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RESEARCH ARTICLE

Macrophomina phaseolina and *Meloidogyne javanica*: Interaction of the pathosystem in soybean

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ABSTRACT

Interactions between soil pathogens, such as those involving fungi and nematodes, can lead to disease complexes that are difficult to manage, intensifying crop losses. *Macrophomina phaseolina*, for instance, has become a recurring problem in soybean (*Glycine max* (L.) Merr.) fields under drought conditions, particularly in nematode-infested areas. This study aimed to investigate the *M. phaseolina-Meloidogyne javanica* pathosystem in soybean to understand how these microorganisms interact with each other and with the plant's defense mechanisms. Three experiments were conducted: (i) A nematode reproduction and disease severity assessment, (ii) a split-root experiment, and (iii) an analysis of plant defense-related enzymes. Soybean plants were subjected to four treatments: Control, inoculation with *M. javanica*, inoculation with *M. phaseolina*, and co-inoculation. The results revealed an antagonistic interaction between pathogens, as co-inoculation reduced *M. javanica* reproduction by up to 68.6% and decreased the severity of injuries caused by *M. phaseolina*. Similar results were observed in the two trials of the split-root experiment, with co-inoculation producing greater reductions in soybean growth than single inoculation. Enzymatic analysis showed an increase in polyphenol oxidase activity at 7 d after inoculation in single-inoculation treatments but not in the co-inoculated group. Peroxidase and glucanase activities increased over time, without differing significantly between treatments. This study contributes to our understanding of pathogen interactions in soybean and demonstrates that, even when pathogens negatively influence each other, their combined presence can exacerbate crop damage.

Key words: Charcoal rot of soybean, defense mechanisms, root-knot nematode, soilborne fungi.

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) holds a central position in the Brazilian and global economies. Brazil is the world's largest exporter and second-largest producer of soybean. Despite its prominence in the agricultural sector, soybean production faces significant challenges due to plant diseases (Ferreira et al., 2018). Soybean diseases can be aggravated by environmental conditions and pathogen adaptation, which hinder effective management and increase financial losses. Plant-parasitic nematodes are the major pathogens responsible for yield losses in soybean crops, estimated at USD 4.8 billion a year (Syngenta, 2022). In Brazil, the most economically important species include the lesion nematode (*Pratylenchus brachyurus*), soybean cyst nematode (*Heterodera glycines*), and root-knot nematodes (*Meloidogyne javanica* and *M. incognita*). In addition to causing direct damage, these pathogens intensify the impact of disease complexes, leading to further reductions in crop yield (Ferreira et al., 2018).

The genus *Meloidogyne* is highly relevant to agriculture, given its broad range of hosts and extensive geographical distribution. Accordingly, *M. javanica* is distributed in more than 40% of the areas cultivated with soybean Brazil (Syngenta, 2022). These nematodes have developed sophisticated mechanisms of parasitism, such as the induction of cell hyperplasia and hypertrophy in host roots, forming galls that function as a metabolic sink, and this feeding process leads to significant changes in root physiology and anatomy, impairing the plant's ability to absorb water and essential nutrients (Guzmán-Piedrahita et al., 2020).

Soil fungi are also associated with soybean losses, especially *Fusarium* spp. and *Macrophomina phaseolina*. *Macrophomina phaseolina* is the causative agent of charcoal rot, a destructive disease that decreases the yield of numerous crops (Zanella et al., 2020; Ruzmetov et al., 2024). The fungus is favored by prolonged periods of low humidity and high temperature (summers) (Marquez et al., 2021; Ruzmetov et al., 2024). It can survive in the form of microsclerotia, which germinate in root exudates and infect roots under favorable conditions (Marquez et al., 2021). Notably, microsclerotia can survive in soil for up to 15 yr as saprophytes evidence of their resilience and adaptability. Such characteristics hinder the management of the disease (Ruzmetov et al., 2024).

