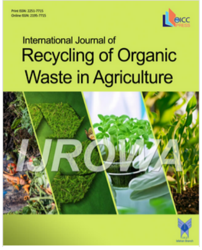

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The inoculum potential of arbuscular mycorrhizal fungi in soil amended with swine slurry

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Abstract:
Purpose: This study was carried out to evaluate the effects of the tillage system and consecutive application of swine slurry for seven years on arbuscular mycorrhizal fungal abundance and infective propagules in Brazilian soil with crop rotation.
Method: The spore density, the external mycelium, and the most probable number of infective propagules of mycorrhizal fungal abundance were assessed in soil samples from an Oxisol in a long-term field experiment in response to soil management (no-tillage and conventional tillage) and following successive application of swine slurry (0, 30, 60, 90 and 120 $m^3 \cdot ha^{-1} \cdot year^{-1}$).
Results: A greater amount of external mycelium was observed in soil under no-tillage, while mycorrhizal fungal abundance spore density was greater in soil under conventional tillage. In soil under no-tillage, mycorrhizal fungal abundance infective propagules were more abundant; however, in soil with both management systems, this variable was reduced with different levels of swine slurry application. In no-tillage, the main source of mycorrhizal fungal abundance propagules is the external mycelium, while in conventional tillage, the main inoculum component is the spores.
Conclusion: In this study, we verified that the application of swine slurry, with high phosphorus contents, reduces arbuscular mycorrhizal fungi propagules. However, these effects are mitigated in no-tillage systems compared to conventional tillage.

Keywords: Swine slurry; No-tillage; Conventional tillage; Mycorrhiza; Infective propagules

1. Introduction

The no-tillage system (NT) is a sustainable management system that improves soil quality by contributing to soil organic matter as it also decreases soil temperature and solar radiation effects and increases water retention. Soil microbiota and beneficial functional groups are favored in the NT system when compared to the conventional tillage (CT) (Srour et al. 2020). Studies comparing these two tillage systems showed that microbial biomass is higher under NT, (Helgason et al. 2010; Balota et al. 2003; Balota et al. 2004a), also increases several microbial enzymes activity (Balota et al. 2004b), nitrogen-fixing bacteria (Torabian et al. 2019) and has a protective effect on arbuscular mycorrhizal fungal community structures (Lu et al. 2018).

Another sustainable management system in agriculture is the incorporation of animal waste, (e.g., swine slurry), as fertilizers into soil, which can contribute to increased agricultural productivity (Sediyama et al. 1998; Vidigal et al. 1997). This increase is probably due to N, P, and K nutrients added together with animal waste, particularly swine slurry (Ceretta et al. 2003). Also, indirect effects are observed by organic matter incorporation, such as the alterations in the activity of the microbial community's structure (Kallenbach et al. 2016; Larkin et al. 2006; Plaza et al. 2007) and, in soil physical and chemical properties (Valarini et al. 2003; Choudhary et al. 1996), reducing the losses of water and soil. Thus, to promote sustainable agriculture, the use of animal waste, such as swine slurry, in soils cultivated under NT, can be a viable alternative for disposal of the waste.

The arbuscular mycorrhizal fungi (AMF), belonging to the Glomeromycota, important symbiotic component of the soil ecosystem, induce in the host plant, mineral nutrition, resistance to water stress, pests, and diseases and, they are favored in the NT system (Wang et al. 2016; Ganeshamurthy et al. 2015; Ganeshamurthy et al. 2018). In agricultural systems, biota answers quickly to disturbance, and AMF propagules are potential bioindicators to measure several stressful factors (Vasconcellos et al. 2016). However, little is known about the effect of swine slurry addition under different soil tillage in the AMF communities in soil. Balota et al. (2014) observed the negative effects of swine slurry on root colonization, spore number, and total external mycelium. On the other hand, authors observed positive effects in the active external mycelium and glomalin-related soil protein content. Thus, the aim of this study was to assess the effect of successive swine slurry application on AMF potential inoculum in soil under no-tillage and conventional tillage.

