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ABSTRACT

Several studies, have reported that glyphosate-based herbicides persist in the soil and are transported into other environmental matrices. This study evaluated the ability of *Aspergillus oryzae* AM2 and *Mucor circinelloides* 166 to remove glyphosate and aminomethylphosphonic acid from agricultural soil under field conditions. The strains are native to Argentinean agricultural soils, and they were assessed separately and in combination in 2 m x 1 m subplots. A completely randomized block design was used (5 treatments with 6 replicates each). The soil was

sprayed with a commercial glyphosate-based herbicide formulation (3 kg ha-1) and inoculated with spores and/or conidial suspensions. Glyphosate and aminomethylphosphonic acid were measured at the beginning of the assay and at the end (150 days) by ultra-high performance liquid chromatography. In all the treatments, residual glyphosate levels were significantly lower at the end than at the start. The most significant removal percentages ($p<0.001$) were 97%, obtained with *A. oryzae* AM2 (106 conidia/mL), and 93%, obtained with the combination of *M. circinelloides* 166 (106 spores mL-1) and *A. oryzae* AM2 (103 conidia mL-1). Aminomethylphosphonic acid decreased significantly (by 32%) in the uninoculated control. The same two treatments that were the most effective at removing glyphosate were the only ones in which the decrease in aminomethylphosphonic acid was higher than in the control (over 70%). This is the first study to demonstrate that these fungal strains can remove glyphosate and aminomethylphosphonic acid under field conditions. Thus, they could be good candidates for the remediation of herbicide-polluted sites.

Keywords: *Aspergillus oryzae*; *Mucor circinelloides*; organophosphate herbicides; tolerant strains.

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Statements and Declaration

Authors contribution

Cecilia Carranza and Melisa Aluffi designed and conducted the experiments and analyzed data with the assistance of Karen Magnoli and Nicolas Benito. Eduardo De Gerónimo and Virginia Aparicio collaborated with the detection and quantification of GP and

AMPA. Carina Magnoli wrote the manuscript with additional support of Carla Barberis and Melisa Aluffi.

Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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obtained with *A. oryzae* AM2 (10^6 conidia mL^{-1}), and 93%, obtained with the combination of *M. circinelloides* 166 (10^6 spores mL^{-1}) and *A. oryzae* AM2 (10^3 conidia mL^{-1}). Aminomethylphosphonic acid decreased significantly (by 32%) in the uninoculated control. The same two treatments that were the most effective at removing glyphosate were the only ones in which the decrease in aminomethylphosphonic acid was higher than in the control (over 70%). This is the first study to demonstrate that these fungal strains can remove glyphosate and aminomethylphosphonic acid under field conditions. Thus, they could be good candidates for the remediation of herbicide-polluted sites.

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1- INTRODUCTION

Glyphosate (N-[phosphonomethyl] glycine) (GP) is the active ingredient in many commercial herbicides that are collectively known as glyphosate-based herbicides (GBHs). Although other herbicides have been developed, GP continues to be widely used as a broad-spectrum weed controller during the direct seeding of transgenic crops such as soybean, maize, and cotton (Muñoz et al. 2023). Contamination with GP, therefore, is attributable mostly to agricultural activity. Additional sources of GP pollution include the filling and cleaning of spraying equipment, improper waste disposal, and accidental spills (Aluffi et al. 2022).

The persistence of GP in the soil, as well as that of its most relevant degradation product (aminomethylphosphonic acid or AMPA), depends mainly on abiotic and biotic factors, e.g., soil composition and environmental conditions (Wimmer et al. 2023). The half-life of AMPA has been reported to last between 20 and more than 800 days. For GP, it is

between 1 and 200 days. The herbicide is strongly adsorbed onto soil colloids through a reversible process that regulates its half-life and mobility. The soil-bound molecules are then transported to other environmental compartments as a result of runoff or leaching. These compartments become important GP reservoirs, with negative consequences for human health and the environment (Meftaul et al. 2020).

