

Use of the *Glomus etunicatum* as biocontrol agent of the soybean cyst nematode

Uso do *Glomus etunicatum* como agente de biocontrole do nematóide de cisto da soja

Uso del *Glomus etunicatum* como agente de biocontrol del nematodo del quiste de la soja

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Abstract

This study investigated the effect of arbuscular mycorrhiza (*Glomus etunicatum* Becker & Gerd.), on the cyst nematode (*Heterodera glycines* Ichinohe), in a greenhouse. Mycorrhizal and non-mycorrhizal soybean plants were exposed to the pathogen at different initial population densities (0, 500, 1000, 2000, and 4000 nematodes eggs). Soybean growth, nematode reproduction, and the arbuscular mycorrhizal fungus's capacity to decrease disease pressure were determined after 60 day-olds. The height of the plants was increased by 26% in the presence of arbuscular mycorrhizal fungus (AMF) despite of a higher initial population of cyst nematodes. The root length was, on average, 32.20% was greater in the presence of AMF. The number of nematodes females found in the root system of mycorrhizal plants was 28.21% lower than in non-mycorrhizal roots. These results suggest that AMF *G. etunicatum* acts indirectly, promoting an improvement in the nutritional plant status creating tolerance to the presence of the pathogen by soybean.

Keywords: Biological control; *Heterodera glycines*; *Glomus etunicatum*; Mycorrhizal fungi.

Resumo

Este estudo investigou o efeito da micorriza arbuscular (*Glomus etunicatum* Becker & Gerd.), no nematóide de cisto da soja (*Heterodera glycines* Ichinohe) em casa de vegetação. Plantas de soja micorrizada e não micorrizada foram expostas ao patógeno em diferentes níveis de densidade populacional inicial (0, 500, 1000, 2000 e 4000 ovos de nematóides). O crescimento da soja, a reprodução do nematóide e a capacidade do fungo micorrízico arbuscular em diminuir a pressão da doença foram determinados após 60 dias da emergência das plantas. A altura das plantas foi aumentada em 26% na presença de fungo micorrízico arbuscular (FMA) apesar do alta população inicial do nematóides de cisto. O comprimento da raiz foi, em média, 32,20% maior na presença de FMA. O número de fêmeas do nematoide encontradas no sistema radicular das plantas micorrizadas foi 28,21% menor do que nas raízes não micorrizadas. Esses resultados sugerem que AMF *G. etunicatum* atua de forma indireta, promovendo uma melhora no estado nutricional da planta e criando tolerância à presença do patógeno na soja.

Palavras-chave: Controle biológico; *Heterodera glycines*; *Glomus etunicatum*; Fungos micorrízicos.

Resumen

Este estudio investigó el efecto de la micorriza arbuscular (*Glomus etunicatum* Becker & Gerd.), el nematodo del quiste (*Heterodera glycines* Ichinohe) en un invernadero. Plantas de soja micorrízicas y no micorrízicas se expuso al patógeno en diferentes niveles de densidad poblacional inicial (0, 500, 1000, 2000 y 4000 huevos de nematodos). Después de 60 días se determinó el crecimiento de la soja, la reproducción de nematodos y la capacidad del hongo micorrízico arbuscular para disminuir la presión de la enfermedad. La altura de las plantas se incrementó en un 26% en presencia de hongo micorrízico arbuscular (HMA) a pesar de la alta poblacion inicial de nematodos quísticos. La longitud de la raíz fue, en promedio, 32,20% mayor en presencia de HMA. El número de hembras de nematodos

encontradas en el sistema radicular de las plantas micorrízicas fue 28,21% menor que en las raíces no micorrízicas. Estos resultados sugieren que AMF *G. etunicatum* actúa indirectamente, promoviendo una mejora en el estado nutricional de la planta y creando tolerancia a la presencia del patógeno en la soja.

Palabras clave: Control biológico; *Heterodera glicinas*; *Glomus etunicatum*; Hongos micorrízicos.

