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mrlegarreta@prodigy.net.mx
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Comparative Analysis among Pathogenic Fungal Species that Cause Gladiolus (*Gladiolus grandiflorus* Hort.) Corm Rot in Mexico

Enrique González-Pérez[1], María de Jesús Yáñez-Morales[2], Héctor Manuel Ortega-Escobar[1], and **Juan Velázquez-Mendoza[3]**, Colegio de Postgraduados, [1]Hidrociencias, [2]Fitopatología, and [3]Forestal, Campus Montecillo, km 36.5 Carr. México-Texcoco, Montecillo, Edo. de México CP 56230. Correspondence to: yanezmj@colpos.mx

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Abstract. An unknown disease causing wilting and death of gladiolus plants was studied in 2004, in Tlacotepec de José Manzo, in the municipality of San Salvador el Verde, located in the region of San Martín Texmelucan, Puebla, Mexico. The objective was to identify and characterize by ITS-PCR the plant pathogen(s) that cause this disease and to comparatively analyze the fungal species involved. Five species of fungi were identified from healthy and symptomatic corms taken in the field and from storage, and also from damaged basal stem and neck tissues of field plants, these were: *Fusarium oxysporum* f. *gladioli*, *F. solani*, *Penicillium* sp.; and two previously reported *Acremonium strictum* and *Gliocladium roseum* which were unknown worldwide. All species were pathogenic, they grouped into three categories for their aggressiveness and differed in cultural characters. The molecular analyses corroborated the species identification and their sequences were deposited in the NCBI GenBank. This was the first formal study of this kind in Mexico.

Additional keywords: Basal stem, neck rot, ITS.

Resumen. Una enfermedad desconocida causante de marchitez y muerte de plantas de gladiolo fue estudiada en 2004, en Tlacotepec de José Manzo, en el municipio de San Salvador el Verde, en la region de San Martín Texmelucan, Puebla, México. El objetivo fue identificar y caracterizar molecularmente por PCR-ITS el (los) patógenos causantes de la enfermedad y analizar comparativamente las especies de hongos involucrados. Cinco especies fungosas fueron identificadas en tejido sano y sintomático de cormos plantados en campo y de almacén; y también en tejido dañado de tallos basales y cuello de plantas de campo, estas fueron: *Fusarium oxysporum* f. *gladioli*, *F. solani*, *Penicillium* sp.; y dos especies reportadas anteriormente, *Acremonium strictum* y *Gliocladium roseum* que eran desconocidas en este cultivo. Todas las especies fueron patogénicas, se agruparon en tres categorías por su agresividad para causar la enfermedad, difirieron en características culturales. Los análisis moleculares corroboraron las especies identificadas y sus secuencias fueron depositadas en el banco de genes del NCBI. Este fue el primer estudio formal de este tipo en México.

Palabras clave adicionales: Tallo basal, pudrición de cuello, ITS.

In the region of San Martín Texmelucan (19° 17´ 00" N, 98° 26´ 00" W; alt. 2360 m), Puebla, Mexico, gladiolus (*Gladiolus grandiflorus* Hort.) is one of the most important ornamental crops. This is due to the large area planted in Puebla State (around 1,400 ha per year) with different varieties and its production which ranks third after chrysanthemum (*Chrysanthemum* spp.) and rose (*Rosa* spp.) (SAGARPA, 2001). Symptoms of wilt and death of gladiolus plants in this region, however, result in estimated losses of about 40 to 80% per ha (personal communication, Aarón González-Morales, member of the Gladiolus Farmer´s Association), yet the causes remain unknown. In other countries such as Belgium, Britain, Holland, Japan, London, Spain, and USA, *Fusarium oxysporum* Schlechtend.:Fr. f. *gladioli* (L. Massey) W.C. Snyder y H.N. Hans. has been reported since 1926 (Buxton and Robertson, 1953; WDCM, 2009a) to cause gladiolus disease; and *Penicillium gladioli* McCulloch and Thom, to cause core rot of gladiolus corms in Australia, Brazil, Canada, India, Japan, several countries from Europe, and the USA (WDCM. 2009b). In these countries, both pathogens are important and well known as causal agents of gladiolus diseases in field and stored corms (Larson, 1992). According to several databases and Mexican literature (CP, 2009), even when gladiolus is an important crop, no research has been conducted in Mexico, although the gladiolus disease is referred to be caused by the same pathogens mentioned above (Gardezi *et al*., 2001; Romero-Cova, 1993). Preliminary sampling in San Martín Texmelucan revealed corm rot and basal stem and neck damage along with fungal signs

