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Rhizophagus clarus controls *Meloidogyne javanica* and enhances the activity of defense-related enzymes in tomato

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ABSTRACT

Root-knot nematodes (*Meloidogyne* spp.) cause severe damage to tomato crop. This study aimed to investigate the biocontrol potential of the arbuscular mycorrhizal fungus *Rhizophagus clarus* compared with that of the fungi *Trichoderma harzianum* and *Pochonia chlamydosporia* against *Meloidogyne javanica* in tomato and assess the activity of defense-related enzymes in mycorrhizal roots. Three experiments were carried out to evaluate mycorrhizal colonization, nematode penetration, development, and reproduction, plant growth, and enzyme activity in mycorrhizal roots. We observed that *R. clarus* colonization of tomato roots increased with time. Although *R. clarus* did not prevent nematode penetration or development in roots, it was efficient in reducing total nematode numbers. *R. clarus*, *T. harzianum* and *P. chlamydosporia* treatments reduced nematode reproduction by 56.5, 54.4, and 56.9%, respectively, compared with the control. Moreover, *R. clarus* increased tomato shoot weight and phenylalanine ammonia-lyase and peroxidase activities.

Keywords: *Trichoderma harzianum*, *Pochonia chlamydosporia*, root-knot nematodes, mycorrhiza, resistance induction.

RESUMO

***Rhizophagus clarus* controla *Meloidogyne javanica* e aumenta a atividade de enzimas relacionadas a defesa em tomateiro**

Os nematoides das galhas (*Meloidogyne* spp.) causam danos severos na cultura do tomateiro. Dessa forma, o presente estudo objetivou avaliar o biocontrole de *M. javanica* mediado pelo fungo micorrízico arbuscular *Rhizophagus clarus* comparado aos fungos *Trichoderma harzianum* e *Pochonia chlamydosporia* em tomateiro e atividade de enzimas relacionadas à defesa vegetal em raízes micorrizadas. Três experimentos foram realizados a fim de avaliar: a colonização micorrízica, a penetração, desenvolvimento e reprodução de *M. javanica*, o desenvolvimento do tomateiro e a atividade de enzimas relacionadas a defesa em raízes micorrizadas. Observou-se que a colonização por *R. clarus* em tomateiro foi crescente com as épocas de avaliação e, apesar de não impedir a penetração e desenvolvimento de *M. javanica* nas raízes, foi eficiente em reduzir o número total do nematoide. Os tratamentos *R. clarus*, *T. harzianum* e *P. chlamydosporia* reduziram a reprodução do nematoide em 56,5, 54,4 e 56,9%, respectivamente, se comparados à testemunha. Além disso, *R. clarus* aumentou as massas de parte aérea do tomateiro e a atividade das enzimas fenilalanina amônia-liase e peroxidase.

Palavras-chave: *Trichoderma harzianum*, *Pochonia chlamydosporia*, nematoide das galhas, micorriza, indução de resistência.

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Tomato (*Solanum lycopersicum*), one of the most important vegetable crops, is widely cultivated around the world. Tomato yields can be affected by root-knot nematode (*Meloidogyne* spp.) infection (Pinheiro *et al.*, 2019). Females of these sedentary endoparasites establish a complex parasitic relationship with hosts, inducing feeding sites characterized by the presence of multinucleated giant cells (Vitela *et al.*, 2019). Adjacent cells undergo hyperplasia and hypertrophy, resulting in the occurrence of nodosities in roots, a symptom also known as galls (Jones *et al.*, 2013). Root galls seriously impair water and nutrient absorption by plants, negatively affecting host development.

