CROP PROTECTION

Selection of filamentous fungi that are resistant to the herbicides atrazine, glyphosate and pendimethalin

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ABSTRACT. The objective of the present study was to isolate fungi from agricultural soils and evaluate fungal growth in culture medium contaminated with atrazine, glyphosate and pendimethalin. Filamentous fungi were isolated from agricultural soils and cultured in a modified culture medium containing 0, 10, 20, 50, and 100 μg mL^{-1} atrazine, glyphosate and pendimethalin for 14 days at 28°C. The fungi that presented optimal and satisfactory growth were plated in Sabouraud culture medium with 4% dextrose and containing the herbicides at concentrations of 0, 10, 20, 50, and 100 μg mL^{-1} for seven days at 28°C. The mean mycelial growth values were submitted to analysis of variance and the Tukey test ($p < 0.05\%$) for comparison and relative growth determination, and maximum inhibition rates were calculated. The isolated fungi *Aspergillus fumigatus*, *Fusarium verticillioides* and *Penicillium citrinum* were shown to be resistant to atrazine, glyphosate and pendimethalin. *F. verticillioides* showed higher mean mycelial growth in the culture media contaminated with atrazine and glyphosate than the other two fungi. In the culture medium contaminated with pendimethalin, *F. verticillioides*, and *A. fumigatus* presented the highest mean mycelial growth values.

Keywords: bioremediation; microorganisms; agricultural soils; pesticides.

Introduction

Herbicides are most commonly applied in vegetable production systems; approximately 2.5 million tons of pesticides are used annually in the world, and in Brazil, the annual consumption has exceeded 300 thousand tons (Moraes, 2019). The high consumption of pesticides is based on the agricultural improvements that these products present, such as a decrease in invasive plants and increased economic efficiency in the production process.

Despite the benefits, the final location of these products is the soil, and they may have negative impacts, such as intoxication to crops cultivated in succession, organic matter degradation, surface and groundwater eutrophication and loss of essential soil functions, including nutrient cycling and environmental buffering power. There may also be a reduction in microbiota and biodiversity (Moreno-Mateos, Meli, Vara-Rodríguez, & Aronson, 2015).

To restore soils that have become polluted and unproductive by herbicide addition, it is necessary to remove contaminants from the area. Among the technologies available for this purpose, we highlight bioremediation that consists of ecological technology to accelerate rates of natural degradation through plants, algae, bacteria and fungi (Alegbeleye, Opeolu, & Jackson, 2017; Khayati & Barati, 2017).

Fungi play an important role in bioremediation due to their metabolic ability to degrade different toxic and recalcitrant compounds. They show efficiency in degradation due to their ability to adapt their metabolism to different carbon sources. This metabolic flexibility occurs due to the production of enzymes, such as oxidative and hydrolytic enzymes, that degrade compounds, including polycyclic aromatic hydrocarbons, plastics (PET) and pesticides, such as atrazine, glyphosate and pendimethalin (Kanagaraj, Senthilvelan, & Panda, 2015; Deshmukh, Khardenavis, & Purohit, 2016).

Atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine) is a pre-emergent and selective systemic herbicide, an important representative of the triazine group. When applied for many years, atrazine

and its residues accumulate in agricultural soils and can be mobilized through cycles between dry and wet periods and then leached. Therefore, considering the high amounts applied annually, together with research demonstrating its high persistence in soils, atrazine represents a potential threat to the environment (Peng et al., 2018; Zhao, Feng, Hu, Wang, & Lu, 2019).

Some microorganisms degrade atrazine by N-dealkylation reactions, wherein alkyl groups attached to the ring nitrogen atom in amines, carbamates or amides are removed by oxidation and conversion to aldehydes, producing desethylatrazine (DEA) and desisopropylatrazine (DIA). These degradation products may further be dealkylated to be converted to desisopropyl desethylatrazine (Coelho & Bernardo, 2017).

Glyphosate (N-(phosphonomethyl) glycine) is a post-emergent desiccant and systemic herbicide. It has high adsorption capacity through the exchange of binders with iron and aluminium oxides and hydrogen bridges formed with humic substances present in the soil. Adsorption reduces the concentration of herbicides in the solubilized fraction of soil, reducing its potential action. Once adsorbed, glyphosate can remain as a bound residue in the environment until complete mineralization occurs (Sidoli, Baran, & Angulo-Jaramillo, 2016; Gill, Sethi, & Mohan, 2017).

Microbial activity is an important factor that determines the presence of glyphosate in the soil, and microorganisms use it as a source of energy, phosphorus and carbon, which are mainly responsible for its degradation (Zhan, Feng, Fan, & Chen, 2018). The pathway of glyphosate degradation by microorganisms involves the action of enzymes, such as oxidoreductases and transaminases, and glyoxylic acid, which cleave the glyphosate molecule at bonds other than those of carbon and phosphorus and may occur under aerobic and anaerobic conditions in the soil profile (Wang et al., 2016).

