Eur J Plant Pathol
https://doi.org/10.1007/s10658-020-01934-w

Arbuscular mycorrhiza protects soybean plants against *Macrophomina phaseolina* even under nitrogen fertilization

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Accepted: 16 January 2020

Abstract The capacity of arbuscular mycorrhizal fungi (AMF) to alleviate the negative effects incited by root pathogens in a range of plant hosts has been established. On the other hand, accumulated evidence also shows that fertilization practices can negatively impact AMF. Nevertheless, the interaction between AMF, pathogens and fertilizers, especially nitrogen (N) fertilizers, has not been previously reported. In this work, the effect of nitrogen on both the severity of the pathogen *Macrophomina phaseolina* (charcoal root rot) and the protection by the arbuscular mycorrhiza fungi (AMF) *Rhizophagus intraradices* was investigated in greenhouse experiments using soybean (*Glycine max*) as a host. The treatments were two levels of N (0 and 92 kg of urea ha^{-1}), inoculation and non-inoculation with the AMF, and infection and non-infection with the pathogen. Soybean was harvested at R4 phenological stage (completed pod formation). Plant biomass, numbers of pods and leaves, plant height, root length, greenness index, mycorrhizal colonization and disease severity were measured. Pathogen infection reduced soybean biomass and negatively affected the greenness index, but co-inoculation with AMF improved these parameters. Nitrogen fertilization reduced AMF colonization but not arbuscules percentage. N fertilization increased disease severity but mycorrhizal symbiosis was able to reduce it. These results demonstrate that severity of charcoal root-rot disease in N fertilized soybean can be reduced by AMF inoculation. The implication of these results is that N fertilization could increase the risk of diseases in soybean but mycorrhiza could contribute to soybean charcoal root rot control even if the crop is under N fertilization.

Keywords Nitrogen fertilization · Biological control · Charcoal rot · Fungal diseases

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10658-020-01934-w) contains supplementary material, which is available to authorized users.

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Introduction

Most terrestrial plants have high requirements of nitrogen (N) and respond to its application. Leguminosae can meet N needs by both N_2 fixation and soil nitrate absorption. Although the inhibitory effect of high levels of soil nitrate on *Rhizobium*-legume symbiosis is known, N fertilization is very often indicated to increase soybean yields. Nitrogen application can be applied early in the crop cycle or late in the growing season, but does not always significantly increase yield (Gutiérrez-Boem et al. 2004; Gai et al. 2017; La Menza et al. 2017). Soybean (*Glycine max*) is an important crop worldwide

Published online: 24 January 2020

since it is present in several industrial products of nutritional and commercial value for human and animal food. Its production is challenged by phytopathogenic fungi, which cause considerable yield and quality losses (Al-Askar et al. 2014). Charcoal root rot, caused by *Macrophomina phaseolina* (Tassi) Goid is one of the most destructive soybean diseases. This fungus is a soilborne necrotrophic pathogen distributed worldwide. Charcoal root rot may be problematic to control because of the nature of the pathogen. The causal agent is a fungus with a broad host range, able to infect more than 800 plant species (Farr and Rossman 2019), including a broad spectrum of crops like soybean, sorghum, cotton, beans, sunflower, and maize, among others. The disease symptoms include yellow leaves, brown coloration of stems and roots, premature senescence and even plant death (Romero Luna et al. 2017). *M. phaseolina* infection develops black specks within the lower plant stem and brown or black streaks in the vascular tissue of the stems (Romero Luna et al. 2017). The control of the disease is challenging because *M. phaseolina* can survive for long periods in soil as sclerotia, and is disseminated by soil, wind and infected crop residues (Romero Luna et al. 2017).