2. Material and methods

2.1 Description of the experimental area and soil sampling

This study is conducted in a long-term field experiment, which was set up in 1996, at the experimental station of the Institute of Rural Development – IDR-PR in Palotina, State of Paraná, Brazil (24° 12′ south latitude; 53° 50′ 30″ west longitude, average temperature of 26°C and 1500 mm of annual precipitation). The soil is a clayey oxisol classified as a eutroferric red latosol according to the Brazilian Soil Classification system, loamy texture with pH ($CaCl_2$ 0.01 M) of 5,2; Al = 0.00, Ca = 6.1, Mg = 1.9 and K = 0.8 in $cmol_c \cdot dm^{-3}$; C = 20 $g \cdot kg^{-1}$; P (Mehlich^{-1}) = 14.8 $mg \cdot dm^{-3}$; clay = 60% silt = 16% and sand = 24%. The experimental area has been cultivated with crop rotation of soybean/maize (*Glycine max* L./*Zea mays* L.) and wheat/forage pea/oats (*Triticum sativum* Lam./*Pisum sativum* L./*Avena sativa* L.) since experiment installation.

The experiment was conducted in a split plot design with a randomized block scheme with four replicates, for seven years. The treatments were: two tillage systems (Plot) and doses of 0, 30, 60, 90, and 120 $m^3 \cdot ha^{-1} \cdot year^{-1}$ of SS (subplot) applied half before summer and winter crop sowing. The average composition of applied SS presented 10.8% of total solids; pH of 8.2; C = 201; N = 30; P = 41; K = 31; Ca = 46; Mg = 21; S = 13 and Na = 14 in $g \cdot kg^{-1}$; Cu = 1206; Zn = 4981; Fe = 718; Mn = 718 and B = 88 in $mg \cdot kg^{-1}$ (IAPAR 1989).

To evaluate the inoculum potential of AMF, in 2004 after the winter crop season in September, soil samples and roots of oats were collected, at a depth of 0 – 10 cm. Samples were stored and refrigerated (4°C) until analysis.

2.2 Evaluation of AMF inoculum potential in laboratory and greenhouse

Extraction and counting of AMF spores in soil

AMF sporulation was determined according to Colozzi-Filho and Balota (1994), in 50 mL soil samples collected in the rhizosphere of oats and sieved in 2 mm mesh (to remove big fragments of roots and other materials). The AMF spores extraction from soil was by wet sieving (Gerdemann and Nicolson 1963) and centrifugation in water at 3000 rpm for 3 minutes, and flotation in sucrose (50%) at 2000 rpm for 2 minutes (Jenkins 1964), proceeded the counting in stereoscopic microscope with zoom up to 40 times.

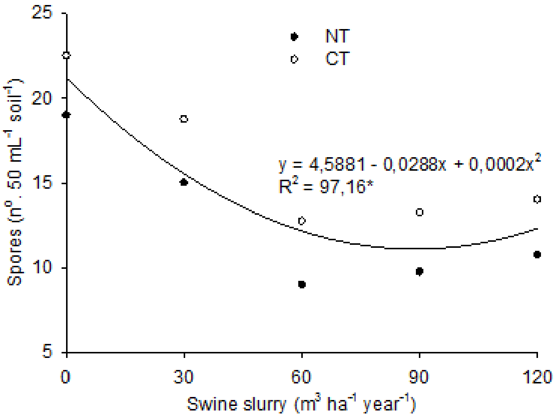

Figure 1. Effect of successive applications of swine slurry for seven years on arbuscular mycorrhizal fungi spore number, in layer 0 – 10 cm, in soil under no till (NT) and conventional tillage (ploughing and harrowing) (CT). Palotina, Paraná, Brazil *Significant to 5% by F test.