The interaction between phytopathogenic fungi and nematodes can intensify the severity of diseases in host plants. Nematodes induce mechanical damage in roots, creating lesions that serve as gateways for soil fungi, facilitating invasion and colonization (Jain et al., 2024). Furthermore, nematode feeding alters the composition of root exudates and affects the plant's biochemical responses, inducing the release of phenolic compounds and sugars that attract pathogenic fungi (Hiremath et al., 2024; Jain et al., 2024). These changes in the root microenvironment can compromise the plant's defense mechanisms, promoting vascular colonization by fungi and affecting the transport of water and nutrients (Archana et al., 2023; Hiremath et al., 2024). Conversely, there is evidence that fungal inoculation can reduce nematode multiplication, suggesting the occurrence of antagonistic interactions (Singh et al., 2012). However, regardless of whether fungus-nematode interactions are synergistic or antagonistic, the damage tends to be additive (Archana et al., 2023). The complexity of fungus-nematode interactions underscores the importance of elucidating their underlying mechanisms to develop effective strategies for integrated disease management.

In view of these considerations, this study aimed to investigate the interaction between *M. javanica* and *M. phaseolina* in soybean crops through a nematode reproduction assay, a split-root experiment, and an assessment of plant defense mechanisms.

MATERIAL AND METHODS

General experimental procedures

The experiments were carried out in a laboratory and a greenhouse at the State University of Maringá (23°47′28.4″ S, 53°15′24.0″ W; 379 m a.s.l.), Paraná, Brazil. A completely randomized design was used. The substrate consisted of a 2:1 mixture of clay soil and sand, previously autoclaved for 2 h at 120 °C.

The *Macrophomina javanica* inoculum used in all experiments was obtained from a pure population multiplied on tomato (*Solanum lycopersicum* L.) 'Santa Clara' under greenhouse conditions. Nematodes were extracted from plant roots by the method adaptions of Boneti and Ferraz (1981). Nematodes were counted in a Peters chamber under a light microscope.

The *M. phaseolina* inoculum was obtained from a commercial soybean (*Glycine max* (L.) Merr.) field in Floresta (23°34′23″ S, 52°04′12″ W), Paraná, Brazil. The fungus was isolated on Petri dishes containing potato dextrose agar medium at 25 °C in the dark. After a pure population was separated, pathogenicity was confirmed by following Koch's postulates, and the morphological characteristics of the fungus were compared with those described in the literature. Plates containing the pure inoculum were stored at 25 °C in the dark in a biochemical oxygen demand incubator. After 14 d of incubation, plates were evaluated for microsclerotial production. For quantification and adjustment of the inoculum concentration, 10 mL autoclaved distilled water containing 0.05% Tween 80 was added to the plates, and microsclerotia were released by using a scraping blade. The resulting suspension was filtered through a double layer of gauze and counted in a Neubauer chamber under a light microscope.

Experiment 1: Effects of *M. phaseolina* on *M. javanica* reproduction

The experiment was conducted in a greenhouse in two periods. Trial 1 lasted from December 2023 to March 2024 and Trial 2 from September to November 2024. The mean minimum and maximum temperatures were respectively 21 and 30 °C during Trial 1 and 19 and 30 °C during Trial 2. The design was completely randomized, with four treatments: Uninoculated control, nematode-inoculated plants, fungus-inoculated plants, and plants inoculated with both pathogens. Each treatment comprised eight replicates.

Seeds of soybean 'BMX Potência RR' were sown directly in pots containing 900 mL substrate. Each pot was planted with a single seed. After 5 d, a 2 mL suspension containing 2000 eggs and eventual second-stage juveniles (J2) of *M. javanica* was inoculated into two holes made in the soil at the base of the plant. On the same day, 2 mL suspension containing 6×10^4 *M. phaseolina* spores were inoculated in the same hole. Inoculation was performed using automatic pipettes. The plants were maintained in a greenhouse and irrigated daily, as needed.