The control of charcoal root rot is complicated because applications of chemical fungicides, soil tillage practices or crop rotation are ineffective for complete management of *M. phaseolina* (Chamorro et al. 2015). On the other hand, chemical products present negative environmental impact and the possibility of development of fungicide-resistant fungal isolates. Consequently, an environmentally acceptable alternative is the use of competitive antagonistic microorganisms as biocontrol agents that are capable of promoting plant growth and controlling diseases (Pascual 2016). Several studies have demonstrated the beneficial effects of antagonist microorganisms on disease control; however most studies have used non obligate biotrophic filamentous fungi, such as *Trichoderma* (Contreras-Cornejo et al. 2016; Sumida et al. 2018). Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs that have a mutualistic association with most land plants and have the potential to control some fungal plant pathogens (Olawuyi et al. 2014; Filho et al. 2016; Eke et al. 2016). *Rhizophagus intraradices* is one of the most studied AMF due to their easy propagation in pot culture (Malbreil et al. 2014). The protective effects of AMF against root pathogenic fungi are the result of complex interactions between pathogen, AMF and the host plant (Harrier and Watson 2004). Several mechanisms are involved in mycorrhizal protection against plant pathogens and may include direct competition with the pathogen for the hosts photosynthetic products and colonization sites, and several indirect mechanisms such as the morphological modification of the plant root, root damage compensation, plant health and nutritional status improvement, induction of plant resistance and alteration of the rhizospheric microbial composition (Majewska et al. 2017). There is limited evidence about the effect of arbuscular mycorrhizal symbiosis on the control of charcoal root rot (*M. phaseolina*) of soybean (Doley and Jite 2012).

It is known that phosphorus and nitrogen fertilization can progressively reduce arbuscular mycorrhizal symbiosis (Alguacil et al. 2008; Liu et al. 2012; Spagnoletti et al. 2018) and also modify fungal disease incidence and severity (Mahmood and Bashir 2011; Spagnoletti et al. 2018). It has been shown that nitrogen fertilization reduced mycorrhizal root colonization in Asteraceae and Poaceae (Blanke et al. 2011) and, on the other hand, in general increased plant disease severity of hemibiotrophic, biotrophic and necrotrophic fungal pathogens (Veresoglou et al. 2013). Moreover, high N concentration reduces the production of phenolic compounds (fungistatic) and lignin content, impairing resistance to pathogens (Marschner 1986). However, plant-pathogen interactions in response to N availability are complex and do not always follow this trend (Lecompte et al. 2010; Huber and Haneklaus 2007).

The aim of the current study was to evaluate the effects of N fertilization on the relationship between the AMF *R. intraradices* and the pathogen *M. phaseolina* in soybean. The effects of N on both the severity of the pathogen and the AMF protection against the charcoal root rot disease were analyzed. The hypothesis tested was that N fertilization increase charcoal root rot severity and negatively affects mycorrhizal symbiosis.

Materials and methods

Microorganism, culture condition and inoculum preparation

The AMF (*Rhizophagus intraradices* VCh 0011) infested soil was obtained from the Fungi Bank of the Microbiology Department of the School of Agriculture,

University of Buenos Aires (UBA). The fungus was propagated in pot culture with *Trifolium repens* and *Sorghum bicolor* plants grown in a sterile sandy loam soil for four months in 250 cm^3 pots. Then, the aerial parts of the plants were cut above the soil surface and the soil was allowed to dry completely in the pot. In order to evaluate the quality of the inoculum, the total number of *R. intraradices* spores (Gerdemann and Nicolson 1963) and the percentage of root colonization were determined (Phillips and Hayman 1970; McGonigle et al. 1990). The AMF inoculums, containing colonized root fragments, spores and rhizospheric soil, was used for inoculating soybean seeds.

The pathogen used in this study was *M. phaseolina* (VCh 0018) stored in the Fungi Bank of the Microbiology Department of the School of Agriculture, University of Buenos Aires (UBA). The pathogen inoculum was cultivated in rice grains according to Spagnoletti et al. (2016). Sterile Erlenmeyer flasks of 500 mL capacity were filled with 100 g of water-soaked rice grains, which were then sterilized at a pressure of 15 lbs. for 20 min. Mycelia discs of 5 mm from the active periphery of a 7-day-old culture of the pathogen grown on potato dextrose agar (PDA) were inoculated on sterilized rice seeds and were incubated for one month at 28 °C ± 1 °C in the dark.

Experimental conditions

Two pot experiments with soybean (cultivar NIDERA 4990, rhizobial free) were conducted from December to February for two consecutive years (2015 and 2016) in a glasshouse located in the campus of the School of Agriculture, University of Buenos Aires (FAUBA), Argentina.

The plastic pots (1000cm^3) contained a sterilized growing mixture of soil:sand:perlite (7:3:2, *v*/v/v). 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 physical composition of the substrate was 18% clay, 11% silt, and 71% sand and the chemical characteristics of the original soil were 17.4 g kg^{-1} of organic carbon (Walkley and Black method), pH 6.9, with 34.7 mg kg^{-1} phosphorus availability (Kurtz and Bray No 1 method) and 0.36 dSm^{-1} electrical conductivity (soil saturation extract) (Sparks et al. 1996).