1. Introduction

Nematodes constitute a diverse group of soil organisms. Plants and animals have free-living genera and parasitic genera (Ferraz & Brown, 2002). Plant-parasitic nematodes are classified as endoparasites or ectoparasites based on their parasitism strategy (Perry & Moens, 2001). Ectoparasites use their stylet to acquire food from the cells of the epidermis or root cortex, while endoparasites enter completely into the root cells and feed within the roots of the host plant. Endoparasites have the most complex feeding strategy among phyto-parasites. They select cells in the central root cylinder, converting them into feeding sites and making them their habitat and food source (Gheysen & Mitchum, 2011). This last group includes cyst nematodes, which, together with root gall nematodes, are considered the parasites most harmful to agricultural plants in the world (Bartlem et al., 2014; Ibrahim et al., 2019; Jones et al., 2013). They can cause significant damage to crop (Wesemael et al., 2011). Estimates indicate that nematode disease causes more than one billion dollars of productivity losses per year in American crops alone (Koenning & Wrather, 2010).

Among the species of cyst nematodes most damaging to crops are the cereal cyst nematode (*Heterodera avenae* Wollenweber), which causes damage at an estimated rate of 20% to 90% in wheat and oats in different locations in the world (Toumi et al., 2018; Wu et al., 2014), and the soybean cyst nematode (*Heterodera glycines* Ichinohe), which is one of the most devastating soybean pests (*Glycine max* (L.) Merrill) (Guo et al., 2020). The latter species of nematode produces a survival structure known as a cyst, which consists of the female's body and contains hundreds of eggs protected by a layer of cuticle. Eggs inside cysts can survive in the soil for more than 10 years and produce viable nematodes (J2) under favorable conditions (Haarith et al., 2020). Production losses caused by plant-parasitic nematodes tend to increase due to changes in climate and intensified production systems (Nicol et al., 2011). Studies show that the density of cyst nematodes increases after consecutive years of soybean monoculture, correlating with the decline in crop productivity (Grabau & Chen, 2016; Strom et al., 2020). The use of nematicides to control nematodes has also been limited due to increased concerns about human health and environmental sustainability, which have led to the prohibition of several chemical molecules (Hol & Cook, 2005; Schouteden et al., 2015).

New nematicide alternatives are being studied (Ruanpanun & Chamswarn, 2016; Wesemael et al., 2011). Several organisms have been tested as potential cyst nematode biocontrol agents, including certain fungi and bacteria (Haarith et al., 2020). One of these new nematicide alternatives is arbuscular mycorrhizal fungi (AMFs), which are obligatory symbionts that can protect their hosts against stress factors, such as plant parasitic nematode infections (Schouteden et al., 2015). There is a consensus in the literature that AMFs can improve host tolerance and increase plant resistance to nematode attacks (Gianinazzi et al., 2010; Hol & Cook, 2005; Pozo & Azcón-Agüilar, 2007; Rasmussen et al., 2019; Schouteden et al., 2015; Singh et al., 2011; Vos et al., 2012; Whipps, 2004). Microorganisms are promising alternative nematode biocontrol agents (Alban et al., 2013; Haarith, et al., 2020; Hol & Cook 2005; Ma et al., 2017; Nimnoi et al., 2020; Ruanpanun et al., 2016;). However, much remains to be studied regarding the mechanisms of this biocontrol (Herrejón-Alvarado et al., 2019). AMFs may have direct effects on the pathogen through competition for space or nutrients, or indirect effects through better plant nutrition or increased root system resistance (Gianinazzi et al., 2010; Sohrabi et al., 2020). These different mechanisms cannot be considered completely independent, as biocontrol probably results from a combination of them (Cameron et al., 2013).

Technological advances in science, such as the genome sequencing of some nematodes (Abad et al., 2008; Cotton et al., 2014) and of some host plants, such as soybeans (Scmutz et al., 2010), have improved the understanding of the

mechanisms involved in these AMF effects (Schouteden et al., 2015). Greater attention has also been paid to the interactions between plant roots and microorganisms (De Coninck et al., 2015) and to the biotic and abiotic factors involved in this process. Thus, this work aimed to evaluate the potential of mycorrhizal fungus *Glomus etunicatum* as a biocontrol agent of the cyst nematode (*H. glycines* race 3) in soybeans.

To that purpose, this study evaluated the response of soybean plants mycorrhized with *G. etunicatum* comparatively with non-mycorrhized soybean plants to the presence of different pressure levels of the soybean cyst nematodes.