At 60 d after inoculation (DAI), plants were harvested and separated into shoots and roots. Shoots were evaluated for height (cm) by using a millimeter ruler. Then, shoots were weighed on a semi-analytical scale to obtain the fresh weight. For determination of the shoot dry weight, shoots were oven-dried at 60 °C for 5 d and weighed. The roots were thoroughly washed, placed on absorbent paper to remove excess water, and weighed on a semi-analytical scale to obtain the fresh weight. Then, a photographic record of the root system was made to better visualize the effects of pathogens.

Macrophomina disease severity was assessed by analyzing plant roots and collar according to a visual scale adapted from Anderson (1986), which measures the degree of lesions and discoloration in the root tissue and stem. The lesion scale ranges from 0 to 5: Grade 0, no visible lesion; grade 1, lesions smaller than 1 cm; grade 2, lesions between 1 and 2 cm; grade 3, lesions between 2 and 3 cm; grade, 4 lesions between 3 and 4 cm; and grade, 5 lesions larger than 4 cm.

Finally, the roots were subjected to nematode extraction according to the method described above. Nematodes were counted in a Peters chamber under a light microscope. The total nematode number was divided by the root fresh weight to obtain the population density (number of nematodes per gram of root).

Experiment 2: Split-root test

A split-root test was performed to investigate the combined effects of *M. javanica* and *M. phaseolina* in soybean and the systemic and local responses of the plant to pathogens. Two trials were conducted in different periods, the first from December 2023 to March 2024 and the second from September to November 2024. The mean minimum and maximum temperatures were 21 and 30 °C (Trial 1), respectively, and 19 and 30 °C (Trial 2), respectively. The experiment was conducted in a completely randomized design, with 10 replicates per treatment.

Seeds of soybean 'BMX Potência RR' were initially seeded in plastic trays containing commercial substrate (HF-01, Mecplant, Telêmaco Borba, Paraná, Brazil). After 14 d, when seedlings measured approximately 10 cm in height, units within the average standard of vigor and height were selected. Seedling roots were separated into two, as described by Olzem (2001). Each root portion of the split-root system was transplanted into Falcon tubes arranged side by side (in parallel) and fixed with latex rubber. Tubes were filled with 50 mL substrate.

Five days after transplantation, representing the period necessary for rooting, each root portion was inoculated with the pathogens described in Experiment 1. Treatments were arranged as follows (tube A × tube B): Un × Un; Un × Mj; Un × Mp; Un × Mj + Mp; and Mj × Mp, where Un represents the uninoculated control, Mj represents *M. javanica*, Mp represents *M. phaseolina*, and Mj + Mp represents co-inoculation with *M. javanica* and *M. phaseolina.*

At 45 d after inoculation, the root fresh weight, disease severity, and total nematode population were determined, as described in Experiment 1.

Experiment 3: Activation of defense mechanisms in plants co-inoculated with *M. javanica* and *M. phaseolina*

The experiment was conducted to elucidate whether co-inoculation of pathogens interferes with the expression of plant defense enzymes compared with sole inoculation. The experiment was installed and inoculations were performed as described in Experiment 1. However, experimental units consisted of cups filled with 400 mL substrate. The assay was conducted in April 2024, with mean minimum and maximum temperatures of 21 and 30 °C, respectively. A completely randomized design with a 3 × 3 (Treatment ×

Evaluation period) factorial arrangement was adopted. Treatments were as follows: Inoculation with *M. javanica* (Mj), inoculation with *M. phaseolina* (Mp), and co-inoculation with both pathogens (Mj + Mp). The evaluation periods were 7, 12, and 17 DAI.