Experimental design

Each greenhouse experiment was considered a block and within them the experimental design was factorial in a completely randomized design (CRD) with ten replications. The air temperature ranged between 25 and 37 °C and the photoperiod varied between 12 and 13 h. During the experiment, the maximum photosynthetic photon flux density (Q) was around 1800 μmol m^{-2} s^{-1}.

The treatments were: non-fertilized (control treatment) and fertilized with an equivalent to 15 kg of N ha^{-1} applied every 15 days as urea; two levels of AMF inoculation (inoculated and non-inoculated), and two levels of infection with *M. phaseolina* (infected and non-infected). Soybean seeds (*Glycine max* L. cv. NIDERA 4990, rhizobial-free) were superficially disinfected 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 non-sterilized deionized water. Before sowing the seeds, 10 g of AMF general inoculant (containing colonized root fragments, rhizospheric soil, and approximately 100 spores g^{-1} dry soil) was added to the corresponding treatment at the depth of 3 cm in soil. The pathogen inoculum was incorporated into the substrate at a rate of 8 g per pot, after 15 d of soybean growth (Spagnoletti et al. 2018). Propagule concentration was determined by plating the inoculum on PDA medium. The population of *M. phaseolina* was 1×10^4 colony-forming units (CFU) g^{-1}. After 70 days (R4 soybean phenological stage; Fehr and Caviness 1977), entire plants (aerial and root biomass) were harvested.

Biomass production, morphological parameters

Plant height and number of leaves were recorded. Aerial biomass was separated into leaves, shoots, pods, and grains. Roots were cut and washed to remove the soil. Root length was determined by the line intersection method according to Tennant (1975). All samples were rinsed with distilled water, dried in a forced hot-air oven at 80 °C until a constant weight was reached then measured.

Symbiotic development

Sub-samples of fresh roots were collected and stained following the protocol of Phillips and Hayman (1970). The percentage of total AMF colonization and the arbuscules percentage were estimated microscopically (Nikon H550S). One hundred randomly selected stained root pieces of 1 cm were mounted on slides and examined microscopically; each root piece was examined in three different microscope fields of view (McGonigle et al. 1990).

Leaf greenness index (SPAD)

Leaf greenness index (SPAD) was measured nondestructively at the end of the experiment, with a portable chlorophyll meter (SPAD-502, Minolta Corp., Ramsey, New Jersey, USA). This index was used to assess plant nitrogen status (Gianquinto et al. 2004). The average of six measurements taken on the upper leaves from soybean plants of each treatment was recorded.

Evaluation of impact of R. intraradices on disease incidence and severity

The percentage of disease incidence in infected soybean plants was calculated (Persson et al. 1997).

Disease severity was determined counting *M. phaseolina* CFUs per gram of root; following the technique described by Mengistu et al. (2007). The roots were dried in an oven at 40 °C for 7 days. The dried material was ground and passed through a 1-mm mesh sieve. Then, 5 mg of each powdered root was placed in Eppendorf tubes (2 ml) with 3% NaOCl for 3 min, and washed three times with sterile distilled water. The disinfected material was poured into 90 mm Petri dishes containing 5 mL of PDA medium previously autoclaved, and amended with rifampicin (100 mg L^{-1}) and metalaxyl (224 mg L^{-1}). The plates were incubated at 28 °C in the dark for 3 days and the numbers of colony forming units of *M. phaseolina* (CFU g^{-1} root) were counted to determinate disease severity.

Statistical analysis

All values presented in the text are expressed as mean ± standard deviation of 20 replicates (10 replicates × 2 experiments). Results were analyzed with INFOSTAT software (Balzarini et al. 2008). Separate multivariate analyses were carried out using principal component analysis (PCA). Analysis of variance (ANOVA) and Tukey's tests were applied to determine the significant differences between treatments. Results were considered statistically significant when $p < 0.05$. In all cases, the assumptions of normality and homogeneity of variance were verified using the INFOSTAT software.

