázquez et al., 2005; Teixeira et al., 2005; González-Pérez et al., 2009), as well as the specific primers for the genus *Fusarium* reported by Adb-Elsalam et al. (2003): ITS-Fu-Fwd (CGCACGATTACCACTAACGA) and ITS-Fu-Rev (CAACTCCCAAACCCCTGTGA). The primers were synthesized by the Sigma Aldrich® Company. Each PCR reaction was carried out in a final volume of 25 µl; 5.5 µl of nuclease-free water were mixed with 2.0 µl of DNA of the fungus problem, 2.5 µl of each ITS and 12.5 µl of Taq 2X Master Mix® (BioLabs*Inc*, New England). The amplification reaction was carried out in a Perkin Elmer® thermocycler (GeneAmp PCR System 2400) with the following program: initial denaturation at 95°C for 5 min; 35 cycles of denaturation, alignment and extension at 95°C for 1 min and 72°C for 2 min. respectively, and a final extension at 72°C for 10 min. The amplified fragments were verified by electrophoresis in 1% agarose gel stained with ethidium bromide (0.1 µg μl^{-1}). The gel was run at 85 V for 40 min and observed in a gel documentation system (G Box®; Syngene, England).

Sequencing

The amplified PCR products were purified using the DNA Clean & Concentrator™-5 Kit (Zymo Research, USA) and analyzed at the Sequencing Laboratory of the Institute of Biotechnology, belonging to the University National Autonomous of México, in Cuernavaca, Morelos. The sequences were aligned and compared with sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (Zhang et al., 2000).

Collection of *J. curcas* seeds

Seeds were collected from *J. curcas* plants being used as a living fence in the following locations: 1) Segunda Sección de la Cebadilla, municipality of Tapachula, Chiapas, 14° 50' 48" North; 92° 17' 00" West, at 109 masl. 2) Villa de Mazatán, Chiapas, 14° 52' 17" North; 92° 27' 04" West, at 26 masl. 3) Ejido la Victoria, municipality of Mazatán, Chiapas, 14° 49' 16" North; 92° 29' 56" West, at 10 masl. Location altitudes were taken with a GPS lll (Garmin, GPS lll Plus model, Ronsey, UK).

Oil extraction

The extraction of the seed oil was performed according to the Soxhlet method (NMX-F-089-S-1978), with petroleum ether and hexane and 8-h reflux time. About 2 to 5 g of ground *J. curcas* seeds were weighed and placed in the extraction thimble. After extraction, the solvent was gently evaporated from the flask and the excess petroleum ether or hexane was removed using a BÜCHI model 114 rotary evaporator (BÜCHI® Labortechnik AG, Switzerland) at 60°C. The calculations to determine the percentage of oil obtained was performed using the formula:

Oil (%) = (P-p/M) × 100

Where: P = mass in grams of the flask with the oil; p = mass in grams of the flask; M = mass in grams of the sample.

Phorbol esters were quantified using the high performance liquid chromatography (HPLC) method described by Martínez-Herrera (2006). Specifically, 20 ml of methanol were added to a 2 g sample, which was sonicated for 2 min. The sample was centrifuged at 3600 rpm for 10 min and the supernatant was evaporated almost to dryness in a rotary evaporator (the extraction was repeated 3 times). The standard used was 12,13-phorbol myristate (Sigma Aldrich®), which showed a retention time of 25 min. The results were expressed as mg g^{-1} of sample equivalent to 12,13-phorbol myristate. This analysis was performed in the food laboratory of the Institute of Animal Production in the Tropics and Subtropics at the University of Hohenheim in Stuttgart, Germany.

Mycelial growth

Only the 11 isolates mentioned above and the reference strain (Fog) were used to evaluate this parameter. A 5-mm disc of the pathogen was placed on 60 × 15 mm Petri dishes containing PDA, Tween 20® and the oil at different concentrations (2.5, 5 and 10 mg ml^{-1}), as well as 4 controls: PDA, petroleum ether (10 mg ml^{-1}), Tween 20® (10 mg ml^{-1}) and Captan® (2 g L^{-1}). They were incubated at 25°C in the dark. Diameter growth was measured daily for 5 days with a Vernier caliper until the control treatments reached the edge of the Petri dish. The experimental design was completely randomized and consisted of 7 treatments with 6 replicates. Analysis of variance (ANOVA) and Tukey's comparison of means test ($P < 0.05$) were performed using the Sigma Stat 3.5 software program designed by STATCON© Witzenhausen, Germany.

Spore germination

Ten milliliters of sterile distilled water were added to Petri dishes containing the growth of each isolate, then the surface was scraped with a bent metal rod and the filtrate was passed through cotton gauze. Of this suspension, 20 µl were placed on PDA discs of 10 mm in diameter and incubated for 6 to 10 h at room temperature (26°C). Later, a few drops of lactophenol methylene blue were added and the number of germinated spores was determined by photo analysis with Image Tool© software for Windows version 2.01 Alpha 4, developed by the Center for Health Sciences at the University of Texas. Germination was evaluated on nine PDA discs. Mean percentage germination and standard deviations were calculated.

