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ORIGINAL ARTICLE

First report of *Aspergillus sydowii* and *Aspergillus brasiliensis* as phosphorus solubilizers in maize

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Abstract

Maize is one of the most important crops worldwide. It provides food for humans and animals and is used in biotechnological and industrial processes to produce a wide variety of products. The phosphorus (P) requirement for its development and production is high, but the absorption efficiency of this nutrient is insufficient to meet its requirements. The use of P-solubilizing fungi can increase this efficiency, thus reducing the amount of fertilizers applied to the crops. Therefore, this study aimed to verify the potential use of *A. sydowii* and *A. brasiliensis* and their effect in the field as bioinoculants when associated with three P fertilization doses. The experiment was carried out in a maize field, and treatments were presence and absence of fungi associated with P mineral fertilization doses. The parameters evaluated were shoot dry matter, P content in the plant, and P content in the soil. *A. sydowii* caused the highest P content in the plant and soil at fertilization doses of 75% and 100%, contradicting the expected results from in vitro assays that indicated that *A. brasiliensis* would provide better rates of P uptake. There was no difference in the other fertilization doses or for dry matter when comparing all treatments. This result strongly suggests that the use of *A. sydowii* can improve the efficiency of P absorption with fertilization application. In addition, the molecular analysis of the two fungi performed in this study contributes novel information on the use of both species during the agricultural process.

Keywords Maize · *Aspergillus brasiliensis* · *Aspergillus sydowii* · Phosphate solubilization · Phosphorous uptake · Fertilization reduction

Introduction

Maize is one of the main cereals cultivated around the world and is used as a food source for human and animals; it is a basic dietary compound among low-income families and in animal feed and is used as a raw material for biotechnological and industrial processes, such as ethanol production (Ranum et al. 2014). In Brazil, it is the second-most produced grain, surpassed only by soybean (Conab 2017).

The high nutritional requirement of maize limits its cultivation on fertile soils or with high fertilizer applications because an inappropriate supply of nutrients leads to a decrease in grain and biomass production by the plant (Malavolta 1997).

Phosphorus (P) is an essential nutrient for the plant. Although it is required in smaller quantities compared to other nutrients, such as nitrogen, it is necessary throughout the crop cycle because it is part of the structure of energy molecules, nucleic acids, and co-enzymes, among others, and participates in all process that involves energy expenditure by the plant. In addition, P deficiencies in the maize plants cause a reduction of the photosynthetic rate, resulting in low productivity and large losses in grain weight (Fornasieri Filho 2007; Patil and Gaikwad 2012).

Brazilian soils contain low levels of available P that can be assimilated by plants. Their origin and the strong interaction that the P may establish with soil particles, especially where a higher proportion of clay is found, results in the major part of this nutrient remaining adsorbed (Raij 1991). In this manner, P becomes one of the most limiting nutrients for agricultural production in tropical soils, creating a huge demand for phosphate supplementation to achieve adequate plant development (Rheinheimer dos Santos et al. 2008).

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The use of agricultural fertilizers is an expensive process, and it has generated growing concerns about its use due to the high recalcitrance and toxicity of many molecules contained in fertilizers. Additionally, studies show that only 10 to 25% of the phosphate supplied by mineral fertilization is efficiently assimilated by plants (Tripti et al. 2017). In this context, the use of plant growth promoting microorganisms as bioinoculants is a promising alternative because they possess the traits to aid the plant development through several processes, including increasing the P availability to the plant.

Microorganisms that exploit the soil P act on inorganic or organic P insoluble forms present in the soil releasing organic acids or producing enzymes (phosphatases and phytases) (Gaind and Singh 2016). These molecules are able to hydrolyze organic or inorganic P, thus making it ready for absorption by the roots (de Carvalho Mendes and dos Reis Junior 2003; Oliveira Mendes et al. 2014).

The genus *Aspergillus* is cosmopolitan and ubiquitous. It is highly diverse, with more than 339 species identified to date, and it has economic and social importance (Samson et al. 2014). Several species of *Aspergillus* are commercially exploited due to their ability to produce and secrete many enzymes and metabolites such as antibiotics and mycotoxins (Volke-Sepulveda et al. 2016).