Results

Morphological parameters and mycorrhizal colonization

Table 1 shows the effects of different treatments on soybean. Morphological parameters were negatively affected by the pathogen, especially aerial biomass and pod numbers. Aerial biomass was significantly lower in plants infected with the pathogen, and higher in N-fertilized, AMF-inoculated plants. No effect of N fertilization was observed in control and co-inoculated plants (AMF + Pathogen). However, co-inoculated plants showed similar aerial biomass to the control treatment ($p > 0.05$). The number of pods followed the same pattern that aerial biomass, being negatively affected by *M. phaseolina* infection. N fertilization increased the detrimental effect of the pathogen ($p < 0.0001$). The number of leaves increased in fertilized AMF-inoculated plants and in co-inoculated plants regardless of nitrogen level; the rest of the treatments showed lower values of this parameter ($p = 0.0276$). Plant height was significantly reduced by the pathogen regardless of nitrogen fertilization ($p = 0.0002$). On the other hand, root biomass was severely affected by the presence of the pathogen. Nitrogen fertilization significantly intensified the negative effect of the pathogen ($p = 0.0036$). No significant differences between treatments were found in root length ($p > 0.05$).

Figure 1 shows AMF colonization in soybean roots. Arbuscular mycorrhizal root colonization was reduced by nitrogen fertilization, by pathogen infection in roots and also by pathogen infection in N-fertilized plants (N + Pathogen) ($p = 0.0079$). The percentage of reduction was about 28% for all treatments (Fig. 1a). A different pattern was found in arbuscules percentage (Fig. 1b), which was higher in *M. phaseolina* infected-plants (116%), that found in the control treatment. On the other hand, nitrogen fertilization and co-inoculated treatment did not modify the arbuscules amount ($p > 0.05$).

Table 1 Effect of nitrogen fertilization (N+), Arbuscular mycorrhizal fungus (*R. intraradices*) and Pathogen (*M. phaseolina*) on morphological parameters of soybean plants

Treatment	Control		AMF		Pathogen		AMF + Pathogen	
	N -	N +	N -	N +	N -	N +	N -	N +
Aerial biomass (g)	3.32 ± 0.39 b	3.66 ± 0.37 b	2.99 ± 0.13 b	4.30 ± 0.96 a	2.27 ± 0.88 c	3.00 ± 0.44 b	3.56 ± 0.63 b	3.76 ± 0.35 b
Pods	14.00 ± 1.49 b	13.68 ± 1.32 b	15.30 ± 2.15 b	18.23 ± 1.27 a	11.10 ± 1.26 c	5.23 ± 1.05 d	13.25 ± 1.15 b	15.47 ± 1.48 b
Leaves	10.00 ± 0.81 b	11.25 ± 0.96 b	11.50 ± 0.57 b	14.75 ± 0.50 a	9.75 ± 1.26 b	10.50 ± 1.00 b	13.50 ± 0.57 a	14.25 ± 0.96 a
Plant height (cm)	30.12 ± 4.09 a	34.12 ± 2.78 a	30.25 ± 1.89 a	33.50 ± 1.08 a	24.00 ± 2.16 b	27.00 ± 2.16 b	32.25 ± 1.19 a	32.62 ± 1.88 a
Root biomass (g)	1.67 ± 0.17 a	1.22 ± 0.46 b	1.37 ± 0.33 b	1.02 ± 0.29 b	0.95 ± 0.51 b	0.60 ± 0.11 c	1.05 ± 0.24 b	1.15 ± 0.13 b
Root length (cm)	29.00 ± 1.41 a	28.00 ± 9.79 a	30.75 ± 5.96 a	23.75 ± 4.85 a	21.25 ± 2.75 a	28.25 ± 6.80 a	27.5 ± 1.91 a	29.25 ± 6.02 a

Each value represents the mean obtained from 20 replicates ± standard deviation. Row values for each variable followed by different letters are significant different according to Tukey ($p < 0.05$)

Leaf greenness index (SPAD)

Figure 2 shows the greenness index (SPAD units), correlated with the chlorophyll content of soybean plants. Fertilization with N resulted in higher greenness index in soybean leaves, while mycorrhizal fertilized plants exhibited the same SPAD values as the N-fertilized control. Pathogen-infected, non-fertilized plants exhibited the lowest SPAD units. However, both co-inoculated treatments (AMF + Pathogen) had SPAD values similar to that of non-fertilized, AMF-inoculated plants ($p > 0.05$).