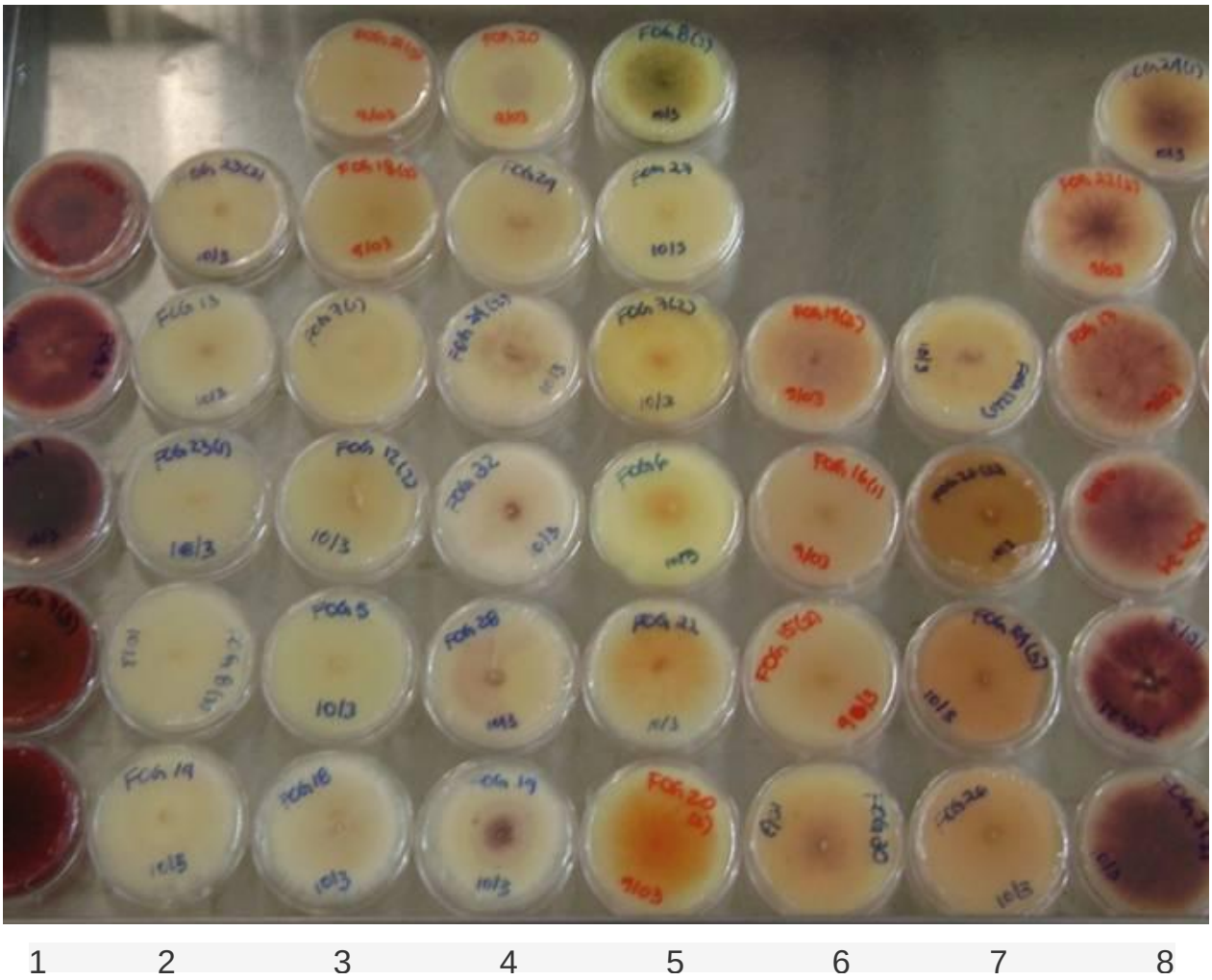

Figure 1. Preliminary classification of isolates according to coloring on PDA medium.

RESULTS

Number of isolates

Their morphological aspect, identified as belonging to the genus Fusarium, obtained a total of 45 isolates. The isolates showed different apparent morphological characteristics, mainly in the color of the culture medium. Nelson et al. (1983) performed a morphological characterization using the coloration on PDA as indicating that each species has a specific color. Based on the coloring obtained, a preliminary classification was conducted, obtaining a total of eight groups as shown in Figure 1.