2. Methodology

The research was descriptive-explanatory, since it characterized and compared the effect of arbuscular mycorrhiza in the control of cyst nematodes, using experimental procedures, in the field and in the laboratory. As for nature, it is a quantitative research (Silveira & Córdova, 2009).

Soybean seeds of cultivar BRS 154 superficially disinfected in 2.5% NaOCl for 2 minutes were pre-germinated in autoclaved sand at 121 °C, 0.15 Mpa, for 120 minutes. The seeds were inoculated with 4 ml of a cell suspension (about 1×10^6 ml) of *Bradyrhizobium elkani*, strains Semia 587 and 5079 (CPAC 15).

The *H. glycines* inoculum was obtained from Embrapa wheat growing in Passo Fundo, the Rio Grande do Sul. A nematode egg solution was prepared by suspending 100 cm³ of soil in water and pouring the suspension through 25 and 100 µm mesh sieves. The material retained in the 100 µm sieve was macerated over a 500 µm sieve and the retained material was collected and counted in a Peters chamber using an optical microscope. The egg concentration was adjusted according to the treatments necessary for inoculation of the plants.

The nematode inoculation was conducted at the time of transplanting pre-germinated soybean seedlings into a substrate containing the inoculum of the AMF *Glomus etunicatum*. This transplanting occurred 10 days after soybean germination, and *G. etunicatum* was chosen because it is the most common AMF species in soybean-producing soils (Khalil et al., 1992; Winkler et al., 1994). The treatments, which included samples with and without soybean cyst nematodes and with and without the presence of *G. etunicatum*, were distributed in a completely randomized design, with 10 treatments and six repetitions. Fifteen days after emergence, the seedlings were transplanted to 500 g ceramic pots containing a substrate composed of a 2:1 (v/v) mixture of sand and soil (23% clay and 1.2% organic matter). The soil pH in water was pH 6.5, and the soil contained Al at 0.0 cmolc L⁻¹, P at 7.2 mg L⁻¹, K at 42 mg kg⁻¹, and Ca at 7.4 cmolc L⁻¹. The substrate was sterilized in an autoclave at 121°C, 0.15 Mpa, for 120 min. The substrate was inoculated with 1 g of *G. etunicatum* spores at a concentration of 120 spores g⁻¹, according to treatments. The plants were kept in the greenhouse for 60 days and irrigated weekly with 10 mL of a nutrient solution containing 1.2 mg.kg⁻¹ ZnCl₂, 0.4 mg.kg⁻¹ H₃BO₃, 0.2 mg.kg⁻¹ MnCl₂.4H₂O, 0.01 mg.Kg⁻¹ (NH₄)₆Mo₇O₂₄.4H₂O, and 9.0 mg.Kg⁻¹ K₂SO₄.

The evaluations were carried out after sixty days. The number of female nematodes in the plant root systems was determined using the method of Tihohod & Santos (1993) and counting with a microscope. The number of cysts in the soil was assessed using 100 cm³ of soil placed in a container and flooded with running water to wash out the cysts. The solution was poured through a set of 25 and 100 µm sieves and the retained material was collected on paper towels in Petri plates. The cysts were counted using a microscope.

Plant height and dry mass were also measured. For dry mass analysis, the aerial part of the plant was detached at the soil level and dried in an oven at 65oC. The dried material was then weighed for determination of the mass. The root length was determined by measuring the length of the primary root from the region of the root-shoot junction to the root tip.

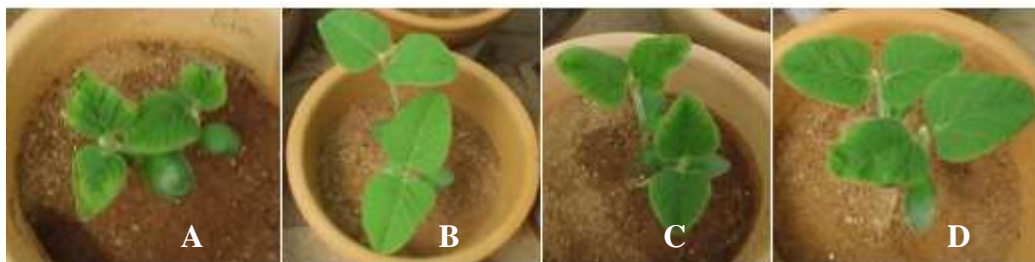
The plant height and root length data were subjected to analysis of variance, with the averages compared by the Tukey test at a 5% probability of error. The numbers of cysts and females in the roots were analyzed by regression analysis. For these

analyses, the data obtained from the evaluations were transformed (square root of $X + 0.5$) to reduce the heterogeneity of the variance. The relationship between the population density of cyst nematode in the treatments with and without AMFs was compared using polynomial regressions.