Nematode control is a complex task, as the main strategies have particular limitations. Crop rotation is considered the ideal practice but is limited by the nematode wide host range (Jones *et al.*, 2013). Genetic control, in turn, reduces the use of chemical nematicides, which can pose risks to human health, present a relatively high cost, in addition to polluting the environment (Pinheiro *et al.*, 2019). The Mi gene in tomato plants confers resistance to Meloidogyne javanica, M. incognita and M. arenaria (Williamson, 1999) and is an important strategy for producers. However, there is thermo-instability of this gene at temperatures above 27°C (Dropkin, 1969). Biological control has been increasingly adopted by farmers. Biocontrol agents, such as arbuscular mycorrhizal fungi (AMF), positively contribute to the management of Meloidogyne spp. (Khan et al., 2017; Sharma & Sharma, 2017). These fungi establish symbiotic associations with host roots.

Mycorrhizal colonization contributes to the absorption of nutrients that, when lacking, limit plant growth, particularly nitrogen and phosphorus (Prasad *et al.*, 2017). Furthermore, plants in mycorrhizal symbiosis undergo biochemical, physiological, and molecular changes (Garcia-Garrido & Ocampo, 2002), such as alterations in root exudate composition that may hinder nematode penetration, as observed in tomato parasitized by *Meloidogyne incognita* (Vos *et al.*, 2012a). AMF may also induce systemic resistance in plants, as observed in tomato infected by *M. incognita* (Vos *et al.*, 2012b), and increase the activity of antioxidant enzymes such as guaiacol peroxidase (POX), polyphenol oxidase (PPO), and superoxide dismutase (SOD) (Sharma & Sharma, 2017). However, such alterations depend on the plant, nematode species, and AMF, as well as on the prevailing environmental conditions of the site (Campos, 2020).

Similar to AMF, the fungi *Trichoderma harzianum* and *Pochonia chlamydosporia* have been shown to antagonize *Meloidogyne* spp. (Martinez-Medina *et al.*, 2017; Ghahremani *et al.*, 2019). Fungi of the genus *Trichoderma* have multiple mechanisms of action against nematodes, including antibiosis and direct egg parasitism, in addition to stimulating growth hormone synthesis and resistance induction in host plants (Freitas *et al.*, 2012; Martínez-Medina *et al.*, 2017). *P. chlamydosporia* is a chitinolytic fungus with potential for controlling sedentary nematodes, acting mainly as an egg parasite (Sankaranarayanan & Hari, 2020).

Although many studies have investigated the relationship between plants, biocontrol fungi, and nematodes, there is a need for research on the action of mycorrhizal species against root-knot nematodes parasitizing tomato as well as comparisons between AMF and other biological control agents, given that mycorrhizal symbiosis may exert variable effects depending on environmental conditions and the host. This study aimed to investigate the potential of *Rhizophagus clarus*, as compared with *T. harzianum* and *P. chlamydosporia*, in the control of *Meloidogyne javanica* and assess the effect of mycorrhizal colonization on the activity of defense-related enzymes in tomato.

MATERIAL AND METHODS

General Information

Three experiments were conducted in a greenhouse at the State University of Maringá (23°78′91″S 53°25′85″W, 430 m altitude), Brazil, using a completely randomized design.

The AMF *R. clarus* was obtained from the Glomales collection of Paranaense University (UNIPAR, Umuarama, PR, Brazil). The fungi *T. harzianum* IBLF006 (Ecotrich®, Ballagro Agro Tecnologia Ltd., 300 g a.i. kg^{-1}, applied at a dose of 0.5 kg ha^{-1}) and *P. chlamydosporia* Pc-10 (Rizotec®, Stoller do Brasil Ltd., 280 g a.i. kg^{-1}, applied at a dose of 2.5 kg ha^{-1}) were also used in the experiments. This *T. harzianum* strain has not been registered as a nematicide; however, it has shown potential in the control of root-knot nematodes and as a plant resistance inducer (Martínez-Medina *et al.*, 2017).

The *M. javanica* inoculum was obtained from a pure population maintained on tomato in a greenhouse. Nematode extraction was performed by the method of Hussey & Barker as adapted by Boneti & Ferraz (1981). Nematodes were counted in a Peter's chamber under an optical microscope.