Pathogenicity tests

Statistical analysis showed that when compared with the reference strain Fog (T25), isolates T1, T15, T20, T36 and T44 were significantly different ($P < 0.01$), whereas highly significant differences ($P < 0.001$) were observed in isolates T9, T11, T12, T20, T24, T30, T32, T34, T35, T39 and T40, which were collected from the municipalities of Yautepec and Cuautla (Figure 2). Isolates that showed highly significant differences in the pathogenicity capacity were used for morphological and molecular characterization and *J. curcas* oil sensitivity testing.

Morphological characterization

Some of the typical structures of the 11 isolates and the reference strain (Fog) are summarized in Table 1.

Molecular characterization

The DNA had a yield of 220 to 670 µg g^{-1} of mycelium, the band of the PCR products containing the primers ITS2-ITS5 was 500 base pairs (bp) and the PCR product for the primers ITS-Fu-Fwd and ITS-Fu-Rev was from

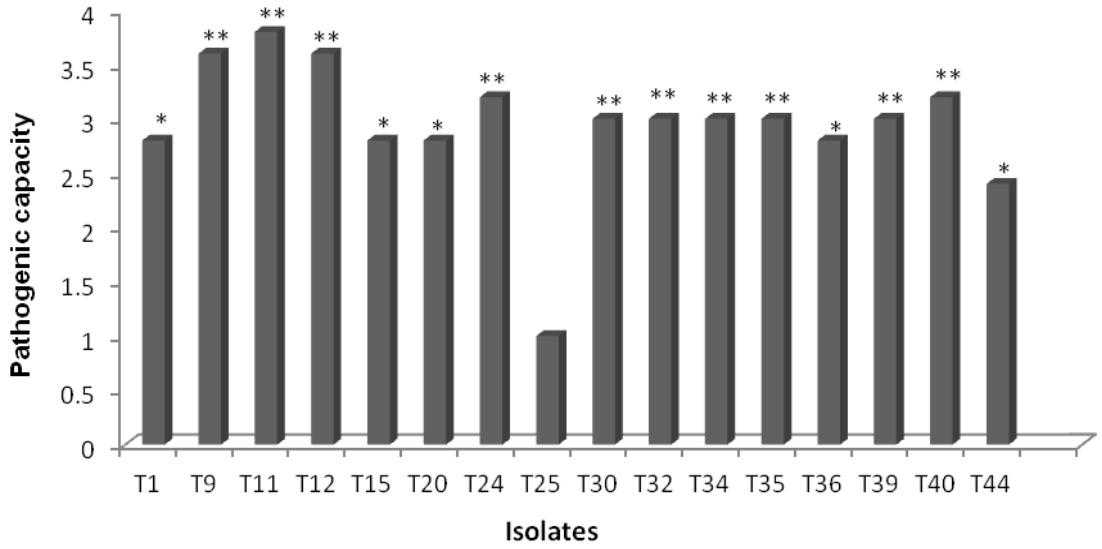

Figure 2. Comparison of means against the reference strain Fog (T25) of the pathogenic capacity of isolates of *Fusarium* spp. Significant difference *P < 0.01; **P < 0.001.

410 to 429 bp (Figure 3). For the multiple alignments, the total amplified portion of the 12-nucleotide sequences is shown in Table 2, which corresponds to the complete sequence of both regions (ITS2 and ITS5). In Genbank, the sequence of *F. oxysporum* f. sp *gladioli* and isolates T30, T35 and T39 showed a similarity index of 99% with *F. oxysporum*. Another five isolates showed a similarity index of 99% with *F. proliferatum*. Isolates T20 and T34 showed no similarity to other sequences (Table 2).

Oil extraction

The oil percentage obtained for both solvents was 61.5%. Phorbol esters in the kernel meal and the oil extracted with petroleum ether were 0.94 and 1.52 mg g^{-1}, respectively, while the oil extracted with hexane was 0.24 and 0.67 mg g^{-1}, respectively.

Mycelium growth

Comparison of means of mycelial growth on the last day of each treatment indicated that the eleven *Fusarium* isolates and the reference strain (Fog) were more sensitive to the concentration of 5 mg ml^{-1}. Isolates T9, T11, T20, T32, T34, T35 and T40 were also sensitive to the concentration of 2.5 mg ml^{-1}. For isolate T9, it was observed that the concentration of 5 mg ml^{-1} showed less mycelial growth than the fungicide Captan®, while for the isolate T11 incubated at the same concentration (5 mg ml^{-1}) and the control with Tween®20 there were no significant differences. The isolate most susceptible to the fungicide Captan® was the reference strain (Fog) (Table 3).

Spore germination

Treatment susceptibility depended on the isolate, as no trend was observed for any specific treatment (Table 4). Isolate T9 presented the lowest germination rate with *J. curcas* oil at concentrations of 2.5 and 10 mg ml^{-1} with a percentage germination of 14.40 and 14.43%, respectively, as compared to 98.44% for the PDA treatment. Isolate T24 also showed a low germination rate, which was 12.62 and 17.28% for the concentrations of 2.5 and 5 mg ml^{-1}, respectively. Isolate T24 was the most susceptible to the treatment with the fungicide Captan®, presenting a germination of 33.79% while the PDA treatment had 99.77% germination.

