Isolation and identification of fungi with bio-herbicidal activity in *Cyperus rotundus*

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ABSTRACT

Objective: to isolate and to identify selected fungi with bio-herbicidal potential in *Cyperus rotundus*.

Design/Methodology/Approach: for this research we collected *Cyperus* spp. plants in four sites of the municipalities Cárdenas and Huimanguillo (Tabasco) Mexico. From various tissues of healthy and diseased plants of *Cyperus*, different strains of fungi were isolated for morphological and molecular characterization. They were then identified through the ITS (Internal Transcribed Spacer) region. Some fungi that were isolated from diseased plants were selected to perform some pathogenicity and severity tests on seedlings of *C. rotundus*

Results: nine genera of fungi were isolated and associated with diseased and healthy plants of *Cyperus* spp. Only *Fusarium*, *Curvularia* and *Pestalotiopsis* were pathogenic in seedlings of *Cyperus rotundus*. These three genera of fungi caused different degrees of symptomatology and severity of damage on *C. rotundus*.

Limitations/Implications of the study: infrastructure and climate variables in the greenhouse could affect the variables evaluated. One clear limitation is found in the high costs of performing the complete molecular identification of selected fungi up to the taxonomic level of species.

Findings/Conclusions: this study provided evidence on the bio-herbicidal potential of fungi from genera *Fusarium*, *Curvularia* and *Pestalotiopsis* in seedlings of *C. rotundus*, However, more detailed studies are required on determining the range of hosts; on the combinations of these fungi, both of spores and extracts; and on the effect on sexual or asexual propagation of different species of *Cyperus*. As well as to evaluate the effect on wild plants considered weeds, and cultivated plants in order to test their full potential as bio-herbicide.

Keywords: weeds, endophyte fungi, phytopathogens, bio-herbicides.

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INTRODUCTION

Around the world, one of the biotic-related problems that negatively impact the agriculture sector is the presence of large numbers of weeds. The species *Cyperus rotundus* L. and *Cyperus esculentus* L. are among the most well-known weeds of global importance. These plants have a great capacity to adapt to different soil conditions, moreover, they disperse rapidly through bulbs or rhizomes. Due to this, these wild plants can establish in various agri-climatic regions, where they can cause losses between 20 and 90% in agricultural crops worldwide (Moncada, 2017; Peerzada, 2017). In general, weed populations are controlled through commercial chemical herbicides, however, these products result in negative impacts on the environment and health when they are used in the long term (Franco *et al.*, 2015; Mohd Ghazi

et al., 2023). Thus, the use of alternatives that are responsible with the environment is suggested, such as integrated weed management (Pavlović *et al.*, 2022).

Recent research focused on the microbial endobiome, in particular of those plants considered as weeds, in search of potential agents for biological control (Saitheja *et al.*, 2024). Thus, for example, endophyte fungi (EF) are found in the plant endobiome, which colonize internal plant tissues without causing apparent symptoms of disease in their host. Nonetheless, under certain conditions (environmental, from stress, or senescence of the plant) they can become pathogenic (Dos Reis *et al.*, 2022).

This study reports the presence of endophyte fungi and fungi with potential as phytopathogens in *Cyperus* spp. Some strains that could have potential as biological control agents were identified; those were evidenced by high level of pathogenicity and virulence to *C. rotundus*. This study set the foundations for future studies with these fungi to expand the characterization of bio-herbicide potential, by determining a range of hosts and by assessing combination with other weed management methods on land evaluations.

MATERIALS AND METHODS

Isolation and characterization of fungi

Sampling in four sites consisted of collecting six plants with a similar size per site, either with or without symptoms of *Cyperus* spp. infection. Sites were the "Benito Juárez" sugar mill (18° 00' 02.7" N, 93° 34' 46.5" W), the C-34 village (17° 57' 56.3" N, 93° 35' 54.5" W), and two plots of "Ejido La Esperanza" (17° 54' 23.4" N, 93° 36' 26.2" W; 17° 54' 22.8" N, 93° 37' 31.1" W).

Collected plants were cut into fragments of roots, stem, leaves and inflorescence which were disinfected with 70% ethanol inside a laminar flow hood for 2 min. They were then immersed in 0.5% sodium hypochlorite for 2 min. Finally, they were rinsed twice with sterile distilled water. Fragments were inoculated in Petri dishes with Potato Dextrose Agar (PDA) medium and left in incubation at 27 ± 2 °C for 12 h light and 12 h dark until hyphae growth was observed. These isolates were classified into morphotypes according to Arnold *et al.* (2001). In addition, monosporic cultures were done following the method of Molina -Cárdenas *et al.* (2021).

