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Phenotypic and molecular characterization of *Fusarium spp.* coming from sesame production zone in Venezuela

Caracterización fenotípica y molecular de *Fusarium spp.* proveniente de la zona de producción de ajonjolí en Venezuela

Caracterização fenotípica e molecular da *Fusarium spp.* origem da área de produção de gergelim na Venezuela

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ABSTRACT

Sesame is an important crop in tropical areas, which is seriously affected by soil-borne fungi such as Macrophomina phaseolina and Fusarium sp. In order to identify and characterize Fusarium spp. causing disease in sesame plants in the field, ten isolates were obtained. Eleven distinctive taxonomic traits were determined, and additionally growth rate was measured in Petri dishes. Random Amplified Polymorphic DNA (RAPD) by means of five primers was also performed. Three Fusarium species were reported: Fusarium oxysporum (2 isolates), Fusarium solani (7 isolates) and Fusarium semitectum (1 isolate). Previous reports indicate that sesame is affected just by F. oxysporum f. sp. sesami, only one previous report indicate that also F. solani causes fusarium wilt in this crop. In vitro growth rates were within a range of 0,21-0,29 mm h^{-1}, with no trend according to the species. Growth of one of the isolates of F. oxysporum was statistically (P<0,05) faster than the others. Fifty RAPD bands (92% polymorphic) were not able to differentiate the three Fusarium species. Similarity Jaccard coefficient ranged between 0,15 and 0,47 with an average of 0,32.

KEYWORDS:

Fusarium wilt, *F. solani*, *F. oxysporum*, *F. semitectum*, RAPD.

RESUMEN

El ajonjolí es un cultivo importante en regiones tropicales, el cual es afectado por hongos del suelo como Macrophomina phaseolina y Fusarium sp. Con el objetivo de identificar y caracterizar a Fusarium spp. como causante de pudrición en plantas de ajonjolí en campo, se obtuvieron diez aislados. Once características distintivas a nivel taxonómico se determinaron sobre los diez aislados, y adicionalmente se midió la velocidad de crecimiento en cápsulas de Petri. A nivel molecular, la caracterización de los diez aislados, se desarrolló utilizando la técnica molecular de ADN polimórfico amplificado al azar (RAPD) mediante el uso de cinco cebadores o "primers". Se identificaron tres especies de Fusarium oxysporum (2 aislados), Fusarium solani (7 aislados) y Fusarium semitectum (1 aislado). Investigaciones anteriores al presente trabajo indican que el ajonjolí es solo afectado por F. oxysporum f. sp. sesami, solo un reporte previo indica que también F. solani causa la pudrición de Fusarium en este cultivo. La velocidad de crecimiento in vitro se ubicó en un rango entre 0,21-0,29 mm h^{-1}, sin ningún patrón identificable de acuerdo a la especie. El crecimiento de uno de los aislados de F. oxysporum fue estadísticamente (P<0,05) más rápido que los otros aislados evaluados. Cincuenta bandas RAPD (92% polimórficas) no pudieron diferenciar las tres especies identificadas de Fusarium. El coeficiente de similitud de Jaccard estuvo entre 0,15 y 0,47 con un promedio de 0,32.

PALABRAS CLAVE:

Pudrición por Fusarium, *F. solani*, *F. oxysporum*, *F. semitectum*, RAPD.

RESUMO

O gergelim, uma cultura importante em regiões tropicais é seriamente afetada pelos fungos do solo Macrophomina phaseolina e Fusarium sp. A fim de identificar e caracterizar Fusarium spp. causadores da podridão em plantas de gergelim no campo, dez isolados foram obtidos. Vários carateres taxonômicos distintos foram determinados nos dez isolados, e a taxa de crescimento

PALAVRAS-CHAVE:

Podridão de Fusarium, *F. solani*, *F. oxysporum*, *F. semitectum*, RAPD.

em placas de Petri foi medida adicionalmente. Para mais detalhes da caracterização dos dez isolados, utilizou-se a técnica molecular de DNA polimórfico amplificado ao acaso (RAPD), através de cinco iniciadores ou "primers". Foram identificadas sob criterio morfológico três espécies; Fusarium oxysporum (2 isolados), Fusarium solani (7 isolados) e Fusarium semitectum (um isolado). Relatórios anteriores indicam que o gergelim é afetado apenas por F. oxysporum f. sp. sesami, no entanto, um relatório anterior indica que F. solani, também pode causar a podridão de fusarium nesta cultura. A taxa de crescimento in vitro foi na faixa entre 0,21- 0,29 mm. h-1, sem qualquer padrão identificável de acordo com a espécie. O crescimento de um dos isolados de F. oxysporum foi estatisticamente (P <0,05) mais rápido que os outros isolados avaliados. Cinquenta bandas de RAPD (92% polimórficas) não conseguiram diferenciar as três espécies de Fusarium identificadas morfologicamente. O coeficiente de similaridade de Jaccard foi entre 0,15 e 0,47 com uma média de 0,32.