DISCUSSION

The highly pathogenic isolates were characterized morphologically, identifying at least 2 species: *F. oxysporum* and *Fusarium solani*, which had different morphological features, both gross and microscopic, and which were associated with the afore mentioned species. González-Pérez et al. (2009) reported that these species caused rot in gladiolus in San Martin Texmelucan, Puebla. They also reported that these species differed in terms of colony color and morphological characteristics, coinciding with the results obtained in this work for descriptions of macro- and micro-conidia, phialides and chlamydospores. For their part, Montiel-Gonzalez et al. (2005) described the morphological characteristics of *F. oxysporum*, *F. solani*, *Fusarium lateritium*, *Fusarium reticulatum*, *Fusarium equiseti*, *Fusarium verticillioides*, *Fusarium culmorum*, *Fusarium crookwellense, Fusarium proliferatum* and *Fusarium sporotrichioides* present in bean roots in five

Table 1. Morphological characteristics of isolates of the genus *Fusarium* collected in gladiolus-growing areas of the state of Morelos.

Isolate	Color		Microconidia	Macroconidia				Chlamydospore	Species
	Anverse	Reverse		Apical	Basal	Size (μm)	# septa		
Fog*	White	Violet	Oval with one septa Oval	Hooked	Distinctly notched	50.50-67.49	3	No	*F. oxysporum*
T9	White	Orange	Oval with one septa Reniform	Blunt	Barely notched	48.72-64.51	3-4	No	*F. solani*
T11	White	Brown	Oval Oval with one septa	Hooked	Distinctly notched	69.63-73.19	3-4	Single and paired verrucose	*F. oxysporum*
T12	White and orange	Brown	Oval with one septa Oval	Blunt	Barely notched	55.37-82.54	3-4	No	*F. solani*
T20	White	Cream	Oval Oval with one septa	Papillate	Barely notched	56.35-67.68	3-4	Single verrucose	*F. solani*
T24	Cream	Cream	Oval Pyriform Globose Oval with one septa	Papillate	Barely notched	54.77-87.51	3-4	Single verrucose Paired smooth-walled	*F. solani*
T30	White	Cream	Obovoid with a truncate base	NO	NO	NO	NO	Single and paired verrucose	*Fusarium* spp.
T32	White	Cream	Oval Obovoid with a truncate base Oval with one septa	Blunt	Foot shaped	57.31-69.52	3	Single verrucose Paired smooth-walled	*F. oxysporum*
T34	White	Cream	Oval Obovoid with a truncate base	NO	NO	NO	NO	Single verrucose Paired	*Fusarium* spp.
T35	White	Violet	Oval Oval with one septa	NO	NO	NO	NO	Single smooth	*Fusarium* spp.
T39	White and orange	Cream	Globose oval Oval with one septa	NO	NO	NO	NO	Chains 2,3 and 4 verrucose	*Fusarium* spp.
T40	Cream	Yellow	Globose Oval Obovoid with a truncate base	NO	NO	NO	NO	Single verrucose	*Fusarium* spp.

*Reference strain.

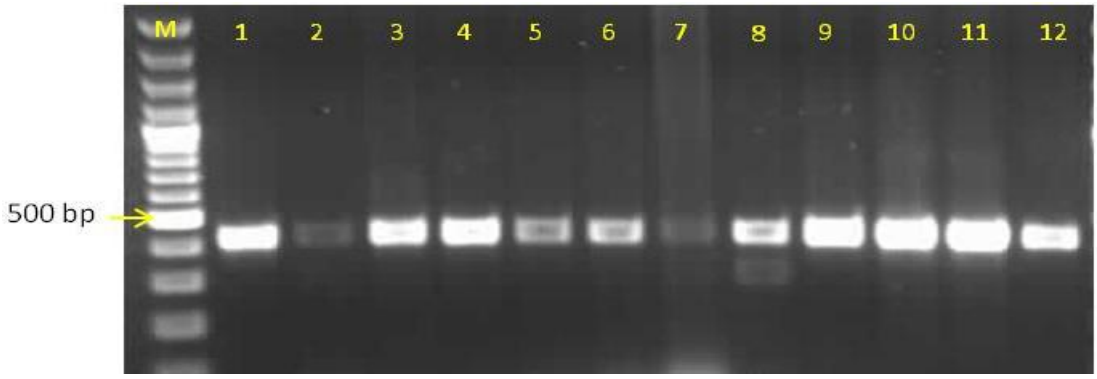

Figure 3. 1% agarose gel electrophoresis of the PCR products of the regions ITS-Fu-Fwd and ITS-Fu-Rev of the isolates of *Fusarium* spp. Line: M) Molecular marker 100 bp (BioLabsInc, New England), 1) Fog, 2) T9, 3) T11, 4) T12, 5) T20, 6) T24, 7) T30, 8) T32, 9) T34, 10) T35, 11) T39, 12) T40.