Morphological identification

The morphological characterization of the fungi was done by observation through an optical compound microscope (Leyca Microsystem DM750). The structures were analyzed following Dugan's (2006) taxonomic identification keys.

Molecular identification

DNA extraction

For DNA extraction, the protocol of Raeder and Broda (1985) was used, with the modifications established at the Pest Microbial-control Lab under Colegio de Postgraduados Campus Campeche. Integrity was observed through 0.7% agarose gels added with ethidium bromide (BrEt). The concentrations of the gDNA (genomic DNA) were quantified with a

NanoDrop (Thermo Fisher). All gDNA concentrations were adjusted to 100 ng μL^{-1} prior to PCR amplification (Castle *et al.*, 2018).

Amplificación por PCR

To perform the PCR amplification of the fungal gDNA, a reaction of 40 μL was performed for each sample with the following concentrations: 100 ng μL^{-1} gDNA, PCR Buffer- $MgCl_2$, 4mM $MgCl_2$, 0.6mM dNTP mix, 0.4 pmol μL^{-1} of each primer ITS1-TCCGTAGGTGAACCTGCGG, or ITS4-TCCTCCGCTTATTGATATGC, 0.1 U μL^{-1} of Taq Polymerase (Invitrogen Cat. No. 10342-020) and sterile ultrapure H_2O.

The C1000TM Thermal Cycler (BIO-RAD) was programmed based on the temperatures mentioned by White *et al.* (1990) plus specific modifications in timing. An initial denaturation at 95 °C for 7 min, 40 cycles with denaturation of 95 °C for 1 min, alignment at 55 °C for 1 min and extension at 72 °C for 1 min, ending with a final extension of 72 °C for 5 min. At the end of the reactions, the PCR products were visualized in 1% agarose gel plus BrEt. Subsequently, the PCR purifications were done with the commercial QIAquick PCR Purification Kit (Quiagen Cat. Num. 28104) and the integrity of the product was verified with Nanodrop.

Preliminary pathogenicity tests in *Cyperus rotundus*

Plants of *Cyperus rotundus* that had the same height and age characteristics were collected and selected. Twelve *C. rotundus* plants were placed per plastic germination tray in rows of three plants for each of the selected fungi; which belong to the genera *Curvularia*, *Pestalotiopsis*, *Fusarium*, *Helminsthosporium*, *Trichoderma*, *Pseudopestalotiopsis* and *Penicillium*. Subsequently, in the greenhouse, inoculations were done following the methodology of Jyothi *et al.* (2010). Plants were under observation until they developed symptoms, affected by the inoculated fungi. Finally, Koch's postulates were tested.

Evaluation of the severity of damage caused by *Pestalotiopsis*, *Curvularia* and *Fusarium* on *Cyperus rotundus*

From the preliminary tests, three fungi (*Fusarium* sp., *Curvularia* sp. and *Pestalotiopsis* sp.) were selected that were able to produce symptoms on *C. rotundus* plants. The conidia solutions were prepared following the methodology of Wang *et al.* (2021) and were adjusted to two concentrations of conidia (1×10^7 and 1×10^8 conidia mL^{-1}). In the greenhouse, two trays were used for each genus of fungus (each inoculated with its respective concentrations of fungi) and one as a control. Subsequently, they were inoculated with 1 mL of the conidia suspension during the evening, when the environment was at a 90% relative humidity. For 20 days we evaluated the height, number of tillers emerged (regrowth) and severity of damage as result of the disease in the plants (Figure 1).

Statistical analysis

A completely randomized design was used with two treatments (conidia concentrations) and six replicates. The response variables of interest were height and number of tillers. Normality and homogeneity tests were performed on the data obtained to run the analysis