AMF external mycelium

The extraction of total external mycelium of soil was carried out according to Filho et al. (1999), modified by Melloni and Cardoso (1999), through wet sieving after vigorous agitation of 10 g of soil sample, with subsequent filtration by vacuous in nitrocellulose membrane of mycelium in suspension and staining with trypan blue. The evaluation of membranes was done under an optical microscope with a zoom up to 100 times, with the aid of a reticulated ocular. The total external mycelium length was estimated according to Schubert et al. (1987).

2.3 Most Probable Number (MPN) of AMF infective propagules

In the same year, 2004, in October, beginning of the summer crop season, a bioassay was conducted in the greenhouse, with corn (*Zea mays* L.) as a trap plant, to evaluate the AMF infective potential of soil according to the procedure described by Daniels and Skipper (1982). The most probable number (MPN) of AMF infective propagules was evaluated in soil samples collected in the field experiment. The soil was sterilized for 90 minutes at 120ºC. Those sterilized soil samples were put in a plastic container of 150 mL and inoculated with the same field soil treatment diluted successive times in the base 4 up to 10^{-8}, as the procedure described by Sieverding (1991). The corn seeds were disinfected and sowed in sterilized soil with one plant per container and inoculated with soil samples from field treatments.

The experimental design was randomized with three replicates. After eight weeks with controlled irrigation, the plant roots were collected, washed, and stored in ethanol 50% for evaluation of AMF root colonization. The AMF root colonization was done by using 1 g of corn roots according to the procedure described by Colozzi-Filho and Balota (1994). The roots were clarified in KOH 10%, stained with trypan blue, and evaluated in the stereoscopic microscope with zoom up to 40 times, for the presence or absence of colonization. To calculate infective propagules, MPN was used through the formula proposed by Sieverding (1991).

2.4 Statistical analysis

The data of spore counting were transformed to $(x+0.5)^{0.5}$ and submitted to variance analysis (ANOVA), considering soil management and SS doses as factors. The averages of total mycelium length were separated by standard error. The values found for MPN data of AMF infective propagule were transformed to $\log_{10}$ and submitted to the polynomial regression analysis. The correlation analysis (Pearson‘s correlation coefficient) was calculated between spore number, external mycelium or infective propagules, and available P of soil in layers 0 – 10 cm. The data analysis was carried out through the SAS statistical program Version 8.1.

3. Results and discussion

There were significant mathematical model adjustments for the behavior of the AMF sporulation in response to the application of slurry doses; however, that behavior was independent of tillage systems. It was verified a decrease until dose 60 $m^3 \cdot ha^{-1} \cdot year^{-1}$ where lesser spore densities were observed in both tillage system soils and a little increase in subsequent doses (Fig. 1). Higher spore density of AMF was observed in CT, with an average of 16 spores in 50 mL of soil, against 12 in NT.

The application effects of SS doses were less pronounced in CT (Fig. 2), there was a 38% negative effect on sporulation in CT, against 45% in NT in relation to the control treatment without slurry.

The application of increasing doses of SS in soil caused a reduction in the total mycelium production in both tillage systems (Fig. 3); however, we did not obtain significant mathematical adjustments. There was significantly higher production of total mycelium in NT, however, the negative effect of the slurry application on the total external mycelium production in soil was greater in NT, with a 25% decrease in relation to the control treatment without slurry and 22% in CT (Fig. 2).

The AMF inoculum potential of soil, infective propagules, measured by the most probable number (MPN) method was affected drastically by increasing doses of SS in both tillage systems, reducing the natural inoculum potential of those fungi in soil (Fig. 4). MPN of infective propagules was higher in NT soil in all slurry doses.

Pearson‘s linear correlation analysis revealed a significant negative correlation between the length of the total external mycelium and P concentration in NT soil ($r = -0.51$, $p < 0.05$, $n = 20$) and between spore number and P concentration in CT ($r = -0.61$, $p < 0.01$, $n = 20$). The addition of SS promoted an increase in P concentration (Melich-1) that varied from 12 to 91 mg dm^{-3} in both tillage systems (Fig. 5).