INTRODUCTION

Sesame is one of the oldest oil crops which grows in tropical and subtropical areas [1]. Sesame oil is highly appreciated by consumers because of good balance of fatty acids [2] and also because of the high antioxidant content [3], which is desirable for human health and for shelf-life.

Sesame, as any other crop, has several biological constraints at the field such as insects pest (e.g. whitefly, *Bemisia tabaci* Gennadius) [4], and pathogenic fungi (e.g. *Macrophomina phaseolina* and *Fusarium oxysporum* f.sp. *sesami*) [5]. *Fusarium oxysporum* is a soil-borne fungus which survives as saprophytic, and affects many crops [6]. When conditions are favorable, fungus mycelium goes into the host plant through the roots until xylem, affecting plant water supply. *F. oxysporum* is considered a complex, and within this complex, more than 150 host-specific Forma specialis have been described [7].

Sesame is one of these many crops that may be affected by *F. oxysporum*, and specific Forma specialis of the fungus is *F. oxysporum* f.sp. *sesami*. According to Pineda (2002) [8], plants affected by *F. oxysporum* f. sp. *sesami* are recognized by leaf yellowness on one side of the plant, and subsequent appearance of a brown stripe and pink powder along the stem (fructification structures of the fungus); in plantlets, disease is identified by wet rot at the neck, plant wilt, and sudden death. Symptoms described are consequence of fungus colonization inside of vascular system in roots and stem.

Strategies design of integrated pathogen management implies knowledge of fungus epidemiology and attributes of population, and these studies should be based on a correct identification of the pathogen. For identifying *Fusarium* species, taxonomic system considers morphological attributes divided in primary and secondary traits. Primary attributes are chlamidospore presence, size and shape of macroconidia, and microconidia; secondary attributes are colony color and morphology, and sporodochium [9, 10].

The objective of this research was to characterize at morphological and molecular level isolates of *Fusarium* spp. coming from symptomatic tissue from sesame production area in Venezuela, in order to have a general idea about genetic diversity of this fungus affecting the crop.

METHODS

Fungus isolation

In sesame commercial fields, infected plants showing the typical symptoms of Fusarium wilt were identified. Pieces of about 1 cm^2 coming from stems of these plants were washed with sodium hypochlorite (1%) and put into Petri dishes containing water agar. Petri dishes were incubated at room temperature (20-28°C) for 5 days, and hyphae tips of typical *Fusarium* mycelia was transfer to new Petri dishes containing potato dextrose agar (PDA), which also was incubated at room temperature for 5 days. Ten Petri dishes containing Fusarium were obtained, and from them, monosporic cultures were prepared by means of addition of 20 µL of conidia suspension in new Petri dishes containing water agar, which was incubated for 24 h. Germinated conidia were identified under microscope, and only one was transferred to a new Petri dish containing PDA, which was incubated for 5 days at room temperature. Ten agar disks for each isolate coming from each of the ten Petri dishes were conserved in sterilized water according to Figueiredo (1967) [11].

Morphological characterization

A single agar disk containing mycelium of *Fusarium* sp. was transferred to SNA medium (synthetischer nährstoffarmer agar, 0,1% K_2HPO_4, 0,1% $NaNO_3$, 0,05% $MgSO_4$.7H2O, 0,05% KCl, 0,02% glucose, 0,2% sucrose and 2% agar) prepared into a Petri dish containing a single piece of carnation (*Dianthus cariphylus)* leaf. It was replicated twice for each isolate. Morphological characterization was carried out by using traits proposed by Nelson *et al.* (1983, 1994) [12, 9]. Taxonomic identification of each isolate was carried out on mycelium growing for 7 days, by using the morphological traits proposed by Leslie and Summerell (2006) [13]: conidiophore, type of conidiogenous cells (monophialide or polyphialide), microconidia (shape, septum presence, arrangement on condinogenous cell), macroconidia (shape, septa number, origin), chlamydospores (number, shape, arrangement on mycelium). Growth rate was also determined for each isolate. A single piece of 1 cm^2 of potato dextrose agar taken from the edge of 48 h fungi cultures, was placed at the center of a Petri dish. It was replicated four times for each isolate. Colony diameter was measured every 12 h until fungus reached the edge of the dish. Colony area was calculated and data subjected to analysis of variance as a factorial design (isolate with 10 levels, measuring time with 10 levels, and interaction). When statistical differences were detected, Tukey test was performed for mean comparison (P<0,01).