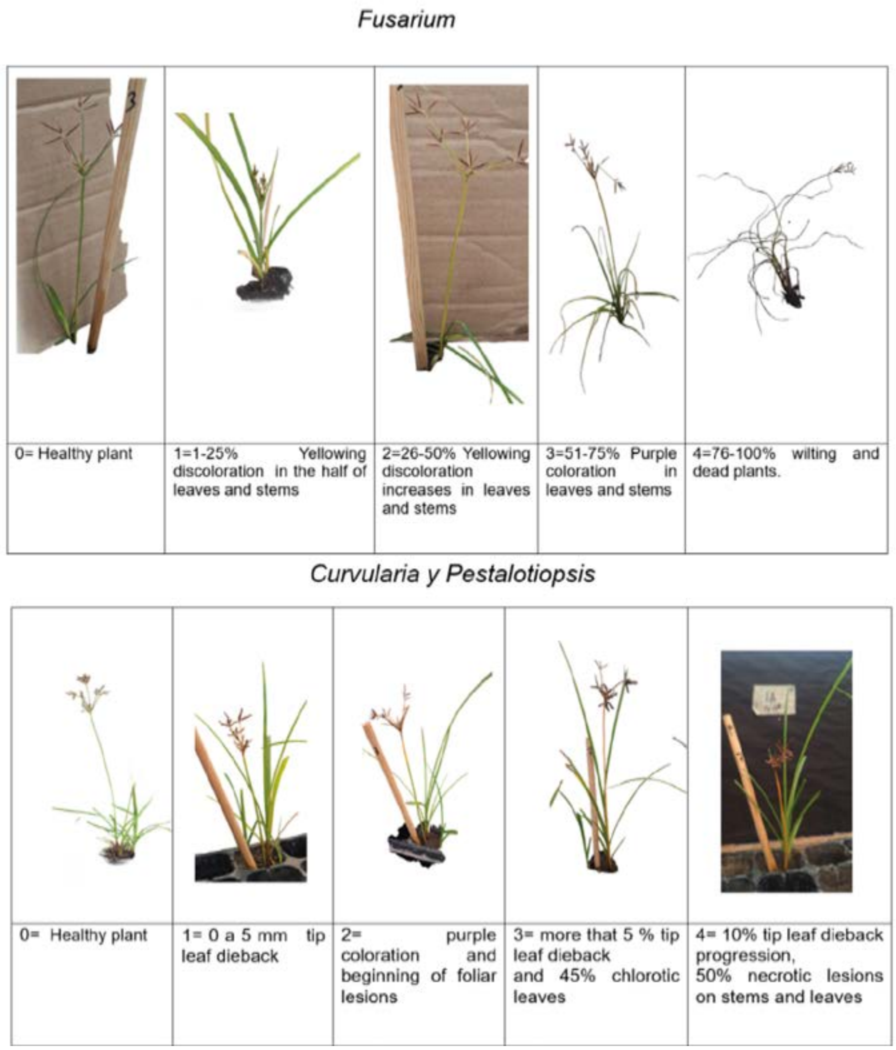

Figure 1. Severity score used in the greenhouse bioassays at Colegio de Postgraduados Campus Tabasco, in 2024.

of variance with the RStudio software version 4.4.1; When this was significant, multiple comparisons of means was done with the Tukey's test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Isolation and morphological identification of fungi

A total of 146 isolates of endophytic and epiphyte fungi were obtained from different parts of *Cyperus* spp. plants with and without symptoms. These isolates were grouped into 20 morphotypes. Seven genera of fungi were isolated from healthy plants, corresponding to the genera *Nigrospora*, *Aspergillus*, *Penicillium*, *Fusarium*, *Pseudopestalotiopsis*, *Curvularia* and *Trichoderma*. On the other hand, in plants with symptoms, the same fungi that were identified in plants without symptoms were found, plus two more genera of fungi, *Pestalotiopsis* and *Helminthosporium* that only were isolated with low frequency. It is well documented that there are many microorganisms that can be found in almost all parts of plants. However, the identification of weed-colonizing fungi is a topic that is little researched and, therefore, little known. In this study, we identified fungi whose colonies were found in different vegetative parts, in both healthy plants and symptomatic plants of *Cyperus rotundus* and *C. esculentus*.

Triolet *et al.* (2022) mentioned that weeds, like cultivated plants, possess and host a large amount of endophytic microflora, micro-epiphytes, and rhizosphere microorganisms. Within the community of microorganisms there are phytopathogenic fungi and some of them with potential for use as bio-herbicides. The starting point for developing a bio-herbicide is the isolation of microorganisms associated with weeds with disease symptoms. In addition, isolation from asymptomatic plants is also necessary to contrast and identify possible fungi with biological control potential in unexpected genera. In this study, the aforementioned principles were taken as a reference and from that it was possible to identify nine genera of fungi associated with *Cyperus* plants. Some authors such as Maharachchikumbura *et al.* (2011) and Kubiak *et al.* (2022) among others, in studies on the diversity of fungi and their possible use as biological weed controls, reported founding genera of fungi similar to those we isolated in this research.