However, the toxicological effects of GP are the subject of some controversy. In 2015, the International Agency for Research on Cancer (IARC) classified the herbicide and its formulated products within Group 2A, i.e., as probably carcinogenic in humans (IARC 2015). In contrast, the United States Environmental Protection Agency (US EPA) considers there are no risks to public health when GP is used in accordance with its current label (US EPA 2020). Nevertheless, several studies associate human exposure to GBHs with a higher risk of developing diseases such as non-Hodgkin lymphoma, multiple myeloma, and other cancers (Zhang et al. 2019; Portier 2020). Adverse effects on the nervous system of mammals, including humans, have also been described (Madani and Carpenter 2022). Other reports indicate that AMPA might be more toxic than GP due its longer persistence in the environment (Meftaul et al. 2020). Concerns about the possible environmental impact of GP have increased in recent years, due to its widespread use and the large amounts applied annually. The literature contains varying values for GP and AMPA levels in the soil and water, depending on location. Very low GP concentrations (up to 2.5 µg L^{-1}) were observed in surface waters in some European countries, while higher concentrations (up to 200 µg L^{-1}) were measured in the US, Denmark, and France (Meftaul et al. 2020). A recent study by Ayoola et al. (2023) found that GP concentrations in water and soil samples from Nigerian farms were higher during the wet season than during the dry season, with the highest concentrations being around 25 mg Kg^{-1} in top soil and 2.5 mg L^{-1} in groundwater. On the other hand, concentrations between 35 and

1,502 mg kg^{-1} for GP and between 299 and 2,256 mg kg^{-1} for AMPA were detected in Argentinean soils (Aparicio et al. 2013). A study that focused on the Mesopotamian Pampas agroecosystem found 8,105 mg kg^{-1} of GP and 38,939 mg kg^{-1} of AMPA in the soil (Primost et al. 2017). Bento et al. (2019) observed that GP and AMPA levels were between 1.1 and 17.5 times higher in water-eroded sediments than in soil, and that whereas AMPA can persist and accumulate in the soil, both GP and AMPA are prone to off-site transport into adjacent fields and surface water through water erosion. Similarly, groundwater has been described to have higher GP and AMPA levels than surface water (Lutri et al. 2020). This is likely because the long-term application of the herbicide within current agricultural schemes far exceeds the potential for its degradation in the soil and the unsaturated zone.

While photodegradation and chemical degradation play a minor part in GP's fate and behavior in the soil, the native microbiota is a key player (Sviridov et al. 2015). Bacterial and fungal strains that can degrade the herbicide have been isolated from soils exposed to pesticides (Singh and Singh 2016; Mohy-Ud-Din et al. 2023), as well as from pristine soils rich in organic matter and fungal saprobes (Correa et al. 2021). The most frequently isolated fungi with these characteristics belong to the genera *Penicillium*, *Aspergillus*, and *Trichoderma*. Given that filamentous fungi can colonize and oxidize various organic substrates, even under adverse environmental conditions, they are powerful biotechnological tools for bioremediation (Vaksmaa et al. 2023).

Earlier, *Aspergillus oryzae* AM2 and *Mucor circinelloides* 166 were isolated from agricultural soil in the south of the province of Córdoba (Argentina). When studied in GP-supplemented media, their growth parameters (lag phase and growth rate) showed that both were able to grow in the presence of GBHs as a source of phosphorus or nitrogen (Carranza et al. 2016; Aluffi et al. 2020). The two strains were then tested separately and

in mixed cultures in microcosm assays (60 days). They survived in the presence of native microbiota and removed GP under nonoptimal humidity conditions (Carranza el al. 2019; Aluffi et al. 2023, personal communication). However, their effectiveness in removing GP had not been investigated *in situ* until now. The present study evaluated different inocula prepared with *A. oryzae* AM2 and/or *M. circinelloides* 166 in terms of their ability to dissipate GP in agricultural soil exposed to GBHs under field conditions. The experiment was carried out in Córdoba, Argentina, from December 2019 to May 2020. The levels of AMPA, the main metabolite of GP degradation, were also quantified.

2- MATERIALS AND METHODS

2.1. Fungal strains and inocula

The two strains used in this study were *Aspergillus oryzae* AM2 and *Mucor circinelloides* 166. In previous studies, they had been isolated from agricultural soil and they proved capable of removing GP from culture media and soil microcosms (Carranza et al. 2019; Aluffi et al. 2020; 2022). The nucleotide sequences for the calmodulin and ß-tubulin genes of the first strain were deposited in GenBank under accession numbers KX298158 and KX306817, respectively. The nucleotide sequences for the ITS gene of the second strain were deposited in GenBank under accession number ON645978. Both strains have been classified as GRAS species (Generally Recognized as Safe) (Han et al. 2004; He et al. 2019).

