# Phosphorus fertilization reduces the severity of charcoal rot (*Macrophomina phaseolina*) and the arbuscular mycorrhizal protection in soybean

**Federico N. Spagnoletti[1,3]*, Micaela Leiva[1], Viviana Chiocchio[1,3],** and **Raul S. Lavado[2,3]**

[1] Cátedra de Microbiología Agrícola. Facultad de Agronomía, Universidad de Buenos Aires. Av. San Martín 4453 C1417DSE Buenos Aires, Argentina
[2] Cátedra de Fertilidad y Fertilizantes. Facultad de Agronomía, Universidad de Buenos Aires. Av. San Martín 4453 C1417DSE Buenos Aires, Argentina
[3] Instituto de Investigaciones en Biociencias Agrícolas y Ambientales - INBA (CONICET/UBA)

## Abstract

The effects of phosphorus (P) application on the relationship between the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* and the pathogen *Macrophomina phaseolina* (charcoal rot) affecting soybean (*Glycine max* L.) are unknown. We evaluated the effects of P on both the severity of the pathogen and the AMF protection against the charcoal rot in soybean. We conducted greenhouse experiments with a randomized multifactorial design with ten replications. The treatments were: two concentrations of P as superphosphate (0 and 50 kg of P $ha^{-1}$), inoculated and non-inoculated with the AMF *R. intraradices*, and infected and non-infected with *M. phaseolina*. Soybean was seeded in pots containing 1 kg of sterilized substrate soil : sand : perlite (7 : 3 : 2). When soybean completed pod formation (R4 phenological stage), the plants were harvested. Plant parameters, mycorrhizal colonization, and disease severity were measured. The presence of *M. phaseolina* negatively affected soybean biomass, but AMF inoculation improved it. Phosphorus reduced AMF colonization but not arbuscules percentage. Moreover, both P and AMF inoculation had a negative effect on disease severity, although P also reduced mycorrhizal protection. These results suggest that phosphorus application could reduce disease severity, but can simultaneously partially reduce the AMF protection against the pathogen. These effects should be considered in agricultural integrated management practices of soybean.

**Key words:** biological control / fungal diseases / *Glycine max* / *Rhizophagus intraradices*

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## 1 Introduction

Phosphorus (P) is a plant nutrient and its deficiency in soils significantly reduces yields and can make plants more susceptible to disease (*Krupinsky* et al., 2002). Also, fertilizers are essential to feed a growing world population, but also show some undesirable effects on the environment (*Youssef* and *Eissa*, 2014). Among these effects, fertilizers can affect the soil biota (*Geisseler* and *Scow*, 2014). One constituent of the soil biota are fungi, which have different functions in eco- and agrosystems, mainly as microsymbionts, decomposers, elemental transformers, soil-borne pests and diseases, and microregulators (*Barrios*, 2007). These fungi may be classified in two opposed groups: pathogenic fungi, which show negative effects on crops, and mycorrhizal fungi, including arbuscular mycorrhizal fungi (AMF), which exhibit in general, positive effects on crops (*Smith* and *Read*, 2010). However, plant responses to AMF may range from positive (mutualism) to neutral (commensalism) and even to negative (parasitism; *Klironomos*, 2003).

In general, the extent to which plants benefit from the association depends on a number of factors, including the genotypes of AMF and the host, and the environmental conditions under which they interact (*Klironomos*, 2003). The arbuscular mycorrhizal symbiosis can improve plant mineral nutrition, plant water relationship, and resistance to contaminants (*Smith* and *Read*, 2010). Also, it was reported that AMF usually increase host resistance to phytopathogens, reduce symptoms and disease severity, and positively affect plant survival and biomass production (*Spagnoletti* et al., 2016). Bioprotection by AMF involves various mechanisms. One of them is the increase in root biomass, which can compensate roots damaged by the pathogen (*Harrier* and *Watson*, 2004). Another mechanism is the competition with the pathogen for colonization sites in the host root, because AMF and soil-borne fungal pathogens occupy similar root tissues (*Wehner* et al., 2010). The third and fourth mechanism is the better nutrient status of the plant and the activation of plant defense mechanisms. The improvement of nutrient status can help to overcome a pathogen infection. *Wehner* et al. (2010) have shown that colonization of root systems by AMF can increase phosphorus and nitrogen nutrition and that of other mineral nutrients such as Cu, Ca, Zn, and Mn. On the other hand, several authors have demonstrated that the establishment of an arbuscular mycorrhizal symbiosis could predispose the