Molecular analyses

Results of molecular identification are consistent with the results of macro and microscopic morphological analyses. Therefore, in all cases it was possible to confirm through the ITS region the genus to which the strains belonged. However, when analyzing the sequences in the NCBI and UNITE databases, the data we obtained only coincided with two strains of *Trichoderma* (*Trichoderma koningiopsis* and *T. harzianum*) which could be identified up to the taxonomic level of species (Table 1). Raja *et al.* (2017) indicated that although the ITS region is considered as the barcode of fungi, it is not widely well accepted. One of the reasons is because it does not work properly to distinguish species from highly specific genera. This is because some fungi have very narrow or no spaces in their ITS regions. Then, to achieve identification, it would be necessary to resort to the use of complementary primers, thus obtaining an accurate identification.

Identification, characterization and pathogenicity of *Fusarium* sp., *Curvularia* sp. and *Pestalotiopsis* sp. in *Cyperus rotundus*

Strains from the genera *Fusarium*, *Curvularia*, *Trichoderma*, *Penicillium*, *Pestalotiopsis*, *Pseudopestalotiopsis*, and *Helminthosporium* were taken from symptomatic plants. Days after inoculation, only *Fusarium*, *Curvularia* and *Pestalotiopsis* managed to cause damage to *C. rotundus* plants (Table 2).

Figure 2 shows the macro and microscopic characteristics of the strain which belongs to the genus *Fusarium*. This strain was isolated from the stem of a *Cyperus* spp. plant which presented symptoms of yellowing and stunting. This colony has a flat, smooth surface with a floccose appearance and creamy-orange color (Figure 2A). We observed the presence of conidia; thin, elongated, smooth, and crescent-shaped macroconidia can be seen, hialine and multi-septated (Figure 2B). These characteristics coincide with those reported by Lestari *et al.* (2021).

Table 1. Identification of fungi isolated from *Cyperus* spp., based on reference data from the NCBI and UNITE databases.

Strain	Identification	Base pairs	Accession	Percentage of identity (%)
HP	*Pestaloptiosis*	592	NCBI: KT459349.1 UNITE: MW404604	100 99.81
H2	*Pseudopestaloptiosis*	548	NCBI: OR588134.1 UNITE: OR588134	99.82 99.82
R1	*Penicillium*	408	NCBI: PP665290 UNITE: PP665290	100 100
RP	*Aspergillus*	555	NCBI: OR354739.1 UNITE: PQ106409	99.46 99.46
R8	*Trichoderma*	578	NCBI: KP965729.1 UNITE: OR2669177	99.83 99.83
R5	*Trichoderma koningiopsis*	580	NCBI: PP647347.1 UNITE: PP647347	100 100
R6	*Trichoderma harzianum*	595	NCBI: KU530201.1 UNITE: MT378439	100 100
T8	*Fusarium*	521	NCBI: MW391509.1 UNITE: PQ039530	100 100
TE	*Curvularia*	272	NCBI: LR994025.1 UNITE: PQ047117	99.26 99.26
TP	*Nigrospora*	413	NCBI: LC704396.1 UNITE: PP939049	99.52 99.52

Table 2. Symptomatology observed after fungal inoculation during the experiment with *Cyperus rotundus*.

Fungus	Symptoms
Pestalotiopsis sp.	Yellowing, spotting, and death on the tips of the leaves.
Curvularia sp.	Brown spots on leaves, yellowing, and death of leaf tips
Fusarium sp.	Yellowing, laxity and necrosis of stems and leaves, from the base of the plant.

On the other hand, Figure 3 shows the observed characteristics that correspond to the descriptions of the genus *Pestalotiopsis*. This strain was isolated from leaves with symptoms of yellowish leaf spots. Colony presents the following macro and microscopic characteristics, semi-elevated surface with a cottony white appearance and curved growth along its entire periphery (Figure 3A), small and circular black acervuli formed by smooth and septate sub-cylindrical fusiform conidia (Figure 3B). The apical and median appendices contained a coloration ranging from brown to dark brown. These characteristics coincide with those reported by Yin *et al.* (2024).

Finally, Figure 4 shows the isolation of the strain belonging to the genus *Curvularia*. This strain was isolated from the stem of a plant that had brown spots and yellow-brown colorations on the stem. It has an elevated cottony, dark brown surface with circular growth (Figure 4A). At the microscopic level, their conidia are straight and septated, it was observed that the conidia had a more marked and pigmented septum in the middle compared to the rest of the septa (Figure 4B). These characteristics coincide with those reported by Santos *et al.* (2018).