Four plants from each treatment were collected at 7, 12, and 17 DAI. The roots were separated from shoots, washed, and stored in aluminum foil at -80 °C until analysis. For sample preparation, roots were macerated in a mortar with liquid nitrogen. The resulting powder was homogenized in 4 mL extraction solution containing 1% polyvinylpyrrolidone (PVP), 0.1 mM EDTA, and 50 mM potassium phosphate buffer (pH 7.0). The mixture was centrifuged at 14 500 rpm for 30 min at 4 °C. The supernatant, corresponding to the enzyme extract, was transferred to 1.5 mL microtubes and stored at -80 °C.

Total proteins were quantified by the Bradford method (Bradford, 1976), using bovine serum albumin as standard. Briefly, 50 µL enzyme extract was added to 2.5 mL Bradford's reagent, and the absorbance was read spectrophotometrically at 595 nm. Polyphenol oxidase (PPO, EC 1.10.3.2) activity was determined by the catechol method (Duangmal and Apenten, 1999). The absorbance was read at 420 nm, and the oxidation of catechol quinone is expressed as Δabs min^{-1} mg^{-1} protein. Glucanase (GLU, EC 3.2.1.6) activity was quantified by the release of laminarin-reducing sugars (β-1,3 glucan), according to Vogelsang and Barz (1993). The reaction mixture consisted of 150 µL enzyme extract and 150 µL laminarin (1.5 mg mL^{-1} in 0.01 M sodium phosphate buffer pH 6.0), incubated at 40 °C for 60 min. After incubation, an aliquot of 30 µL was removed, added to 1.5 mL PAHBAH solution, incubated at 100 °C for 5 min, and transferred to an ice bath for 3 min to stop the reaction. The readings were taken at 410 nm, and the results are expressed as mg glucose h^{-1} mg^{-1} protein.

Guaiacol peroxidase (POX, EC 1.11.1.7) activity was determined by the guaiacol method, which measures the conversion of guaiacol to tetraguaiacol in the presence of hydrogen peroxide (H_2O_2). The reaction mixture consisted of 2.9 mL substrate (7.25 µL guaiacol and 8.874 µL H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0) and 100 µL enzyme extract. The reaction was conducted at 30 °C, and readings were taken at 470 nm for 1 min. The results are expressed as Δabs min^{-1} mg^{-1} protein (Lusso and Pascholati, 1999).

Finally, phenylalanine ammonia lyase (PAL, EC 4.3.1.5) activity was determined by the conversion of L-phenylalanine to *trans*-cinnamic acid, according to Umesha (2006). Briefly, 100 µL enzyme extract, 400 µL 0.025 M Tris-HCl buffer (pH 8.8), and 500 µL 0.05 M L-phenylalanine were mixed and incubated at 40 °C for 2 h. The reaction was stopped with 60 µL 5 M HCl, and the absorbance was read at 290 nm. The results are expressed as µg *trans*-cinnamic acid min^{-1} mg^{-1} protein (Umesha, 2006).

Statistical analysis

Experimental data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene tests, respectively. For normality assumptions to be met, it was necessary to transform total nematode number (reproduction experiment) and the results of the slit-root root test to $\sqrt{x}$. One-way ANOVA was performed to examine the effects of treatments on nematological and vegetative variables ($p < 0.05$). Two-way ANOVA was performed to determine the effect of interactions, using the same level of significance. Differences between treatments were compared by the Tukey test ($p < 0.05$). Analyses were performed using SISVAR software (Ferreira, 2014).

RESULTS

Experiment 1: Effects of *M. phaseolina* on *M. javanica* reproduction

In Experiment 1, sole inoculation of *M. javanica* resulted in a higher total nematode number than co-inoculation with *M. phaseolina* in both trials (Table 1). Co-inoculation reduced *M. javanica* reproduction by 57.6% and 68.6% in Trials 1 and 2, respectively. The results for nematode population density were similar to those observed for total nematode number. Whereas in Trial 1, nonsignificant differences were observed between treatments, in Trial 2, nematode reproduction decreased by 68.68% with co-inoculation. In Trials 1 and 2, disease severity was higher in plants inoculated with *M. javanica* only (grade 3 on the Anderson scale, 1986) and lower in plants co-inoculated with nematodes and fungi (grade 2.5).