Root colonization by mycorrhizae

Root colonization was determined in mycorrhizal plants from Experiments 1 and 2. For analysis, 2 g of root fragment was collected and stained according to the method proposed by Phillips & Hayman (1970). Root colonization percentage was determined by analyzing 100 root segments per replication (McGonigle *et al.*, 1990) under a light microscope at 100× magnification. Colonized sites were identified by the presence of fungal hyphae, arbuscules, and vesicles.

Experiment 1: Penetration and development of *M. javanica* in tomato treated with *R. clarus* or nematophagous fungi

The experiment was conducted between February and March 2020 (mean maximum, average, and minimum temperatures of 31.01, 25.15, and 18.87°C, respectively) in a completely randomized design with a 4×3 factorial arrangement and 4 replications per treatment. The first factor was biological treatment (*R. clarus*, *T. harzianum*, *P. chlamydosporia*, and untreated control) and the second factor was evaluation time.

First, seedlings of tomato 'Santa Clara' were grown in trays containing commercial substrate. Plants in the mycorrhizal treatment group were sown in substrate containing 250 mycorrhizal spores per 100 g soil. Seeds were placed in direct contact with fungal spores.

At 20 days after germination, plants were transplanted to polystyrene pots containing 1 kg of a mixture of soil and sand (2:1 v/v). Before use, the substrate was autoclaved twice at 120°C for 2 h with a 24 h interval between cycles. *T. harzianum* and *P. chlamydosporia* treatments were applied via in-furrow application at the time of transplanting. Untreated plants were used as control. One day after transplanting, plants were inoculated with a suspension (1 mL) containing 500 eggs and eventual second-stage juveniles (J2) of *M. javanica*. The inoculum was deposited in four open holes made in the soil around the base of the plant.

At 5, 10, and 15 days after inoculation (DAI), plants were carefully removed from pots and their roots separated from shoots, washed under running water, weighed, and stained with acid fuchsin (Byrd Junior *et al.*, 1983). Subsequently, all root fragments were used to prepare temporary slides, which were evaluated for presence and number of nematodes under an optical microscope. Nematodes were classified into the following developmental phases: J2, third-stage juveniles (J3), fourth-stage juveniles (J4), and adult females.

Experiment 2: Effect of *R. clarus* on *M. javanica* reproduction

Experiment 2 was conducted between February and April 2020 (mean maximum, average, and minimum temperatures of 30.02, 24.87, and

18.44°C, respectively). Treatments consisted of *R. clarus*, *T. harzianum*, *P. chlamydosporia*, and an untreated control, with eight replications each. The experiment was conducted as described in Experiment 1 but using an initial nematode population of 2000 eggs + eventual J2 and an inoculum volume of 2 mL per pot.

At 60 DAI, plants were harvested and separated into shoots and roots. The roots were washed and weighed. Then, 2 g of root fragment treated with *R. clarus* was collected from each plant for evaluation of mycorrhizal colonization. The rest of the root system was subjected to nematode extraction by the above-mentioned method, and the total number of nematodes was determined by using a Peter's chamber under an optical microscope. This value was divided by the root weight (g) to obtain the population density (number of nematodes per gram of root). The nematode reproduction factor (RF) was calculated by the equation RF = Final population/Initial population (Oostenbrink, 1966).

Shoots were evaluated for height (cm) and fresh weight (g). Then, shoot samples were placed in paper bags, dried in a forced-air oven at 65°C for 72 h, and weighed to obtain the shoot dry weight (g).

Experiment 3: Defense enzyme analysis

The third experiment was conducted between February and March 2020 (in parallel with Experiments 1 and 2) to investigate defense-related enzyme activities in mycorrhizal tomato roots. The experiment followed a completely randomized design with a 2×3 factorial arrangement, comprising two biological treatments (*R. clarus* and an untreated control), three evaluation periods, and four replications per treatment. Experimental procedures were the same as those described for Experiment 1.

At 5, 8, and 11 DAI, plants were carefully removed from pots. The roots were washed under running water and dried with paper towels to remove excess water. Then, 0.5 g of root tissue was collected from each treatment and stored in liquid nitrogen in a freezer at -5°C until use.