Table 2. Morphological and molecular characterization of highly pathogenic isolates from gladiolus corms.

Isolate	Color		Species				
	Anverse	Reverse	Morphological characterization	Molecular characterization	Molecular size (bp)	Similarity percentage (%)	Accession number NCBI
FOG*	White	Violet	*Fusarium oxysporum*	*Fusarium oxysporum*	537	99	GU724514.1
T9	White	Orange	*Fusarium solani*	*Fusarium solani*	538	99	EU982942.1
T11	White	Brown	*Fusarium oxysporum*	*Fusarium proliferatum*	553	99	EU839366.1
T12	White	Brown	*Fusarium solani*	*Fusarium solani*	539	86	EU625405.1
T20	White	Cream	*Fusarium solani*	No identified	435		
T24	Cream	Cream	*Fusarium solani*	*Fusarium solani*	456	78	GU355660.1
T30	White	Cream	*Fusarium* spp	*Fusarium oxysporum*	526	99	GU445378.1
T32	White	Cream	*Fusarium oxysporum*	*Fusarium solani*	541	99	FJ460589.1
T34	White	Cream	*Fusarium* spp	No identified	433		
T35	White	Violet	*Fusarium* spp	*Fusarium oxysporum*	516	99	GU724514.1
T39	White	Cream	*Fusarium* spp	*Fusarium oxysporum*	515	99	GU724514.1
T40	Cream	Yellow	*Fusarium* spp	*Fusarium solani*	557	99	EU625405.1

states of central Mexico. The descriptions made for the species isolated in this study also agreed with those reported by these authors. Molecular taxonomic results by PCR: ITS confirm the morphological identification of ten isolates, which corresponded to *F. oxysporum*, *F. solani* and *F. proliferatum*. The two isolates that showed no similarity when aligning them using BLAST could be *Fusarium* species, where genetic variation has occurred due to the indiscriminate use of fungicides in the region. Alternatively, these unidentified isolates could be species not yet reported. The percentage of *J. curcas* seed oil reported by Martínez-Herrera et al. (2010) coincides with the values obtained in this research in seeds from the state of Chiapas, with a value of 60.4%. The same author reports phorbol ester values of 2.03 mg g^{-1} in oil and 0.16 mg g^{-1} in kernel meal. The differences in phorbol ester

Table 3. Effect of *J. curcas* oil on mycelial growth of isolates of *Fusarium* spp.

Treatment	Isolate											
	Fog*	T9	T11	T12	T20	T24	T30	T32	T34	T35	T39	T40
PDA	4.08^{a} (0.82)	4.51^{a} (0.82)	4.95^{a} (1.01)	4.19^{a} (0.87)	4.76^{a} (0.96)	3.70^{a} (0.79)	4.26^{a} (0.84)	4.68^{a} (0.96)	4.16^{a} (0.88)	4.91^{a} (0.99)	4.23^{a} (0.90)	4.80^{a} (0.99)
Tween®20	3.45^{a} (0.61)	3.10^{bc} (0.53)	2.86^{c} (0.47)	3.55^{bc} (0.5)	2.91^{d} (0.48)	2.61^{bc} (0.46)	3.56^{ab} (0.89)	3.0^{bc} (0.56)	3.56^{abc} (0.70)	3.30^{bc} (0.59)	2.95^{b} (0.54)	3.76^{b} (0.75)
Captan®	0.78^{b} (0.08)	1.31^{d} (0.17)	1.43^{d} (0.20)	1.43^{d} (0.19)	1.50^{e} (0.21)	1.08^{d} (0.13)	1.23^{d} (0.19)	0.90 ^{e} (0.09)	0.98^{e} (0.09)	1.41^{e} (0.23)	1.66^{d} (0.26)	1.33^{d} (0.20)
Petroleum ether	3.91^{a} (0.75)	3.0^{bc} (0.56)	3.93^{b} (0.76)	3.11^{bc} (0.6)	3.78^{bc} (0.74)	3.03^{ab} (0.53)	3.96^{ab} (0.81)	3.66^{b} (0.72)	3.31^{bcd} (0.66)	3.91^{ab} (0.79)	3.51^{ab} (0.74)	3.63^{b} (0.70)
Oil 2.5 mg mL^{-1}	3.61^{a} (0.72)	2.78^{c} (0.54)	2.81^{c} (0.47)	3.23^{bc} (0.6)	3.55^{cd} (0.70)	3.33^{ab} (0.68)	3.60^{ab} (0.68)	2.23^{cd} (0.40)	2.76^{cd} (0.52)	2.8^{cd} (0.49)	3.55^{ab}(0.70)	3.51^{b} (0.67)
Oil 5 mg mL^{-1}	1.28^{b} (0.17)	1.26^{d} (0.15)	1.83^{d} (0.28)	1.92^{d} (0.28)	2.06^{e} (0.34)	1.95^{cd} (0.28)	2.61^{c} (0.45)	1.23^{de} (0.14)	2.56^{d} (0.46)	1.98^{de} (0.41)	2.48^{c} (0.42)	2.33^{c} (0.42)
Oil 10 mg mL^{-1}	3.39^{a} (0.72)	3.70^{ab} (0.77)	3.11^{bc} (0.61)	3.67^{ab}(0.6)	4.50^{ab} (0.92)	3.05^{ab} (0.61)	3.38^{bc} (0.68)	3.35^{b} (0.66)	3.78^{ab} (0.79)	3.58^{bc} (0.73)	3.68^{ab} (0.76)	3.55^{b} (0.76)