* Correspondence: F. N. Spagnoletti; e-mail: spagnole@agro.uba.ar

 

plant to respond more rapidly to pathogenic attack through a pre-activation of plant defense responses (*Pozo* et al., 2010; *Wehner* et al., 2010; *Spagnoletti* et al., 2016). Other important mechanisms are changes in the anatomy and architecture of the root system. When AMF colonize plant roots, morphological changes, such as an increase in cell wall lignification, can occur within the host plant, and these may aid bioprotection (*Al-Askar* and *Rashad*, 2010). The protection by AMF is a cumulative result of all these mechanisms either together or separately (*Harrier* and *Watson*, 2004; *Pozo* et al., 2010; *Wehner* et al., 2010). However, it is known that the impact of the arbuscular mycorrhizal symbiosis in terms of resistance to pathogens differs among AM fungal isolates for a given plant–pathogen interaction and, moreover, such impact can be modulated by environmental conditions (*Pozo* et al., 2010).

On the other hand, phytopathogenic fungi contribute substantially to the overall yield losses in crop plants. Plant resistance to diseases can be reduced by any of several stresses, such as drought, temperature extremes, compaction, physical plant injury, excess moisture, and nutrient imbalances (*Prabhu* et al., 2007). One of the most important pathogens is *Macrophomina phaseolina* (Tassi) Goid, the causal agent of charcoal rot, which is considered to be a major plant pathogen worldwide (*Mengistu* et al., 2007). The pathogen affects a wide range of cultivated and wild species in warm, temperate, and tropical regions and causes significant economic losses in soybean (*Glycine max* L.) (*Mengistu* et al., 2007). This disease can appear at any stage of plant growth affecting seeds, seedlings and adult plants, and its severity increases as the air and soil temperatures increase and when soil moisture is limited. Disease-ridden plants show brown discoloration of the pith in roots and shoots, and sufficient dark microesclerotia in roots can cause premature death of the plant (*Mengistu* et al., 2007).

Some agronomic practices, such as fertilization, can affect soil microorganisms and even could affect the relationship between pathogens and their host (*Veresoglou* et al., 2013). The effects of P fertilization on plant disease is difficult to predict (*Dordas*, 2008), either increasing disease severity in some cases or decreasing it in others (*Huber*, 1980; *Reuveni* et al., 2000). Some authors have reported that the severity of take-all of wheat (*Gaeumannomyces graminis*) decreases with increasing P application (*Brennan*, 1995), whereas other authors showed that P has a moderately suppressive effect on wheat leaf rust (*Puccinia recondite*; *Sweeney* et al., 2000) or little to no significant effect on gray leaf spot of corn (*Cercospora zeae-maydis*; *Okori* et al., 2004).

Phosphorus deficiency in soils significantly reduces yields of crop plants. Similarly to some pathogens, AMF development may be inhibited by P application exceeding certain rates (*Balzergue* et al., 2011), with possible impact on the mycorrhizal benefits in agroecosystems. Inhibition of arbuscular mycorrhizal development by P is a systemic effect that depends on the nutritional status of the plant (*Nouri* et al., 2014). Since AMF are carbon-costing, the suppression of arbuscular mycorrhizal symbiosis by high P levels may be an energy-saving negative feedback mechanism (*Nouri* et al., 2014). The effect of P application on *M. phaseolina* severity has been studied, but the results were inconsistent. *Csöndes* et al. (2008) demonstrated that P application reduced the disease severity, although *Mengistu* et al. (2016) did not detect any effect of P fertilization on that parameter. Nevertheless, there are no reports that evaluate the effect of AMF inoculation in P-fertilized soils and in *M. phaseolina*-infected soybean plants. Thus, the objective of this study was to determine effects of P application on the relationship between the AMF *R. intraradices* and the pathogen *M. phaseolina* (charcoal rot) in soybean. We evaluated the effects of P on both the severity of the pathogen and the AMF protection against the charcoal rot. Our hypothesis was that P fertilization not only causes a reduction in the arbuscular mycorrhizal symbiosis and in the disease severity but also reduces arbuscular mycorrhizal protection in *M. phaseolina*-infected plants.