Pendimethalin (N-1-(ethyl propyl)-2,6-dinitro-3,4-methyl-toluidine) is a non-systemic herbicide belonging to the selective, pre- and post- emergent dinitroaniline chemical group. After application of this herbicide to soil, it may dissipate through evaporation, leaching and runoff. It has low adsorption capacity, which makes it more mobile in the environment; in groundwater, as in surface water, its presence has already been detected (Kpagh, Sha'Ato, Wuana, & Tor-Anyiin, 2016).

The degradation of pendimethalin in soil can occur under both aerobic and anaerobic conditions. The aerobic degradation route occurs by amino group dealkylation, followed by the reduction of the nitrile group. Under anaerobic conditions, there is a sequential reduction of nitro groups (Tobler, Hofstetter, & Schwarzenbach, 2007).

Therefore, fungi are an attractive alternative for herbicide biodegradation processes, which makes them convenient for application in bioremediation processes. The objective of the present study was to isolate fungi from agricultural soils and evaluate fungal growth in culture medium contaminated with atrazine, glyphosate and pendimethalin.

Material and methods

The experiments were conducted at the Laboratory of Environmental Biotechnology (LABITEC) of the Center of Exact, Natural and Technological Sciences belonging to the State University of the Tocantina Region of Maranhão (UEMASUL), Brazil.

Soil samples were collected on three farms that use agrochemicals located in the city of Imperatriz, Maranhão. The first area (5°31'31" S, 47°26'38" W) grows vegetables and uses glyphosate and pendimethalin; the second area (5°35'22" S, 47°25'35" W) applies atrazine and glyphosate and grows corn; the third (5°31'13" S, 47°26'53" W) is an experimental agricultural area and uses the herbicide glyphosate.

In each area, 12 soil subsamples were collected with the help of a dutch piercer at a depth of 20 cm. Each subsample set was mixed to form a composite sample (Chan-Cupul, Heredia-Abarca, & Rodríguez-Vázquez, 2016). Filamentous fungi were isolated by serial dilution; first, 12.5 g of each composite soil sample was weighed and then added to an Erlenmeyer flask containing 125 mL sterilized mineral medium (NaCl 0.85%), forming a 1:10 suspension that was agitated for 30 minutes on a shaker table. Then, 1 mL of each suspension was diluted in 9 mL mineral medium to obtain a 1:1000 ratio (Oliveira, Lima, Ambrósio, Bezerra Neto, & Chaves, 2017).

Subsequently, in a laminar flow hood, aliquots of 1 mL of each final dilution were inoculated into sterile Petri dishes containing Sabouraud culture medium with 4% dextrose, and tetracycline and chloramphenicol were added to inhibit bacterial growth during incubation in an oven at 28°C for seven days. The isolation sequence was performed using the scratch marks technique. Ten fungi were obtained and were preserved in inclined Sabouraud culture medium with 4% dextrose at 4°C.

The selection of fungi that were resistant to atrazine, glyphosate and pendimethalin was carried out by inoculating the microorganisms in a modified culture medium (0.5 g K_2HPO_4, 0.5 g $NaNO_3$, 0.0125 g $MgSO_4.7H_2O$, 0.005 g $CaCl_2.2H_2O$, 10 mL trace solution (10 mg $ZnSO_4.7H_2O$, 3 mg $MnCl_2.2H_2O$, 30 mg H_3BO_3, 20 mg $CoCl_2.6H_2O$, 1 mg $CuCl_2.2H_2O$, 2 mg $NiCl_2.6H_2O$, 3 mg $NaMoO_4.H_2O$, and 1 l distilled water), 13 g agar, and 1 l distilled water, pH 7.0) proposed by Ma et al. (2017) and contaminated with atrazine, glyphosate or pendimethalin at concentrations 0, 10, 20, 50, and 100 µg mL^{-1}. The Petri dishes were incubated at 28°C for 14 days.

The qualitative selection of the fungi was based on macroscopic observations of fungal colony growth after 14 days of incubation: poor growth (5 mm), reasonable growth (10 mm), satisfactory growth (20 mm) and optimum growth (40 mm). At this stage, 10 fungi, three herbicides (atrazine, glyphosate, and pendimethalin), five concentrations, and triplicate analysis were used, resulting in 450 experimental units.

The fungi that presented optimal and satisfactory growth were considered resistant to the herbicides and sent to the URM Microbiology Department of the Biological Sciences Center of the Federal University of Pernambuco, Brazil, for identification. The resistant fungi were inoculated in sterile Petri dishes containing Sabouraud solid culture medium with 4% dextrose, contaminated with atrazine, glyphosate or pendimethalin at concentrations of 0, 10, 20, 50, and 100 µg mL^{-1} and incubated for seven days at 28°C.