Regarding vegetative variables (Table 2), in Trial 1, plants inoculated with *M. javanica* only exhibited the highest root fresh weight, followed by plants co-inoculated with the microorganisms. Plants inoculated with *M. phaseolina* only had the lowest root fresh weight (Table 2). In Trial 2, the same pattern was observed: Root

fresh weight was highest in the Mj treatment, followed by Mj + Mp and, finally, Mp. These findings underscore the negative impact of the fungus on root development (Figure 1).

Table 1. Total nematode number, nematode population density, and disease severity index (DSI) of soybean inoculated with *Meloidogyne javanica* (Mj), *Macrophomina phaseolina* (Mp), or both microorganisms (Mj + Mp) in two periods (Trials 1 and 2). Means within columns followed by the same lowercase letter are not significantly different from each other by Tukey's test at $p < 0.05$. ^{ns}Nonsignificant; CV: coefficient of variation.

	Total nematode number		Population density (nematodes g^{-1} root)		DSI	
Treatment	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Mj	10.056^{a}	214.500^{a}	12.566^{ns}	11.179^{a}	-	-
Mp	-	-	-	-	3.0^{a}	3.0^{a}
Mj + Mp	4.262^{b}	67.200^{b}	11.610	4.487^{b}	2.5^{b}	2.5^{b}
CV, %	14.23	15.20	29.21	17.39	28.32	26.63

Table 2. Root fresh weight (RFW), plant height, and shoot dry weight (SDW) of soybean inoculated with *Meloidogyne javanica* (Mj), *Macrophomina phaseolina* (Mp), or both microorganisms (Mj + Mp) in two periods (Trials 1 and 2). Means within columns followed by the same lowercase letter are not significantly different from each other by Tukey's test at $p < 0.05$. ^{ns}Nonsignificant; CV: coefficient of variation.

	RFW (g)		Plant height (cm)		SDW (g)	
Treatment	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Mj	6.13^{a}	19.67^{a}	32.00^{a}	46.72^{ns}	3.25^{b}	5.51^{ns}
Mp	4.65^{b}	12.01^{b}	33.32^{a}	41.78	7.40^{a}	5.74
Mj + Mp	5.67^{b}	15.56^{b}	28.71^{b}	45.43	4.87^{b}	5.20
CV, %	38.17	25.76	16.56	13.21	42.33	27.46

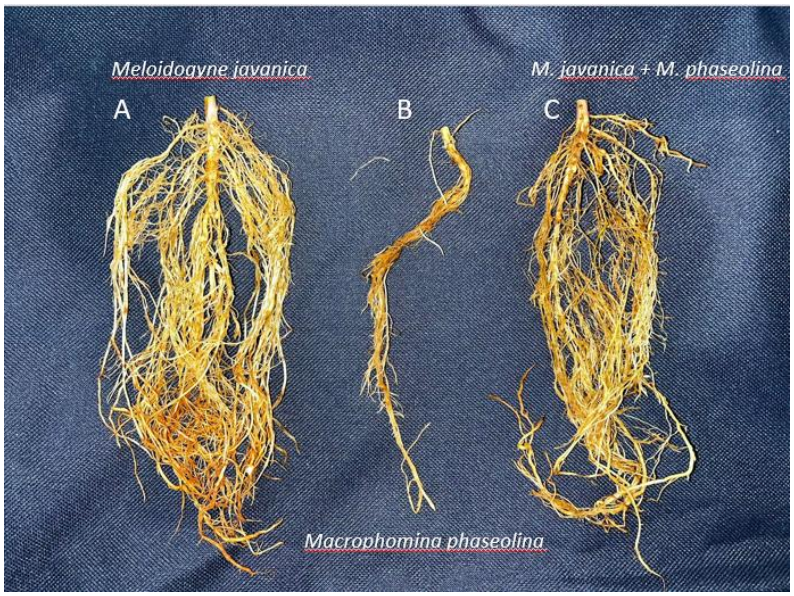

Figure 1. Effect of pathogens on root development in soybean: Roots of soybean inoculated with *Meloidogyne javanica* (A), inoculated with *Macrophomina phaseolina* (B), and inoculated with both pathogens (C).