To prepare the inocula, the strains were grown on malt extract agar (MEA) for 7 days at 25°C. Spore suspensions of *M. circinelloides* 166 and conidial suspensions of *A. oryzae* AM2 were obtained by harvesting the spores and conidia from the surface of the MEA plates, and placing them inside tubes containing distilled water and 0.1% Tween 80 (dispersing agent). Then, they were filtered through sterile glass wool to remove hyphal

fragments and conidia clumps (i.e., to make the suspensions homogeneous). The spores and conidia were counted in a hemocytometer chamber (Boeco, Germany), and the water volume was adjusted to reach concentrations of 10^6 or 10^3 spores/conidia mL^{-1}, depending on the strain. The viability of the suspensions was confirmed by the standard plate count method on MEA (Pitt and Hocking 2009).

2.2. Experimental design

The experiment was performed in an arable field that belongs to the National University of Río Cuarto (Córdoba, Argentina) (33°06'05''S 64°17'16''W). The soil has a fine sandy loam texture (Gorgas 2006) and has been physicochemically characterized (Walkley and Black 1965; Sparks 1996). At the beginning of the assay, it contained 242.4 kg ha^{-1} of extractable phosphorus, 95.4 kg/ha of nitrate nitrogen, 11.13 kg ha^{-1} of sulfur sulfate, 60.48 kg ha^{-1} of organic matter, and 3.36 kg ha^{-1} of nitrogen. All these nutrients were likely to become mineralized during the summer. The climate in the area is temperate and semiarid. For the duration of the assay, there was little rainfall and the mean air temperature was between 18 and 28°C (BCCBA 2019; 2020).

For the assay, a 10 m x 2 m plot was selected and divided into 2 m x 1 m subplots, demarcated with wooden stakes. The sector where this plot is located has a long history of exposure to pesticides. In the past, corn was grown there through conventional tillage, with the pre-emergence application of atrazine and metolachlor and the post-emergence application of GP. For this reason, GP and AMPA residues were measured in samples taken before the start of the experiment (Aparicio et al. 2013; De Gerónimo et al. 2028). Their values were respectively 0.5 mg kg^{-1} and 2.0 mg kg^{-1}. Moreover, at the time of the experiment corn was being cultivated in proximity to the experimental plot. The crop was at the V4 stage.

Using a manual sprayer (Crossmaster, Buenos Aires, Argentina), all the subplots were sprayed at a distance of 35 cm from the soil surface with a commercial aqueous formulation of GP (N-phosphonomethyl-glycine, 72% amine salt, Monsanto Laboratories). The chosen dose (3 kg ha^{-1}) is within the standard GBH dose range recommended for the control of weeds in agricultural soils (Diaz and Prado 2018). The control subplot was sprayed with the same volume of distilled water.

The herbicide was allowed to become adsorbed onto soil particles for two days. After that, each subplot was inoculated with the conidia and/or spore suspensions (200 mL). Inoculation was implemented according to a completely randomized three-block design, which involved 5 treatments with 6 replicates each. Six replicates were also done for the control, and the entire experiment was repeated twice. The treatments were as follows: (a) 10^6 conidia mL^{-1} of *A. oryzae* AM2; (b) 10^6 spores mL^{-1} of *M. circinelloides* 166; (c) 10^6 conidia mL^{-1} of *A. oryzae* AM2 and 10^3 spores mL^{-1} of *M. circinelloides* 166; and (d) 10^6 spores mL^{-1} of *M. circinelloides* 166 and 10^3 conidia mL^{-1} of *A. oryzae* AM2. The fifth treatment (control) consisted of 200 mL of distilled water and 0.1% Tween 80.

The assay lasted 150 days. This duration was chosen considering the half-life for GP and AMPA reported in the province of Córdoba (Bento et al. 2019). Immediately after inoculation (initial time) and at the end of the assay (150 days), ten samples were randomly collected from each subplot at a 0–10 cm depth, following a diagonal direction (Buduba et al. 2004). In the laboratory, these samples were homogenized, air-dried at 25–30 °C, and sieved to separate soil from debris. After being used for each sample, the sieve was disinfected with 1% sodium hypochlorite, rinsed with sterile distilled water, and dried. This was done to prevent cross-contamination with fungi and/or pesticides.

The samples from each subplot were organized into two pools of approximately 2 kg each: one consisting of the ten samples obtained at the initial time, and another made up

of the ten samples obtained after 150 days. Given that the entire experiment was repeated twice, there were four pools for each subplot in total. Subsamples (300 g each) were taken in triplicate from each pool, sealed in a plastic bag, and stored at−20°C until the analysis was performed. Figure 1 summarizes the main steps of the methodology.