(morphological structures). From this research, *Acremonium strictum* W. Gams and *Gliocladium roseum* Bainier were reported for the first time worldwide as the causal agents of gladiolus corm rot in Mexico (González-Pérez *et al*., 2008). The present study examined whether this disease occurs as a result of corm rot caused by several fungi, and if it is being introduced to the field by planted corms. The objectives were to identify and molecularly characterize the causal agents of corm rot in stored and field samples using an internal transcribed spacer: polymerase chain reaction (ITS:PCR) (White *et al*., 1990), and compare all the species involved, including the two previously reported by González-Pérez *et al*. (2008).

MATERIALS AND METHODS

Material sampled. Corm and plant samples (basal stem with necks) from two gladiolus cultivar were taken from plant material in storage and from the field in San Martín Texmelucan in the community of Tlacotepec de Jose Manzo in the municipality of San Salvador el Verde, Puebla, Mexico.

Samples from storage. Twenty corms of cultivar Lupe stored for two months were collected by directed sampling during January, 2004, and grouped in five categories (four corms per category) according to percentage of damage. Categories were: apparently healthy (0% damage), and 25, 50, 75, and 100% damage. Four tissue samples from each corm were plated on Petri dishes with potato-carrot-agar (PCA) (20 g potato, 20 g carrot, 20 g agar L^{-1} distilled water) after disinfestation with 1.5% sodium hypochlorite for 3 min, rinsing three times with sterilized distilled water and drying. Dishes were incubated for one week at 21°C under continuous white light with 40 W lamps (36 $\mu E\ cm^{-2}S^{-1}$).

Field samples. A mixture of twenty-seven flowering-stage plants of cultivars Borrega Blanca (BB; 14 plants) and Lupe (13 plants), showing symptoms of wilt or death were collected in July from three commercial lots (Bentudero, Potrero, and Tlayehualco; 9 per plot). All plants showed damage of basal stem, neck, and corms. The tissues were plated as indicated previously.

Fungal isolation. Eight days after plating all corm tissues from storage and field samples, fungal colonies were quantified, selected, and purified by monosporic culture or hyphal-tip method. Some cultures were stored in slant tubes with potato-dextrose-agar (PDA) (200 g potato, 20 g dextrose, 20 g agar L^{-1} distilled water) and in sterile mineral oil; conidial suspensions of the remainder fungal cultures were stored in 20% glycerol at -85°C.

Identification. Keys and morphological descriptions by Massey (1926), McCulloch (1944), Raper and Thom (1949), Booth (1971), Domsch (1980), and Nelson *et al*. (1983) were used for the identification. Some conidial structures were induced in PCA microculture (Jones *et al*., 1960) and incubation at 28°C under continuous white light with 40 W lamps. Other structures such as sclerotia were induced in malt-agar extract (MEA) (10 g malt extract, 20 g agar L^{-1} distilled water) at 20 and 25ºC and under the light conditions previously mentioned. Morphological observations and measurements were performed using a compound microscope at 40X, in 50% glycerol acidified with HCl 12N.