3. Results and Discussion

The beneficial action of the mycorrhizal association with *Glomus etunicatum* was evident by the development and health of the treated soybean plants (Figure 1). Several authors have described the efficiency of these fungi in reducing the severity of various diseases (Dehne, 1982; Vos et al., 2012; Rasmussen et al., 2019).

Figure 1 - Action of the fungus *Glomus etunicatum* on soybean plants, 20 days old, growing in a greenhouse. A = plants infected with 2000 eggs from the cyst nematode *Heterodera glycines*



B = plants inoculated with *G. etunicatum* and then infected with 2000 cyst nematode eggs. C = plants treated with 4000 eggs from the cyst nematode. D = plants inoculated with *G. etunicatum* and then infected with 4000 cyst nematode eggs. Source: Authors.

The presence of AMF increased the plant height by 6.93% (Table 1). AMFs are known to have a stimulatory effect on plant growth, including that of soybeans, because of improved P nutrition and water transport (Baum et al., 2015; Parniske, 2008). This increase in height was also in agreement with the findings of Ceustermans et al. (2018), who also found increases in plant growth and weight in the presence of AMFs. Similarly, Hol & Cook (2005) found an average increase of 8% in plant height in response to AMFs in plants under attack by cyst nematodes. In the present study, plants treated with a high level of cyst nematode inoculum were 26% shorter in height, on average, in the absence than in the presence of AMFs (Table 1). This result was similar to that reported by Todd et al. (2001), who found a decrease of 29% in the average plant weight in the presence of the nematodes and an increase of 36% in the average plant weight in the presence of an AMF.

Table 1 - Effect of the arbuscular mycorrhizal fungus (*Glomus etunicatum*) on the height of 60-day-old soybean plants infected with the soybean cyst nematode (*Heterodera glycines*) under greenhouse conditions.

| <i>Number eggs</i> | Height plant (cm) | |
|--------------------|--------------------------|----------|
| | Without FMA | With FMA |
| 0 | 29.34 a | 29.64 a |
| 500 | 23.52 ab | 20.20 ab |
| 1000 | 20.22 ab | 20.38 ab |
| 2000 | 16.90 b | 26.80 ab |
| 4000 | 15.68 b | 15.96 b |

The averages in the column do not differ by the Tukey test ($p < 0.05$ probability of error). Source: authors.

The stimulatory effect of AMFs on the growth of soybean plants may be a result of an increase in plant nutrition;