In Trial 1, sole inoculation of *M. phaseolina* resulted in the highest plant height (33.32 cm), being 13.8% higher than that of the Mj + Mp treatment (Table 2), which resulted in the lowest height. Treatments did not differ in plant height in Trial 2.

Shoot dry weight was highest in plants inoculated with *M. phaseolina* only (Trial 1). Thus, Mj and Mj + Mp treatments reduced shoot biomass accumulation by 56.08% and 34.10%, respectively. Shoot fresh weight did not differ between treatments in either assay. The means ranged from 3.25 to 5.51 g for plants inoculated with *M. javanica*, from 5.74 to 7.40 g for plants inoculated with *M. phaseolina*, and from 4.87 to 5.20 g for plants co-inoculated with *M. javanica* and *M. phaseolina* (data not shown).

Experiment 2: Split-root test

In Trial 1 of Experiment 2 (Table 3), the Un × Mj + Mp treatment afforded an 85.7% lower total nematode number than Un × Mj. Similarly, the total nematode number of the Mj × Mp treatment was 65.8% lower than that of the Un × Mj treatment. In Trial 2, Un × Mj + Mp reduced total nematode number by 72.6% compared with Un × Mj, whereas Mj × Mp reduced the variable by 90%.

In Trial 1, nematode population density was highest in the Un × Mj treatment; the Un × Mj + Mp treatment reduced this variable by 63.4%. Similarly, in Trial 2, Un × Mj + Mp reduced nematode population density by 82.4% compared with Un × Mj, which achieved the highest value. In both trials, disease severity was highest in the Un × Mp treatment (grade 3) and lowest in Un × Mj + Mp and Mj × Mp (grade 2).

In Trial 1, the Un × Mj treatment afforded the highest root fresh weight, possibly due to the formation of galls. In Trial 2, the highest root fresh weight was also observed in the Un × Mj treatment. The Un × Mp treatment resulted in a 50.9% reduction in root fresh weight compared with the uninoculated control, demonstrating the negative impact of the fungus on root development.

Table 3. Total nematode number, nematode population density, and disease severity index (DSI), and root fresh weight (RFW) of soybean inoculated with *Meloidogyne javanica* (Mj), *Macrophomina phaseolina* (Mp), or both microorganisms (Mj + Mp) in split-root experiments conducted in two periods (Trials 1 and 2). Means within columns followed by the same lowercase letter are not significantly different from each other by Tukey's test at $p < 0.05$. ^{ns}Nonsignificant. Un: uninoculated; CV: coefficient of variation.

	Total nematode number		Population density (nematodes g^{-1} root)		DSI		RFW	
Treatment	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Un × Un	-	-	-	-	-	-	0.52^{bc}	0.87^{ab}
Un × Mj	13650^{a}	636^{a}	9374^{a}	491^{a}	-	-	1.48^{a}	1.08^{a}
Un × Mp	-	-	-	-	3^{ns}	3^{ns}	0.21^{c}	0.53^{c}
Un × Mj + Mp	1950^{c}	174^{b}	3425^{b}	86^{b}	2	2	0.61^{bc}	0.82^{b}
Mj × Mp	4667^{b}	60^{b}	6408^{ab}	265^{ab}	2	2	0.83^{b}	0.84^{ab}
CV, %	15.13	45.01	20.64	17.94	20.41	37.04	11.48	12.56

Experiment 3: Activation of defense mechanisms in plants co-inoculated with *M. javanica* and *M. phaseolina*

Treatment and evaluation period exerted significant interaction effects on PPO activity (Table 4). Higher PPO activity was observed at 7 DAI in Mj and Mp treatments compared with Mj + Mp. In the other evaluation periods, PPO activity did not differ between treatments.