Extracts were prepared by homogenizing 0.1 g of sample in 4 mL of 0.1 M sodium phosphate buffer (pH 6.5) added with 0.05 g of polyvinyl pyrrolidone. The homogenized sample was centrifuged at 21,400 G for 30 min at 4°C. The supernatant was used to determine total soluble proteins (Bradford, 1976) and enzyme activity.

POX (EC 1.11.1.7) activity was determined by the conversion of guaiacol to tetraguaiacol in the presence of hydrogen peroxide. The absorbance was read spectrophotometrically at 470 nm. Results are expressed as $\Delta_{abs470nm}$ min^{-1} mg^{-1} protein (Lusso & Pascholati, 1999). Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity was determined by the difference between the absorbance of the sample and that of the control (without PAL). A standard curve for *trans*-cinnamic acid was constructed, and results are expressed as mg *trans*-cinnamic acid h^{-1} mg^{-1} protein (Umesha, 2006).

Statistical analysis

Data were analyzed by one-way or two-way analysis of variance (ANOVA). When *F*-values were significant, means were compared by Tukey's test ($p<0.05$). Shapiro–Wilk and Levene's tests were previously performed to assess normality and homoscedasticity, respectively. Nematode numbers and tomato growth data were square-root transformed before ANOVA. All analyses were performed using SISVAR software (Ferreira, 2011).

RESULTS AND DISCUSSION

R. clarus root colonization percentage increased with time (Figure 1), being highest (31.17%) at 60 DAI. In Experiment 1, there were no significant interaction effects of evaluation time and biological treatment on J2 number, J3 number, or population density. However, J3 number and population density were influenced by the main effects of evaluation time; the parameters were highest at 15 DAI (data not shown). Significant interaction effects were observed on J4 number, female number, and total nematode number. The highest means were observed at 15 DAI in *R. clarus*-treated plants and the control (Table 1).

At 60 DAI, all biological treatments were efficient in reducing nematode multiplication compared with the control (Table 2): *R. clarus*, *T. harzianum*, and *P. chlamydosporia* reduced total nematode number by 56.5, 54.4, and 56.9%, respectively. Population density did not differ significantly among treatments, but the RF on untreated plants was about two times higher than that on treated plants.

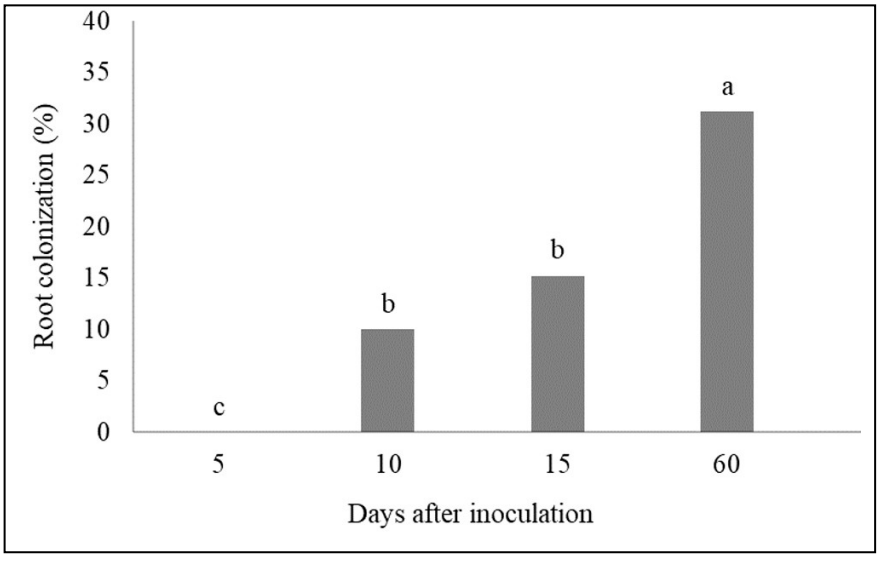

Figure 1. Mycorrhizal colonization percentage at different evaluation times. Bars followed by the same letter do not differ at $p<0.05$ by Tukey's test. Umuarama, UEM, 2020.