The mean mycelial growth (MCM) of the colonies, which indicates the degree of adaptability of the fungi as a function of time, was evaluated after the first, third and seventh days. The relative growth rates (TCR) demonstrated the growth of each fungus after three days, considering only the herbicide as a carbon source, and the maximum inhibition rates (TIM) that were related to the growth inhibition of each fungus after 24 hours of exposure to herbicides were calculated using Equations 1 and 2 (Costa et al., 2015):

$$T_r = \frac{T_t}{T_{z3}} \times 100 \tag{1}$$

$$T_i = \frac{T_{zi} - T_t}{T_{zi}} \times 100 \tag{2}$$

where: T_r is the relative growth rate, T_i is the maximum inhibition rate, T_t is the minimum growth rate with atrazine, glyphosate or pendimethalin (µg mL^{-1}), T_{z3} is the growth rate at a concentration of 0 µg mL^{-1} after the third day, and T_{z1} is the growth rate at a concentration of 0 µg mL^{-1} after the first day.

The fungal mycelial growth test was performed in a factorial scheme, with the factor levels including the herbicide resistant fungi (three microorganisms), herbicides (atrazine, glyphosate and pendimethalin), five herbicide concentrations (0, 10, 20, 50, and 100 µg mL^{-1}) and five replications, resulting in 225 experimental units. The results were submitted to analysis of variance and the Tukey test ($p < 0.05\%$) to compare the means using the statistical program Sisvar (Ferreira, 2019).

Results and discussion

Filamentous fungi that are resistant to the herbicides atrazine, glyphosate, and pendimethalin

The fungi classified as optimal and satisfactory, after macroscopic observations of colony growth, were identified by the Micoteca URM of the Mycology Department of the Biological Sciences Center of the Federal University of Pernambuco and considered resistant to the tested herbicides (Table 1).

Table 1. Fungi identified and incorporated into the Micoteca URM Culture Collection of the Center for Biological Sciences of the Federal University of Pernambuco, Brazil.

Species name	Sample code (URM)
Aspergillus fumigatus Fresen.	8070
Fusarium verticillioides (Sacc.) Nirenberg	7954
Penicillium citrinum Thom	8069

Applications of fungal mycelial growth in culture media contaminated with atrazine, glyphosate and pendimethalin

In culture medium contaminated with atrazine, *F. verticillioides* differed from *A. fumigatus* and *P. citrinum*, showing a higher MCM (Figure 1). The 0 µg mL^{-1} concentration was the most adequate for fungal growth, followed by the 10 and 20 µg mL^{-1} concentrations, the growth at which did not differ from each other (Figure 2).
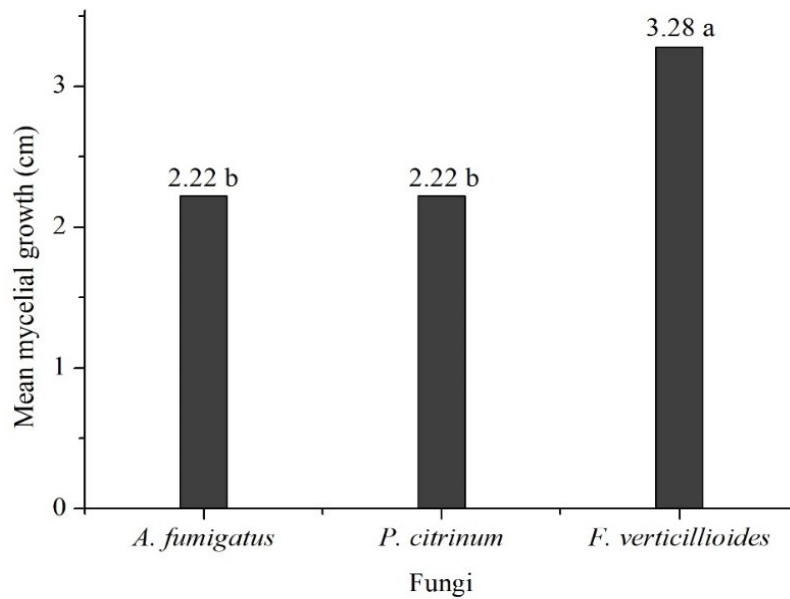
Figure 1. Mean mycelial growth values (cm) in culture medium contaminated with atrazine. Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.
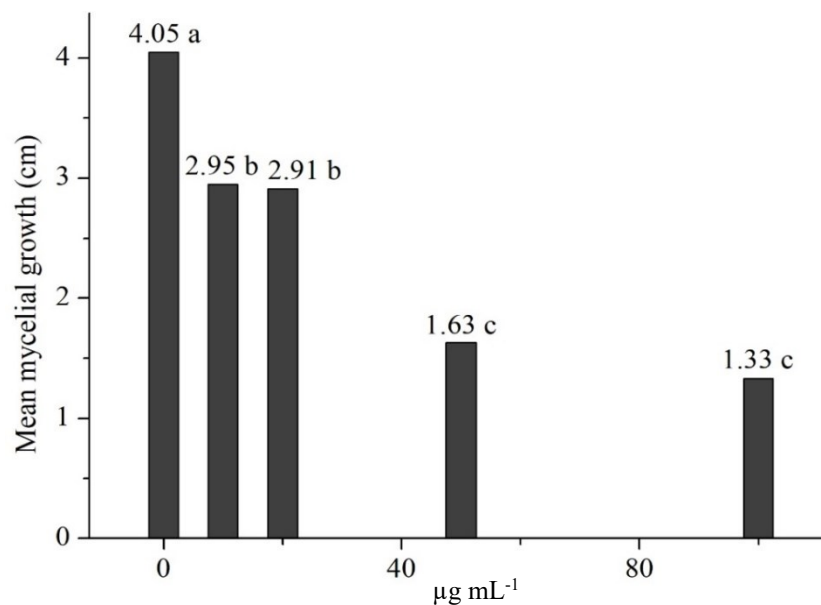
Figure 2. Mean mycelial growth values (cm) at different atrazine concentrations. Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.