The POX activity was influenced by the main effect of evaluation period: The activity was higher at 17 DAI than at 7 or 14 DAI, when activities were 68% and 57% lower, respectively (Table 5). A similar result was observed for GLU, with the highest activity recorded at 17 DAI. Interaction effects were nonsignificant for POX, GLU, or PAL activities (Table 6). The delayed response of these enzymes suggests a possible role in plant defense at more advanced stages of infection.

Table 4. Polyphenol oxidase (PPO) activity in soybean roots at 7, 12, and 17 d after inoculation (DAI) of *Meloidogyne javanica* (Mj), *Macrophomina phaseolina* (Mp), or both microorganisms (Mj + Mp). Means within columns followed by the same lowercase letter and means within rows followed by the same uppercase letter are not significantly different by Tukey's test at $p < 0.05$. CV: Coefficient of variation.

	PPO activity (Δabs_{420} min^{-1} mg^{-1} protein)		
Treatment	7 DAI	12 DAI	17 DAI
Mj	24.97^{aA}	6.77^{aB}	7.58^{aB}
Mp	23.48^{aA}	7.69^{aB}	7.07^{aB}
Mj + Mp	0.21^{bA}	7.30^{aA}	6.93^{aA}
CV, %		43.10	

Table 5. Peroxidase (POX) and glucanase (GLU) activities in soybean roots at 7, 12, and 17 d after inoculation (DAI) of *Meloidogyne javanica* (Mj), *Macrophomina phaseolina* (Mp), or both microorganisms (Mj + Mp). Means followed by the same lowercase letter are not significantly different from each other by Tukey's test at $p < 0.05$. CV: Coefficient of variation.

Period	POX activity	GLU activity
	Δabs_{470} min^{-1} mg^{-1} protein	Δabs_{410} min^{-1} mg^{-1} glucose mg^{-1} protein
7 DAI	13.01^{b}	0.09^{b}
12 DAI	17.34^{b}	0.21^{ab}
17 DAI	40.91^{a}	0.30^{a}
CV, %	66.42	93.78

Table 6. ANOVA for the effects of evaluation period, pathogen inoculation, and their interaction on polyphenol oxidase (PPO), peroxidase (POX), glucanase (GLU), and phenylalanine ammonia-lyase (PAL) activities in soybean roots (Experiment 3).

	Evaluation period (P)			Pathogen inoculation (I)			P × I		
Enzyme activity	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
PPO	2	14.515	< 0.0001	2	9.475	0.0011	4	11.449	0.0000
POX	2	10.860	0.0003	2	2.339	0.1157	4	0.922	0.4659
GLU	2	3.820	0.0346	2	1.359	0.2740	4	0.778	0.5495
PAL	2	0.841	0.4424	2	0.952	0.3986	4	0.879	0.4894

DISCUSSION

The results of reproduction and split-root experiments demonstrated the impact of the root disease complex on soybean and indicated antagonism between *Meloidogyne javanica* and *Macrophomina phaseolina*. Nematode number and disease severity were lower in plants co-inoculated with the microorganisms. For instance, nematode number decreased by 57.6% to 68.6% with co-inoculation. In contrast to what was observed here, previous studies reported that parasitism by soil fungi increased nematode reproduction. In tomato seedlings, for example, co-inoculation with *M. incognita* and *Fusarium oxysporum* increased nematode reproduction and gall formation (Ozdemir, 2022). In soybean, inoculation with *H. glycines* prior to *M. phaseolina* led to an increase in nematode number (Lopez-Nicora et al., 2023). Nevertheless, other studies on different pathosystems corroborate the findings of the current study (Maqsood and Khaliq, 2022; Ozdemir, 2022). Inoculation of *F. oxysporum* f.sp. *lycopersici* before or concurrently with *M. incognita* led to a reduction in gall number and nematode reproduction on tomato, in addition to decreasing the fecundity of female nematodes (Ozdemir, 2022).