## 2 Material and methods

### 2.1 Pathogenic fungal isolate and culture conditions

*Macrophomina phaseolina* was isolated from soybean plants exhibiting charcoal rot symptoms in early summer 2015 in a cropped plot located near Pergamino city, Buenos Aires Province, Argentina (34°18′ S 61°18′ W). The infected roots of the plants were taken to isolate and characterize *M. phaseolina*. Infected roots were washed with tap water and 0.5 cm sections were surface-sterilized with 3% NaOCl solution to remove surface contaminants, washed with sterilized distilled water, and placed in 90-mm Petri plates containing potato dextrose agar (PDA) medium supplemented with streptomycin 200 mg $L^{-1}$. The plates were incubated in the dark at 28°C for 4 d. Single sclerotia were removed using a stereomicroscope (Zeiss Stemi 2000-C) and transferred to a new PDA plate. The morphology of mycelia and microsclerotia were monitored under light microscopy (Nikon Eclipse 50i). These investigations as well as those of the characteristics of colonies allowed the classification of isolates using standard literature (*Agarwal*, 2010). A pure culture of the isolate was maintained on PDA medium in a refrigerator at 4°C. The isolate was incorporated to the collection of the Fungi Bank of the Microbiology Department of the School of Agriculture, University of Buenos Aires (UBA), Buenos Aires, Argentina.The pathogen inoculum was mass-multiplied in rice grains according to *Spagnoletti* et al. (2016).

### 2.2 Arbuscular mycorrhizal fungi

The AMF inoculum (*Rhizophagus intraradices*) was originally obtained from the Campus of the School of Agriculture. Currently, the strain (VCh 0011) belongs to the Fungi Bank of the Microbiology Department of the School of Agriculture, UBA. The fungus was propagated in pot culture with *Trifolium repens* and *Sorghum bicolor* plants grown in a sterile sandy loam soil in 250 $cm^3$ pots for 4 months. The general inoculum containing colonized root fragments, spores, and rhizospheric soil was prepared to inoculate soybean seeds.

 

### 2.3 Greenhouse experiment and data assessments

Two pots experiments (in summer 2015 and 2016) with soybean (*Glycine max* L. cv. NIDERA 4990, rhizobial-free) were carried out in a greenhouse located in the Campus of the School of Agriculture, UBA. The substrate used was a mix of sterilized soil : sand : perlite (7 : 3 : 2). The soil used for the preparation of the substrate was a loamy A horizon of a Typic Argiudoll (US Soil Taxonomy) from Solís, Buenos Aires province, Argentina (34°18′ S, 59°20′ W). The particle size distribution of the substrate was 18% clay, 11% silt, and 71% sand, and the chemical composition of the substrate was: 17.4 g $kg^{-1}$ of organic carbon (Walkley and Black method), pH 6.9, 34.7 mg $kg^{-1}$ bioavailable P (Kurtz and Bray N° 1 method), and 0.36 dS $m^{-1}$ electrical conductivity (soil saturation extract; *Sparks* et al., 1996).

Each experiment was considered a block and within them the experimental design was factorial at completely randomized design with ten replications: two concentrations of P (0 and an equivalent to 50 kg of P $ha^{-1}$, *i.e.*, a dose twice the regular rate) as superphosphate, two levels of inoculation with the mycorrhizal fungus *R. intraradices* (inoculated and non-inoculated), and two levels of infection with the pathogenic fungus *M. phaseolina* (infected and non-infected).