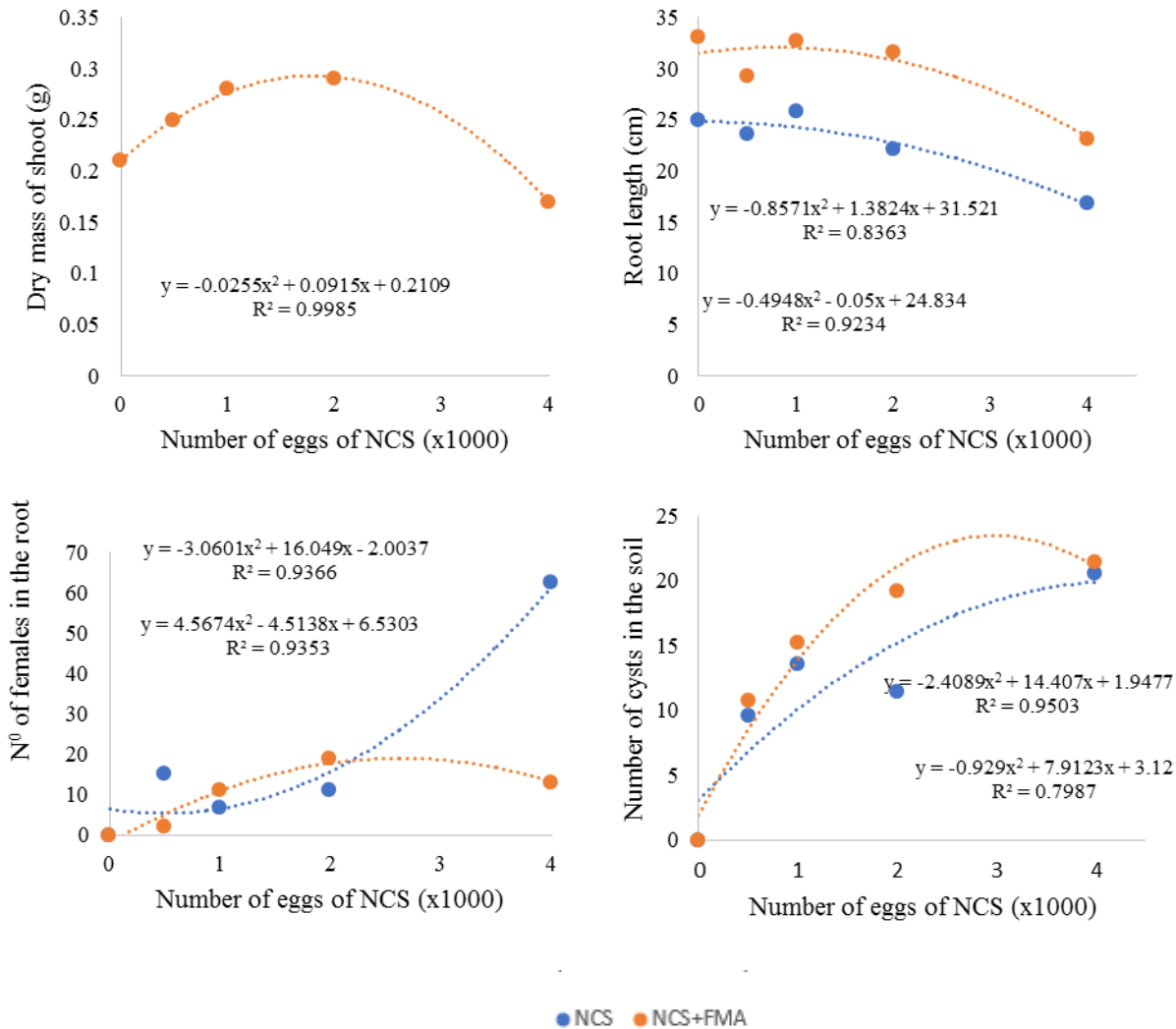
however, some studies have indicated that the suppression of cyst nematodes by the presence of AMFs occurs due to biological phenomena and not just nutritional improvement (Cameron et al., 2013; Schouteden et al., 2015). This suppression of the nematodes may be a consequence of competition for nutrients and space between organisms (Vos et al., 2014). AMFs rely on photosynthesis from the host in the early stages of development for root colonization. This period of intense biosynthesis by the AMFs can also coincide with the period of initial establishment of the nematode feeding sites. Since the fungus requires at least 10 days to establish itself at the root (Kellam & Shenck, 1980), an insufficiency of nutrients could result in decreased nematode reproduction. This competition for nutrients, with an emphasis on carbon, has been suggested as an AMF-mediated biocontrol mechanism, although evidence is sparse in the literature (Jung et al., 2012). Current estimates indicate that the transfer of carbon from the host plant to the AMF varies between 4 and 20% of the total assimilated carbon (Hummer et al., 2011).

Treatments that involved subjecting the plants to a high pathogen level (4000 cyst nematode eggs) eliminated the improved growth, such as greater plant height and root length, seen in response to AMF (Figure 2 and Table 1), indicating that the presence of the high level of inoculum of the pathogen prevented the AMF response. These results are in line with other studies that claimed that the protection conferred by the AMF depends on the level of colonization and the specificity of the isolated AMF. The degree of improvement in tolerance of the host plant to the pathogen also depends on the degree of presence of the pathogen and the rate of association with the mycorrhizal fungus (Ceustermans et al., 2018; Chen et al., 2017; Harrier and Watson, 2004; Ruscitti et al., 2017).