2.3. GP and AMPA determination

To measure residual GP and AMPA, 10 g were taken from each of the 300 g subsamples prepared in the previous step. Two g out of this quantity were enriched with isotopically-labeled GP (1.2-^{13}C, ^{15}N, Sigma-Aldrich, Argentina) and allowed to stabilize for 30 min. Then, 25 mL of an extraction solution (100 mM $Na_2B_4O_710H_2O$/100 mM K_3PO_4, pH=9) were added, and the mix was sonicated and centrifuged to obtain different phases. Two ml of the liquid phase were derivatized with the same volume of 9-fluorenylmethyl chloroformate (FMOC-CL) (Sigma Aldrich, Argentina), and incubated overnight in the dark. After that, 4.5 mL of dichloromethane (CH_3Cl_2) were added, followed by vigorous mixing to eliminate organic impurities and excess FMOC. The mix was centrifuged at 3,000 rpm for 10 min. The aqueous fraction was collected, filtered (0.22 µm Microclar, Buenos Aires, Argentina), and transferred into a vial for analysis. This was conducted by ultra-high performance liquid chromatography (UPLC ESI MS/MS) (Waters Inc., Milford, MA, USA) coupled to triple quadrupole mass spectrometry, using a source of electrospray ionization (ESI) with a Z-spray design, following Aparicio et al. (2013) and De Gerónimo et al. (2018).

UPLC ESI MS/MS is a widely used method to measure GP and AMPA in environmental matrices such as soil, sediments, and water (Aparicio et al. 2013; 2018; Lupi et al. 2019; Pérez et al. 2021; Gen et al. 2021). Standard curves were prepared with GP and AMPA (PESTANAL®, 99.9%, Buenos Aires, Argentina). To evaluate the analytical efficiency

of the method, each point in the curve was fortified with the same amount of isotopically-labeled GP as the samples. The limit of detection (LD), defined as the lowest concentration that the analysis can reliably differentiate from background levels, was estimated for a signal-to-noise ratio of three from the chromatograms of standards at low concentration levels (0.05–1 μg L^{-1}). The limit of quantification (LQ) was established as the lowest concentration for which the method was fully validated using spiked samples with satisfactory recovery (between 70% and 120%) and precision (RSD⩽20%). The LQ values were 0.0119 mg kg^{-1} for GP and 0.0016 mg kg^{-1} for AMPA. The LD values were 0.0036 mg kg^{-1} for GP and 0.0041 mg kg^{-1} for AMPA.

Separation was carried out with a Waters® ACQUITY® UPLC (C18 column 1.7 μm x 50, 2.1 mm, Teknokroma Analytical SA, Spain). The mobile phase consisted of Milli-Q grade water with 5 mM ammonium acetate (phase A), with a flow rate of 0.4 mL min^{-1}, and a methanol concentration gradient modified with 5 mM ammonium acetate (phase B). For MS/MS analysis, the cone gas and desolvation gas flows (both nitrogen) were optimized at 2 and 600 L h^{-1}, respectively. Gaseous argon (99.99%, PRAXAIR, Buenos Aires, Argentina) was used as collision gas at 4.04 x 10^{3} mbar in the T-Wave cell for ion fragmentation. The gas solvation temperature was set at 400°C and the source temperature at 120°C. Instrumental operation and data acquisition and analysis were performed on Masslynx NT v 4.1 (Waters, Manchester, UK).

2.4. Statistical analysis

The GP and AMPA data were subjected to analysis of variance. Means were compared with a linear mixed model and Fisher's protected least significant difference (LSD) test. Significant differences were determined between the GP and AMPA means measured for

different treatments. The data were statistically analyzed on InfoStat v 2017 (Di Rienzo et al. 2017).

3- RESULTS AND DISCUSSION

At the beginning of the assay (two days after a commercial GP formulation had been applied), the mean GP and AMPA levels in the samples from the fumigated subplots ranged from 31.65 to 67.6 mg kg^{-1} and from 970.5 to 2,755 mg kg^{-1}, respectively. At the end (150 days), GP levels were significantly lower in all the treatments. The most significant reductions ($p<0.001$) were 97%, which was obtained after inoculation with *A. oryzae* AM2 (10^6 conidia mL^{-1}), and 93%, which corresponded to the combination of *M. circinelloides* 166 (10^6 spores mL^{-1}) and *A. oryzae* AM2 (10^3 conidia mL^{-1}), i.e., treatments (a) and (d). Other treatments associated with high removal percentages were *M. circinelloides* 166 (10^6 spores mL^{-1}) and the uninoculated control, which respectively reduced GP levels by 84 and 74%. In contrast, no significant differences with respect to the uninoculated control ($p<0.001$) were recorded in the subplot treated with the combination of *A. oryzae* AM2 (10^6 conidia mL^{-1}) and *M. circinelloides* 166 (10^3 spores mL^{-1}) (Figure 2).