Soybean seeds were superficially sterilized using ethanol 70% and sodium hypochlorite 3% for 3 min each, rinsed several times with sterilized distilled water, and seeded in each pot containing 1 kg of substrate, which was kept watered (average 70–80% of its water-holding capacity) using deionized water. Before sowing the soybean seeds in pots, 10 g of AMF general inoculum (containing colonized root fragments, rhizospheric soil, and approximately 100 spores $g^{-1}$ dry soil) was added to the corresponding treatment at a soil depth of 3 cm. The pathogen inoculum was incorporated into the substrate of each pot at a rate of 8 g, after 15 d of soybean growth. Propagule concentration was determined by plating the inoculum on PDA medium. The population of *M. phaseolina* was $1 \times 10^4$ colony-forming units (CFU) $g^{-1}$. After 70 d (R4 phenological stage; *Fehr* and *Caviness*, 1977), all plants (aerial and root biomass) were harvested. Aerial biomass was divided into leaves, stems, and pods (including immature grains). All samples were rinsed with distilled water, dried at 80°C for 72 h, and then weighed. Plant height (main shoot only) was recorded. Parts of root samples were stained with lactophenol-Trypan Blue (*Phillips* and *Hayman*, 1970) to determine the percentage of mycorrhizal colonization and the presence of arbuscules. One hundred randomly selected stained root pieces were mounted on slides and examined by light microscopy (Nikon H550S) to estimate mycorrhizal root colonization (%) and percentage of arbuscules, expressed as a percentage of the colonized root (*McGonigle* et al., 1990).

### 2.4 Disease severity

The disease severity caused by *M. phaseolina* was determined counting the colony-forming units (CFU $g^{-1}$ root) following the technique described by *Mengistu* et al. (2007).

### 2.5 Statistical analysis

Results were analyzed with INFOSTAT software (*Balzarini* et al., 2008). Analysis of variance (ANOVA) and Tukey's tests were applied to determine significant differences between treatments. Results were considered statistically significant when $P < 5\%$. All results are expressed as the mean of ten replicates. Both greenhouse experiments showed similar results, therefore only parameters of the second experiment are shown.

## 3 Results

### 3.1 Effect of treatments on plant biomass and morphological parameters

Phosphorus fertilization did not affect soybean aerial (leaves, stems, and pods) or root biomass production ($P > 0.05$). AMF inoculation did not affect aerial biomass ($P > 0.05$), but increased (39%) root biomass, compared to non-inoculated plants ($P < 0.01\%$). *M. phaseolina* infection diminished both aerial (31%) and root (43%) biomass ($P = 0.07\%$), while co-inoculated plants (AMF + *M. phaseolina*) showed biomass values similar to those of AMF-inoculated ones (Tab. 1). Plant height showed a pattern similar to that of its aerial biomass. The number of leaves was greater in AMF-inoculated plants, with and without *M. phaseolina* infection (37% and 15% respectively), while P application did not modify this morphological parameter ($P > 0.5\%$). However, soybean leaves were reduced by 30% in fertilized *plus M. phaseolina*-infected plants. Phosphorus fertilization showed no significant effects on the number of pods ($P > 5\%$), while AMF inoculation increased (7%) this parameter ($P > 0.7\%$). The pathogen negatively affected root length of soybean plants. *M. phaseolina* infection had significantly reduced root length (25%), while AMF-inoculated plants were similar to the control treatment (Tab. 1). No significant differences were detected in the P application ($P > 5\%$).

### 3.2 Mycorrhizal colonization

No mycorrhizal colonization was found in non-inoculated treatments (Control and *M. phaseolina*-infected plants). Phosphorus fertilization reduced (54%; $P < 0.01\%$), while the pathogen infection did not affect ($P > 5\%$) the mycorrhizal colonization. However, the percentage of arbuscules was affected by a P × R.i × M.p interaction ($P < 0.01\%$). The percentage of arbuscules was increased by P fertilization without *M. phaseolina* (204%), while its lowest values were found in plants of the unfertilized or AMF-inoculated treatments. Co-inoculated plants with both fungi (with and without P fertilization) showed high arbuscules percentage (Fig. 1a, b).