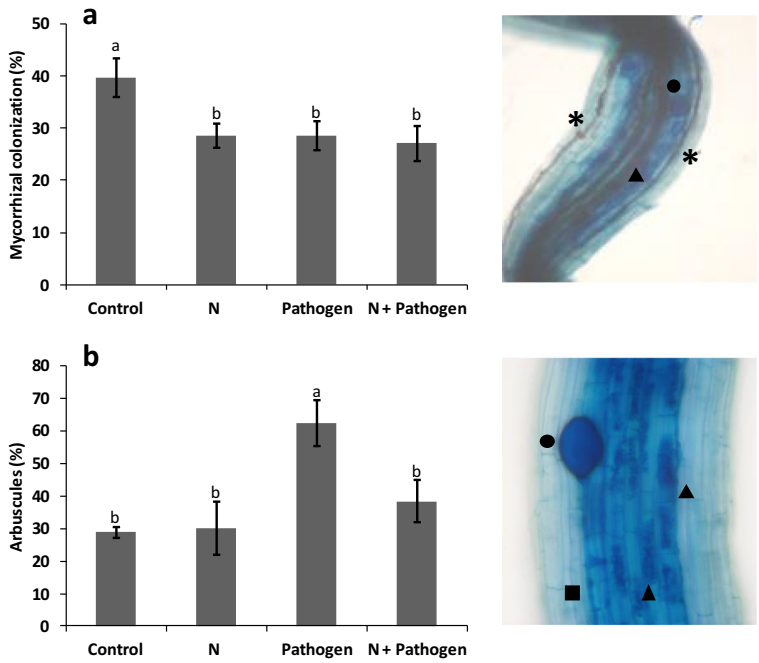

Fig. 1 Arbuscular mycorrhizal colonization (**a**) and arbuscules percentage expressed as a percentage of the colonized root (**b**) with fertilization of N and the presence of *M. phaseolina*. Each value represents the mean value obtained from 20 replicates. Vertical bars represent standard deviation. Different letters indicate significant differences (Tukey test $p < 0.05$). Photographs from Trypan blue stained roots under stereoscopic microscope (100X and 400X) showing soybean root colonization. Symbols: black squares denote the presence of *R. intraradices* intraradical hyphae; triangles denote arbuscules, circles denote vesicules and asterisks denote the presence of *M. phaseolina* hyphae

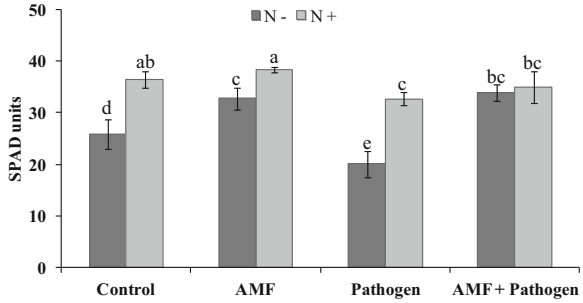

Fig. 2 Mean SPAD units, correlated with chlorophyll content of soybean plants under different treatments: Control, arbuscular mycorrhizal fungi (AMF) inoculated plants, *M. paseolina* infected plants (Pathogen) and Co-inoculated plants (AMF + Pathogen). Dark columns denote non fertilized plants (N-) and grey columns denote fertilized plants (N+). Data represent means (± standard deviation) of six observations. Different letters indicate significant differences (Tukey test $p < 0.05$)

Disease incidence and severity

Figure 3 shows the disease incidence and severity as a result of inoculation of soybean plants with *M. phaseolina*. The incidence of disease was 100% in pathogen infected plants, regardless of the fertilization treatment. On the contrary, AMF inoculation reduced the incidence to 80% in non-fertilized plants and to 90% in N-fertilized treatment (Fig. 3a).

The CFU counts in soybean roots showed that root rot disease increased in N-fertilized plants around 41% with respect to non-fertilized ones. Additionally, AMF inoculation reduced the amount of pathogen CFU at all N evaluated levels ($p = 0.0174$). The percentages of disease control by AMF were 42% in plants without fertilization and 49% in N-fertilized plants (Fig. 3b).