Some studies reported that the action of mycorrhizae on *Meloidogyne* spp. depends on the degree of colonization of host roots (Talavera *et al.*, 2001; Vos *et al.*, 2012b). Such a hypothesis is corroborated by the results of the current study, as nematode numbers (J2 to J4 and adults) in the roots of tomato treated with *R. clarus* did not differ from the control from 5 to 15 DAI (Table 1), during which the colonization percentage was low (Figure 1). On the other hand, mycorrhizae reduced nematode number at 60 DAI (Table 2), when the colonization percentage increased.

It is important to note that other mechanisms might be involved in nematode suppression. The findings can be explained by the reproduction behavior of sedentary nematodes, such as *Meloidogyne* spp. As reported by Silva *et al.* (2021), root-knot nematodes complete their life cycle in about

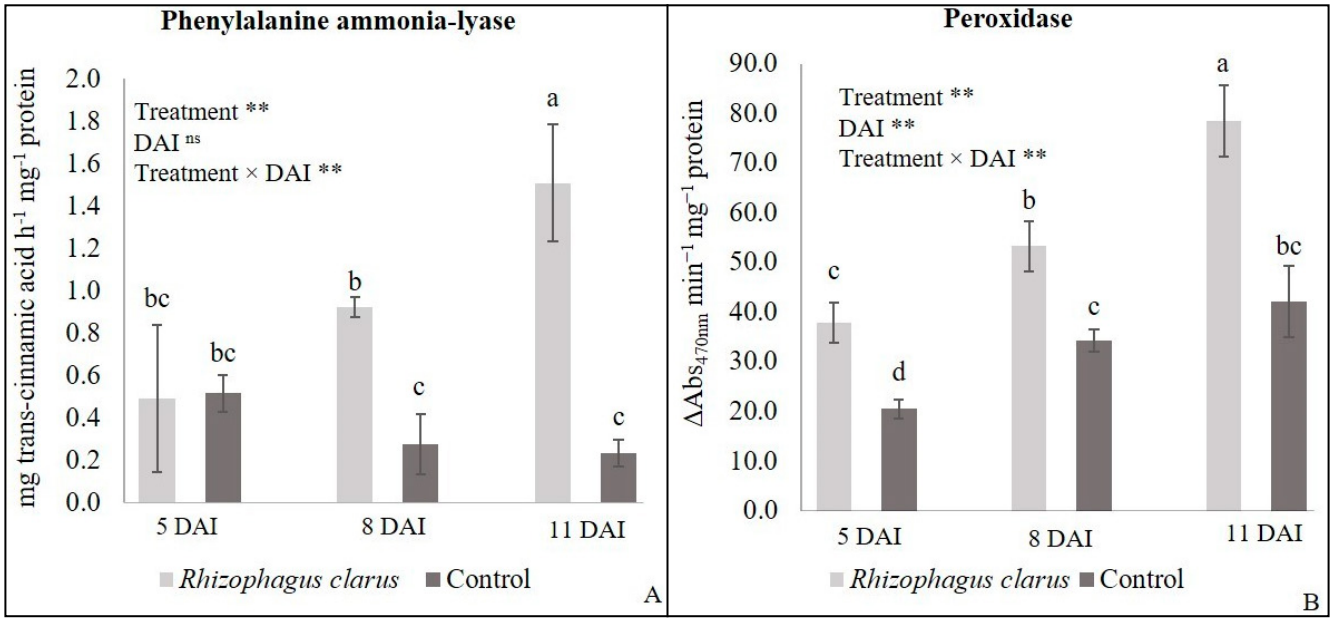

Figure 2. Treatment × Evaluation time interaction effects on (A) phenylalanine ammonia-lyase and (B) peroxidase levels in tomato roots at 5, 8, and 11 days after inoculation (DAI) of *Meloidogyne javanica*. Bars followed by the same letter do not differ at $p<0.05$ by Tukey's test. Data are expressed as mean (columns) ± standard error (error bars). **significant at $p<0.01$; ns= not significant. Umuarama, UEM, 2020.