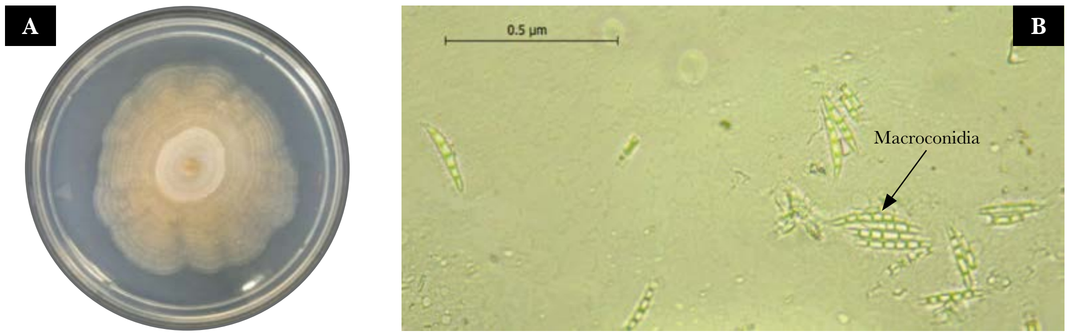

Figure 2. *Fusarium*. A: colony on PDA medium; B: microscopic structures observed using a compound optical microscope at Colegio de Postgraduados Campus Tabasco, Laboratorio de Control biológico.

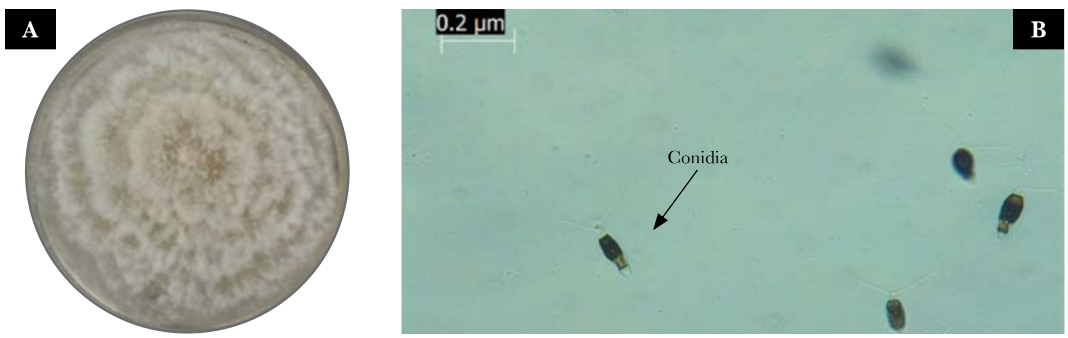

Figure 3. *Pestalotiopsis*. A: colony on PDA medium; B: microscopic structures; Compound optical microscopy at Colegio de Postgraduados Campus Tabasco, Laboratorio de Control biológico.

Figure 4. *Curvularia*. A: colony on PDA medium. B: microscopic structures; Compound optical microscopy at Colegio de Postgraduados Campus Tabasco, Laboratorio de Control biológico.

Severity of damage caused by fungi of *Curvularia*, *Fusarium* and *Pestalotiopsis* genera

Only three fungi were selected since those were pathogenic to *Cyperus rotundus* plants in greenhouse. These strains were selected to test for severity of damage analyses and to evaluate the effects of inoculum under semi-controlled conditions. After 20 days from inoculation, the damage shown in Figure 5 was observed. Damages were observed in most of the plants, in addition to stem necrosis and damage to the leaves (necrotic spots or dead leaves).