### 3.3 Disease severity

Figure 2 presents the disease severity. The disease severity was high in *M. phaseolina*-infected plants, but P fertilization reduced the disease by 60% ($P < 0.01\%$). AMF inoculation in the non-fertilized treatment also reduced (81%) the charcoal rot severity ($P < 0.01\%$), but AMF inoculation did not diminish the disease severity in the P fertilized soils. In soybean plants

 

**Table 1**: Effects of phosphorus fertilization (P+), *R. intraradices* (R.i), and *M. phaseolina* (M.p) on morphological parameters of soybean plants. Each value represents the mean value obtained from 10 replicates ± SD. Row values of each variable followed by different letters mean significant differences according Tukey ($P < 5\%$).

| Treatments | Control | | R. i | | M. p | | R. i + M. p | |
|---|---|---|---|---|---|---|---|---|
| | P – | P + | P – | P + | P – | P + | P – | P + |
| Aerial Biomass (g) | 3.32 ± 0.38 a | 3.03 ± 0.94 a | 2.99 ± 0.24 a | 3.48 ± 0.70 a | 2.28 ± 0.96 b | 1.83 ± 0.73 b | 3.56 ± 0.73 a | 2.71 ± 0.22 a |
| Pods | 13.50 ± 2.79 ab | 12.70 ± 2.86 ab | 14.40 ± 2.45 a | 15.00 ± 2.62 a | 10.20 ± 3.46 b | 7.80 ± 1.81 b | 8.60 ± 1.95 b | 9.50 ± 2.59 b |
| Leaves | 10.00 ± 0.71 b | 9.25 ± 1.48 bc | 11.50 ± 0.50 ab | 11.50 ± 0.58 ab | 9.50 ± 2.06 bc | 7.00 ± 1.00 c | 13.75 ± 0.83 a | 11.25 ± 0.50 ab |
| Plant height (cm) | 30.12 ± 3.54 a | 30.25 ± 2.87 a | 30.25 ± 1.64 a | 34.37 ± 3.94 a | 24.50 ± 2.50 b | 26.12 ± 2.32 b | 34.75 ± 3.68 a | 31.37 ± 1.88 a |
| Root biomass (g) | 1.67 ± 0.20 b | 1.45 ± 0.39 b | 2.32 ± 0.36 a | 2.43 ± 0.44 a | 0.95 ± 0.37 c | 0.60 ± 0.14 c | 1.88 ± 0.39 b | 2.05 ± 0.37 b |
| Root length (cm) | 29.00 ± 1.22 a | 24.00 ± 3.56 a | 28.25 ± 3.63 a | 25.50 ± 3.06 a | 21.75 ± 2.03 ab | 19.13 ± 1.75 b | 30.00 ± 5.83 a | 31.00 ± 3.51 a |

simultaneously inoculated with AMF and fertilized with P, the effect of the arbuscular mycorrhizal symbiosis was negatively affected: the reduction of the disease was about 46% compared to co-inoculated plants without phosphorus application (Fig. 2).

## 4 Discussion

Adequate mineral nutrition is important for plant resistance to disease (*Krupinsky* et al., 2002; *Csöndes* et al., 2008). Our data also suggest that P fertilization may increase the resistance of soybean to *M. phaseolina*. This positive effect of P could be related to the role of this nutrient in plant development. One of the best defenses against root and other diseases is a vigorous and healthy developed plant root system, and it can be achieved by P fertilizing (*Armstrong*, 1999). In accordance with *Mengistu* et al. (2016), charcoal rot severity depends mainly on environmental factors such as the variation in drought and water excess.