Species of the genus *Aspergillus* have already been characterized by their P solubilization capacity and their potential for use as solubilizers of different phosphorus sources in the soil (Nahas and de Assis 1992; Souchie et al. 2006; Pacheco and Damasio 2014; Oliveira Mendes et al. 2014). Schneider et al. (2010) reported the ability to synthesize organic acids and produce high quantities of citric acid, which is one of the main factors responsible for the solubilizing characteristic of these fungi.

A. brasiliensis has a relatively recent characterization (Varga et al. 2007). It is clustered in the black aspergilli section and produces large amounts of citric acid and enzymes such as xylanases, thermostable β-xylosidases, α-glucosidase, and amylases (Varga et al. 2007; Pedersen et al. 2007; Miyazaki et al. 2011; Volke-Sepulveda et al. 2016; de Almeida et al. 2017). In addition, the *Aspergillus* species in the section *Nigri*, which includes 19 species such as *A. niger*, *A. awamori*, *A. oryzae*, *A. nidulans*, and *A. brasiliensis* (Varga et al. 2007), are considered GRAS (generally regarded as safe) by the US FDA.

A. sydowii is a common saprotrophic fungus found in soils (Rypien and Andras 2008). Soil strains of this species are considered harmless, but several studies were published describing the relation of marine strains with an epizootic disease on sea fan corals (Alker et al. 2001; Marfenina et al. 2013; Yarden 2014). Some *Aspergillus* strains clustered in the section *Versicolores* can act as opportunistic pathogens in humans and animals (Siqueira et al. 2016). However, as found for *A. brasiliensis*, *A. sydowii* has been the target of numerous studies on its ability to produce enzymes, mainly cellulases and ligninases, correlating this fungus to biomass degradation for biotechnological processes similar to bioethanol production (Matkar et al. 2013; Cong et al. 2017).

Neither fungus has been tested for its potential use in agriculture. This, together with the abilities described for *A. brasiliensis* and *A. sydowii* that have highlighted their economic importance, motivated this study, which is the first in this area.

Thus, this study aimed to evaluate the effect of the inoculation of *A. brasiliensis* and *A. sydowii* associated with different concentrations of P mineral fertilization during field conditions to determine their capacity of plant growth promotion by increasing the P availability for maize plants.

Materials and methods

Fungal strains

The strains used in this assay are part of the fungal culture collection of the Laboratory of Soil Microbiology at the Plant Production Department in the School of Agricultural and Veterinarian Sciences, São Paulo State University, Jaboticabal, Brazil.

Molecular characterization

Genomic DNA was extracted from fungal mycelia previously grown on PDA (Potato Dextrose Agar- Acumedia®) for 5 to 7 days with a Quick DNA Universal kit (Zymo Research) following the manufacturer's instructions. A previous step of cellular lysis was carried out using glass microspheres (Sigma) and 500 μL of lysis buffer TES (Tris 100 mM, EDTA 10 mM, SDS 2%) in 1.5 mL microtubes. The tubes were vortexed for 5 min and were incubated in a water bath at 65 °C for 60 min. They were vortexed again for 5 min. The tubes were centrifuged at 10,000×*g* for 15 min, and the supernatant was collected to start the extraction procedures of the kit.

DNA amplification was carried out using the primers ITS1 and ITS4 (White 1990) for the ITS region of the ribosomal DNA. The amplicons were generated as follows: 94 °C/3 min, followed by 30 cycles at 94 °C/30 s, 55 °C/30 s, 72 °C/1 min, 10 °C/∞. The sequencing reaction was carried out using the same primers and amplification program. Sequences were obtained using automated sequencing in an ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA) and then were aligned and edited using BioEdit software, 7.0.5.3 version (Hall 1999) and compared to sequences deposited at the NCBI (National Center for Biotechnology Information) and CBS database – Fungal Biodiversity Centre (The Netherlands).

Phylogenetic studies were carried out using the Clustal W software (Larkin et al. 2007) for sequence alignment and MEGA version 5.2 (Tamura et al. 2011) for phylogenetic tree construction.

Phosphorus solubilization assay in vitro

Fungi were grown on Petri dishes with PDA (Acumedia®) for 7 days. Disks of approximately 6 mm were taken from plates and inoculated into Erlenmeyer flasks (250 mL) with 50 mL of liquid medium containing, per liter, 0.1 g NaCl, 1 g NH_4Cl, 0.2 g KCl, 0.1 g $CaCl_2.2H_2O$, 1.2 g $MgSO_4.7H_2O$, 10 g glucose, and 0.5 g yeast extract (Nahas et al. 1994) supplemented with 5 g of rock phosphate (fluorapatite) as the only source of available phosphorus. The flasks were inoculated with three disks with each one in triplicate and were incubated on a rotatory shaker at 110 rpm at 27 °C for 7 days. After incubation, the medium was vacuum-filtered, and the quantification of soluble phosphorus was performed by spectrophotometric analysis according to the Ames method (1966).