Pathogenicity tests and inoculum preparation. Thirteen fungal isolates showing differences in colony color were selected as different species, and inoculated using two separate techniques and conducted at one year interval. Test 1. Corms of Borrega Roja (BR) cultivar were disinfested with 1.5% sodium hypochlorite, then planted in a greenhouse, four plants per pot, in sterile substrate (peat-moss). They were then inoculated with one of the following 12 isolates obtained from corms and diseased plants: *Fusarium* (n = 7 isolates), *A. strictum* (n = 1) and *G. roseum* (n = 4) (Table 1). For inoculum preparation, 13 flasks containing 25 g millet seed (*Panicum miliaceum* L.) in 125 mL distilled water were autoclaved at 120°C for 20 min every 24 h for three consecutive days. Twelve flasks were inoculated with two discs (5 mm in diameter) of each isolate on PCA. The remaining flask was used as a control and contained autoclaved seed only. All flasks were incubated at 20°C under diffuse and continuous fluorescent white light for eight days. Conidial suspension of 10.9 x 10^6 (*Acremonium*), 2.1-7.2 x 10^5 (*Gliocladium*), 3.2 x 10^5 to 2.4 x 10^6 (from seven isolates of *F. oxysporum* f. *gladioli*) or 5.6 x 10^5 [*Fusarium solani* (Mart.) Sacc.] conidia/mL was prepared in 50 mL of distilled sterile water, then four seedlings were inoculated on their new corm with each isolate (two corms with wounds on the basal periphery and two without wounds). The conidial suspension was added to the basal stem of all potted plants, 24 h later the remaining inoculum which consisted of colonized millet seeds, was added around the seedlings and covered with substrate. Incubation was conducted in a moist chamber for 15 days at 85-90% relative humidity (RH) and 28-31°C, and for 15 more days at 25-27°C with 35-45% RH. Plants were lightly irrigated twice a week. Controls were inoculated with distilled sterile water only and the autoclaved seeds. Disease symptoms evaluation was conducted 30 days after inoculation. Test 2. A set of healthy seedlings produced as described before was kept in an aseptic moist chamber (four per chamber). Corms were inoculated with six isolates selected from *A. strictum* (n = 1 isolate), *F. oxysporum* f. *gladioli* (n = 2), *F. solani* (n = 1), and *G. roseum* (n = 2). Conidial suspensions were prepared in 10 mL distilled sterile water and adjusted to 3.5 x10^5 conidia/mL for each isolate. Four seedlings per isolate were inoculated making a wound on the basal periphery of their new corm. Two milliliters of conidial suspension were added around the basal stem of each seedling; controls were inoculated with sterile distilled water only. Disease evaluation was conducted 20 days after inoculation.

Penicillium inoculations. Each of eight desinfested seedlings and germinated corms (BB variety) produced as in Test 2, were inoculated with *Penicillium* sp., one of the 13 selected fungal isolates (Table 1). Three wounds were made in each of four corms around the emerging hypocotyl. A disc of PCA

Table 1. Percentage of fungal species isolated from stored corms and field samples showing wilting and death symptoms in San Martín Texmelucan in the community of Tlacotepec de José Manzo in the municipality of San Salvador el Verde, Puebla, Mexico, in 2004.

Damaged tissue	*Acremonium strictum*	*Fusarium oxysporum* f. *gladioli*	*F. solani*	*Gliocladium roseum*	*Penicillium* sp.
Stored corms[x]					
0[y]	0.0	31.3	0.0	12.5	31.2
25	0.0	53.3	0.0	6.7	0.0
50	0.0	50.0	0.0	16.6	0.0
75	0.0	20.0	0.0	40.0	20.0
100	0.0	0.0	0.0	0.0	0.0
Field plants[z]					
Basal stem	5.5	71.1	6.6	36.6	27.7
Neck	0.0	45.4	6.3	27.7	27.2
Corm	22.2	7.1	20.0	100.0	72.2

[x]Twenty corms of cultivar Lupe.
[y]Percent damage.
[z]Twenty seven plants of cultivars Borrega Blanca and Lupe.

containing the fungus was then placed on each wound, and on an unwounded area of healthy tissue around the same corm. Incubation was conducted at 28-30°C with 100% RH for ten days, and then at 45-55% RH for ten more days. One year later, four corms were inoculated as previously described. In each case, four additional corms were used as controls and inoculated with PCA discs without the fungus.