Negative impacts were observed in the AMF infective propagules under SS application (Fig. 1 and 3). Despite being a source of nutrients for plants, toxic concentrations of present metals in slurry, applied consecutively for 7 years, can affect the AMF. Although in soils contaminated with heavy metals, AMF may be used for environmental remediation and contribute to developing tolerance and producing high plant biomass (Riaz et al. 2021), AMF propagules seem to reduce in the presence of these metals in the soil. Gattai et al. observed that AMF benefited the growth of the plant *Erythrina velutina* in Pb-contaminated soils; however, authors also detected a reduction in infective propagules and colonization by AMF in those contaminated soils (Gattai et al. 2011).

Christie and Kilpatric observed that the application of doses up to 200 $m^3 \cdot ha^{-1} \cdot year^{-1}$ of SS in *Lollium perenne* L. for several years resulted in significant increments of Cu and total and extractable Zn in soil, finding significant negative correlations between these and the mycorrhizal infections, besides, observing decreases in the external mycelium production around 94% (Christie and Kilpatric 1992). In a study carried out by Val et al. (1999b) in heavy metals polluted soils, authors observed that AMF spore number decreased significantly with the increase of metal concentrations in soil. Metal-polluted soils also reduce the mycorrhizal colonization and the external mycelium production of *Glomus* sp and *G. mosseae* (Val et al. 1999a).

It is well known that heavy metals interfere with soil AMF community. Many studies highlight that the presence of heavy metals such as lead, cadmium, and zinc can have negative effects on the abundance and diversity of AMF (Dietterich et al. 2017; Atakan et al. 2018) and inhibits the spore germination, the mycelium growth and reduces the mycorrhizal colonization of plants (Bartolome-Esteban and Schenck 1994). Chen et al. found that high concentrations of cadmium (Cd) in soil significantly reduced the abundance and diversity of AMF communities (Chen et al. 2022). Mitra et al. (2022) found that lead (Pb) contamination had a negative impact on the diversity and composition of arbuscular mycorrhizal fungi (AMF) communities in the rhizosphere of rice plants. Mi et al. (2023) have also shown that heavy metal pollution can alter the composition and diversity of AMF communities in soil. Similarly, Chen et al. observed that high concentrations of heavy metals such as cadmium (Cd) and zinc (Zn) have been shown to inhibit AMF infec-

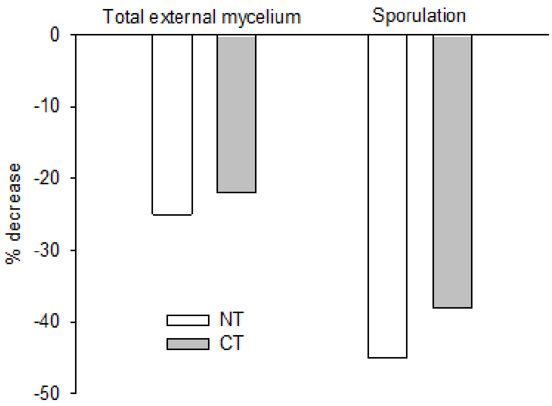

Figure 2. Decrease percentage (%) of the total external mycelium and the sporulation of arbuscular mycorrhizal fungi, due to consecutive application for seven years of swine slurry in soil cultivated under no till (NT) and conventional tillage (ploughing and harrowing) (CT).
The calculation done with average data of different doses of swine slurry in relation to the control treatment (without swine slurry). Palotina, Paraná, Brazil.