Molecular characterization

Each isolate was grown for 7 days on PDA, and mycelium was taken for DNA extraction, which was carried out following the Hoisington *et al.* (1994) [14] procedure. DNA was purified with 10 mg mL^{-1} of RNAase, and concentration was estimated by visualization in agarose gel (0,8%) under UV light, after electrophoresis (30 minutes, 100 V) and staining with ethidium bromide. Concentration of all DNAs extracted was standardized in approximately 10 mg mL^{-1}. DNA was amplified in a thermal cycler Perkin Elmer 2400 using 5 primers (OPA10, sequence GTGATCGCAG, OPM-1 sequence GTTGGTGGCT, OPO-16 sequence TCGGCGGTTC, OPP-1 sequence GTAGCACTCC, OPP-17 sequence TGACCCGCCT), using the same procedure of Zamani et al. (2004) [15]. PCR products were subjected to electrophoresis on agarose gels (1,5%) for 80 minutes at 100 V, and were visualized under UV light after staining with ethidium bromide. All PCR reactions were carried out twice. Data was recorded as presence or absence of bands for each isolate (presence of bands was considered when it appeared in both PCR reactions), and Jaccard similarity coefficient was calculated between each pair of isolates. Coordinate analysis was used for visualizing genetic relationship among isolates. For that, correlation coefficient was calculated between all pairs of bands, afterward matrix was doubly centered and eigenvalues and eigenvectors were calculated, which were projected in a two dimension space. Analyses were performed with NTSYS pc v. 2.02.

RESULTS

Three *Fusarium* species were identified from the ten isolates according to Leslie and Summerell (2006) [13] and Nelson *et al*. (1994) [9] criteria. Two isolates were identified as *Fusarium oxysporum* because of the presence of oval microconidia, most of them two-celled in false head on a short monophialide which in most of the cases was close to another long monophialide; macroconidia had 3-5 septa, formed on a sporodochium; chlamydospores were terminal or intercalar, smooth and ornamented In the culture this species was characterized by abundant white (slightly pink) mycelium in both surface and deep. Seven isolates were identified as *Fusarium solani*, which had white mycelium, hyaline two-celled microconidia formed on a long and lonely monophialide in false head; macroconidia had 3-5 septa on branched conidiophore; rounded to oval and slightly wrinkled chlamydospores were intercalar. This species was characterized by abundant and white mycelium. One isolate was identified as *Fusarium semitectum*, which presented an uniform slighty pink color in the mycelium; many branched conidiophores with monophialialide in shape of V, with many 3-5 septa macroconidia; sporodochium of pink color having few chlamydospores and macroconidia.

Growth rate was statistically different (P<0,05) for interaction Isolate x Measuring Time. Figure 1 displays the growth rate for each isolate during 5 days. Table 1 shows that covered area in Petri dishes at fifth day resulted also in statistical differences (P<0,05) among isolates, according to Tukey test.

Molecular characterization by means of RAPD resulted in 50 bands, 46 of them (92%) polymorphic. Table 2 indicates number of bands obtained for each primer.

Jaccard coefficient for the 45 possible pairwise comparison between the 10 isolates had an average of 0,32; in a range between 0,15 (*Fusarium oxysporum* 001 and *Fusarium solani* 4) and 0,47 (*Fusarium oxysporum* 002 and *Fusarium semitectum*). Three-dimension plot generated by principal coordinates analysis (Figure 2), according to the eigenvalues, explained 58% of variation.

