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Original Article

Amazonian soil fungi are efficient degraders of glyphosate herbicide; novel isolates of *Penicillium*, *Aspergillus*, and *Trichoderma*

Os fungos do solo amazônico são degradadores eficientes do herbicida glifosato; novos isolados de Penicillium, Aspergillus e Trichoderma

L. O. Correa[a,b], A. F. M. Bezerra[a], L. R. S. Honorato[a,b], A. C. A. Cortez[b], J. V. B. Souza[b*] and E. S. Souza[a,c]

[a]Universidade do Estado do Amazonas – UEA, Escola Superior de Ciências da Saúde, Manaus, AM, Brasil
[b]Instituto Nacional de Pesquisas da Amazônia – INPA, Laboratório de Micologia, Manaus, AM, Brasil
[c]Universidade do Estado do Amazonas – UEA, Escola Superior de Tecnologia, Manaus, AM, Brasil

Abstract

Pesticide residues that contaminate the environment circulate within the hydrological cycle can accumulate within the food chain and cause problems to both environmental and human health. Microbes, however, are well known for their metabolic versatility and the ability to degrade chemically stable substances, including recalcitrant xenobiotics. The current study focused on bio-prospecting within Amazonian rainforest soils to find novel strains fungi capable of efficiently degrading the agriculturally and environmentally ubiquitous herbicide, glyphosate. Of 50 fungal strains isolated (using culture media supplemented with glyphosate as the sole carbon-substrate), the majority were *Penicillium* strains (60%) and the others were *Aspergillus* and *Trichoderma* strains (26 and 8%, respectively). All 50 fungal isolates could use glyphosate as a phosphorous source. Eight of these isolates grew better on glyphosate-supplemented media than on regular Czapek Dox medium. LC-MS revealed that glyphosate degradation by *Penicillium* 4A21 resulted in sarcosine and aminomethylphosphonic acid.

Keywords: amazonian soil fungi, *Aspergillus*, bioremediation, degradation of xenobiotics, glyphosate, *Penicillium*, recalcitrant compounds, *Trichoderma*.

Resumo

Resíduos de agrotóxicos que contaminam o meio ambiente circulam no ciclo hidrológico, podendo se acumular na cadeia alimentar e causar problemas tanto à saúde ambiental quanto humana. Por sua vez, microrganismos são bem conhecidos por sua versatilidade metabólica e capacidade de degradar substâncias quimicamente estáveis, incluindo xenobióticos recalcitrantes. O estudo atual se concentrou na bioprospecção nos solos da floresta amazônica para encontrar novas linhagens de fungos capazes de degradar com eficiência o herbicida onipresente na agricultura e no meio ambiente, o glifosato. Entre os 50 fungos isolados (usando meio de cultura suplementado com glifosato como única fonte de carbono), a maioria eram isolados do gênero *Penicillium* (60%) e os outros eram isolados de *Aspergillus* e *Trichoderma* (26 e 8%, respectivamente). Todos os 50 isolados de fungos foram capazes de usar glifosato como fonte de fósforo. Oito desses isolados cresceram melhor em meio suplementado com glifosato do que em meio Czapek Dox regular. LC-MS revelou que a degradação do glifosato por *Penicillium* 4A21 resultou nos metabólitos sarcosina e ácido aminometilfosfônico.

Keywords: fungos do solo amazônico, *Aspergillus*, biorremediação, degradação de xenobióticos, glifosato, *Penicillium*, compostos recalcitrantes, *Trichoderma*.

1. Introduction

The need for global food security is now more urgent than at any time in human history, and yet the need to protect environmental and human health are both necessary and challenging (Sharma et al., 2019). These, sometimes opposing, forces underlie the conflict between the need to intensify agriculture while avoiding poisoning the Earth's ecosystems, including the food supply chain with pesticides and other agricultural chemicals (Sharma et al., 2019). There are over 14.1 million new cancer cases each year worldwide, many of these because of exposure to xenobiotics in the environment and food chain (Torre et al., 2015). For some decades, we have known that these xenobiotics circulate within the hydrological cycle and so contaminate diverse geographical regions indiscriminately, including otherwise

*e-mail: joao.souza@inpa.gov.br
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pristine environments (Gasnier et al., 2009; Giesy et al., 2000; Monsanto, 2005; Van Bruggen et al., 2018). In some cases, it can influence the loss of biodiversity in different biomes, affecting the physiological performance of native plants not resistant to herbicides (Batista et al., 2018).