The root system of the plants was 32.20% longer, on average, in the presence of AMFs (Figure 2). In addition to improving nutritional status, mycorrhizal plants generally show increased root growth and branching (Gamelero et al., 2010; Gutjahr & Paszkowski, 2013). This factor can have a positive effect on the host plant, by counterbalancing the decrease in root branching caused by migratory endoparasitic nematodes. For sedentary endoparasitic nematodes, such as the cyst nematode, the zones of root elongation and the sites of lateral root formation are the preferred sites of penetration, probably because of the enhanced root exudation in these zones (Curtis et al., 2009).

According to Jung et al. (2012), a competition factor may be involved in the interaction between nematodes and AMFs. A competition for space may explain the fact that the number of female nematodes found in the root was 28.21% lower in the mycorrhizal plant than in the non-mycorrhizal plant, since the number of cysts in the soil was 20.65 % higher in the presence of the AMF than in the absence of the fungus (Figure 2). The increase in the population density of the nematode cysts in the soil, as observed in this work, is also in agreement with the findings of Tylka (1991), who stated that the population density of the cyst nematode is generally higher in sites colonized by AMFs. These results support the suggestion that a reduction in the penetration of juveniles occurred in the roots of soybeans.

Figure 2 - Effect of inoculation of the fungus *Glomus etunicatum* on the dry mass of the shoot (A), the root length (B), the number of *Heterodera glycines* female nematodes in the roots (C), and the number of cysts in the soil (D) surrounding the soybean plants at 50 days after fungal inoculation.



Source: Authors.

The negative effects on the nematode by the fungus are explained by the fact that mycorrhizal bushes are formed exclusively in the cortex, where the migratory endoparasitic nematodes also feed (Schouteden et al., 2015). In the case of sedentary endoparasites, such as the cyst nematode, this factor could be important only if the feeder cells extend into the cortex prior to the nematode inoculation (Schouteden et al., 2015). However, as mycorrhizal bushes are short-lived structures, distinguishing whether the AMF or the nematode colonized the same root first is difficult (Javot et al., 2011; Schouteden et al., 2015). The findings of the present study can neither confirm nor disprove that this effect occurred.

The mycorrhizal association may also affect the reproduction of nematodes by reducing oviposition and the number of individuals in the plant root system. This effect of AMF on nematode reproduction is considered to depend on a high percentage of root colonization by the mycorrhizal fungus (Cofcewicz et al., 2001). The observed reduction in the number of nematodes in the root, considering the greater number of cysts in the soil in the mycorrhizal plant (Figure 2), may be due to a reduction in the penetration of juveniles because of the competition factor. Alternatively, it may reflect a pathogenic effect of *G. etunicatum* on the reproduction of the cyst nematode, in agreement with other work in the literature (Hol & Cook, 2005;

Vos et al., 2012). The AMF *G. etunicatum* therefore appears to have good potential for the biocontrol of soybean cyst nematode (*H. glycines*, race 3) in soybean, thereby corroborating the initial hypothesis of the present study.

4. Conclusion

This study evaluated the potential of the mycorrhizal fungus *Glomus etunicatum* in the biological control of the cyst nematode (*Heterodera glycines* race 3) in soybean. Overall, the findings indicated that:

- i) the presence of AMF *G. etunicatum* favors an increase in the length of roots and height of plants.
- ii) the mycorrhizal fungus *G. etunicatum* reduces the number of female cyst nematode (*H. glycines*) is reduced in the presence of the mycorrhizal fungus *G. etunicatum*.
- iii) the presence of the *G. etunicatum* improves the nutritional status of the plant.
- iv) the presence of the *G. etunicatum* in soybean decreases the nematode penetration in root due to the competition for space between the organism species.

Our results suggest that the fungus acts indirectly to improve the nutritional status of the plant, thereby favoring better tolerance of the plant to the presence of the pathogen. Further studies may be carried out in order to verify the effect of the potential of *G. etunicatum* on the biological control of the NCS, considering its effect on the yield of the culture. In addition, studies are needed to elucidate the interaction between the NCS, the mycorrhizal fungus and the host plant at a physiological and biochemical level for a better understanding of the interaction process.

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