When observing the fungi at each atrazine concentration, there was no significant difference between the, at 0 µg mL^{-1}. *A. fumigatus* and *F. verticillioides* showed statistically significant MCM values at 0, 10 and 20 µg mL^{-1}. *F. verticillioides* showed a higher MCM at concentrations of 50 and 100 µg mL^{-1} than at the lower atrazine concentrations (Table 2).

Table 2. Fungal growth at each atrazine concentration.

Fungi	Concentrations (µg mL^{-1})				
	0	10	20	50	100
A. fumigatus	3.88 a	3.02 a	2.70 a	1.18 b	0.32 b
F. verticillioides	4.00 a	3.58 a	3.40 a	2.56 a	2.84 a
P. citrinum	4.26 a	2.26 b	2.62 b	1.16 b	0.82 b

Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.

When observing the atrazine concentrations for each fungus, there were no significant differences at 0 µg mL^{-1}. At concentrations of 0, 10 and 20 µg mL^{-1}, *F. verticillioides* showed statistically equal MCM, but at 10 and 20 µg mL^{-1}, the fungi did not differ (Table 3).

Table 3. Mean mycelial growth as a function of atrazine concentration for each fungi.
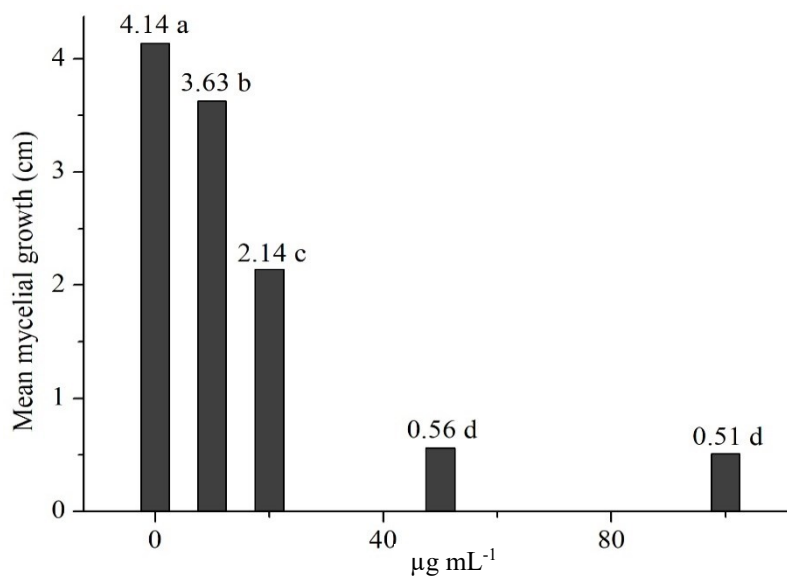
Concentrations (μg mL^{-1})	Fungi		
	A. fumigatus	*F. verticillioides*	*P. citrinum*
0	3.88 a	4.00 a	4.26 a
10	3.02 b	3.58 ab	2.62 b
20	2.70 b	3.40 ab	2.26 b
50	1.18 c	2.84 bc	1.16 c
100	0.32 d	2.56 c	0.82 c

Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.