The current study was carried out towards easing this pollution load via an approached based bio-prospecting for novel microbial strains capable of effective and efficient degradation of xenobiotics as demonstrated in previous studies. We focused on glyphosate, a recalcitrant substance that has been applied as an herbicide that targets broad-leaved plants and grasses since 1974, throughout both temperate- and tropical regions (and is marketed under the name Roundup®). Glyphosate is the agrochemical with the biggest sale in Brazil, totaling 217 metric tons per year (Krzyśko-Lupicka et al., 1997; Sviridov et al., 2015; Zhan et al., 2018). In 2015, analyses by the World Health Organization's International Agency for Research on Cancer and the European Food Safety Authority resulted in glyphosate being classed as genotoxic and cancer risk (Torre et al., 2015). However, the exact impacts on human health are subject to ongoing review and further research.

Microbes identified as glyphosate degraders are commonly isolated from contaminated soils; these include some bacteria but are mostly fungi species (Singh and Singh, 2016; Zhan et al., 2018). However, the phylogenetic range of strains/species known to degrade this herbicide and its toxic breakdown product, aminomethylphosphonic acid (AMPA), is limited, and the degradation is slow (Fu et al., 2017; Kaczyński and Łozowicka, 2015; Van Bruggen et al., 2018). The majority of the glyphosate-degrading fungi have been isolated from microbial habitats that are impoverished in terms of microbial diversity, such as cultivated agricultural soils (Arfarita et al., 2016; Klimek et al., 2001; Krzyśko-Lupicka et al., 1997). Therefore, we focused our bio-prospecting activities on pristine, high organic-matter soils of the Amazon rainforest, likely the most phylogenetically rich source of soil-based fungal saprobes in Earth's biosphere. We cast the net wide using a culture medium, supplemented with glyphosate to isolate any strain that can degrade this xenobiotic. The specific aims were to find fungi able to tolerate and degrade glyphosate. We discussed implications for glyphosate bioremediation and the potential for bio-prospecting for microbes capable of degrading other recalcitrant pollutants.

2. Material and Methods

2.1. Glyphosate and analytical reagents

Roundup® WG-Glyphosate (containing 0.720 kg glyphosate/kg) was obtained from Monsanto Agricultural Products Inc. (Creve Coeur, Missouri, U.S). AMPA and all other chemicals were obtained of the highest purity of analytical reagent grade commercially available (Sigma-Aldrich - St. Louis, Missouri, United States).

2.2. Sampling soils and isolation of fungi

Four soil samples were collected from the surface (1-10 cm depth) of the forest located at the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil (Latitude-South 3° 5' 36" and Longitude West 59° 59' 16").

Each soil sample (500 g) was homogenized with glyphosate (0.1 g) and incubated in a plastic box (30×40×30 cm) for 20 days, 25 °C. After this period, the isolation of glyphosate-tolerant fungal strains was carried by Successive Serial Dilutions of soil in distillated water. Dilutions (100 µL per dilution) were plated on the surface of Potato Dextrose Agar supplemented with glyphosate (2.35 g/L) and the plates were incubated for 7-10 days at 25 °C. Fungal colonies were transferred and cultivated on Czapek Dox Agar. Micromorphological identifications were conducted according to taxonomy keys and literature (Fassatiowa, 1983; Pitt, 1979; Raper and Fennel, 1965).