Field assay

The experiment was carried out at the Teaching, Research and Extension Farm in the School of Agricultural and Veterinarian Sciences, on an area of 610.5 m^2. Maize seeds were from the Agroeste Company, variety AS 1633 PRO2 Cruizer.

The experimental design was a factorial 3 × 3 randomized blocks with nine treatments and four repetitions. The total area of 610.5 m^2 was divided into 36 plots. Each plot was composed of five rows of six meters, with each row containing a total of 15 plants. The treatments varied between the presence and absence of fungi associated with P mineral fertilization doses of 50%, 75%, and 100% of that recommended for maize according to a soil analysis (Table 1).

Fertilization was carried out during seed sowing using rock phosphate. The dose considered to be 100% contained 40.0 kg ha^{-1}. For nitrogen and potassium, urea and potassium chloride were applied, and in all treatments, their concentrations were the same: 30.0 kg ha^{-1} of urea and 20.0 kg ha^{-1} of potassium chloride, according to Raij et al. (1997) for maize crop.

Table 1 Treatments carried out in the field assay

Treatment	Inoculum	Fertilization dose
1	Absent	100%
2	Absent	75%
3	Absent	50%
4	*A. brasiliensis*	100%
5	*A. brasiliensis*	75%
6	*A. brasiliensis*	50%
7	*A. sydowii*	100%
8	*A. sydowii*	75%
9	*A. sydowii*	50%

At the end of the experiment, the parameters evaluated were P content in the plant according to Sarruge and Haag (1974), P content in the soil (Watanabe and Olsen 1965), and shoot dry matter with $n = 3$ plants collected from each plot and dried in a forced ventilation oven for approximately 3 days until they presented a constant weight in a semi-analytical balance.

The data obtained were submitted to a variance analysis with an F test, and the means were compared with a Tukey test ($P < 0.05$). The software Agroestat (Barbosa and Maldonado Junior 2010) was used.

Fungus cultivation and inoculation in the field

Aspergillus brasiliensis was cultivated on Petri dishes containing PDA (Acumedia®) and incubated in a microbiological oven at 25 °C for 7 days. A conidial suspension was prepared from plate scrapings with 5 mL 0.1% Tween 80 solution added to each plate. Then, the suspension produced was filtered; the excess mycelium was withdrawn, and the volume was brought to 10 L using the same Tween 80 solution.

A. sydowii was cultivated on a large scale on parboiled rice based on the methodology of Alves and Pereira (1989) with modifications: the rice was washed for approximately 40 min in water at room temperature and was then sifted to remove the excess water. The wet rice was placed into autoclavable plastic bags containing from 300 to 400 g of wet rice. The bags were sealed with bobby pins and autoclaved at 121 °C for 40 min. After cooling, the rice was inoculated.

The inoculum was a conidial suspension of *A. sydowii* at a concentration of 5.10^7 conidia per milliliter (conidia mL^{-1}) prepared from scraping Petri dishes with the addition of 5 mL of 0.1% Tween 80 solution. The plates were previously prepared by cultivating the fungus for 7 days on PDA medium (Acumedia ®). With the aid of a needle and a syringe, the suspension was inoculated inside the plastic bags with previously prepared, sterile parboiled rice. The bags with inoculated rice were incubated at 25 °C for 20 days.

After the incubation period, the bags were opened in a sterile room, and the colonized rice was placed in trays, cleaned, and sterilized with ultraviolet radiation for 15 min, before being allowed to dry for 3 to 4 days. After drying, the rice was cleaned with 10 L 0.1%Tween 80 solution, and this suspension was taken to the field for inoculation.

Before the field inoculation, both suspensions were counted in a Neubauer chamber, and the conidial concentrations were adjusted to 7×10^7 conidia mL^{-1}. In the field, 0.8 L suspension was applied per plot in the soil near the roots of

the maize plants. Two applications of fungi were conducted throughout the experiment. The first inoculation was carried out 30 days after sowing, and the second occurred 60 days after sowing.