Table 1. Mean number of fourth-stage juveniles (J4), adult females, and total individuals of *Meloidogyne javanica* at 5, 10 or 15 days after inoculation (DAI) in the roots of tomato treated or not with fungi. Umuarama, UEM, 2020.

DAI	Treatment	J4	Females	Total
5	Control	0.0 b	0.0 b	1.25 c
	Rhizophagus clarus	0.0 b	0.0 b	1.0 c
	Trichoderma harzianum	0.0 b	0.0 b	0.25 c
	Pochonia chlamydosporia	0.0 b	0.0 b	0.0 c
10	Control	0.0 b	0.0 b	2.25 c
	R. clarus	0.0 b	0.0 b	7.50 c
	T. harzianum	0.0 b	0.0 b	0.50 c
	P. chlamydosporia	0.0 b	0.0 b	1.0 c
15	Control	15.2 a	3.2 a	38.0 ab
	R. clarus	11.5 a	2.2 a	38.5 a
	T. harzianum	1.5 b	0.0 b	19.0 bc
	P. chlamydosporia	3.2 b	0.0 b	14.7 c
MSD/SE		3.93/0.79	1.51/0.30	19.13/3.89
Significance	DAI	$F = 130.131$**	$F = 26.889$**	$F = 59.857$**
	Treatment	$F = 22.656$ **	$F = 9.556$**	$F = 5.840$**
	DAI × Treatment	$F = 22.656$**	$F = 9.556$**	$F = 2.808$*

Means in a column followed by the same letter do not differ at $p<0.05$ by Tukey's test. MSD= minimum significant difference; SE= standard error; *significant at $p<0.05$; **significant at $p<0.01$.

Table 2. Total nematode number, population density (nematodes g^{-1} root), reproduction factor (RF), plant height (cm), shoot fresh weight (SFW, g), shoot dry weight (SDW, g), and root fresh weight (RFW, g) of tomato inoculated with *Meloidogyne javanica* and subjected to different fungal treatments. Umuarama, UEM, 2020.

Treatment	Total number	Population density	RF	Height	SFW	SDW	RFW
Control	30938 a	4250^{ns}	15.47 a	46.96^{ns}	12.81 b	2.68 b	7.44^{ns}
Rhizophagus clarus	13470 b	2248^{ns}	6.73 b	48.91^{ns}	18.78 a	3.97 a	6.01^{ns}
Trichoderma harzianum	14115 b	2899^{ns}	7.06 b	44.41^{ns}	12.96 b	2.65 b	5.44^{ns}
Pochonia chlamydosporia	13345 b	2736^{ns}	6.67 b	46.70^{ns}	11.49 b	2.28 b	6.07^{ns}
MSD/SE	26.9/6.8	19.7/4.9	0.6/0.1	0.4/0.1	0.4/0.1	0.2/0.07	0.4/0.1

Means in a column followed by the same letter do not differ at $p<0.05$ by Tukey's test. MSD= minimum significant difference; SE= standard error; ns= not significant. Original means were transformed by a square root function ($\sqrt{x}$) before analysis.

four weeks and lay eggs on the root surface; therefore, reinfection will depend on the ability of juveniles to overcome the barriers imposed by mycorrhizae, such as exoderm lignification (Sankaranarayanan & Hari, 2020) and alterations in root exudate composition (Vos *et al.*, 2012a). Khan *et al.* (2017) found that eggplant roots treated with *Funneliformis mosseae* (formerly *Glomus mosseae*) had about 16 J2 after four weeks of *M. incognita* inoculation, whereas untreated plants had about 186, demonstrating that nematode reinfection was hindered in mycorrhizal plants.