The fungus *F. verticillioides* presented higher MCM values in culture medium contaminated with glyphosate than in the other media (Figure 3). The highest MCM values were at glyphosate concentrations of 0 and 10 μg mL^{-1} (Figure 4).
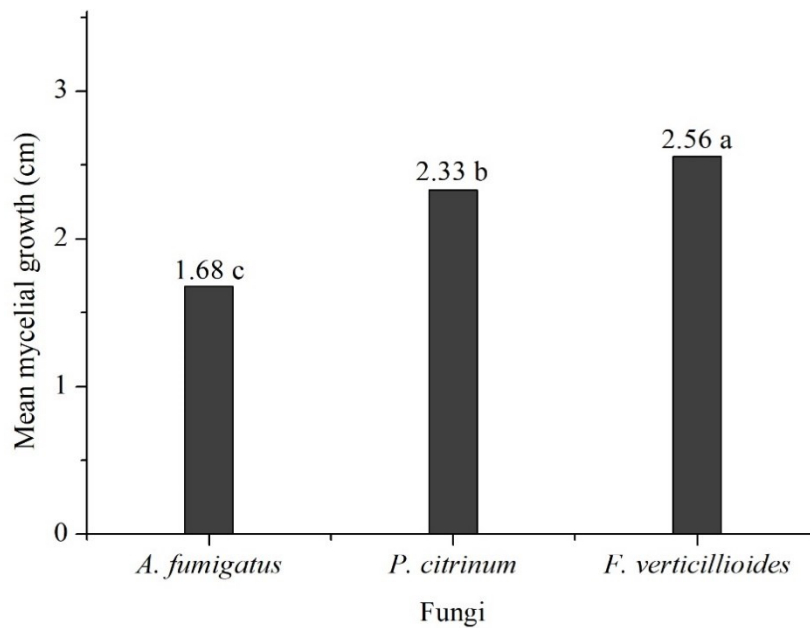
Figure 3. Mean mycelial growth values (cm) in culture medium contaminated with glyphosate. Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.

Figure 4. Mean mycelial growth values (cm) in glyphosate concentrations. Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.

When evaluating the fungi at each glyphosate concentration, *P. citrinum* presented a higher MCM at 10 μg mL^{-1} than the other two fungi. At 20 μg mL^{-1}, *P. citrinum* and *F. verticillioides* presented higher MCM values

than *A. fumigatus* and were not statistically different. At 50 and 100 µg mL^{-1}, *F. verticillioides* was the only fungus to develop mycelia (Table 4).

Table 4. Evaluation of fungi at each glyphosate concentration.

Fungi	Concentrations (µg mL^{-1})				
	0	10	20	50	100
A. fumigatus	3.88 a	3.12 b	1.42 b	0.00 b	0.00 b
F. verticillioides	4.34 a	2.90 b	2.40 a	1.52 a	1.68 a
P. citrinum	4.20 a	4.86 a	2.60 a	0.00 b	0.00 b

Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.

When observing glyphosate concentrations for each fungus, there were no significant differences at 0 and 10 µg mL^{-1}. At 20 µg mL^{-1}, *F. verticillioides* presented a higher MCM than at the other concentrations and was the only fungus able to grow at 50 and 100 µg mL^{-1} (Table 5).

Table 5. Evaluation of glyphosate concentrations for each fungus.

Concentrations (µg mL^{-1})	Fungi		
	A. fumigatus	*F. verticillioides*	*P. citrinum*
0	3.88 a	4.34 a	4.86 a
10	3.12 b	2.90 b	4.20 b
20	1.42 c	2.40 b	2.60 c
50	0.00 d	1.68 c	0.00 d
100	0.00 d	1.52 c	0.00 d

Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.

In culture medium contaminated with pendimethalin, *F. verticillioides* and *A. fumigatus* presented higher and statistically equal MCM values (Figure 5). The MCM values were highest at 0 µg mL^{-1}, followed by those at the 10 µg mL^{-1} concentration (Figure 6).
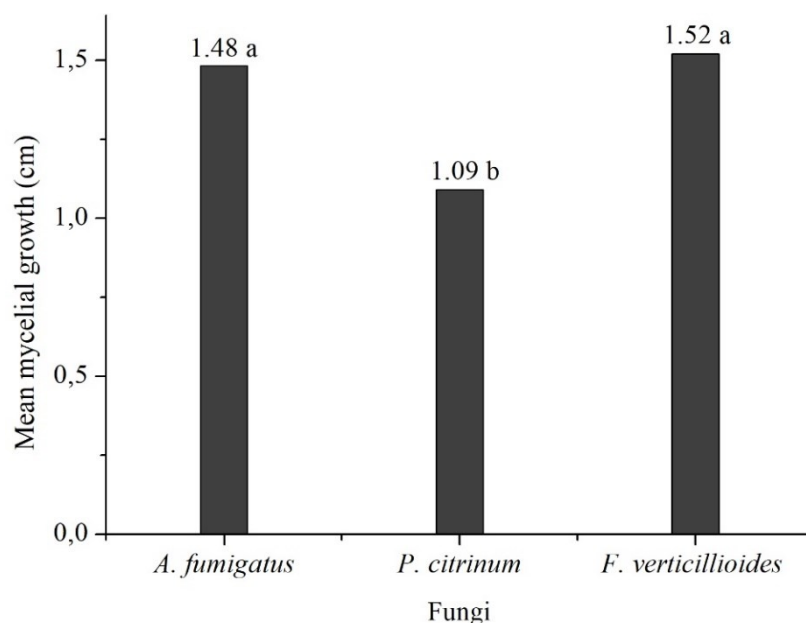
Figure 5. Mean mycelial growth values (cm) in culture medium contaminated with pendimethalin. Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.