2.3. Evaluation of glyphosate assimilation as a phosphorus substrate source

The evaluation of glyphosate assimilation as a phosphorus substrate source was based on the method previously described (Krzyśko-Lupicka et al., 1997; Arfarita et al., 2011) with some modifications. All the growth studies were carried out on Czapek Dox medium, which consisted of saccharose (30 g/L), $MgSO_4.7H_2O$ (0.5 g/L), KCl (0.5 g/L), $NaNO_3$ (3 g/L), $FeSO_4$ (0.01 g/L) and KH_2PO_4 (1 g/L). The same medium, deficient in KH_2PO_4, was used as a control. When the glyphosate was used as the sole source of phosphorus, KH_2PO_4 in Czapek medium was replaced by glyphosate at a final concentration of 7.4 mM. If the starting pH of the medium was 6.0 or higher, it was not adjusted; when it was lower than 6.0 it was adjusted to 6.0 with 1 M sodium hydroxide solution.

Bioprocess was carried out in 125 mL conical flasks containing 50 ml standard or modified Czapek liquid medium. The cultures were inoculated with a spore suspension (2×10^7 spores/mL) and incubated at 25 °C on a shaking platform at 100 rev./min. After 14 days of incubation, the cultures were filtered and dry mass of the mycelium and pH of the medium was determined (g/L). The average values were obtained from three repetitions.

2.4. Evaluation of glyphosate degradation

2.4.1. Ninhydrin reaction for glyphosate quantification

The method used was proposed by Nagaraja and Bhaskara (2006), with some modifications. A calibration curve was prepared from a stock solution of 500 mg/L glyphosate. Aliquots ranging from 40 to 140 µL were transferred to test tubes, and 500 µL of 5% ninhydrin and 500 µL of 5% sodium molybdate were added to each of the tubes. The tubes were sealed and kept in a water bath at 85-95 °C for 12 minutes. Then the samples were cooled to room temperature and were quantitatively transferred to 5 mL volumetric flasks. The volume was completed with distilled and deionized water. Then, the reading was performed by MultiSpec-1501, SHIMADZU spectrophotometer, at 570 nm. We obtained a calibration

curve with the absorbance as a function of glyphosate concentration ranging from 4 to 14 mg/L. For the instrument's baseline, 500 µL of ninhydrin and sodium molybdate solution was used, to a total volume of 5 mL.

2.5. LC-MS/MS analysis

Shield RP C18 column (3.5-µm particle size, 150 mm in length, 2.1-mm i.d.). The mobile phases consisted of Methanol: H_2O (7:3). Initial conditions were re-established in 1 min for a total run time of 15 min. The column temperature was 35.0 °C, and the flow rate was 0.4 mL/min. We obtained the optimization of the ionization and fragmentation conditions for the analytes by the infusion of the individual analytes' solutions. Optimum responses were obtained by electrospray ionization in negative ion mode using the following source parameters: capillary voltage of 3.5 kV, cone voltage of 20 V, source temperature of 120 °C, desolvation gas temperature of 350 °C and gas flow of 160-200 L/h (cone) and 580-600 L/h (desolvation).

2.6. Statistical analysis

All data were expressed as mean±standard error means (SEM). Statistical variance analyses were performed by Student's t-test using SPSS 20.0 for Windows (SPSS Inc., Chicago, IL). Differences at $P = 0.05$ were considered statistically significant.

3. Results

Soil samples contaminated with glyphosate were diluted and transferred to culture media in order to isolate glyphosate tolerant fungi. The isolated microorganisms belonged to the Phylum Ascomycota, specifically the genus *Penicillium* (60.0%), *Aspergillus* (26%) and *Trichoderma* (8%). It was not possible to identify 6% of the isolates (Table 1).

In order to investigate the ability of these isolates in using glyphosate as nutrient source, the isolates were grown in three different culture media (conventional Czapek medium, Czapek without KH_2PO_4, and Czapek without KH_2PO_4 but supplemented with glyphosate (2.35 g/L) (Table 1).

For this test interpretation, the fungi that produced more biomass in Czapek supplemented with glyphosate than in Czapek without KH_2PO_4 (control) are considered capable of using the herbicide formulation as a source of phosphorus. All isolates demonstrated the ability to use the herbicide formulation as a phosphorus source in the present work. Of these, eight isolates produced more biomass in Czapek supplemented with glyphosate than in conventional Czapek.