Fusarium oxysporum f. sp. *sesami* has been reported as the causal agent of fusarium wilt in sesame (*Sesamum indicum* L.) [16-19], however, this research reports other species associated to this disease. Isolates of the fungus were obtained from sesame stem tissue presenting symptoms of the disease, therefore, all the species identified in this research should be recognized as causal agents, or at least to be present as opportunists in the lesions caused by *Fusarium* sp. Sesame is sown in Venezuela as rotation crop to maize in the dry-season, as well as sorghum and sunflower. *F. semitectum* has been reported as a pathogen to sorghum, but *F. solani* is not a pathogen to sunflower or sorghum; therefore, maybe *F. semitectum* could be considered as occasional pathogen to sesame, it could attack sorghum and occasionally sesame, but *F. solani* seems to be nowadays a frequent pathogen to sesame, it does not have another host plant in the area. Li *et al*. (2012) [20]reported that fusarium wilt in sesame is not only caused by *F. oxysporum* f.sp. *sesami*, but also by *Fusarium solani*, however they indicate pathogenicity is stronger for *F. oxysporum* than for *F. solani*. These results agree this study in having evidence that fusarium wilt in sesame is caused by *F. oxysporum* and *F. solani*. Results of the present research indicate that also *F. semitectum* may be present in fusarium wilt lesions in sesame. Recent reports indicate that also *F. proliferatum* may attack sesame [21].

In the case that Fusarium species identified on the sesame tissue are not opportunists, these results must be taken into account to consider two options: coevolution plant-pathogen could be permitting to some *Fusarium* species different of *F. oxysporum*, to take advantage of sesame plants to get the necessary nutrients; it means to cause disease on sesame plants. The other option is that fusarium wilt is caused by a *Fusarium* complex, and maybe aggressiveness will depend on which fungus species are involved. In any case, it is remarkable that two *Fusarium* species of the four most important *Fusarium* species in plant pathology [22] may attack *Sesamum indicum*.

Table 1. Tukey test for covered area (cm^2) in Petri dishes after five days for ten *Fusarium* isolates.

Isolate	Mean (covered area in cm^2)
Fusarium oxysporum (001)	9,30 a
Fusarium solani (001)	7,84 b
Fusarim semitectum	7,65 b
Fusarium solani (002)	7,60 b
Fusarium solani (006)	7,59 b
Fusarium solani (003)	7,59 b
Fusarium oxysporum (002)	6,33 c
Fusarium solani (005)	6,28 c
Fusarium solani (004)	5,28 c
Fusarium solani (007)	5,24 c

Note: means followed by the same letter do not differ statistically (P<0,05)

Table 2. Number of RAPD bands obtained for each primer used in a study of 10 *Fusarium* isolates.

Primer name	Number of bands	Number of polymorphic bands	Number of monomorphic bands
OPA-10	7	3	4
OPM-01	11	11	0
OPO-16	12	12	0
OPP-01	4	4	0
OPP-17	16	16	0
Total	50	46	4

In vitro growth rate of the isolate *Fusarium oxysporum* 001 was higher than the other isolates, which could be indication of higher ability than the other isolates for colonizing plant tissue in less time, it means, more aggressiveness. When growth rate is expressed linearly, isolates of *F. oxysporum* had a rate between 0,24 and 0,29 mm h^{-1}, lower than other previously reported in the same culture medium (PDA) and similar incubation temperature, such as 0,59 mm h^{-1} [23] for *F. oxysporum* strain isolated from the soil, 1,11 mm h^{-1} [20] for *F. oxysporum* f.sp. *fragariae*, and 0,53 mm h^{-1} [24] for *F. oxysporum* causing soybean root rot. Isolates of *F. solani* resulted in a growth rate between 0,22 and 0,26 mm h^{-1}, similar to the reported by Rehman *et al.* (2012) [25] for *F. solani* isolated from citrus plants.

Figure 1. Area covered by ten Fusarium isolates in Petri dishes.