*Reference strain. Means followed by different letters in each column are significantly different by Tukey test at (α 0.05). Values in parenthesis indicate growth rate (mm day^{-1}).

Table 4. Effect of *J. curcas* oil on percentage conidial germination of isolates of *Fusarium* spp.

Isolate	Treatment						
	PDA	Tween® 20	Captan®	Petroleum ether	*J. curcas* oil (mg ml^{-1})		
					2.5	5	10
Fog*	97.92 ± 3.77	97.56 ± 5.20	96.26 ± 5.15	96.05 ± 4.68	61.34 ± 18.52	90.06 ± 14.62	39.87 ± 20.86
T9	98.44 ± 1.66	68.44 ± 14.27	65.35 ± 28.05	36.44 ± 25.43	14.40 ± 9.45	44.64 ± 30.46	14.43 ± 11.44
T11	97.55 ± 3.95	95.55 ± 3.12	83.33 ± 17.37	97.11 ± 2.84	96.00 ± 3.00	96.24 ± 4.69	98.22 ± 2.90
T12	99.55 ± 0.88	44.44 ± 22.70	83.33 ± 35.35	97.17 ± 5.65	44.44 ± 22.75	90.74 ± 18.84	97.77 ± 6.66
T20	99.55 ± 0.88	93.96 ± 6.04	41.95 ± 15.71	42.15 ± 10.42	91.55 ± 7.46	52.41 ± 19.90	98.66 ± 1.73
T24	99.77 ± 0.66	86.00 ± 12.84	33.79 ± 21.46	34.25 ± 11.09	12.62 ± 15.46	17.28 ± 6.03	82.66 ± 30.53
T30	97.55 ± 3.97	92.22 ± 7.03	92.07 ± 4.77	90.00 ± 7.28	89.33 ± 5.19	91.85 ± 7.78	55.37 ± 26.10
T32	99.55 ± 0.88	44.44 ± 22.70	83.33 ± 35.35	97.17 ± 5.65	44.44 ± 22.75	90.74 ± 18.84	97.77 ± 6.66
T34	99.77 ± 0.66	97.77 ± 2.10	91.66 ± 17.67	91.31 ± 8.52	89.13 ± 7.54	64.53 ± 33.37	89.58 ± 6.40
T35	100.0 ± 0.00	98.44 ± 2.51	100.00 ± 0.00	100.00 ± 0.00	98.22 ± 3.93	97.22 ± 4.39	91.15 ± 11.99
T39	99.22 ± 1.16	92.06 ± 7.67	99.77 ± 0.66	96.92 ± 4.89	96.33 ± 4.03	60.81 ± 15.04	94.41 ± 5.29
T40	99.77 ± 0.66	98.20 ± 2.18	89.94 ± 12.53	98.97 ± 2.06	91.00 ± 16.78	70.17 ± 20.16	90.54 ± 12.54

*Reference strain. Means and SD.

content for each solvent, obtained in this study, could be because the two solvents have a different boiling point (68.85 for hexane and 60°C for petroleum ether) and because the phorbol esters in the oil are thermolabile, which means that by applying a higher temperature to obtain the oil with the solvents used, the phorbol esters are degraded. The greatest effect on mycelial growth for the isolates evaluated was with the *J. curcas* oil treatment at the concentration of 5 mg ml^{-1}. Growth rates between 0.140 and 0.462 mm day^{-1} were obtained, when compared with 0.820 to 1.018 mm day^{-1} for the PDA control. Some authors reported that the effect on the mycelial growth of the compounds present in the essential oils may be due to