The glyphosate degradation (culture medium without KH_2PO_4 but supplemented with glyphosate) was investigated using ninhydrin reaction to quantify the glyphosate. *Penicillium* 4A21, *Aspergillus* 2B112 and *Penicillium* 2A31 promoted glyphosate degradations of 42.72±0.02 (%), 36.48±0.01 and, 34.91±0.02 in 14 days, respectively. LC-MS/MS demonstrated that glyphosate, AMPA and sarcosine could be found in culture filtrate of *Penicillium* 4A21 after 14 days of exposure to glyphosate (Figure 1).

4. Discussion

Our results demonstrated that most isolates from genera *Penicillium*, *Aspergillus* and *Trichoderma* probably used the herbicide formulation as a nutrient source. About 8% of the isolates grew better in the medium supplemented with the herbicide than in the conventional culture medium (Czapek). In addition, it was showed that the isolate *Penicillium* 4A21 produced AMPA and Sarcosine as metabolites from glyphosate degradation. This is the first study of screening for glyphosate degradation carried out with fungi isolated from the Amazon Forest soil.

It must be pointed out that extensive use of GP resulted in its accumulation in soil and water environments (Giesy et al., 2000; Monsanto, 2005; Van Bruggen et al., 2018). Moreover, negative effects of GP on animal metabolism have been reported, in particular, with respect to chronic and remote effects (Van Bruggen et al., 2018). In the present work, all the 50 isolates were able to use glyphosate as phosphorus source. The biomass assay described by Arfarita et al. (Arfarita et al., 2011) have previously been used as a robust methodology for the screening of microorganisms able to use glyphosate as nutrient source. A similar conclusion about this methodology was reached out by other workers with similar design (Arfarita et al., 2016; Carranza et al., 2016, 2017).

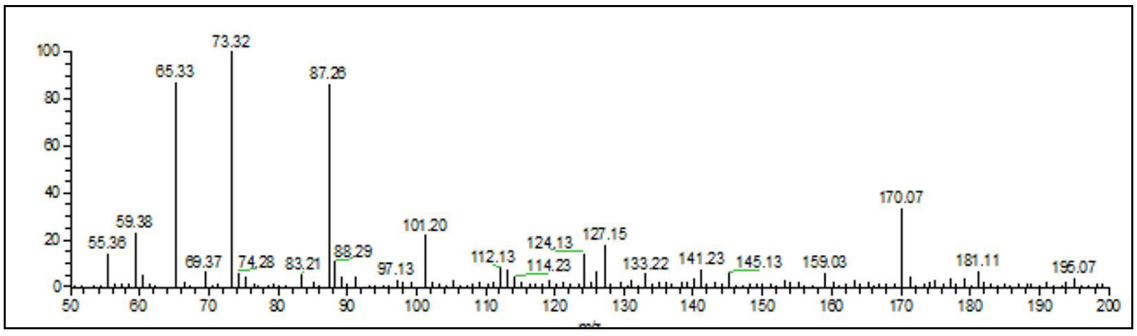

Figure 1. Mass spectrum resulting from the HPLC-MS of the isolated *Penicillium* 4A21 filtered. The filtrate presents possible peaks of glyphosate (170.07), AMPA (112.13) and sarcosine (89).

Table 1. Biomass production after a 14-day incubation (30 °C) by the fungal isolates in three different culture media: a) conventional Czapek medium, b) Czapek without KH_2PO_4 and c) Czapek without KH_2PO_4 but supplemented with glyphosate (2.35 g/L). The fungal strains were isolated from soil collected from Amazon forest.