Evaluation. After inoculations (30 days for test 1 and 20 days for test 2 and for *Penicillium*), the symptoms produced were described. After the second pathogenicity test, corm damage was measured in cm and transformed to a percentage in relation to the whole area of each corm. The area was measured as the depth of damage multiplied by the diameter. For this evaluation, each corm was cut vertically through its center and the exposed surface was measured; all procedures were performed aseptically. From each inoculated corm, fungi were re-isolated from lesion margins and plated out onto PCA to complete Koch´s postulates.

Data analysis. Treated corms (six isolates from Test 2, plus the *Penicillium* isolate) and their controls (four corms per treatment) were assessed for the percent damaged area per corm, following a completely randomized design by variance analyses using SPSS 10.0 for windows, series 657180 (SPSS, 2001). Comparison was performed using Tukey test ($p < 0.05$).

Molecular characterization. DNA extraction of 13 monoconidial isolates was made according to the methodology of Ahrens and Seemüller (1992). The quality was evaluated by electrophoresis in agarose gel (0.8%, and Lambda Hind III DNA marker) and quantified using a Perkin Elmer spectrophotometer (Lambda BIO 10, USA). Amplification of the internal regions ITS4 and ITS5 of ribosomal genes (RNA) 18S-5.8S and 5.8S-28S was performed with the universal primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) (White *et al*., 1990) by PCR. PCR reactions were performed with a final volume of 25 µL reaction mixture composed of the following: 13.22 µL sterile ionized water, 1.0 µL DNA at 80 ng, 2.5 µL 1X buffer solution, 2.08 µL $MgCl_2$ at a concentration of 1.5 mM, 2.0 µL dNTPs mixture at a concentration of 0.2 mM, 2.0 µL of each primer at a concentration of 20 pM each, and 0.2 µL Taq-DNA polymerase at 1.0 Unit (University Biotecnologies®, USA). A Perkin Elmer thermocycler (CT 2400 ICA, USA) was used with the following program: initial denaturalization at 95ºC for 2 min, 30 denaturalization cycles at 95ºC for 1 min, annealing at 50ºC for 30 sec, and extension at 72ºC for 2 min; and a final extension at 72ºC for 10 min. The PCR products were analyzed by electrophoresis in an agarose gel (1%), stained with ethidium bromide, bands were visualized using a transilluminator (Gel Doc 2000, BIO RAD®, USA). Purification was performed with a QIAquick PCR Purification Kit (Qiagen, USA) according to the manufacturer´s protocol. Products were sequenced directionally (5´-3´ and 3´-5´) with the same ITS primers as in an ABI PRISM 3700 sequencer (Applied Biosystems, USA). The sequences were analyzed using Lasergene® 2001, Version 5 software (DNASTAR Inc., USA) (DNASTAR, 2001), and aligned with the GenBank database of the National Center for Biotechnology Information (NCBI, USA). A phylogenetic tree was obtained using the Neighbor-Joining method, Kimura-2-parameter, with 5000 bootstrap replicates with the MEGA 3.1 program (Kumar *et al*., 2004). A GenBank AY803935 sequence of *Rhizopus microsporus* var. *rhizopodiformis* (Cohn) Zopf. was used as the outgroup sequence. Sequence data were deposited in GenBank.

RESULTS

Identification of isolates. A total of five species of fungi were identified (Table 1): *Fusarium oxysporum* f. *gladioli*, *F. solani*,

Table 2. Comparison of differential morphological structures (in µm) among the fungal species causing gladiolus corm rot, wilt, and death of gladiolus (*Gladiolus grandiflorus*) plants in San Martín Texmelucan, Puebla, Mexico.