two factors: the first involves inhibition of extracellular enzyme synthesis, and the second the alteration of the cell wall structure (Tripathi et al., 2009). Siva et al. (2008) evaluated the antifungal effect of aqueous, acetone and ethanol extracts of 20 medicinal plants against *F. oxysporum* f. sp. *melongenae*. *J. curcas* was one of the plants evaluated and showed inhibition percentages of 100% for all extracts used. Donlaporn and Suntornsuk (2010) evaluated the antifungal activity of ethanol extracts of *J. curcas* seeds and the importance of phorbol esters present in the extracts on *F. oxysporum*, *F. semitectum*, *Colletotrichum capsici*, *C. gloeosporioides*, *Pythium aphanidermatum*, *Lasiodiplodia theobromae* and *Curvularia lunata*, using concentrations of 0 to 10,000 mg L^{-1}. Concentrations from 6,000 mg L^{-1} showed 100% inhibition of mycelial growth for all tested pathogens. These authors are the first to report that phorbol esters are responsible for the fungicidal activity of the extracts, as they note that by removing these compounds from the extracts there were no significant differences as compared to the control. As for the germination of conidia, the three concentrations used in this study were effective for only two isolates (T9 and T24) with germination between 12.62 and 17.28%. Ogbebor and Adekunle (2008) assessed the germination of *Drechslera heveae* conidia on PDA with *J. curcas* extracts, attaining a 20% germination rate with the 100% concentration. The three species identified showed symptoms of the disease in the plant, such as leaf yellowing, epinasty and some-times late flowering in the field. It is suggested that in different parts of Morelos, corm rot in gladiolus is caused by three fungal species, which showed morphological, molecular and pathogenic differences, plus different sensitivity to *J. curcas* oil. All three species were capable of causing the disease. The results of this research demonstrate the antifungal potential of *J. curcas* oil to control fungi that cause diseases in ornamental plants.

ACKNOWLEDGEMENTS

This work was funded by the Secretary of Postgraduate and Research (Projects 20090234 and 20100783) and by the Commissions of Operation and Support to Academic Activities from the National Polytechnic Institute.

REFERENCES

Abd-Elsalam AK, Aly IN, Abdel-Satar MA, Khalil MS, Verreet JA (2003) PCR identification of *Fusarium* genus based on nuclear ribosomal-DNA sequence data. Afr. J. Biotechnol. 2:82-85.

Aiyelaagbe OO, Adeniyi BA, Fatunsin OF, Arimah BD (2007) *In Vitro* antimicrobial activity and phytochemical analisis of *Jatropha curcas* roots. Int. J. Pharmacol. 3:106-110.

Alves-Santos FM, Benito EP, Elsava AP, Díaz-Mínguez JM. (1999) Genetic diversity of *Fusarium oxysporum* strains from common bean fields in Spain. Appl. Environ. Microbiol. 65:3335-3340.

Baayen RP (2000). Diagnosis and detection of host-specific forms of *Fusarium oxysporum.* EPPO Bull. 30:489-491.

Barrera-Necha L, García-Barrera L (2008) Antifungal activity of essential oils and their compounds on growth of *Fusarium* sp. isolated from papaya (*Carica papaya*). Rev. UDO Agric. 8:33-41.

Barrera-Necha L, Garduño-Pizaña C, García-Barrera LJ (2009) *In vitro* antifungal activity of essential oils and their compounds on mycelia growth of *Fusarium oxysporum* f.sp. *gladioli* (Massey) Snyder and Hansen. Plant Pathol. J. 8:17-21.

Booth C (1985) The genus *Fusarium*. Ed. Commonweath Mycological Institute. p. 237.

Donlaporn S, Suntornsuk W (2010) Antifungal Activities of Ethanolic Extract from *Jatropha curcas* Seed Cake. J. Microbiol. Biotechnol. 20:319-324.

Doyle JJ, Doyle JL (1990). A rapid total DNA preparation procedure for fresh plant tissue. Focus 12:13-15.

Flores-Olivas A, Martínez-Soriano JP, Martínez-Espinoza AD (1997) Use of technology new in detection and genetic analysis of phytopathogens. Phytopathology 32:96-111.

Garduño-Pizaña C, Barrera-Necha LL, Ríos-Gómez MY (2010). Evaluation of the fungicidal activity of leaves powders and extracts of fifteen Mexican plants against *Fusarium oxysporum* f. sp. *gladioli* (Massey) Snyder & Hansen. Plant Pathol. J. 9:79-87.

González-Pérez E, Yañez-Morales MJ, Ortega-Escobar H, Velázquez-Mendoza J (2009) Comparative Analysis among Pathogenic Fungal Species that Cause Gladiolus (*Gladiolus grandiflorus* Hort.) Corm Rot in Mexico. Mex. J. Phytopathol. 27:45-52.

Haan LAM, Numansen A, Roebroeck EJA, van Doorn J. (2000) PCR detection of *Fusarium oxysporum* f.sp. *gladioli* race 1, causal agent of *Gladiolus* yellows disease, from infected corms. Plant Pathol. 49:89-100.

Katan T, Di Primo P (1999). Current status of vegetative compatibility groups in *Fusarium oxysporum*: Supplement (1999). Phytoparasitica 27:1-5.