Isolate	Biomass (g/L)			
	CZ Medium	CZ Medium without KH_2PO_4	CZ Medium without KH_2PO_4 supplemented with glyphosate	Ratio (CZ Medium without KH_2PO_4 supplemented with glyphosate) / CZ Medium)
Penicillium 4A21	5.7±0.1	4.4±0.4	7.9±0.6	1.32
Aspergillus 2B112	5.7±0.3	4.1±0.5	7.1±0.1	1.24
Penicillium 2A31	5.2±1.2	4.4±0.4	6.2±0.1	1.19
Penicillium 6B221	6.6±1.4	4.6±0.6	7.6±0.8	1.15
Penicillium 6B112	4.7±0.6	4.2±0.3	5.2±0.4	1.10
Penicillium 4A31	5.8±0.3	2.8±0.3	6.3±0.4	1.08
Penicillium 4A211	6.91±0.02	3.6±0.4	7.1±0.4	1.02
Penicillium 2A3	6.4±0.9	5.2±0.8	6.4±0.4	1.00
Penicillium 2B21	5.61±0.05	4.1±0.2	5.5±0.8	0.98
Penicillium 6B21	4.9±0.3	3.9±0.4	4.8±0.4	0.97
Penicillium 4A212	6.7±0.1	4.31±0.07	6.5±0.1	0.97
Aspergillus 3B32	6.6±0.3	6.3±1.4	6.4±1.4	0.96
Aspergillus 5B21	6.4±0.4	4.2±0.3	6.2±0.1	0.96
Aspergillus 3B411	6.3±0.8	4.3±0.3	6.1±0.2	0.96
Penicillium 3B21	5.6±0.4	4.3±0.5	5.4±0.2	0.96
Penicillium 5B114	6.6±0.1	2.9±0.5	6.3±0.3	0.95
Trichoderma 2B11	6.6±0.1	4.0±1.0	6.3±0.4	0.95
Penicillium 5B221	6.5±0.2	4.2±0.2	6.2±0.2	0.95
Mycelium steridium 6B11	6.6±0.1	4.5±0.2	6.2±0.3	0.93
Penicillium 5A11	6.5±0.2	4.2±1.2	6.1±1.5	0.93
Aspergillus 5B123	6.3±0.3	4.2±1.4	5.9±0.5	0.93
Penicillium 5B112	6.7±0.1	4.4±0.2	6.2±1.1	0.92
Aspergillus 5B12	6.6±1.1	4.8±1.1	6.1±0.8	0.92
Penicillium 2B41	6.4±0.4	3.0±1.0	5.9±0.7	0.92
Aspergillus 2B51	6.9±0.1	3.9±0.6	6.3±0.8	0.91
Aspergillus 3A53	6.6±1.1	4.0±1.0	6.0±1.0	0.90
Aspergillus 3B13	8.3±2.3	5.3±0.2	7.5±0.3	0.90
Aspergillus 3B42	6.2±0.2	4.8±0.1	5.6±0.1	0.90
Penicillium 2B311	6.8±0.3	3.6±0.3	6.1±0.6	0.89
Penicillium 6B1	4.8±0.7	2.8±0.4	4.3±0.2	0.89
Penicillium 4C12	6.9±0.6	3.6±1.1	6.1±0.3	0.88
Aspergillus 3B23	4.61±0.02	3.4±0.3	4.0±1.0	0.86
Penicillium 4A213	6.5±0.1	4.2±0.1	5.6±0.1	0.86
Penicillium 5B23	6.1±0.4	4.2±0.2	5.2±0.2	0.85
Trichoderma 3C12	6.6±0.6	4.1±0.4	5.6±0.2	0.84
Penicillium 3B41	5.8±0.3	4.6±0.6	4.9±0.4	0.84
Aspergillus 3B231	6.4±1.5	2.7±0.6	5.3±0.6	0.82
Penicillium 3B22	7.4±0.5	4.2±0.2	6.1±0.1	0.82

Table 1. Continued...

Isolate	Biomass (g/L)			
	CZ Medium	CZ Medium without KH_2PO_4	CZ Medium without KH_2PO_4 supplemented with glyphosate	Ratio (CZ Medium without KH_2PO_4 supplemented with glyphosate) / CZ Medium)
Mycelium steridium 4B12	7.6±0.4	4.5±0.4	6.2±0.1	0.81
Penicillium 5B2	6.9±0.4	3.3±0.6	5.55±0.05	0.79
Trichoderma 3C2	7.7±0.7	5.9±0.9	6.1±1.4	0.79
Penicillium 3C11	6.1±0.3	4.5±0.9	4.8±0.3	0.78
Trichoderma 3C21	7.3±0.8	3.3±0.7	5.7±0.5	0.78
Penicillium 4B23	8.2±0.4	4.3±0.1	6.3±0.2	0.76
Penicillium 5C21	6.5±0.3	4.4±0.1	4.9±0.3	0.75
Penicillium 6C221	8.1±0.5	3.9±0.5	6.1±0.4	0.75
Penicillium 5C211	8.7±2.8	6.4±1.8	6.5±0.8	0.74
Penicillium 3C113	6.5±0.2	3.8±0.4	4.8±1.3	0.73
Aspergillus 5B31	6.5±0.2	4.3±0.3	4.6±0.1	0.70
Mycelium steridium3C112	6.3±0.5	3.1±0.4	3.9±0.6	0.61