Morphological structures	*Acremonium strictum*	*Fusarium oxysporum* f. *gladioli*	*F. solani*	*Gliocladium roseum*	*Penicillium* sp.
Microconidia	-	7.3-19.4 x 3.0-4.1, hyaline, one celled, ovoid, in false heads	10-15 x 2.1-3.2, hyaline, one celled, in false heads	-	-
Macroconidia	-	26.7-38.9 x 2.5-3.7, hyaline, 3-7 septa	31.5-46.1 x 4.5-5.6, only in slant tubes	-	-
Conidia	4.8-5.7 x 1.2-1.3, hyaline, one celled, cylindrical, in slime heads	-	-	5.5-8.1 x 2.5-4.3, hyaline, in slime heads, unicelular	2.5-3.8 x 2.5-3.1, hyaline (single) or greenish in mass, one celled
Monophialides	bulbose base	short	large, 45-62	verticillated	terminal
Conidiophores	-	hyaline, branched	-	straight	hyaline, large, branched
Sporodochia	-	in some cultures	-	-	-
Chlamydospores	-	rare, hyaline, single or in pairs	hyaline, single or in pairs	-	-

Penicillium sp., *A. strictum* and *G. roseum* (González-Pérez *et al*., 2008). Three in the stored corms: *F. oxysporum* f. *gladioli* (with red, purple, and purple-violet colonies) (Booth, 1971; Massey, 1926; McCulloch, 1944), *G. roseum* (with salmon, white, and yellow colonies) (Domsch, 1980), and *Penicillium* sp. (Jackson, 1961; Raper and Thom, 1949), and two in corms from field plants: *A. strictum* (light pink, inmerse mycelium, cream consistency and slow growth rate of colonies), and *F. solani* (light brown colonies). All species were isolated from symptomatic tissues. *Fusarium oxysporum* f. *gladioli*, *G. roseum*, and *Penicillium* sp. were also isolated from healthy corm tissues, but none of the five species were found in corms showing 100% necrosis. The same five species were also isolated from symptomatic aerial tissues from field plants. Comparatively, all these species showed differences in colony color, as well as morphological differential structures. Some typical structures are listed in Table 2.

Pathogenicity tests. Test 1. All 13 isolates were pathogenic and caused infection mainly on wounded tissues. In descending ranking of damage, plants showed the following symptoms: *F. oxysporum* f. *gladioli* infection, basal stem rot and rot in new and old corms; *G. roseum*, light foliar chlorosis, rot in basal new corm, and basal leaf necrosis. Both species finally caused foliar wilt and plant death. *A. strictum*, basal stem and corm rot; *F. solani*: some necrotic spots (5 x 3 mm; length x width) around new corms; *Penicillium* sp., dry rot under the inoculated zone (about 6 x 3 mm; diameter x depth), with damage slowly advancing internally (foliar wilt was not observed). Test 2. All seven isolates caused infection with the same symptoms already described. According to the percent area of corm rot, statistical differences were detected among isolates (Tukey, $p < 0.05$). Infection was classified into three groups: high, moderate, and low of corm rot (Fig. 1). On average, the species causing the highest percent corm rot (35.8-43.2%) were two isolates of *F. oxysporum* f. *gladioli*

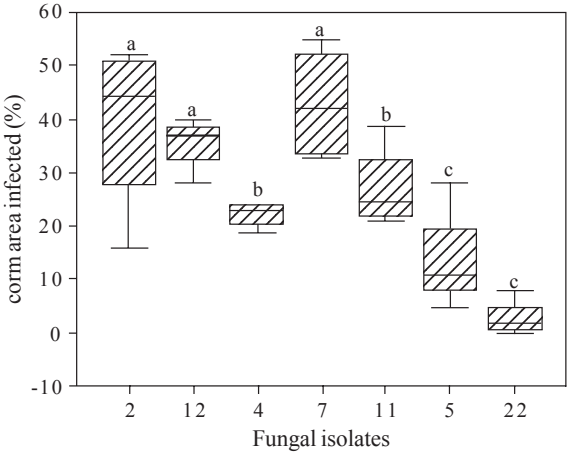

Fig. 1. Percentage of gladiolus corm area infected by inoculation with seven fungal isolates (2, 12: *Fusarium oxysporum* f. *gladioli*; 4: *Acremonium strictum*; 7: *F. solani*; 11, 15: *Gliocladium roseum*; 22: *Penicillum* sp.). Statistical differences were analyzed with SPSS 10.0 for windows. The horizontal black line in each box represents the median value of four corms. Different letters indicate significant differences among treatments ($p < 0.05$).