Leslie J, Summerell BA (2006). The *Fusarium* laboratory manual. Ed. Blackwell publishing. p. 388.

Martínez-Herrera J (2006) Genetic and nutritional diversity of piñón (*Jatropha curcas* L.) in México. PhD dissertation, Instituto Politécnico Nacional, México.

Martínez-Herrera J, Martínez-Ayala AL, Makkar H, Francis H, Becker K (2010) Agroclimatic conditions, Chemical and Nutritional Characterization of Differents provenances of *Jatropha curcas* L. from Mexico. Eur. J. Sci. Res. 39:396-407.

Mendoza-Zamora C (1997). Certificación de estudios de efectividad biológica de plaguicidas. Ed. Cecilio Mendoza Zamora-Universidad Autonóma de Chapingo. Texcoco, Mexico. 279 p.

Montiel-González L, González-Flores F, Sánchez-García BM, Guzmán-Rivera S, Gámez-Vázquez FP, Acosta-Gallegos JA, Rodríguez-Guerra R, Simpson-Williamson J, Cabral-Enciso M, Mendoza-Elos M. (2005). Species of *Fusarium* occurring on bean (*Phaseolus vulgaris* L.) roots affected with rots in five states of Central México. Mex. J. Phytopathol. 23:1-7.

Moreno-Velázquez M, Yáñez-Morales MJ, Rojas-Martínez RI, Zavaleta-Mejía E, Trinidad-Santos A, Arellano-Vazquez JL (2005). Diversity of fungi of amaranthus (*Amaranthus hypochondriacus* L.) seed and their molecular characterization. Mex. J. Phytopathol. 23:111-118.

Nelson P E, Toussoun T A, Marasas WFO (1983) *Fusarium* species: an illustration manual for identification. University Park, Pennsylvania State University Press, Pensylvania, USA. p. 193.

Ogbebor ON, Adekunle AT (2008). Inhibition of *Drechslera heveae* (Petch) M. B. Ellis, causal organism of Bird's eye spot disease of rubber (*Hevea brasiliensis* Muell Arg.) using plant extracts. Afr. J. Gen. Agric. 4:19-26.

Ortoneda M, Guarro J, Madrid M, Caracuel Z, Roncero MI, Mayayo E, Di Pietro A. (2003). *Fusarium oxysporum* as a Multihost Model for the Genetic Dissection of Fungal Virulence in Plants and Mammals. Infect. Immun. 72:1760–1766.

Pauli A, Knobloch K (1987) Inhibitory effects of essential oil components on growth of food-contamining fungi. Z. Leb.-Unt. und-For. 185:10-13.

Pintore G, Chessa M, Boatto GP, Cerri G, Usai M, Trillini B (2002) Essential oil *Hypericum perforatum* L. var. *angustifolium* DC growing wild in Sardinia (Italy). J. Essential Oil Res. 17:533-535.

Rahman M, Ahmad SH, Mohamed MTM, Rahman MZA (2011). Extraction of *Jatropha curcas* fruits for antifungal activity against anthracnose (*Colletotrichum gloeosporioides*) of papaya. Afr. J. Biotechnol. 10:9796-9799.

SAGARPA (2006). Manual Técnico fitosanitario del cultivo de gladiolo. Ed. Comité Estatal de Sanidad Vegetal del Estado de Morelos. p. 13.
Siva N, Ganesan S, Banumathy N, Muthuchelia (2008). Antifungal Effect of Leaf Extract of Some Medicinal Plants Against *Fusarium oxysporum* Causing Wilt Disease of *Solanum melogena* L. Ethnobot. Leaflets 12:156-163.
Srivastava S, Kumar R, Sinha A. (2012). Antifungal Activity of Oil Against Some Seed-borne Fungi. Plant Pathol. J. 11:120-123.
Teixeira M, Figueira G, Sartoratto A, Garcia V, Delarmelina C (2005). Anti-*Candida* activity of Brazilian medicinal plants. J. Ethnopharmacol. 97:305-311
Tripathi A, Sharma N, Sharma V (2009). *In vitro* efficacy of *Hyptis suaveolens* L. (Poit.) essential oil on growth and morphogenesis of *Fusarium oxysporum* f.sp. *gladioli* (Massey) Snyder & Hansen. World J. Microbiol. Biotechnol. 25:503-512.
White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Chapter 38. PCR protocols: A guide to methods and applications. Ed. Academic Press Inc, pp. 315-323.
Wink M, Koshmieder C, Saverwein M, Sporer F (1997). *Phorbol esters of Jatropha curcas*. In G.M. Gübitz, M. Mittelbach, & M. Trabi (Eds.), Biofuels and Industrial products from *Jatropha curcas* Austria, Dbv-Verlag Univ. pp.160-166.
Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 7:203-14.