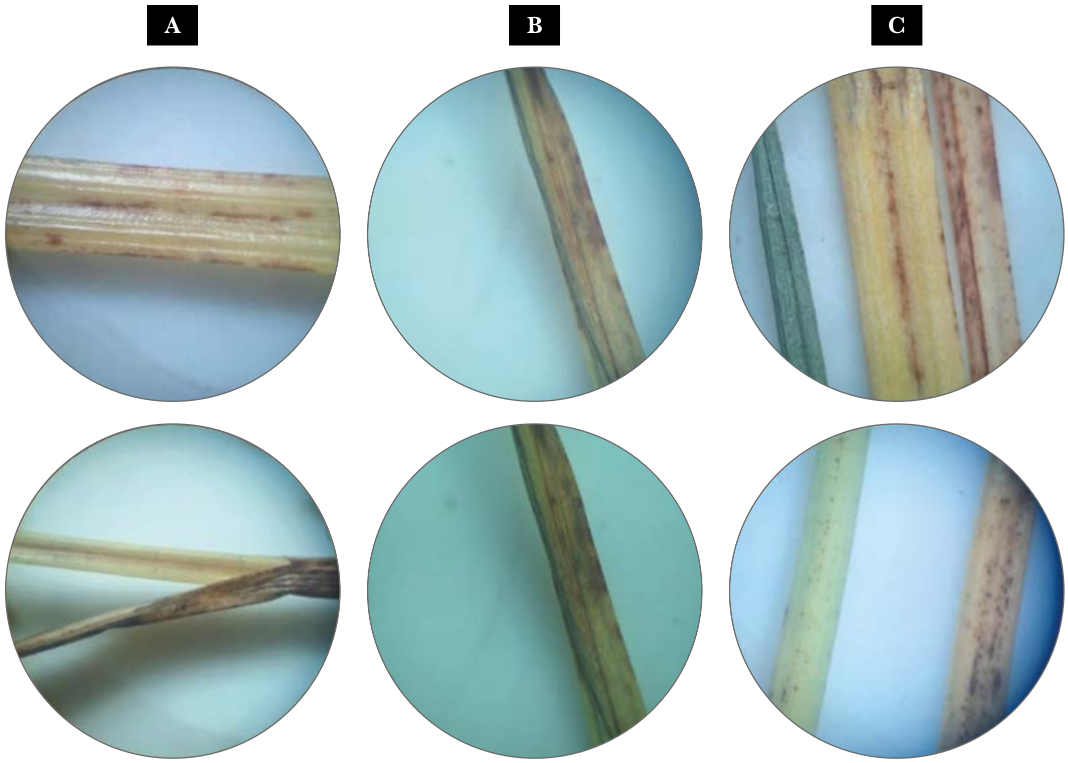

Figure 5. Damage on *Cyperus rotundus* 20 days after inoculation with A: *Fusarium* sp., B: *Curvularia* sp. and C: *Pestalotiopsis* sp.

Height of *C. rotundus* plants was affected after 20 days since inoculation (Figure 6). The inoculated control (with only distilled water) showed greater growth than the plants infected with fungi. Although non-significant statistical differences were found between both concentration treatments, there were statistical differences among the control and the treatments with fungal inoculation at two different concentrations. In addition, treatments C1, F2, P1, and P2 displayed greater dispersion of data compared to the treatments

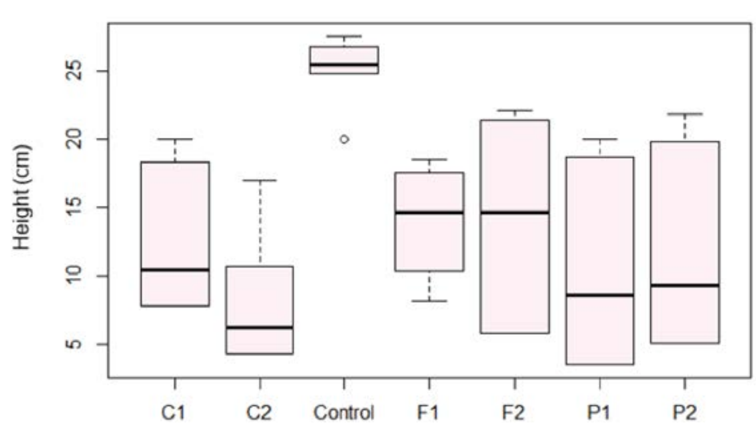

Figure 6. Effect of fungal concentrations (conidia mL^{-1}) on the height of *Cyperus rotundus* 20 days after inoculation. F1: *Fusarium* sp. 1×10^8; F2: *Fusarium* sp. 1×10^7; C1: *Curvularia* sp. 1×10^8; C2: *Curvularia* sp. 1×10^7; P1: *Pestalotiopsis* sp. 1×10^8; P2: *Pestalotiopsis* sp. 1×10^7.

with *Curvularia* 1×10^7 conidia mL^{-1} and *Fusarium* 1×10^8 conidia mL^{-1}, which had less dispersion and homogeneous damage.

For the variable of number of tillers, Figure 7 shows the trend in the emergence of new tillers on six different dates over a 20-day period. The control showed a higher number of tillers sprouted per tray cavity (4.5), while plants with fungal treatments had fewer tillers (2.5 and 3.5).

Non-significant statistical differences were found between the treatments with the fungi evaluated (Table 3). The results obtained from the analysis of variance indicated that there were significant differences ($p\leq0.05$) between the control and the concentrations of conidia used.

Regarding the severity of damage caused by the three fungi and the two concentrations, the reference values used were those in the severity score presented in the materials and methods section (Figure 1). Results obtained showed that there was a negative effect of fungi on plants of *C. rotundus*. The *Pestalotiopsis* strain at a concentration of 1×10^8 conidia mL^{-1} proved to be more aggressive to the plants inoculated with it, causing the damage described above in a shorter period of time (Figure 8). In the case of Curvularia, both inoculated concentrations managed to cause damage to the plants in a period of time

Table 3. Effect of fungi of *Fusarium*, *Curvularia* and *Pestalotiopsis* genera on the height (cm) of *Cyperus rotundus* plants.