Results

Figure 1 depicts a phylogenetic tree showing the molecular identification of the tested strains. As can be verified, the analysis of the ITS region of the ribosomal DNA allowed us to precisely classify them as *A. sydowii* and *A. brasiliensis*.

Figure 2a presents the content of P solubilized in vitro using rock phosphate (fluorapatite) as the only P source for 1 week of incubation. *A. brasiliensis* solubilized 82.10 μg P/mL filtrate, and *A. sydowii* solubilized 22.50 μg P/mL filtrate.

The highest P content in the plants was found for those that received *A. sydowii* inoculation (10.58 μg of P g^{-1} of dry matter) followed by plants that received *A. brasiliensis* (9.31 μg of P g^{-1} of dry matter) and the control (6.58 μg of P g^{-1} of dry matter). Statistical analysis showed a significant difference between both fungal treatments compared to the control (Fig. 2b).

Comparing the three levels of fertilization tested simultaneously in the presence or absence of the fungi, the P content of the plants was significantly higher in the plants that received 75.0% of the recommended dose according to the soil chemical analysis (10.58 μg of P g^{-1} of dry matter), followed by plants that received 100.0% of fertilization (9.73 μg of P g^{-1} of dry matter) and plants that received 50.0% (6.16 μg of P g^{-1} of dry matter) (Fig. 2c).

For the plants that received 100.0% of the recommended dose of P fertilization, the statistical data reveal that the P content was significantly superior only for the plants that received *A. sydowii* (15.50 μg of P g^{-1} of dry matter) (Fig. 2d). When the maize plants received 75.0% or 50.0% of the recommended P dose, there was no statistical significance of the P content among the different inocula within the same level of fertilization. However, in both doses, higher values were obtained for *A. sydowii*, followed by *A. brasiliensis* and the control (Fig. 2e, f).

There were no significant differences for total dry matter data among treatments that received *A. sydowii* (51.05 g), *A. brasiliensis* (50.66 g), or the control (48.62 g). The same was observed in relation to the different doses of fertilization: 100% (46.72 g), 75% (50.86 g), or 50% (52.75 g).

Statistical analysis of the P content in the soil showed no difference between inoculation and fertilization. However, the highest levels of P were found at the treatments that were inoculated with *A. sydowii* (28.85 mg kg^{-1}), being 18.89% higher than those that received *A. brasiliensis* (23.40 mg kg^{-1}) and 21.66% higher than the control (22.60 mg kg^{-1}). When 50% of the recommended dose of