However, our results did not agree with those of *Mengistu* et al. (2016), who found that disease severity produced by *M. phaseolina* was not significantly affected by P application in soybean grown under field conditions. This discrepancy could be due to the different experimental conditions. We found that disease severity also depended on different levels of P availability to plants (Fig. 2). Also the presence of mycorrhiza in soybean roots led to significantly lower infection levels of *M. phaseolina* than those observed in non-mycorrhizal plants. Bioprotection of AMF-colonized plants against soilborne pests, like various root diseases, is commonly observed (*Whipps*, 2004; *Spagnoletti* et al., 2016). Some studies have demonstrated mycorrhizal protection of soybean plants against the root pathogens (*Doley* and *Jite*, 2012; *Li* et al., 2013; *Qian* et al., 2015; *Spagnoletti* et al., 2016). Other researchers, such as *Doley* and *Jite* (2012), who used the AMF *Glomus fasciculatum*, and *Spagnoletti* et al. (2016), who used *R. intraradices*, have found results similar to ours.

When beneficial and pathogenic fungi act simultaneously, they interact with each other, either reducing or promoting their effects on crops (*Li* et al., 2013). Several studies in other crops than soybean have shown that the pathogen reduces crop aerial and root biomass and that AMF inoculation can reverse this effect (*Doley* and *Jite*, 2012; *Spagnoletti* et al., 2016). In the present study, plant height and root length reductions were also reversed by the mycorrhiza. Several authors have suggested that the protective effect of AMF against pathogens may be operative through different mechanisms. The principal mechanisms are the competition for carbon, nitrogen and other growth factors and for root space (*Wehner* et al., 2010). Mycorrhizal root colonization also induces changes in the root system architecture, morphology and root exudates, which may alter the dynamics of the pathogen infection (*Pivato* et al., 2008). Another mechanism playing a central role in the induction of resistance by arbuscular mycorrhiza involves the activation of the jasmonate pathway (*Pozo* et al., 2010).

Finally, as has been known for several years (*Schubert* and *Hayman*, 1978), we found that P fertilization reduced mycorrhizal colonization. *Treseder* (2004) showed that a higher concentration of P in the plant reduced the production of arbuscular my-

 

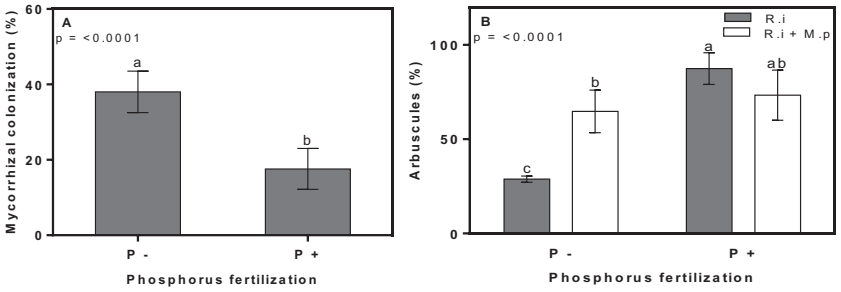


**Figure 1:** Mycorrhizal colonization (A) and Arbuscules percentage expressed as a percentage of the colonized root (B) with fertilization of P and the presence of *M. phaseolina*. R.i = *R. intraradices*; M.p = *M. phaseolina*. Vertical bars represent the standard deviation. Each value represents the mean value obtained from ten replicates. Different letters indicate significant differences (Tukey test P < 5%).