and *F. solani*; moderate (22.7-27.8%): *A. strictum* and one isolate of *G. roseum* (# 21); and low: another isolate (# 5) of *G. roseum* (14.1%), and *Penicillium* sp. (2.7%). The same species were re-isolated from infected tissues, but not the controls. According to Figure 1, comparatively, the two species of *Fusarium* were the most aggressive, *G. roseum* isolates were less aggressive and showed almost 50% difference between isolates; *Penicillium* was the least aggressive pathogen.

Molecular characterization. The DNA had a molecular weight of 23,130 bases and the band of the ITS:PCR product was 600 base pairs (bp) long. The two sequenced directions of each isolate had 100% similarity. By multiple alignment, the amplified total portion of the 13 nucleotide sequences was 572 bp (including alignment gaps), which corresponded to the complete sequences of both regions ITS1 and ITS2. In GenBank, the sequences of *A. strictum*, *F. oxysporum* f. *gladioli*, *F. solani* and *G. roseum* were aligned with sequences of the same species and showed to have 100% similarity indexes, except *A. strictum* (99.8%) and *G. roseum* (99.6%); and *Penicillium* sp. (100%) with the same genus (*Penicillium*) (Table 3). The phylogenetic tree showed 91-100% bootstrap support for all isolates listed in Table 3 (Fig. 2). The isolates of *F. solani* and *G. roseum* differed in two nucleotides and *A. strictum* in one nucleotide in relation to the aligned isolates (94 and 100% bootstrap support). Newly generated accession numbers were DQ279790- DQ279803 (Table 3).

DISCUSSION

Isolates of *F. oxysporum* f. *gladioli* and *Penicillium gladioli* from 18 countries are deposited in several International Culture Collections and held by the "World Federation of Culture Collections" (WFCC, 2009), but no isolates have been registered from Mexico. Of the five fungal species identified in the present study, two were unknown worldwide. *Acremonium strictum* has not been reported in gladiolus or other plants in the same family; however, it is known to cause wilt in seedless *Chrysanthemum maximum* hort. (Koike and Wilen, 2009), and has been detected in the rhizosphere of this

Table 3. Molecular characterization according to alignment of the 13 identified isolates obtained from gladiolus (*Gladiolus grandiflorus*) in San Martín Texmelucan in the community of Tlacotepec de José Manzo, municipality of San Salvador el Verde, Puebla, Mexico, with the species held in the NCBI database.

Number				Most related species with accession number (Blast-GenBank)[u]		Index[w]		Geographic origin
Order	Isolate	N[t]	Identified species		H/S[v]	I	S	
Stored corms								
1	1	504	*Fusarium oxysporum* f. *gladioli* DQ279796	*F. oxysporum* AY728210	Pepper	991	100	Mexico
2	3	520	*F. oxysporum* f. *gladioli* DQ279800	*F. oxysporum* AY728210	Pepper	991	100	Mexico
3	2	515	*F. oxysporum* f. *gladioli* DQ279795	*F. oxysporum* FJ545244	-------[y]	952	100	EUA
4	5[x]	545	*Gliocladium roseum* DQ279793	*Gliocladium roseum* AJ608977	Plastic	1065	99.6	UK
5	6	542	*G. roseum* DQ279790	*G. roseum* AJ608977	Plastic	1059	99.6	UK
6	22	553	*Penicillium* sp. DQ279802	*Penicillium simplicissimum* DQ026013	Amaranth	1086	100	Mexico
Field plant								
7	4[z]	537	*Acremonium strictum* DQ279801	*Acremonium strictum* AY138846	Patient	1015	99.8	EUA
8	8	512	*F. oxysporum* f. *gladioli* DQ279799	*F. oxysporum* AY728210	Pepper	991	100	Mexico
9	12	509	*F. oxysporum* f. *gladioli* DQ279797	*F. oxysporum* AY728210	Pepper	991	100	Mexico
10	10	516	*F. oxysporum* f. *gladioli* DQ279794	*F. oxysporum* FJ233193	Fruit	953	100	China
11	7	526	*Fusarium solani* DQ279803	*F. solani* EU727452	-------	966	99.6	Brazil
12	9	542	*G. roseum* DQ279791	*G. roseum* AJ608977	Plastic	1059	99.6	UK
13	11	541	*G. roseum* DQ279792	*G. roseum* AJ608977	Plastic	1057	99.6	UK