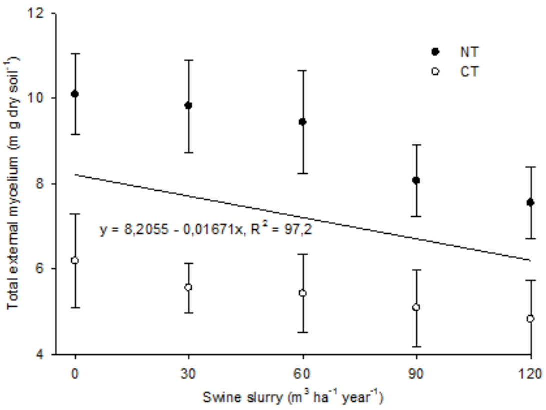

Figure 3. Effect of successive application for seven years of swine slurry on total external mycelium length of fungi, in layer 0 – 10 cm, in soil under no till (NT) and conventional tillage (ploughing and harrowing) (CT). Palotina, Paraná, Brazil.
Standard error bar, n = 4.

tion in maize roots and reduce the abundance and diversity of AMF communities (Chen et al. 2022). Contents of heavy metals in the soil, such as Zn, Cu, Cd, and Pb, are negatively correlated with both species' richness of AMF and glomalin contents (Leal et al. 2016). These studies indicate that metal toxicity may impair the survival and reproduction of AMF in contaminated soil, leading to a reduction in the availability of infective propagules, which can in turn affect plant growth and nutrient uptake.

The impact of heavy metal pollution on soil microorganisms, including AMF, is an important area of research. Understanding the mechanisms by which heavy metals affect soil microorganisms can help to develop strategies for mitigating the negative effects of pollution and promoting healthy soil and plant growth.

Contrary to our results on AMF, some studies have demonstrated the application of SS in the soil increases microbial activity and bacteria, actinomycetes, and fungi populations (Larkin et al. 2006; Jauregi et al. 2021). Also, the application of SS to soil can result in an increase in microbial biomass and activity, associated with the supply of easily available nutrients (Rieke et al. 2018; Lim et al. 2018). Besides, SS can stimulate other microbial populations, as predators, parasites, or antagonists of AMF, as well as other fungi types, such as the saprophytic, that would be interfering in AMF's infective potential. According to Moreira and Siqueira (2006), many genera of fungi like *Rhizidiomycopsis*, *Phlyctochytrium*, *Anquillospora*, *Humicola*, *Stachybotrys* are parasites of the AMF, as well as countless bacteria, actinomycetes, collembola and aphids also inhibit, parasitize, prey or graze the AMF propagules (hyphae and spores) (Fitter and Sanders 1992; Friede et al. 2016). This can directly influence the dynamics of the AMF in the soil. These findings highlight the complexity of interactions in the soil ecosystem and emphasize the importance of considering the various effects of agricultural practices, including the application of swine slurry (SS), on microbial populations, particularly AMF.

According to the results obtained in the present study, it can be inferred that in soil under NT, the major source of infective propagules (natural inoculum potential of AMF) was external mycelium. On the other hand, in soil tilled with ploughing and harrowing (CT), the spores represented the natural inoculum potential of AMF, as a greater spore number was observed in relation to NT (Fig. 2).

The results on the total mycelium production obtained in this study (Fig. 3) agree with other studies that have demonstrated the negative effects of soil management on external mycelium production (Borie et al. 2006; Kabir et al. 1997; Kabir et al. 1998). The soil under NT, without ploughing, allied the humidity conditions, temperature, and organic matter are more favorable to the biological activity (Gajda and Przewłoka 2012), and they make possible permanence of the external hyphal net of AMF in soil and also colonized roots, which maintain the high natural infective potential of soil (Filho et al. 2001). When the conditions are favorable to the mycorrhiza establishment, as in NT, which leads to more balanced relationships among the microbial community, the fungi do not need to produce a lot of spores to guarantee their survival. Conversely, soil tillage (CT) can alter the ability of AMF to colonize roots, breaking up their hyphal network and reducing root colonization, which induces the fungus to form resistance structures in the soil (Filho et al. 2001). The spores are resistance structures, so a higher spore density in CT soil (Fig. 2) indicates the unfavorable and stressful conditions for the fungi and the mycorrhization due to soil ploughing.