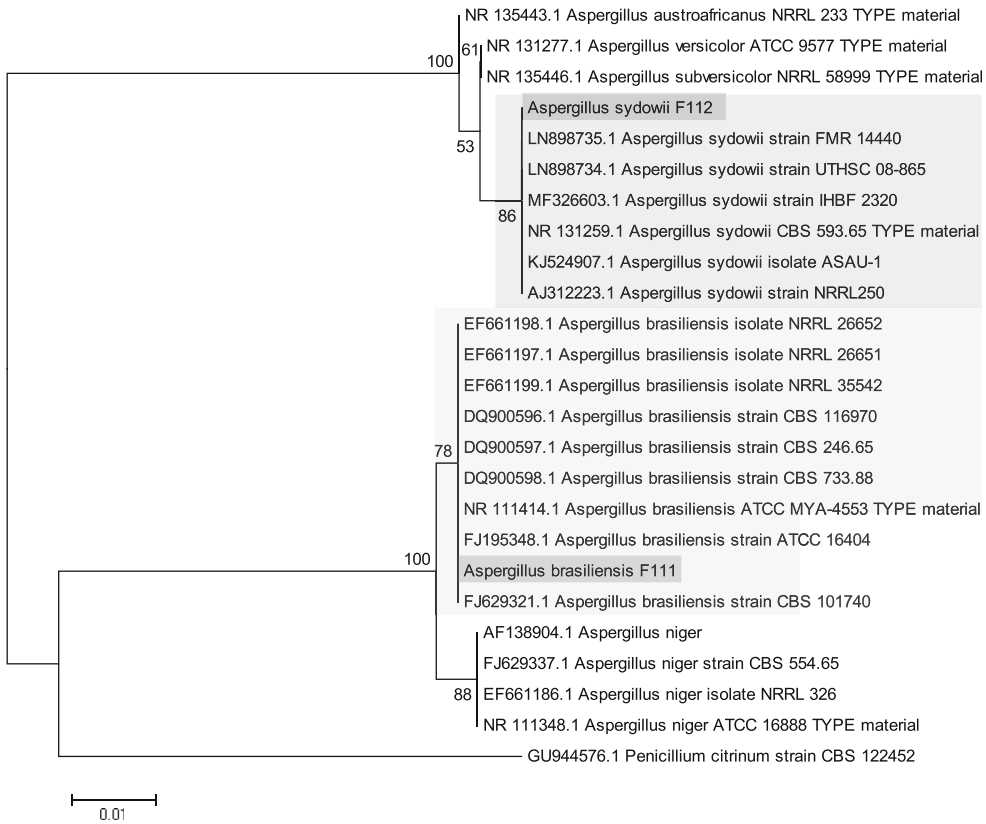

Fig. 1 Phylogenetic analysis of the ITS region from the ribosomal DNA of the fungi tested. The phylogenetic tree was constructed based on the maximum likelihood method, with the evolutionary distances calculated according to the Kimura-2 parameter. Bootstrap values are presented as a percentage for 1000 repetitions. Tree branches presenting bootstrap values higher than 70 were considered highly consistent for identification. *Aspergillus brasiliensis* F111 and *Aspergillus sydowii* F112 represent the strains tested in this study. It could be observed that they are consistently grouped with other strains of the same species, including type strains. The numerical codes before the species names refer to their accession number at the GenBank (NCBI). "TYPE material" refers to DNA sequences from type strains of the species