[t]Nucleotide number.
[u]National Center for Biotechnology Information.
[v]Host/Substrate.
[w]Identity generated by NCBI GenBank alignment. Similarity value generate using the Lasergene program.
[x]Two nucleotides different.
[y]Unknown.
[z]It differs in one nucleotide.

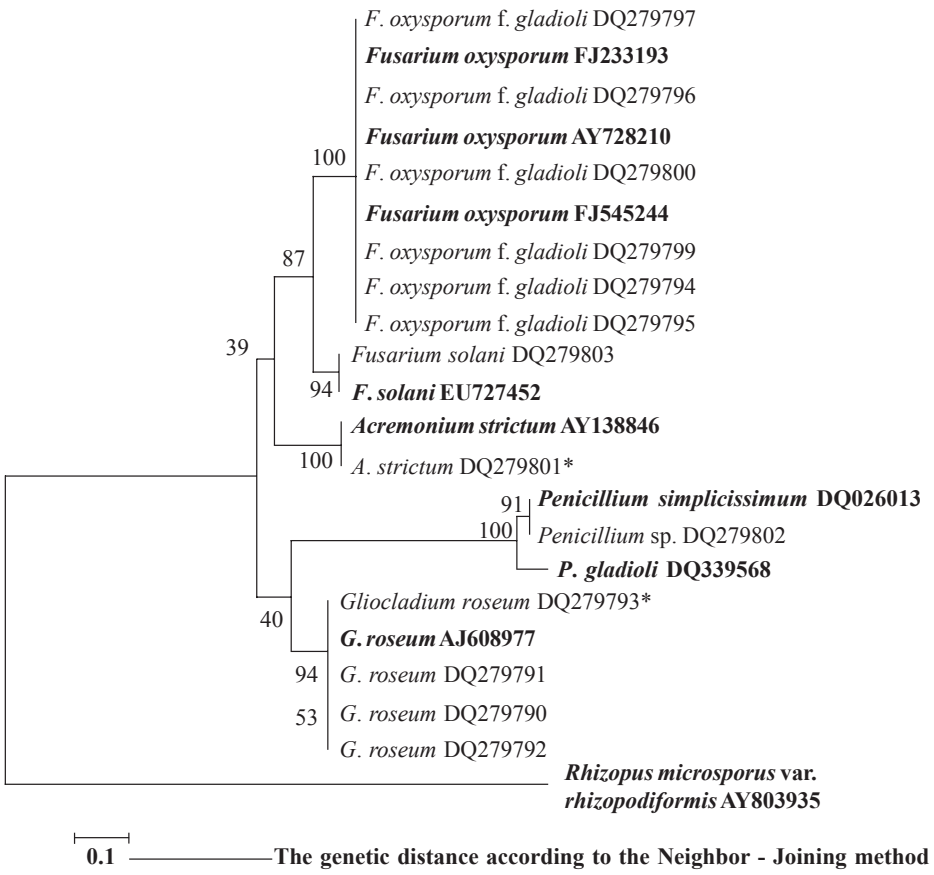

Fig. 2. Phylogenetic tree showing ITS sequence alignment of the 13 isolates obtained from stored corms and field samples showing foliar wilting. The tree was obtained according to the Kimura-2-parameter substitution method and constructed with the neighbor-joining algorithm using MEGA 4.1. Software. Confidence was assessed by bootstrap analysis based on 5000 replications. The tree was rooted to *Rhizopus microsporus* var. *rhizopodiformis* from the GenBank. Boldface sequences were from the NCBI and the sequences with asterisk from González-Pérez *et al*. (2008).