As for AMPA, the most remarkable decreases after 150 days were obtained with the same treatments that proved most successful in removing GP, and the removal percentages were similar. *A. oryzae* AM2 (10^6 conidia mL^{-1}) reduced AMPA levels by 79%, and *M. circinelloides* 166 (10^6 spores mL^{-1}) combined with *A. oryzae* AM2 (10^3 conidia mL^{-1}) lowered them by 73%. These values significantly exceeded ($p<0.001$) the one obtained in the uninoculated treatment, which was itself significant with respect to the control (32%) (Figure 3).

An analysis of variance was performed to find out the effect of single factors (treatment and time) and their two-way interactions on the results. It revealed that both factors on their own and all their interactions were statistically significant (p<0.001) in relation to the GP and AMPA levels detected (Tables 1 and 2).

The findings show that the inocula tested here differed in their ability to reduce GP levels under field conditions. Two treatments, *A. oryzae* AM2 (10^6 conidia mL^{-1}) alone and the combination of *M. circinelloides* 166 (10^6 spores mL^{-1}) with *A. oryzae* AM2 (10^3 conidia mL^{-1}), were the most effective in reducing GP levels and those of its degradation metabolite (AMPA) 150 days after being inoculated. Both of them outperformed natural GP attenuation by the native microbial species (73%), whose activity was assayed in the uninoculated control. Nevertheless, although the two inocula were highly efficient, the results obtained with the mix of strains did not improve upon those achieved with *A. oryzae* AM2 alone. The opposite had occurred in microcosms subjected to hydric stress (30% field capacity) and contaminated with 30 mM of GP (Aluffi et al. 2023, personal communication). In that assay, a co-culture of *M. circinelloides* 166 (10^6 spores mL^{-1}) and *A. oryzae* AM2 (10^3 conidia mL^{-1}) removed 80% of the herbicide, while *A. oryzae* AM2 (10^3 conidia mL^{-1}) by itself removed 57%. This demonstrates the importance of field studies to ascertain the real remediating ability of fungal strains.

In the last decades, numerous studies worldwide have reported potentially concerning GP and AMPA levels in soils and surface water (Primost et al. 2017; Aparicio et al. 2018; Bento el al. 2018; Lupi et al. 2019; Lutri et al. 2020; Meftaul et al. 2020; Pérez et al. 2021). One way of dealing with this issue would be to treat contaminated sites with microorganisms that can remove the herbicide. Several bacterial taxonomic groups isolated from soils have proven able to degrade GP in microcosms or soil columns assays. Fungal strains isolated from the same ecosystems and with the same ability are restricted

only to a few taxa, which for the most part use the herbicide as a phosphorus source and degrade it through the AMPA pathway (Zabaloy et al. 2022).

In situ studies have used abiotic controls to demonstrate the crucial role of native soil microbiota in GP degradation, and the influence of temperature, soil moisture and other soil properties on their metabolic activity (Bento et al. 2016; 2019; Tang et al. 2019; Sun et al. 2019; Muskus et al. 2020). However, most of what is known about the ability of inoculated microbial cultures to stimulate this natural attenuation has been discovered by using bacterial strains *in vitro* or in microcosms. Little information is available on what occurs with inoculated microorganisms under field conditions (Ermakova et al. 2010; Shushkova et al. 2010; Li et al. 2022; Nguyen et al. 2022; Zhang et al. 2022; Mohy-Ud-Din et al. 2023), although some *in situ* research has corroborated that many of the factors that affect degradation *in vitro* also do so on the field, e.g., soil type, texture, and physicochemical characteristics; climatic conditions; native biota, etc. (Guijarro et al. 2018; Mercado and Mactal 2021). In our study, as mentioned before, supplementation of the soil with *A. oryzae* AM2 (10^6 conidia mL^{-1}) on its own or with the combination of *M. circinelloides* 166 (10^6 spores mL^{-1}) and *A. oryzae* AM2 (10^3 conidia mL^{-1}) led to a noticeable improvement in the removal rate of the native microbiota under field conditions.