Also, the increase of sporulation, observed in both systems starting from dose 90 $m^3 \cdot ha^{-1} \cdot year^{-1}$, can be explained by the fact that the environment begins to select and favor AMF species more adapted to stressful conditions. The stimulus of sporulation in the soil is an alternative for fungi to maintain themselves in the system, while the hyphae production is inhibited (Filho et al. 2001).

Despite the effect of SS dose, the AMF inoculum potential of soil in NT was higher than CT (Fig. 4). The maintenance of favorable conditions, (*e.g.* temperature, water retention,

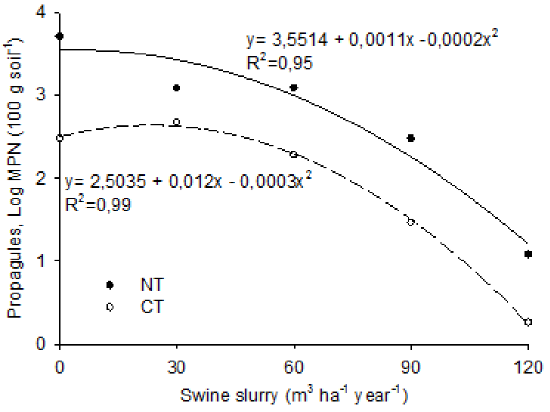

Figure 4. Most probable number (MPN) of arbuscular mycorrhizal fungi infective propagules, in layer 0 – 10 cm, in soil under no till (NT) and conventional tillage (ploughing and harrowing) with the application of increasing doses of swine slurry. Palotina, Paraná, Brazil.

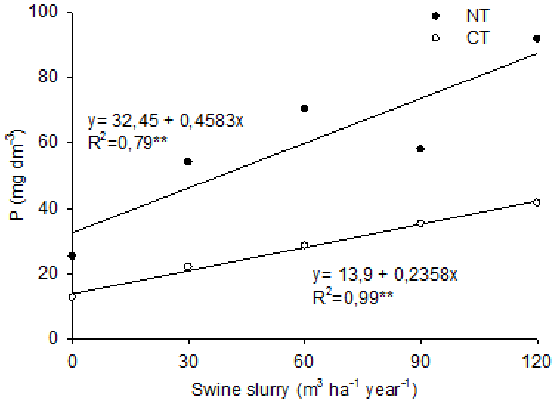

Figure 5. Phosphorus (P Melich-1) concentrations in soil under no till (NT) and conventional tillage (CT) with ploughing and harrowing and successive application of swine slurry for seven years. Soil sampling at 0 to 20 cm layer. Palotina, Paraná, Brazil.

soil stability) in soil under NT (Shen et al. 2018) may reduce the negative effect of slurry on microbiota. Therefore, it is important to consider the potential impacts of tillage systems on AMF propagules and develop appropriate recommendations for the use of tillage systems to promote healthy soil and plant growth while minimizing the risk of environmental pollution (Alguacil et al. 2008).

The lesser MPN of AMF infective propagules in soil under CT (Fig. 4) reflects alterations caused by soil ploughing and harrowing that the mechanical action makes propagules unfeasible. Our results provide evidence that tillage can have a negative impact on the natural infective potential of AMF. Soil tillage can disrupt the hyphal network of AMF propagules, leading to a decrease in soil mycorrhizal infectivity (Kabir 2005) and can have detrimental effects on AMF colonization and diversity in soil. The physical disruption caused by soil ploughing can negatively impact not only AMF but also other beneficial soil microorganisms involved in nutrient cycling and plant-microbe interactions (Alguacil et al. 2008; Brito et al. 2012; Säle et al. 2015). Colozzi et al. showed that soil preparation can negatively affect phosphate solubilizers, rhizobia, and hydrolytic enzymes producing bacteria that facilitate penetration of roots by the AMF, affecting the mycorrhization (Filho et al. 2001). According to Borie et al. (2006), soil management practices affect in a differentiated way the effectiveness of mycorrhizal symbiosis due to alterations that cause on the AMF propagules, as well as on the community structure. To increase the natural inoculum potential of soil, and active propagule number, like mycelium, spores, and colonized root fragments by AMF, better conditions of agricultural practices should be adopted.