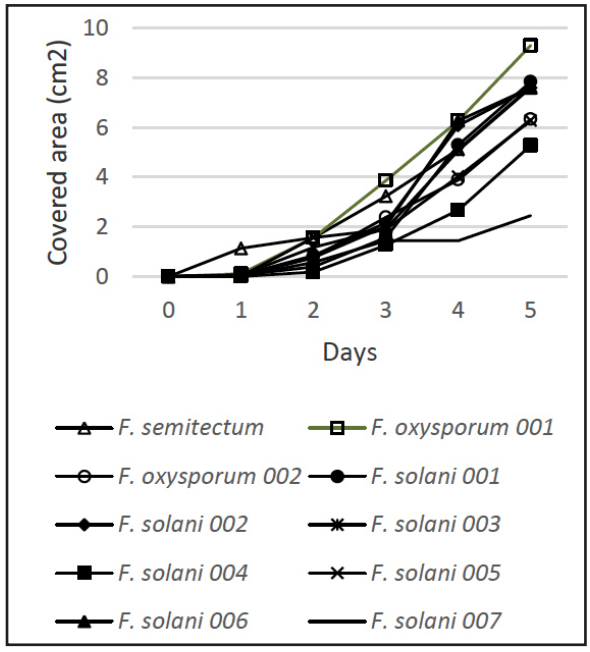
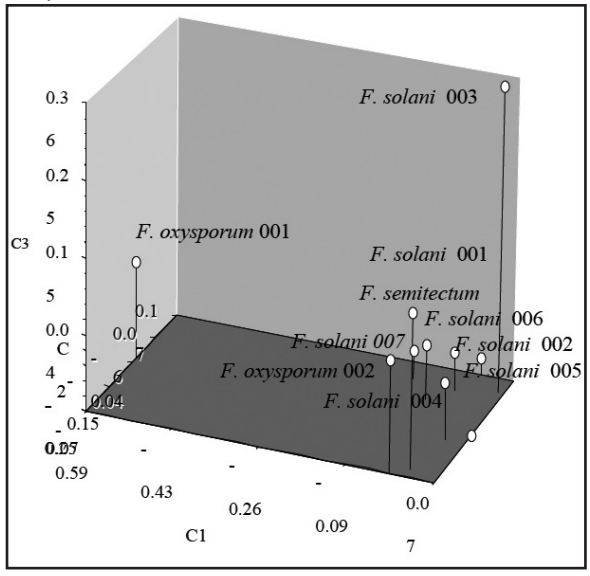


Figure 2. Three-dimension plot from principal coordinates analysis of ten Fusarium isolates based on 50 RAPD.



Single isolate of *F. semitectum* had a rate of 0,26 mm h^{-1}, similar to 0,27 mm h^{-1} reported by Li (2011) [26] when determined growth rate for an isolate coming from soybean. Differences in growth rate of *F. oxysporum* isolates evaluated in this research as compared to the other used in previous research may be explained by a broad genetic diversity of the species, moreover when they are other *Forma specialis* or at least other isolates. In Venezuelan sesame production the main pathogen according to the frequency of disease at fields is *Macrophomina phaseolina*. Martínez-Hilders and Laurentin (2012) [27] determined for this fungus an *in vitro* average growth rate of 0,80 mm h^{-1}, higher than the one found for *F. oxysporum*. This could be the explanation why *F. oxysporum* (or *Fusarium* complex) appears less frequently than *M. phaseolina*, maybe *M. phaseolina* has more ability to colonize sesame plants faster than *F. oxysporum*.

Ten bands per primer were obtained in this molecular characterization, which is lower than the 13 per primer reported by Luna *et al.* (2004) [28] when characterized 41 isolates of *F. oxysporum* f. sp. *ciceri* using 5 primers; however results of the present research are similar to the reported in cucumber [29] (11 band per primer when characterized 106 isolates of *F. oxysporum* from cucumber) and pigeonpea [30] (9 bands per primer when characterized 14 isolates of *F. oxysporum* and *F. udum*). High polimorphic ratio (higher than 85%) was a common factor for all these researches and the present one. The fifty bands produced by PCR reactions were not enough to discriminate among isolates according to the species. Isolate *Fusarium oxysporum* 002 has more similarities with *F. solani* isolates than with the other isolate of *F. oxysporum*, or isolate *Fusarium solani* 006 has more similarities with *F. semitectum* than with the other isolates of *F. solani*. RAPD is a technique based on a random sampling in the DNA, therefore there is a high probability that the 50 bands are no related to the DNA sections responsible of the species differentiation. However, results of the high variability of *F. oxysporum* as compared to *F. solani* may be indicating a long coevolution story with sesame, which results in a large genetic differentiation within the species, and may be this is not the case for *F. solani*.

CONCLUSION

Fusarium spp. isolates coming from sesame production area were different at morphological level, which allow to get identification of three species: *F. oxysporum*, *F. solani and F. semitectum*. Growth speed was not different among species, but it was statistically higher for one isolate. Molecular characterization was not able to group isolates according to species;

probably more primers should be used to get some discrimination among species.

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