Unlike *R. clarus*, *T. harzianum* and *P. chlamydosporia* adversely affected *M. javanica* development (Table 1) and minimized nematode reproduction (Table 2). The protection provided by *T. harzianum* against root-knot nematodes seems to consist of three phases: induction of salicylic acid production (suppression of infection), followed by induction of jasmonic acid production (suppression of reproduction and fecundity) and induction of salicylic acid production (suppression of root infection by next-generation J2) (Martínez-Medina *et al.*, 2017). This model is also valid for *P. chlamydosporia* (Ghahremani *et al.*, 2019). Both fungi are chitinolytic, that is, they parasitize nematode eggs, reducing inoculum amount (Freitas *et al.*, 2012; Ghaheremani *et al.*, 2019; Sankaranarayanan & Hari, 2020).

Fungi did not influence plant height or root fresh weight (Table 2). On the other hand, shoot fresh and dry weights were higher in plants treated with *R. clarus*, demonstrating a positive effect on the accumulation of photosynthetic tissues compared with the other treatments and control (Table 2). The positive effect of *R. clarus* on tomato development was previously reported by Silva *et al.* (2017) in an experiment assessing plants with and without mycorrhizae. Mycorrhizae have shown potential to promote vegetative development in tomato infected by *Meloidogyne* spp. (Talavera *et al.*, 2001; Sharma & Sharma, 2017).

The promotion of vegetative development by AMF might be due to an increase in nutrient absorption by plants, especially that of growth-limiting nutrients, such as phosphorus and nitrogen. Such an effect is provided by the extension of hyphae in soil (Prasad *et al.*, 2017). Nematode parasitism results in the disorganization of the central cylinder of plant roots (Wanderley & Santos, 2004; Vitela *et al.*, 2019), thereby limiting the absorption of soil solution. *R. clarus* increased plant phytomass even in the presence of nematodes, evidence of the benefits that the fungus may provide in the control of *M. javanica*.

In Experiment 3, significant interaction effects were observed on PAL and POX activities, which increased or remained the same over time (Figure 2). The highest enzyme activities were found in *R. clarus*-treated plants at 11 DAI (Figure 2).

PAL is a key enzyme in the synthesis of various secondary compounds related to plant defense, such as phenols and lignins (Borges *et al.*, 2017). It is also the first enzyme in the phenylpropanoid pathway, responsible for the formation of *trans*-cinnamic acid (Fukasawa-Akada *et al.*, 1996). This acid is a precursor of several types of phenolic compounds associated with the elimination of excess reactive oxygen species (ROS) (Borges *et al.*, 2017).

Some isoforms of POX can suppress hydrolytic enzymes and oxidize nematode-derived toxins that are essential for the spread of infection (Zinov'eva *et al.*, 2004). Furthermore, POX catalyzes the last stages of lignin biosynthesis, that is, polymerization of monolignols and subsequent lignin formation (Marjamaa *et al.*, 2009). Lignin modifies cell wall structure and function, serving as a physical barrier against nematode attack (Holbein *et al.*, 2016). The slow increase in PAL and POX activities might explain the lack of effect on J2 penetration, as the peak occurred at 11 DAI. However, enzymes might have contributed to eliminating ROS, avoiding cell damage under nematode infection. Such an assumption is evidenced by the activation of enzymatic and non-enzymatic antioxidant defense systems in infected tomatoes as a strategy to eliminate ROS after nematode infection (El-Beltagi *et al.*, 2012).

Therefore, it is possible to conclude that *R. clarus* did not prevent *M. javanica* penetration or initial development in tomato. However, it reduced nematode reproduction and increased shoot fresh and dry weights as well as the activities of defense-related proteins, such as PAL and POX. Thus, the mycorrhiza studied here can contribute to a more sustainable management. It is important to highlight

that the mechanism of action of AMF against root parasites is complex, given the high specificity of fungi to the host and phytopathogenic agent; thus, nematode control may not always be positive, especially in cases where mycorrhizae do not have competitive advantage (Talavera *et al.*, 2001; Silva *et al.*, 2021). It is evident that the mechanisms involved in nematode-plant-mycorrhiza relationships are not yet fully elucidated and that such relationship may result in unique responses under different conditions.

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