Another study carried out in the field (Ermakova et al. 2010) found that GP levels in the soil, which were initially tenfold higher than the recommended doses, had decreased dramatically within 14 days after the inoculation of two native bacterial strains. *Achromobacter* sp. Kg 16 lowered GP by 75.2%, and *Ochrobactrum anthropi* GPK 3 did so by 61.5%. In both cases, these percentages were two- to threefold the degradation rate of the native microbial community. The two most efficient fungal inocula in our experiment were responsible for even higher GP removal percentages, but the period of

time assayed was also much longer. Similar GP degradation percentages to those of the present study were obtained by Mohy-Ud-Din et al. (2023), although their research involved rhizobacterial strains and was carried out in potted soil. Most of the strains they evaluated degraded 97 to 100% of the herbicide in pots containing 100 mg kg^{-1} GP. At the highest concentration assayed (200 mg kg^{-1} GP), the degradation percentages were under 40%.

Despite having their own limitations with respect to *in situ* research, microcosm assays approximate real-world degradation more closely than *in vitro* experiments in pure cultures. Guo et al. (2022) used soil microcosms (pots containing 60 mg kg^{-1} GP) to examine the degrading capabilities of *Fusarium verticillioides* strain C-2. After 28 days of incubation, the fungus removed 89% of the herbicide in unsterilized soil and 72% in sterilized soil. The first percentage is similar to the ones obtained with the two most effective inocula in the present study.

On the other hand, assessing AMPA levels in the soil is just as important as measuring GP levels, since it can provide information not only about potential pollution risks but also about how GP degradation takes place. Most GP-degrading microorganisms appear to use it as their sole phosphorus source, whereas a few use it as a source of nitrogen or carbon (Feng et al. 2020). According to Guo et al. (2022), *F. verticillioides* C-2 used GP as the only source of carbon for its growth. Data gathered *in vitro* about the strains in the present study showed that *A. oryzae* AM2 used GP as a source of phosphorus and nitrogen (Carranza et al. 2019), and that *M. circinelloides* 166 grew best in mineral media supplied with GP as the only phosphorus source (Aluffi et al. 2020). Nonetheless, as stated earlier, the co-inoculation containing *M. circinelloides* 166 was effective in removing GP and AMPA on the field, but not as much as *A. oryzae* AM2 alone.

There are two known pathways for the degradation of GP. The first consists of cleaving the C–P bond in the GP molecule, and produces sarcosine. It is usually induced upon exogenous phosphorus deficiency, which rarely occurs in agricultural soils. In the second pathway, the C–N bond in the herbicide molecule is cleaved by glyphosate oxidoreductase (GOR). This yields AMPA and glyoxylate (Sviridov et al. 2015). In several fungal species, AMPA is an intermediate metabolite of GP degradation. *A. oryzae* A-F02, for instance, was reported to metabolize AMPA into methylamine, and then into other simple and less toxic products (Fu et al. 2017). AMPA can also be metabolized into phosphono formaldehyde by transaminase, then into formaldehyde by phosphonatase, and later enter the central microbial metabolism (Singh et al. 2020).

The decrease in GP and AMPA observed in the present study after inoculation with *A. oryzae* AM2 and *M. circinelloides* 166 could have been due to degradation via one of those metabolic pathways. Given that GP degradation was not associated with a concomitant increase in AMPA levels in the subplots that received the two most successful treatments, the C-P lyase pathway that generates sarcosine is more likely to have been deployed (Okada et al. 2019). Alternatively, synergic interactions between the inoculated fungi and the native microbiota might have contributed to the degradation of the metabolite as well as to that of the parent molecule (La Cecilia and Maggi 2018). Wirsching et al. (2022) evaluated the degradation of GP by the native microbial community in an arable field in southern Germany. They did so by quantifying the abundance and expression of functional genes involved in the two known GP degradation pathways: *goxA* (for the AMPA pathway) and *sarc* (for the sarcosine pathway). Degradation through the AMPA pathway predominated at first, as evidenced by an increase in AMPA, in *goxA* transcription, and in *goxA*-harboring microorganisms. The authors suggest that this might have been due to fungi rapidly initiating degradation, with

Gram positive bacteria taking over later and at a slower rate. Something similar could have occurred in the present work, in which a significant decrease was observed not only in GP but also in AMPA levels after inoculation with *A. oryzae* AM2 (10^3 conidia mL^{-1}) alone and with the combination of *M. circinelloides* 166 (10^6 conidia mL^{-1}) and *A. oryzae* AM2 (10^3 spores mL^{-1}). To better elucidate this, the specific metabolic pathways for GP and AMPA degradation should be studied in these two fungal strains.