When evaluating the fungi at each pendimethalin concentration level, *A. fumigatus* and *F. verticillioides* presented MCMs that were statistically equal at 0, 10 and 20 µg mL^{-1}. There was no mycelial growth for any fungi at 50 and 100 µg mL^{-1} (Table 6).

When observing the pendimethalin concentrations for each fungus, the highest MCMs occurred at 0 and 10 µg mL^{-1}. The fungi presented no MCM at 50 and 100 µg mL^{-1}. At the 20 µg mL^{-1} concentration, *P. citrinum* did not develop (Table 7).
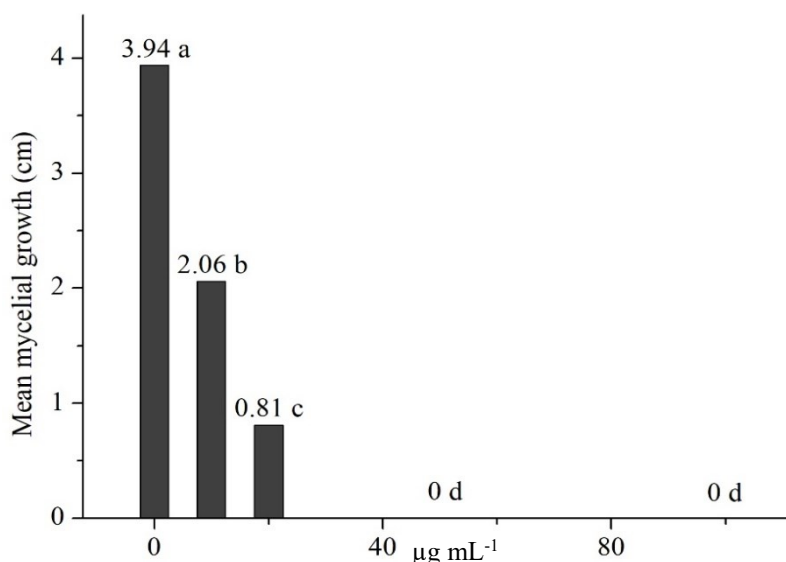
Figure 6. Mean mycelial growth values (cm) in pendimethalin concentrations. Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.

Table 6. Evaluation of fungi at each pendimethalin concentration.

Fungi	Concentrations ($\mu g\ mL^{-1}$)				
	0	10	20	50	100
A. fumigatus	3.80 a	2.46 a	1.32 a	0.00	0.00
F. verticillioides	4.10 a	2.20 a	1.10 a	0.00	0.00
P. citrinum	3.92 a	1.52 b	0.00 b	0.00	0.00

Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.

Table 7. Evaluation of pendimethalin concentrations for each fungus.

Concentrations ($\mu g\ mL^{-1}$)	Fungi		
	A. fumigatus	*F. verticillioides*	*P. citrinum*
0	3.80 a	4.10 a	3.92 a
10	2.46 b	2.20 b	1.52 b
20	1.32 c	1.10 c	0.00 c
50	0.00 d	0.00 d	0.00 c
100	0.00 d	0.00 d	0.00 c

Mean values followed by the same letter do not differ according to the Tukey test at a 5% significance level.

Relative growth

The results of the relative growth rates (TCR) show that the MCM tended to decrease with increasing concentrations of the herbicides tested in this study (Figure 7). The 20 $\mu g\ mL^{-1}$ concentration was most favourable for fungal growth in culture medium contaminated with atrazine, except for *F. verticillioides*, which showed the highest growth at 10 $\mu g\ mL^{-1}$ (Figure 7 A). The 10 $\mu g\ mL^{-1}$ concentration was the most favourable for the microorganisms in culture media contaminated with glyphosate or pendimethalin, as seen in Figure 7 (B and C).

Maximum inhibition

The results described in Figure 8 demonstrate the maximum inhibition rates (TIMs); negative signs indicate growth stimulation. In the culture media contaminated with atrazine and glyphosate, the fungi presented TIMs at 50 $\mu g\ mL^{-1}$, and an increase in growth inhibition was observed at 50 and 100 $\mu g\ mL^{-1}$, according to Figure 8 A and 8 B. In culture medium contaminated with pendimethalin, the concentration with the highest TIM was 20 $\mu g\ mL^{-1}$, with total growth inhibition at concentrations 50 and 100 $\mu g\ mL^{-1}$ (Figure 8 C).