plant (Farr *et al*., 1989). In the region where this study was conducted, chrysanthemum plants were cultivated in a greenhouse using the same soil used for gladiolus crops, although it is unknown if *Acremonium* affects other plants grown in the greenhouse. *F. solani* has also been isolated from corms in the USA (Garcia and Alfaro, 1985); however, pathogenicity was not shown, although *Fusarium* sp. and possibly *F. solani*, that cause corm rot and yellowing have been observed in several US regions (Farr *et al*., 1989). Symptoms and severity caused by this species are likely to be the same as those caused by *F. oxysporum* f. *gladioli* when environmental conditions are optimal. The symptoms induced by the latter species in gladiolus in the present study were similar to those reported by Massey (1926) and McCulloch (1944). *G. roseum* is common in soil, can cause postharvest damage, and colonizes rotten roots (Domsch, 1980). For the pathogen conditions already mentioned, in this study, this fungus probably caused infection only through wounded tissues. The isolated species of *Penicillium* was different from *P. gladioli*, since this last species produce sclerotia in MEA as mentioned by Raper and Thom (1949). Although, this was not supported by ITS sequence analysis using NCBI alignment. The fact that some of the observed fungi were also isolated from healthy corms supports the observations made by several other authors (Jackson, 1961; Larson, 1992; Massey, 1926; McCulloch, 1944) who mentioned that even healthy corms can have latent pathogenic fungi. This is important because it suggests that each year farmers contribute to the spread of the disease by producing their own corms; corms that are used in the following planting season. This also confirms one of the proposed hypotheses. The molecular taxonomic results by PCR:ITS corroborated the morphological identification of the isolates. *F. oxysporum* has several hundreds of "forma specialis" (abbreviations: f. sp. or f.) (CBS, 2009; Kirk *et al*., 2001). This means the range of hosts that this particular species is able to infect and from which the "f. sp." takes the name. Fot this species, including *F. oxysporum* f. *gladioli*, some of their f. sp. are listed by

MycoBank Fungal Databases (CBS, 2009). This is just a physiological relationship (Kirk *et al*., 2001). The amplification of the ITS region of rRNA genes is used as a tool that allows the taxonomist to differentiate organisms to the species level. However, it has some limitations. If some fungal species are morphologically close (for example some species of *Alternaria*, *Penicillium*, soybean rust, *Stemphylium*, *etc*.), the ITS region does not show nucleotide differences. Then, other genes should be amplified by applying different molecular techniques which are available for fungal species (Kirk *et al*., 2001). This was the case of *Penicillium* sp. Its sequence aligned with *P. simplicissimum* (Oudem) Thom., but they are not the same species. In fact, *Penicillium* sp. showed morphologically and molecularly to be a species different even from *P. gladioli* and for these reasons is currently under study. The one or two nucleotide differences among the aligned sequences are probably related to their population´s genetic variability, and perhaps influenced by geographic origin, host range, substrate, *etc*., as shown in Table 3. From all the aligned sequences in the GenBank, only those with the highest identity value were included in the phylogenetic tree. This tree grouped each of the five species in a different branch, as expected for the molecular taxonomic purpose, and due to their strong morphological differences. All the five identified species were also shown to be responsible for wilt and death symptoms of gladiolus field plants. Thus, the findings suggest that in the region of San Martin Texmelucan, gladiolus diseases are caused by five fungal species which affect corms, and not just from one or two species as reported before (Gardezi *et al*., 2001; Romero-Cova, 1993). Two of these were previously reported by González-Pérez *et al*. (2008), and were unknown worldwide in gladiolus. In addition, the comparative analysis showed that the five species were morphologically, culturally, molecularly, and pathogenically different, even though they are able to cause the same disease. All the isolates were deposited in the Culture Collection of Colegio de Postgraduados, Campus Montecillo, Montecillo-Texcoco, Mexico State.

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