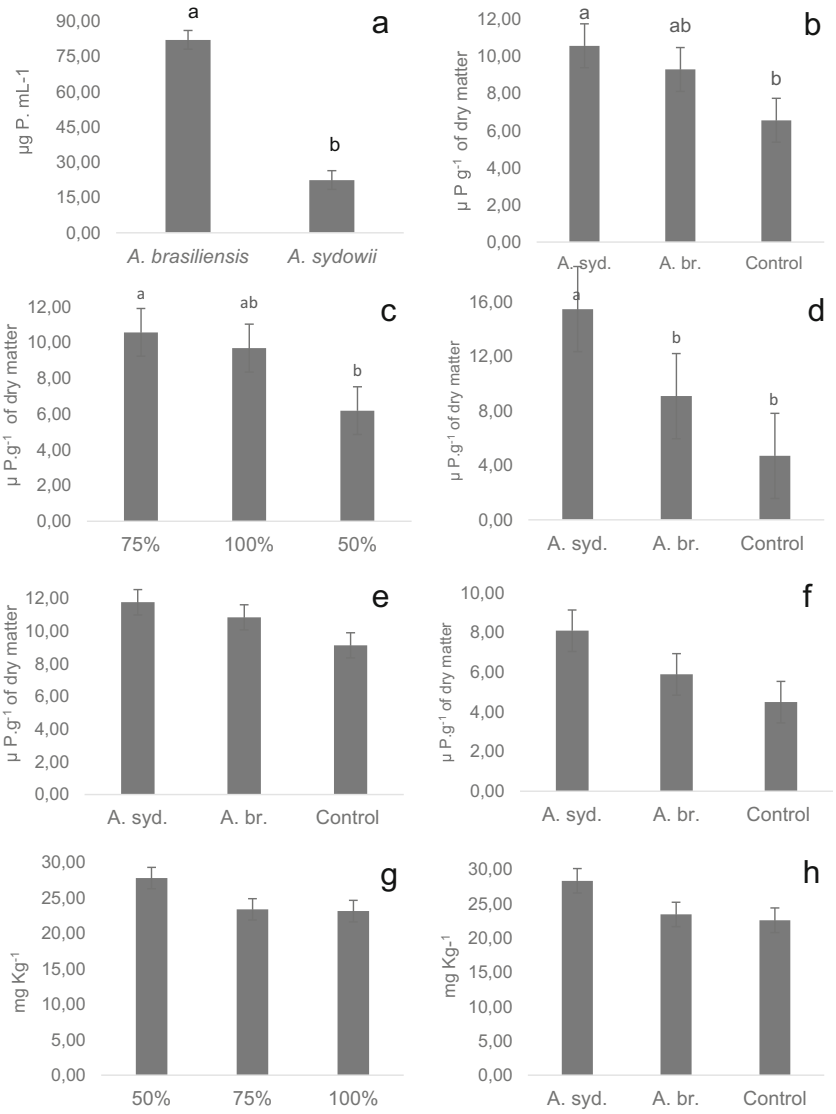

Fig. 2 **a** In vitro assay of P solubilization with fluorapatite as the sole P source. **b** P content added to maize plants after field assay based on each fungal inoculum and **c** each fertilization dose. **d**–f P content in the maize plants after field assay in the fertilization doses of 100%, 75%, and 50%, respectively. P content in the soil depending on the different fertilization doses and the inoculation of *A. sydowii* (**g**) and *A. brasiliensis* (**h**). Different letters above the bars indicate significant differences in Tukey's test with $\alpha = 0.05$. The absence of letters indicates no significant differences among the treatments. *A. syd.*, *Aspergillus sydowii*; *A. br.*, *Aspergillus brasiliensis*

fertilizer was supplied, more P was obtained from the soil (27.83 mg kg^{-1}), followed by the 75% (23.41 mg kg^{-1}), and 100% doses (23.16 mg kg^{-1}) (Fig. 2g, h).

Discussion

Commonly, only the aspects of morphology and physiology were used as taxonomic tools to describe *Aspergillus* species. However, the difficulty in discriminating closely related species requires the use of molecular biology to support their classification.

It is of crucial importance to know the biological agents that are being tested because their application must be safe for both the environment and people. Because of the concerns about biosecurity aspects, which are considered relevant for this research, both isolates of the genus *Aspergillus* had their ribosomal DNA sequenced before their application and were identified as *A. brasiliensis* and *A. sydowii* (Fig. 1). Only later, they were tested as bioinoculants and as P suppliers under field conditions, which is an important characteristic that must be found in a microorganism that is intended to be used as a plant growth promoter.

Of additional importance is that the molecular characterization allowed the novel information of the use of these fungal species as bioinoculants in agriculture. Both *A. brasiliensis* and *A. sydowii* already have a great deal of biotechnological potential due to their production of enzymes and other substances of interest, in addition to the fact that *A. sydowii* acts as an epizootic of sea fan corals. However, their biological importance of the interactions that these fungi can have with plants and the potential benefits that they could provide to them have never been exploited.

It is well recognized that microorganisms can aid in plant growth via several mechanisms. Here, we focused on the importance of P acquirement because it is one of the essential nutrients but its content in tropical soils is generally low compared to other soils in temperate climates. Even though plants require a high amount of P, their efficiency to take up this nutrient from the soil is low (Mahamuni et al. 2012). This problem occurs because there is a large quantity of P that remains adsorbed on soil clay and oxides and becomes unavailable to the plants (Karandashov and Bucher 2005). When microorganisms produce organic acids and enzymes, they can act on different P sources by solubilizing the nutrient and increasing its uptake efficiency by the plants (Priyadharsini and Muthukumar 2017). *Aspergillus* species are recognized for their capacity to produce organic acids, mainly citric acid, and being able to synthesize phosphatases. Different organic acids, depending on their form and amount released, can result in a higher or lower effectiveness of P solubilization in the soil; at this point, citric acid is considered one of the most effective acids (Richardson et al. 2009).

This study tested the potential of the P solubilization of *A. brasiliensis* and *A. sydowii* in vitro and in field conditions. *A. brasiliensis* demonstrated a greater ability to solubilize P from fluorapatite than *A. sydowii* did, with the former providing approximately three times more soluble P (82.10 μg P/mL filtrate) than *A. sydowii* (22.50 μg P/mL filtrate) (Fig. 2a). This result indicates that *A. brasiliensis* could have better performance in field because its ability to solubilize P under the test conditions was superior. Varga et al. (2007) described *A. brasiliensis* and inferred that this fungus can be a citric acid producer similar to *A. niger*, the main black aspergilli. This fact could justify the higher rate of fluorapatite solubilization. No information characterizing the organic acid production by *A. sydowii* or phosphatase secretion by both species could be found in the literature.

Although the in vitro test of solubilization suggested the use of *A. brasiliensis*, both strains were applied in the field. Under this condition, the effect of added fungi was combined with three P fertilization doses (100%, 75%, and 50% of the recommended dose) (Table 1) based on rock phosphate use. Unexpectedly, opposite results to the in vitro results were obtained, and *A. sydowii* had better performance in improving the P uptake from the soil to the maize plants.