Seeing that GP and AMPA pose substantial risks of soil and water contamination, strategies are necessary to stimulate their natural degradation rate in the soil. The results presented here contribute to the characterization of *A. oryzae* AM2 and *M. circinelloides* 166 as potential candidates for bioaugmentation aimed at improving natural GP and AMPA attenuation in agricultural soils. The effects of inoculating these strains (separately and mixed) for GP and AMPA removal after the fumigation season should be studied further, especially considering their immobilization in one substrate, the activity of native microbiota, and the influence of changing redox conditions in the soil and persistent droughts as a consequence of climate change.

4- CONCLUSIONS

This study provides first-time evidence on the ability of *A. oryzae* AM2 and *M. circinelloides* 166, two fungal strains native to Argentinean agricultural soils, to remove GP and AMPA under field conditions. Both have great potential for the implementation of bioaugmentation strategies in sites polluted with herbicides.

FIGURE CAPTIONS

Fig. 1 Main steps of the methodology.

Fig. 2 Effect of inoculation with *A. oryzae* and *M. circinelloides* on glyphosate (GP) levels at the beginning of the assay and after 150 days. Control: without inoculation. Inocula 166: 10^6 spores mL^{-1} *M. circinelloides* 166. Inocula AM2: 10^6 conidia mL^{-1} *A. oryzae* AM2. Inocula 166/AM2: 10^6 spores mL^{-1} *M. circinelloides* 166 and 10^3 conidia mL^{-1} *A. oryzae* AM2. Inocula AM2/166: 10^6 conidia mL^{-1} *A. oryzae* AM2 and 10^3 spores mL^{-1} *M. circinelloides* 166. Mean values with different letters indicate significant differences according to Fisher's LSD test ($p<0.001$).

Fig. 3 Effect of inoculation with *A. oryzae* and *M. circinelloides* on aminomethylphosphonic acid (AMPA) levels at the beginning of the assay and after 150 days. Control: without inoculation. Inocula 166: 10^6 spores mL^{-1} *M. circinelloides* 166. Inocula AM2: 10^6 conidia mL^{-1} *A. oryzae* AM2. Inocula 166/AM2: 10^6 spores mL^{-1} *M. circinelloides* 166 and 10^3 conidia mL^{-1} *A. oryzae* AM2. Inocula AM2/166: 10^6 conidia mL^{-1} *A. oryzae* AM2 and 10^3 spores mL^{-1} *M. circinelloides* 166. Mean values with different letters indicate significant differences according to Fisher's LSD test ($p<0.001$).

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Ethical Statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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Statements and Declaration

Consent to participate

All authors agreed with the content, and they gave explicit consent to participate in this manuscript.

Consent for publication

All authors reviewed, revised, and approved the final draft for publication.

Ethical approval

Not applicable.

Data and material availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Table 1 Analysis of variance on the effect of treatment. time and their interactions on GP levels

Source of variation	df[a]	MS[b]	F[c]	p-value
Model	9	7.63877485	82.7619051	0
Time	1	44.9101893	486.5770884	0*