Bonfleur, Tornisielo, Regitano, & Lavorenti (2015) reported that atrazine toxicity causes stress to the soil microbiota. Marinho, Barbosa, Rodrigues, Aquino, & Pereira (2017) tested the effect of increasing atrazine concentrations in *Aspergillus niger* AN 400, and the fungus was able to grow at concentrations up to 30 mg L^{-1}. The authors concluded that this fungus is very resistant to atrazine.
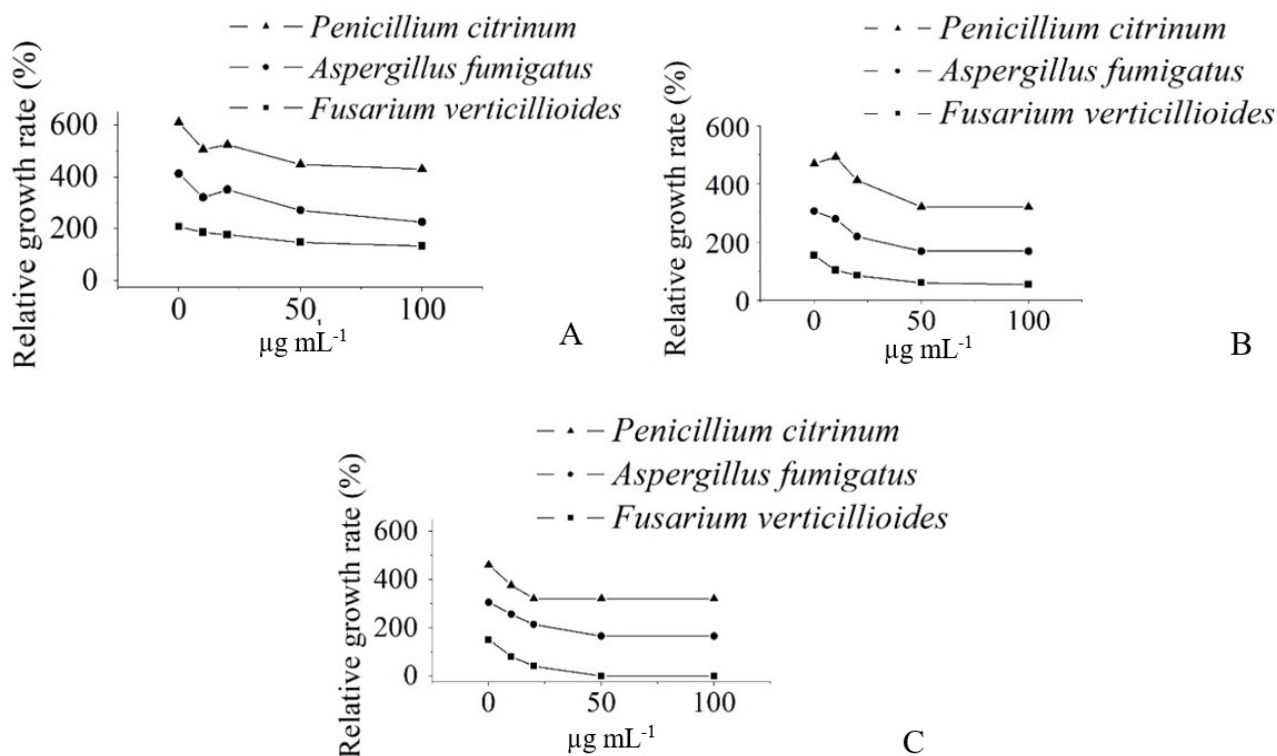
Figure 7. TCR (%) of fungi in culture media contaminated with atrazine, glyphosate or pendimethalin at 0, 10, 20, 50 and 100 μg mL^{-1} after 3 days of incubation. (A) TCR (%) of fungi in culture medium contaminated with atrazine (B) TCR (%) of fungi in culture medium contaminated with glyphosate (C) TCR (%) of fungi in culture medium contaminated with pendimethalin.
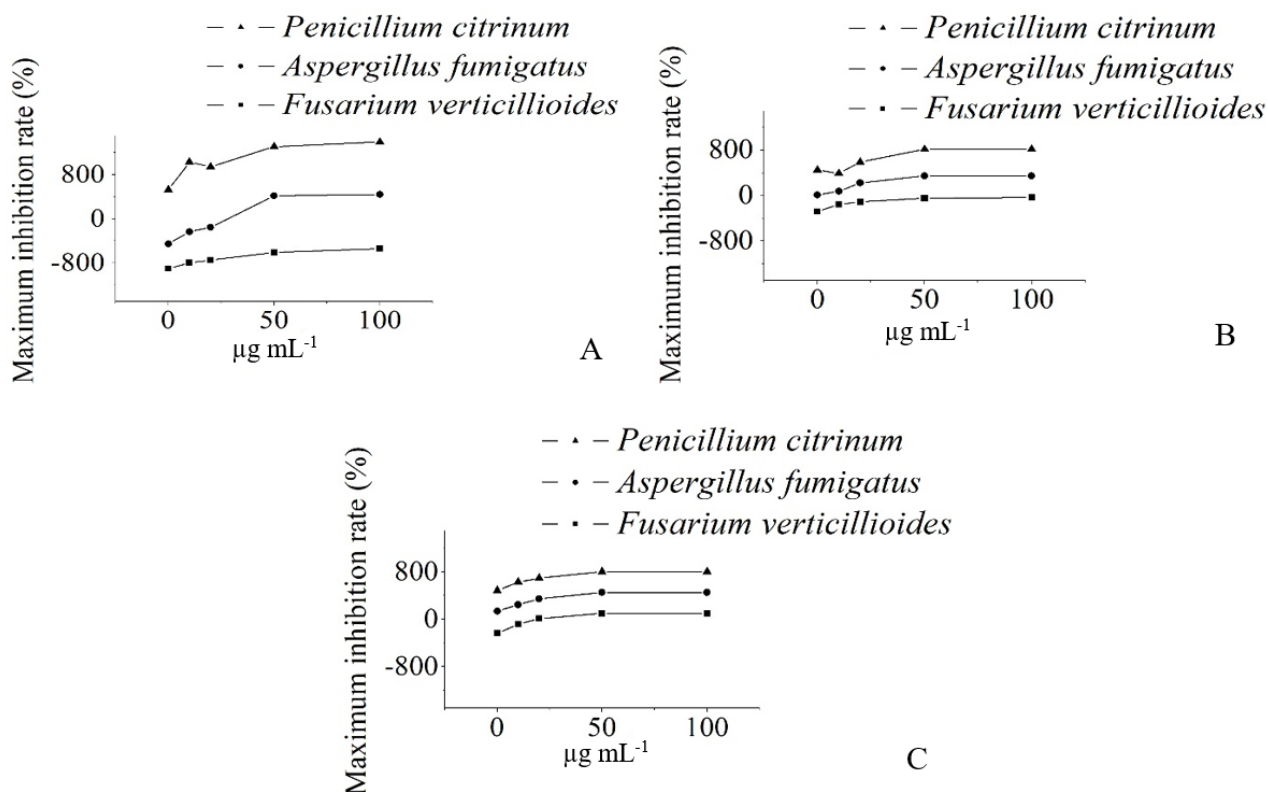
Figure 8. TIM (%) of fungi in culture media contaminated with atrazine, glyphosate or pendimethalin at concentrations of 0, 10, 20, 50 and 100 μg mL^{-1} after 1 day of incubation. (A) TIM (%) of fungi in culture medium contaminated with atrazine. (B) TIM (%) of fungi in culture medium contaminated with glyphosate. (C) TIM (%) of fungi in culture medium contaminated with pendimethalin.