Multivariate analysis

Principal component analysis (PCA) showed that the first two components explained 71.8% of the variation of the different treatments with respect to all parameters. The distribution of different treatments and study parameters in the space of components 1 and 2 are shown in Fig. 4. Plants inoculated with *R. intraradices* were located on the positive side of PC1, while treatments without inoculation with mycorrhizal fungus were located on the negative side of the same axis. Moreover, the treatments inoculated with *M. phaseolina* were located on the negative side of PC2, while the non-inoculated treatments with the pathogen were located on the positive side. The disease severity was located towards the negative side of PC1 (50.5%), leaving the rest of the parameters on the positive side of the same axis. On the other hand, the radical biomass, root length and the number of pods were grouped on the positive side of PC2 (21.3%). The contributions of study parameters are shown in Supplementary Table 1.

Discussion

The results of this study clearly indicate that the symbiotic relationship between soybean and AMF can be established in the presence of *M. phaseolina.* Arbuscular mycorrhizal fungal inoculation reduced the disease severity even in N-fertilized soybean plants. Moreover, AMF inoculation improved the morphological parameters of soybean plants grown in N-fertilized substrate. The higher number of leaves observed in AMF-inoculated plants likely increased the photosynthetic rate which, in turn, increased the number of pods. Furthermore, mycorrhizal inoculation reduced the negative effects of *M. phaseolina* in several morphological parameters. These results are in line with Akhtar and Siddiqui (2008) who found that inoculation with *G. intraradices* significantly increased shoot dry mass and number of pods of *M. phaseolina*-inoculated chickpea plants.

In the current study, nitrogen fertilization had a significant and negative effect on plant symbiosis with the mycorrhizal fungus. Maximum mycorrhizal colonization percentage was observed in AMF-inoculated plants without N fertilization or pathogen infection. Previously, Liu et al. (2012) showed a progressive reduction in AMF colonization in *Elymus nutans* (Poaceae) plants supply with N. Cornejo et al. (2008) showed that nitrogen fertilization influences plant growth and rhizospheric properties, affecting the persistence and functionality of AMF in several crop systems. In contrast, Cornejo et al. (2009) reported that mycorrhizal colonization was not affected by addition of N in wheat and even observed increased mycorrhization in oat. Such differences in the effect of N fertilization on mycorrhizal colonization could be due to phosphorus (P) levels. Johnson et al. (2003) studied the AMF responses to experimental N enrichment at five grasslands distributed across North America and showed that when P is not limiting, N enrichment generally results in a decline in AMF biomass in soil, while in P deficient soils, N enrichment increased AMF biomass. In the current