Treatment (conidia mL^{-1})		Mean	SEM
Control		24.954 a	2.371
Curvularia	1×10^8	12.447 b	2.371
Curvularia	1×10^7	8.093 b	2.371
Fusarium	1×10^8	13.935 b	2.371
Fusarium	1×10^7	14.035 b	2.371
Pestalotiopsis	1×10^8	10.443 b	2.371
Pestalotiopsis	1×10^7	11.708 b	2.371

SEM: standard error of the mean; Tukey ($p\leq0.05$), n=8 plants.

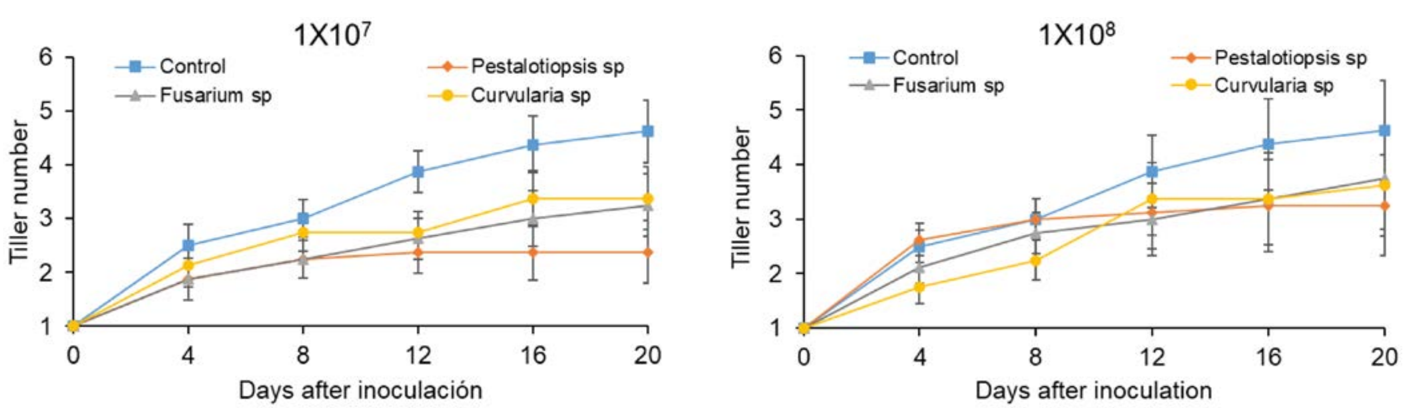

Figure 7. Effect of inoculation and concentration (conidia mL^{-1}) of the solutions with fungi *Fusarium* sp., *Curvularia* sp., and *Pestalotiopsis* sp. on tiller emergence in *Cyperus rotundus* plants.

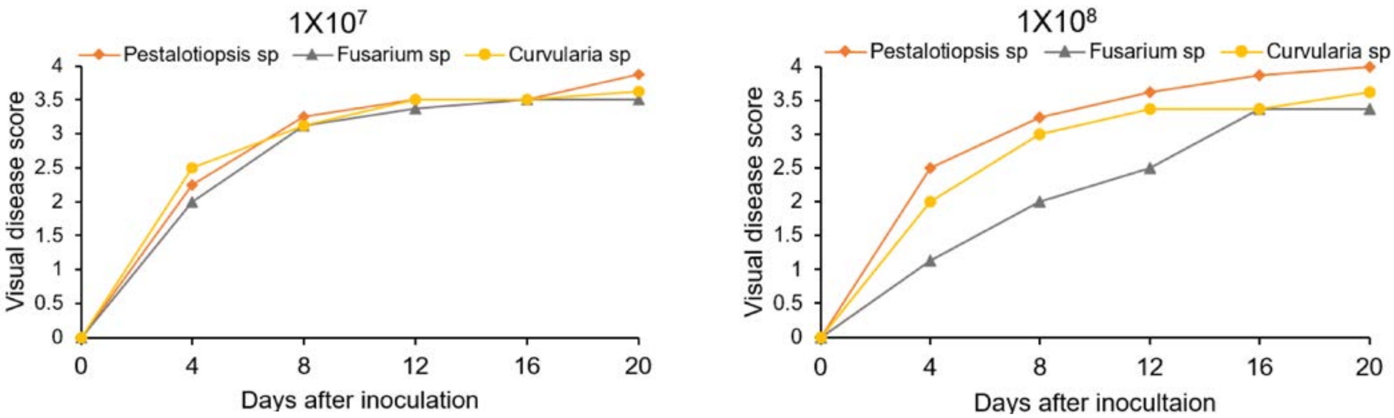

Figure 8. Severity of damage by *Pestalotiopsis* sp., *Curvularia* sp. and *Fusarium* sp. (1×10^8 and 1×10^7 conidia mL^{-1}) in *Cyperus rotundus*.

similar to that of Pestalotiopsis. On the other hand, concentrations of 1×10^8 conidia mL^{-1} of *Fusarium* caused less severe damage to *C. rotundus*. Therefore, despite the effects caused on *C. rotundus* plants, *Fusarium* did not demonstrate that it has the potential to cause significant damage under the experimental conditions reported here.