Chan-Cupul et al. (2016) concluded that the genera *Aspergillus* and *Penicillium* are capable of degrading atrazine, indicating that these fungi can be used in bioremediation studies of soils contaminated by this

herbicide. Barberis, Carranza, Magnoli, Benito, and Magnoli (2018) indicated that *Aspergillus oryzae* was tolerant to high atrazine levels and may be considered a potential bioremediation agent. The fungus *A. fumigatus* was tolerant to all changes that occurred in this study.

Under natural conditions, the degradation of glyphosate in soil depends on microbial degradation. Therefore, it is necessary to identify degrading microorganisms and confirm their potential for the bioremediation of glyphosate-contaminated environments (Yu et al., 2015).

Fu et al. (2017) concluded that the fungus *Aspergillus oryzae* degrades glyphosate through the AMPA and methylamine pathways. Carranza, Barberis, Chiacchiera, and Magnoli (2016) established in their studies that *Aspergillus flavi* and *Aspergillus niger* may develop in vitro in the presence of glyphosate, especially when it is used as the sole source of phosphorus or nitrogen.

The concentrations of 50 and 100 μg mL^{-1} glyphosate limited the mycelial growth of *A. fumigatus*; however, the fungus *F. verticillioides* showed excellent mycelial growth in the presence of glyphosate, using the herbicide as a carbon source.

A study by Rodríguez-Liébana, ElGouzi, and Peña (2017) stated that the dissipation of pendimethalin is related to the endogenous biota of the soil. Microbial metabolism is the most important factor in the degradation of pendimethalin in soil (Ni, Li, Qiu, Chen, & He, 2018). The fungi *A. fumigatus* and *F. verticillioides* presented satisfactory results in this study; however, the growth of these microorganisms was limited at concentrations of 50 and 100 μg mL^{-1}.

Conclusion

The isolated fungi *A. fumigatus*, *F. verticillioides* and *P. citrinum* were resistant to the herbicides atrazine, glyphosate and pendimethalin.

The fungus *F. verticillioides* showed a higher mean mycelial growth in culture medium contaminated with atrazine at 10 and 20 μg mL^{-1} and that contaminated with glyphosate at 10 μg mL^{-1}. In the culture medium contaminated with pendimethalin, the fungi *F. verticillioides* and *A. fumigatus* presented the highest mean mycelial growth values, and the growth was highest at 10 μg mL^{-1}.

Acknowledgements

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