The natural AMF inoculum potential of the soil, which is crucial for successful plant colonization, can be compromised by soil tillage (Tatewaki et al. 2023). Therefore, to enhance the natural inoculum potential of soil and promote optimal mycorrhizal symbiosis, it is important to adopt agricultural practices that create favorable conditions for AMF. Soil management practices that minimize soil disturbance and promote a conducive soil environment are essential for enhancing the natural inoculum potential of AMF. By adopting appropriate agricultural practices, we can create conditions that support the establishment and functioning of mycorrhizal associations, ultimately benefiting plant growth and nutrient uptake.

The addition of SS promoted an increase in P concentration in soil (Fig. 5). Those increments result in the accumulation of consecutive additions of swine liquid residues that contain high P concentration. Boitt et al. (2018) showed that the application of SS can increase the concentration of P in the soil, mostly in the surface layers, and have positive effects on soil quality, such as improving soil fertility and increasing the organic matter content of the soil (Lee et al. 2006). Pratt observed soils that received SS for three consecutive years presented P concentrations up to 15-fold higher than soil without application (Pratt 2008). According to Eghball et al. (2005), 66% of the total P of SS can be or is to be converted, in a short period of time, into an available form for plants in soil. The P availability is the edaphic factor that affects arbuscular mycorrhiza more, and there is an inverse relationship with the mycorrhizal dependence (Nogueira and Cardoso 2000). Those authors observed a decrease in the root colonization in soy (*Glycine max* L.) with an increase of P doses up to 200 mg·kg^{-1}, and a decrease in the total external mycelium production, although they had not obtained significant mathematical adjustments.

Continuous application of SS can lead to a high P content in the soil (Gatiboni et al. 2021). The negative effect of SS on the AMF propagules confirms that these are sensitive and answer quickly to any changes in the agrosystem, impairing the benefits of mycorrhizal association to nutrition and productivity of cultures and sustainability of soil quality (Kabir 2005). Although a lower frequency of AMF with SS addition was observed in both systems, in this study we understand that NT mitigated the negative effects of SS application (Fig. 4). Overall, the relationship between AMF and soil quality is complex and can be affected by various factors, including the use of agricultural fertilizers such as swine residue and soil management.

Therefore, it is important to understand the factors that affect the symbiosis between AMF and crops in soil conditions under swine residue use as an agricultural fertilizer. AMF plays an important role in optimizing nutrient bioavailability and reducing the use of agrochemicals for maintaining sustainable agroecosystems (Ebbisa 2022). According to our results and the importance that AMF carries out in agricultural soils, what becomes important is the knowledge of factors that affect this symbiosis in soil under those conditions, to a more appropriate recommendation of swine residue discard or use as an agricultural fertilizer.

4. Conclusion

After a long term, the consecutive application of increasing doses of swine slurry, with high phosphorus con-tents, reduces the natural inoculum potential of arbuscular mycorrhizal fungi in soil. Doses up to 30 m^3·ha^{-1}·year^{-1} drastically reduced AMF propagules.

However, these effects are mitigated in no-tillage systems compared to conventional tillage. Soil under conventional tillage presents higher AMF sporulation independent of the amount of swine slurry applied. No-tillage system presents higher production of total external mycelium and AMF infective propagules.

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Author Contributions:
ASN and ACF designed experiments, field survey, sample collection, performance of experimental work and analysis. ASN wrote the first draft of the manuscript. ASN, GSM and ASS performed interpretation of data and was involved in writing the manuscript. All authors contributed, read and approved the final manuscript.

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Compliance with ethical standards
Conflict of interest: The authors declare that there are no conflicts of interest associated with this study.

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