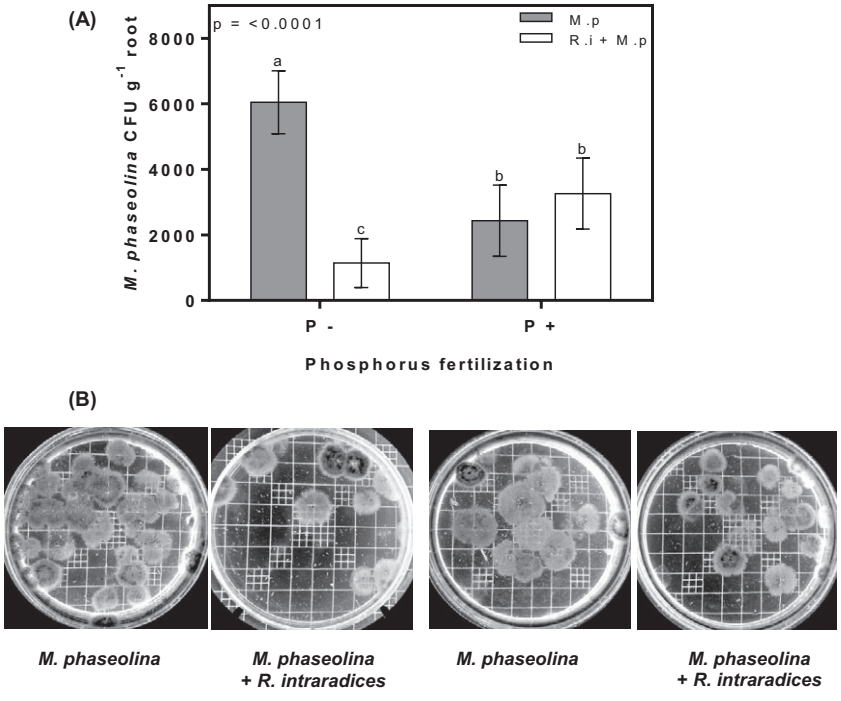


**Figure 2:** (A) Estimation of disease severity measured by counting of CFU $g^{-1}$ soybean roots in the presence of AMF *R. intraradices* and P fertilization. R.i = *R. intraradices*; M.p = *M. phaseolina*. Vertical bars represent the standard deviation. Each value represents the mean value obtained from ten replications. Different letters indicate significant differences (Tukey test P < 5%). (B) CFU of *M. phaseolin*a from root tissues of infected plants on semi-selective medium. This counting was used as a measure of disease severity.

corrhizal spores and external mycorrhizal hyphae. Therefore, it is possible that these parameters have also been negatively affected in our experiment. Anatomical differences in AMF infections, particularly a decrease in arbuscule formation, associated with P fertilization have been reported (*Trinick*, 1977). However we found increases in arbuscules percentage in fertilized plants, which could be due to a high P demand at the soybean growth stage. Moreover, *Schreiner* and *Scagel* (2016) showed that arbuscule frequency is reduced by boosting P status only in plants grown under low light when carbon is limited. Our soybean plants were grown under optimal light conditions, which could also explain the high percentage of arbuscules. On the other hand, P reduced but not suppressed the mycorrhizal root protection against the pathogen. The effect of P application on AMF may be caused by reductions in mycorrhizal entry points, alteration of characteristics of root colonization, and markedly diminution AMF biomass per plant, including both biomass in soil and in roots (*Trinick*, 1977; *Smith* and *Read*, 2010). Analyzing the pathogen, the improvement in mineral nutrition could be the reason that P fertilization reduced disease severity (*Krupinsky* et al., 2002). These interactions (AMF + P fertilization; *M. phaseolina* + P fertilization), *plus* environmental factors, such as temperature, moisture and soil nutritional status, could explain the discrepancy between our results and those of the others authors. Our research is the first study that analyzes the simultaneous effects of P fertilization, AMF inoculation, and *M. phaseolina* infection on soybean.

## 5 Conclusion

Phosphorus fertilization and AMF negatively affected the pathogenic fungus and its relationship with soybean plants. Phosphorus is a crucial nutrient for soybean that could reduce its susceptibility to *M. phaseolina* infection. However, P reduced the development of the mycorrhiza and the arbuscular mycorrhizal colonization, thus partially decreasing its protection against the phytopathogenic fungus. The simultaneous effect of P fertilization and AMF on the protection against the damage caused by *M. phaseolina* in soybean should be taken into account in integrated management practices, because an excess in P fertilization could reduce the arbuscular mycorrhizal protection against charcoal rot.

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