When the fertilization conditions were compared separately during the statistical analysis, the highest P content in the plants was found for those that received 75.0% of phosphate fertilization (Fig. 2c) and the fungus *A. sydowii* as inoculum (Fig. 2b). These results strongly suggest that this fungus was able to increase the efficiency of P uptake from soil and provide it to the plants. In addition, they indicate a possible reduction in 25% of P mineral fertilization in the soil that resulted in a considerable cost reduction and the mitigation of environmental impacts. The fungus *A. sydowii* is not classified as a mycorrhizal fungus, but with these results, it is possible to conclude that, similar to mycorrhizal fungi, it can interact in an associative way with the maize roots and transfer P from the soil to the plants using its hyphae. Usually, mycorrhizal fungi show high P fluxes in their hyphae, ranging from 2 to 20×10^{-6} mol m^{-2} s, with bidirectional protoplasmic flows, as measured by the cellular particle movement (vacuoles, nuclei, fat droplets, organelles, granules), ranging from 3.0 to 4.3 μm s (Giovannetti et al. 2004). An in-depth study could provide information about how this process occurs in *A. sydowii*.

Comparing the presence of *A. brasiliensis* and *A. sydowii* within each phosphate fertilization dose (Fig. 2d–f), a statistically relevant P content was observed only in those plants that received the fungus *A. sydowii* at 100% of the recommended dose of rock phosphate fertilization. At this dose, the application of *A. brasiliensis* did not differ from the control (Fig. 2d). Considering the effect of the dose of fertilization with each inoculum and the control, *A. sydowii* resulted in a significant improvement in P uptake when combined with 75% or 100% of the recommended dose of rock phosphate fertilization. These results suggest the possibility that *A. sydowii* can only solubilize P when a certain amount of P (rock phosphate) is available in the soil, indicating that the lowest dose tested in this experiment was insufficient to stimulate and activate the biosynthetic pathways of the acids and/or enzymes responsible for acquiring P from the soil. The solubilization process occurs under stress conditions and involves the expenditure of cell energy, so it is possible that only in the presence of more raw material did the production of molecules for solubilization becomes a viable strategy.

There was no significant difference when comparing the dry matter of maize plants with fungal inoculation or fertilization dose. The results showed that these fungi did not promote plant growth by increasing biomass. The amount of P fertilizer was calculated according to soil chemical analysis; therefore, the provision of P for plants was adequate at dose of 100%, and this nutrient was undersupplied at doses of 75% and 50%. The use of the fungi was supposed to perform phosphate solubilization and improves P uptake by plants, which not necessarily would promote biomass increase.

The fact that there was no difference regarding dry matter among plants that received 100%, 75%, and 50% of P rates suggests that the lower amounts provided did not affect the dry matter content; however, it did not reflect the health status of the cell metabolism, which was certainly benefited from P increase caused by the presence of the fungi.

Current agricultural systems strongly depend on continuous applications of fertilizers, primarily nitrogen (N), phosphorus (P), and potassium (K). The use of synthetic fertilizers has several undesired effects on both biotic and abiotic components of ecosystems, such as the contribution to the decline of biological soil fertility. In particular, the use of phosphate

fertilizers has increased from approximately 5 million tons of P per year in 1961 to approximately 20 million tons in 2013 (Chen and Graedel 2016). Over the next decades, the major challenge for agriculture will be the sustainable production of enough food crops to meet the growing global demand (Karandashov and Bucher 2005; Battini et al. 2017). This challenge can be faced by using microorganisms that can enhance P uptake from soil to plant, and the use of *A. sydowii* could be an effective alternative to ameliorate this problem.

Conclusions

In an effort to avoid P deficiency in plants, many fertilizers are usually applied to the soil. Some studies showed high losses of P in these cases, demonstrating that up to 90% of this P ends up in rivers and lakes, thus promoting water eutrophication. In addition, the cost of P fertilization can reach up to 50% of the total amount of fertilizer utilized in crop production. A more sustainable system of crop production has been sought and researched in recent years. The use of fungi in the agricultural process to benefit plants is recent and has yet to be exploited. Studies such as this one are important because of their contribution to the practical knowledge on the use of fungi that present a real potential for field application and can become the target of further research. Additionally, this study demonstrates the interesting relation between the behavior in vitro and during field conditions. *A. brasiliensis* was a better solubilizer in vitro. However, *A. sydowii* was more promising for solubilizing and increasing the content of P in maize in the field. *A. sydowii* facilitated the P uptake by the plants, but for this increase to result in a yield increase, more research is needed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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