The selected isolate *Penicillium* 4A21 degraded the herbicide glyphosate by over 42% in just 14 days in submerged fermentation. In soil, glyphosate degradation has different degradation rates. Wauchope et al. (1992) found that the half-life of glyphosate in soil can go up to 174 days. Carranza et al. (2019) reported that 50% of glyphosate was degraded by *Aspergillus oryzae* in soil microcosms in 28 days and 90% within 90 days. Monsanto (2005) conducted a study with agricultural and forest soils from five countries, and reported that the glyphosate half-life in soil ranges from two to 197 days, and AMPA 76 to 240 days. This suggests that the rate of the degradation of the glyphosate by filamentous fungi depends on culture media and bioprocess conditions.

At this stage of understanding, we believe *Penicillium* 4A21 is a potential strain for glyphosate biodegradation, but other characteristics are important for a bioremediation microrganism, according to Maqbool et al. (2016) and Boopathy (2000). The microrganism used in bioremediation assays must have: (1) low toxicity and lack of pathogenicity, (2) high viability immediately after the introduction into environment in combination with low long-term survival rates, (3) high efficiency of GP degradation independent of external conditions, and (4) ability to GP mineralization not associated with AMPA accumulation in the environment.

The analytical methodologies allowed us to detect sarcosine and AMPA as metabolites from glyphosate degradation by *Penicillium* 4A21. Nowadays, studies have been suggesting the existence of C–P lyases that cleaves only the C–P bond of GP to produce sarcosine and Pi; the sarcosine pathway serves to utilize GP as a source of Pi (Selvapandiyan and Bhatnagar, 1994). However, the cleavage of the C–P bond in the GP molecule strongly depends on the concentrations of exogenous and endogenous Pi, and is therefore usually induced under conditions of a phosphorus deficiency, which rarely occurs in natural environments (Bazot and Lebeau, 2008). In other well-known GP degradation pathway, the herbicide molecule is first attacked by the enzyme known as glyphosate oxidoreductase (GOR), which cleaves the C–N bond in GP molecules, yielding stoichiometric quantities of AMPA and glyoxylate (Klimek-Ochab et al., 2007).

AMPA resulting from glyphosate degradation can be a problem since AMPA can be considered toxic (Sviridov et al., 2012). However, previous work demonstrated that AMPA is an intermediary step in the degradation of glyphosate by fungi. *Aspergillus oryzae* A-F02 metabolizes AMPA into methylamine and then into other simple and less toxic products (Fu et al., 2017). AMPA can also be metabolized through phosphoformaldehyde transamination, mediated by aminotransferase enzymes. Then, phosphoformaldehyde is metabolized to formaldehyde for later metabolism (Sviridov et al., 2012; Kertesz et al., 1994). Thus, the complete study of the metabolic pathways of glyphosate degradation by the isolate *Penicillium* 4A21 should be deeply investigated.

The study has great biotechnological importance by presenting isolated and identified fungi from soil of the Amazon rainforest, with great potential for glyphosate herbicide degradation. The limitations include: a) necessity of taxonomy studies of *Penicillium* 4A21 isolate; b) study the ability of *Penicillium* 4A21 to degrade glyphosate "*in situ*"; c) detection and toxicity of biodegradation metabolites, and d) studies optimizing bioremediation process.

5. Conclusion

In the present work, we found strains of fungi capable of using herbicide formulation as a source of phosphorus and others capable of growing more in the medium containing glyphosate as a phosphorous source than in the conventional medium. Some isolates highlighted in the present study have potential as glyphosate bioremediation agents.

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