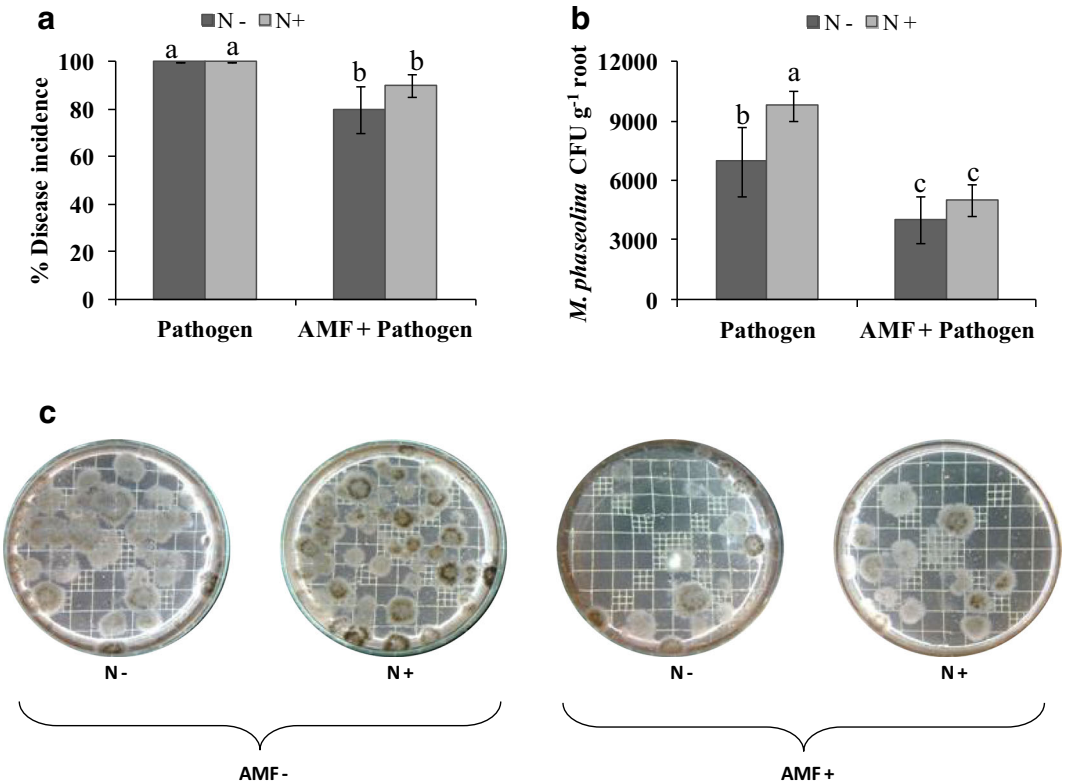

Fig. 3 Estimation of disease incidence (**a**) and severity (**b**) measured by counting of *M. phaseolina* CFU g^{-1} soybean roots in the presence of arbuscular mycorrhiza (AMF) and nitrogen (N) fertilization. Control treatment and AMF inoculated plants did not present incidence or severity of disease. Each represents the mean obtained from 20 replicates. Vertical bars represent the standard deviation. Different letters indicate significant differences (Tukey test $p < 0.05$). **c** CFU of *M. phaseolina* from root tissues of infected plants on semi-selective medium

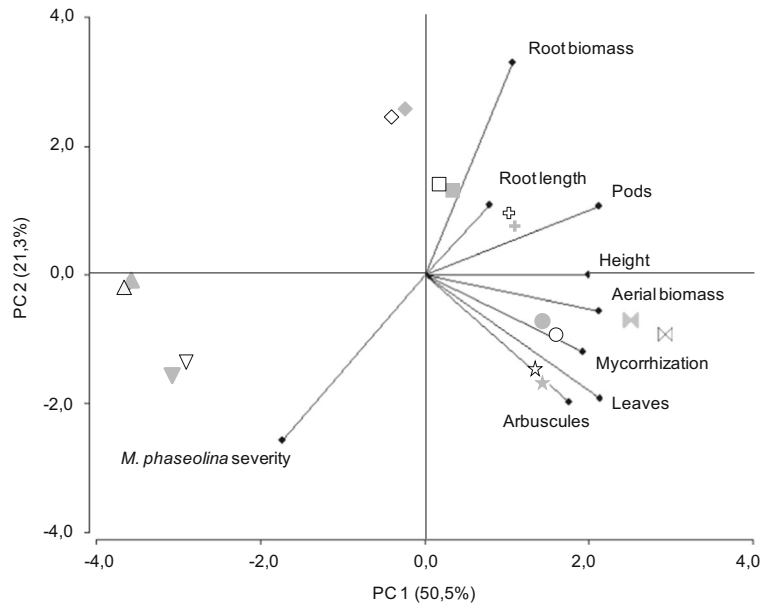

Fig. 4 Principal component analysis of analyzed parameters. Empty and filled symbols correspond to the mean of experiment one and two, respectively. Shape of symbol represents treatments: Control (diamonds), Arbuscular mycorrhiza fungus (cross), Pathogen (triangles), Nitrogen (N) (squares), AMF + Pathogen (stars), N + AMF (keys), N + Pathogen (inverted triangles), N + AMF + Pathogen (circles)

study, the content of P in the substrate was not deficient (see soil properties); therefore the amount of applied nitrogen could be the reason for the low root colonization percentage found. In contrast, Tian et al. (2013) established that AMF colonization of maize roots was not affected by N fertilization rate but was strongly influenced by plant phenological stage. In our experiments, we found that N fertilization did not reduce arbuscule percentage. This could be due that intraradical structures are less responsively to N fertilization than extraradical structures. According to Johnson et al. (2003), under soil high nutritional conditions, plants invest relatively more resources in above ground biomass at the expense of root biomass; so number of arbuscules and extraradical hyphae can be reduced. Hart and Reader (2002) showed that the Glomeraceae family, including *R. intraradices*, produced four times less extraradical hyphae than Gigasporaceae, which could make it less susceptible to N enrichment (Johnson et al. 2003).