In the results of these tests, it was observed that the fungi evaluated have the ability to decrease the development of *C. rotundus* plants; however, they were not able to inhibit the growth of new tillers. Although non-significant statistical differences were found between the fungal inoculums, the *Fusarium* sp. strain at a concentration of 1×10^8 conidia mL^{-1} had a lower rank on the severity score compared to the rest of the treatments.

In a study by Raghavendra *et al.* (2013) on *Centaurea stoebe*, they mentioned that the infection of seeds caused by the *Fusarium* fungus subsequently produced a decrease in the rate of reemergence of this weed. This agrees with our results since a lower rate of emergence of regrowth was observed, compared to control plants. On the other hand, Tan *et al.* (2002) evaluated the effect of *Fusarium* sp. on *Alternanthera philoxeroides* they reported that the fungus is capable of producing an effect on that weed, but the efficiency of the bio-herbicide varies depending on the concentration of the inoculum; better results were obtained when the concentration reached high densities of spores.

Regarding the genus *Pestalotiopsis* sp., there are few studies referring to it as a potential agent for biological control of weeds. Santos *et al.* (2008) conducted studies on the bio-herbicide potential of *Pestalotiopsis guepinii*. Their results showed that its chemical compounds had inhibitory potential in the germination of *Senna obtusifolia* seeds. In another study, it was reported that *Pestalotiopsis vismiae* showed high suppression both in the germination and in the reduction of rhizome length of different weeds (Radi and Hamedi, 2017). This may be consistent with our results, since we did not observe abundant presence of tillers emerged from the rhizome of *C. rotundus* due to the effect of the evaluated fungi.

In another study on weeds, in which De Luna *et al.* (2002) tested the bio-herbicide effect using *Curvularia*, those authors reported two isolates (*Curvularia tuberculata* and *Curvularia oryzae*) obtained from diseased plants of *Cyperus difformis* and *Cyperus iria* in Philippines in

1993. When these fungi were inoculated at a concentration of 1×10^8 conidia mL^{-1} with a dew period of 24 h at 28 °C, rapid and severe blighting of the leaves was observed, which caused the death of the seedlings within two weeks after inoculation with fungi. In the case of our study, although inoculation in *Cyperus rotundus* is reported, symptoms and virulence were different. Since indeed there was death of *C. rotundus* plants, but not all the plants in the treatments died.

Our results in this research are consistent with what was reported by those authors, since necrosis and dieback were observed starting at the tips of the leaves of *C. rotundus* plants, but no sporulation was observed. This may be partly due to the experimental conditions, since we applied the inoculations during the dry season when the temperatures inside the greenhouse were around or above 30-35 °C. In contrast, it could be interpreted that our results on the height of the plants were favorable; however, there was a negative impact on the emergence of tillers or a regrowth delay which may be a sign that there was an effect on rhizomes. More experiments are required to evaluate, quantify and thoroughly report these significant effects of fungi with bio-herbicidal potential in the reduction of the wild populations of *C. rotundus*.

CONCLUSIONS

In this research, we identified fungi associated with *Cyperus* plants. Morphological and molecular identification techniques in the ITS region allowed us to identify and accurately verify nine genera *Fusarium*, *Curvularia*, *Trichoderma*, *Aspergillus*, *Penicillium*, *Pestalotiopsis*, *Pseudopestalotiopsis*, *Helminthosporium* and *Nigrospora*. Moreover, two of them (*Curvularia* and *Pestalotiopsis*) showed to be related with potential phytopathogenic activity to *Cyperus rotundus*. And three isolates, *Fusarium* sp., *Curvularia* sp., and *Pestalotiopsis* sp. proved to be pathogenic to *C. rotundus*.

Findings in this study contribute to the knowledge of the diversity of fungi from wild plants in Mexico and set the foundations for future research aimed at the biological control of invasive weeds. Also, this study adds to the research aiming to explore ecological interactions among groups of microorganisms that inhabit the endobiome and rhizosphere of weeds.

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