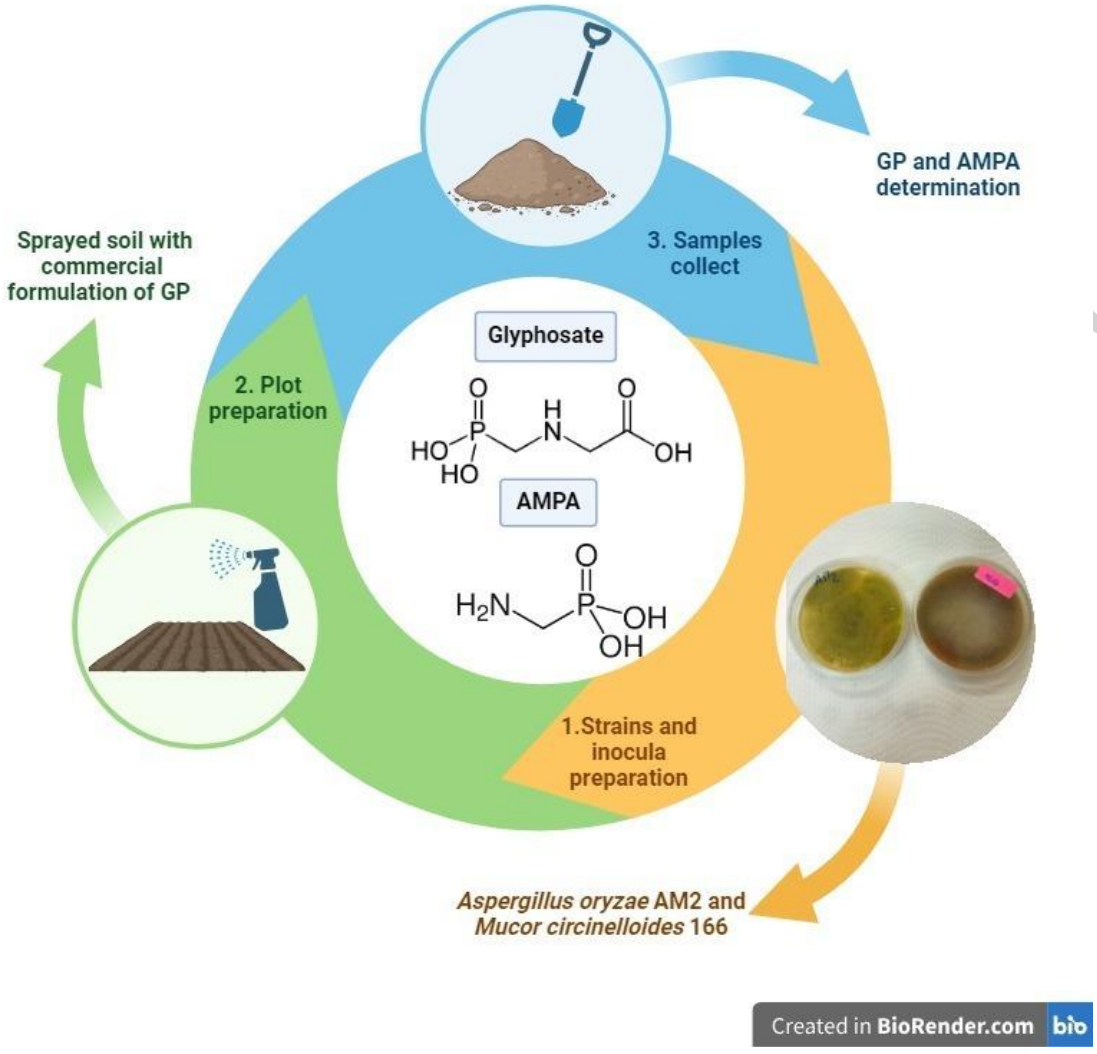
Treatment	4	3.83094577	41.50618087	8.1098E-12*
Time x treatment	4	2.12875031	23.0638335	8.6773E-09*

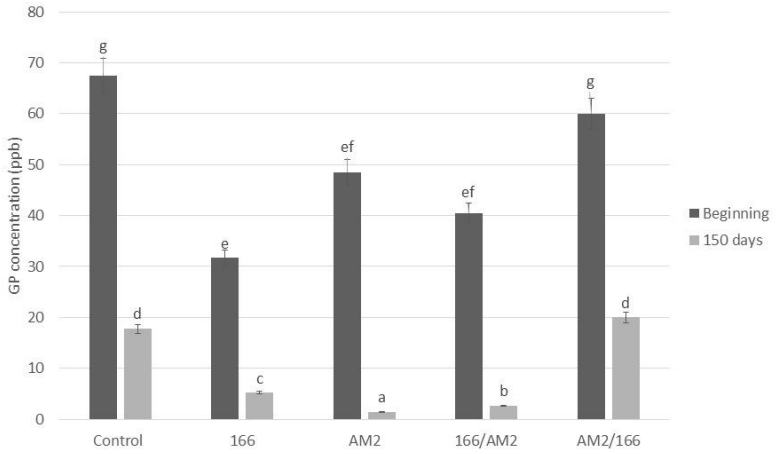
[a] Degrees of freedom.
[b] Mean square.
[C] F-Snedecor.
∗Significant $p < 0.001$.

Table 2 Analysis of variance on the effect of treatment, time and their interactions on AMPA levels

Source of variation	**df[a]**	**MS[b]**	**F[c]**	**p-value**
Model	9	2847310.11	9.22	0
Time	1	4386677.82	14.2	0*
Treatment	4	4499325.21	14.57	0*
Time x treatment	4	810453.08	2.62	0,0005*

[a] Degrees of freedom.
[b] Mean square.
[C] F-Snedecor.
∗Significant $p < 0.001$


GP and AMPA determination
3. Samples collect
Sprayed soil with commercial formulation of GP
Glyphosate
2. Plot preparation
AMPA
1.Strains and inocula preparation
Aspergillus oryzae AM2 and Mucor circinelloides 166
Created in BioRender.com


GP concentration (ppb)
80
70
60
50
40
30
20
10
0
g
d
e
c
ef
a
ef
b
g
d
Control
166
AM2
166/AM2
AM2/166
Beginning
150 days