The negative effect of *M. phaseolina* on AMF colonization of soybean roots has been recognized for several years ago (Zambolim and Schenck 1983). In the current study it was found that the percentage of arbuscules was increased in co-inoculated plants (*M. phaseolina* + AMF) as compared with plants inoculated with only AMF. This result indicated that the presence of a pathogen may not negatively affect the succes of mycorrhizal symbiosis (arbuscule percentage). Since arbuscles are the site of nutrient exchange between the plant and the fungus, that suggests that there is an exchange activity occurring between soybean and *R. intraradices*. These results are in accordance with Spagnoletti et al. (2017) and with Slezack et al. (2000) who found in *Pisum sativum* a similar arbuscule percentage (*G. mosseae*) in *Aphanomyces euteiches*-infected as uninfected plants. Nevertheless, the current results are in contrast to Doley and Jite (2012), who found a decrease in the percentage of arbuscules of *G. fasciculatum* in peanut plants infected by *Sclerotium rolfsii* and *M. phaseolina.* The discrepancy in these results could be related to the time of mycorrhizal inoculation (before or after pathogen infection) and the complex interaction between AMF-pathogen-host and their interaction with the environment.

Higher SPAD readings were observed in plants inoculated with AMF; this is likely to be correlated with an improved plant nitrogen status (Gianquinto et al. 2004) and was in agreement with results shown previously (Vicente-Sánchez et al. 2014). It is known that mycorrhizal symbiosis can enhance nutrient uptake, such as N (Smith and Read 2010); this would explain the high nutritional status observed in mycorrhizal plants in the current study. Moreover, low leaf greenness in *M. phaseolina* infected plants was observed; this could be due to the etiology of 'charcoal rot'. Symptoms of the disease include necrotic root and crown rot accompanied by plant wilting and chlorosis of leaves. The positive effect of *R. intraradices* was also shown in the higher SPAD readings that are highly correlated with chlorophyll content, in co-inoculated treatments.

Higher nitrogen levels in crop tissue have frequently been associated with increased levels of diseases, particularly in roots (Drinkwater et al. 1995). Sanchez-Bel et al. (2016) stated that high N fertilization may contribute to the activation of increased virulence of *Botrytis cinerea* in tomato. The results of the current study were in line with these studies since N fertilization increased charcoal rot disease caused by *M. phaseolina*. Similar results were found in tomato, potato and wheat, where a significant increase of disease caused by *Colletotrichum phomoides* (Williams 1965), *Verticillium albo-atrum* (Wilhelm 1950) and *Fusarium graminearum* (Lemmens et al. 2004), respectively, was observed with increasing N input. In the current study, *R. intraradices* improved plant growth of *M. phaseolina*-infected plants by reducing pathogen multiplication as shown for other AMF (Doley and Jite 2012). The current results showed that the root-rot severity of plants inoculated with the pathogen was also reduced by *R. intraradices* demonstrating that AMF can effectively protect soybean plants against *M. phaseolina.* This is in agreement with the work of Smith and Read (2010) who reported AMF as one of the most important groups of soil organisms playing a critical role in the plant protection against pathogens, under greenhouse and field conditions. Several reports indicate that AMF is an effective biocontrol agent against pathogens (Cordier et al. 1998; Berg et al. 2007; Spagnoletti et al. 2017), supporting the current results. In accordance with Sikes (2010) who studied the effect of *Fusarium oxysporum* in *Setaria glauca* and in a review of Wehner et al. (2010), disease reduction by *R. intraradices* might be related to competition between the two fungi (*M. phaseolina* and *R. intraradices*) for common resources in the roots, such as space, infection sites and photosynthates. However, other mechanisms are likely involved in mycorrhizal protection against pathogens (Majewska et al. 2017).

Conclusion

The present study demonstrates that the AMF *R. intraradices* has the potential to control soybean charcoal root rot caused by *M. phaseolina* even if the crop is N fertilized. Moreover, N fertilization could increase the risk of this disease in soybean. This approach helps in minimizing fungicide application for management of fungal root diseases in soybean crop. Nevertheless, it is necessary for further research into the possible interactions with environment and other crop practices such as soil management and soybean genotype.

Acknowledgments This work was funded by the Agencia Nacional de Promoción Científica FONCYT (PICT-2857) and the Universidad de Buenos Aires (UBA) (UBACYT 068/2011).

Compliance with ethical standards

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

Informed consent All authors consent to this submission.

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