Some factors may explain these results. Firstly, pathogens have distinct parasitic habits. For instance, *M. phaseolina* is a necrotrophic fungus (Ruzmetov et al., 2024). Early or severe infection by this fungus can cause partial or total death of host root tissues, directly affecting the activity of biotrophic organisms or obligate parasites, which feed exclusively on living tissues (Sumbul and Mahmood, 2020). Thus, given that *M. javanica* is an obligatory parasite (Guzmán-Piedrahita et al., 2020), fungi-induced damage to host roots may destroy nematode feeding sites, affecting access to food and indirectly impacting reproduction.

Fungal infection can also alter root exudates (Hiremath et al., 2024), possibly compromising the recognition of roots by nematodes, which locate hosts by following the concentration gradient of root exudates (Sikder and Vestergård, 2019). Although this study did not investigate changes in the root exudates of soybean seedlings infected with *M. phaseolina*, previous studies have shown that soil fungi, such as those of the genus *Fusarium*, can alter exudates, making them less attractive or even repellent to nematodes. Such effects were observed in tomato and rice co-inoculated with *F. oxysporum* and *M. incognita* (Maqsood and Khaliq, 2022; Lopez-Nicora et al., 2023; Hiremath et al., 2024).

A disease complex encompassing fungi and nematodes can result in the decline of nematode populations, owing to competition for root space and nutrients (Costa et al., 2020). In a study with root-knot nematodes, it was observed that *M. phaseolina* directly invaded giant cells, interrupting nematode feeding (Sharma and Khan, 2013). Furthermore, the presence of *M. phaseolina* may increase the time it takes for *M. javanica* to complete its life cycle, thereby reducing reproduction (Sharma and Khan, 2013).

In addition to the negative effect on nematode reproduction, co-inoculation with the fungus led to a decrease in disease severity. These findings disagree with those of studies demonstrating that nematodes increase the severity of fungal diseases (Maqsood and Khaliq, 2022). However, in some experiments, nematode infection (*M. incognita*) preceded that of fungi (*M. phaseolina*), which might have influenced the results (Abdel-lateif and Bakr, 2018). One of the possible causes for the increased severity of fungal diseases in cases of nematode and fungal co-infection is the damage caused to roots by nematodes, facilitating the entry of fungi (Husain et al., 2019). Nematode parasitism can disrupt the physiological balance of plants toward energy expenditure, weakening plants and affecting their ability to respond to pathogen attack (Maqsood and Khaliq, 2022).

In the present study, the decrease in fungal disease severity may be attributed to the direct competition for space and nutrients (Lopez-Nicora et al., 2023). Research demonstrated that nematodes could reduce fungal activity and influence the expression of charcoal rot symptoms in soybean, thereby minimizing the severity of fungal infection (Ozdemir, 2022). Nematode parasitism can lead to the release of secondary metabolites that inhibit fungal growth or alter pathogenicity (Lopez-Nicora et al., 2023).

Vegetative variables were mainly affected by inoculation with *M. phaseolina* alone or combined with *M. javanica*, as compared with sole inoculation of nematodes. It is known that *M. javanica* can cause losses in soybean yield (Khan et al., 2022), but it seems that *M. phaseolina* may be responsible for even greater losses. In fact, the fungus has been cited as a determinant of soybean production in several regions of Brazil, particularly in periods of drought, when yield losses can amount to 75% or more (Zanella et al., 2020). This fungus is known to cause root rot in many crops; symptoms of its infection include damage to roots and stems, wilting, yellowing, and, in more severe cases, plant death (Marquez et al., 2021). Additionally, *M. phaseolina* produces sclerotia inside conductive tissues, causing mechanical blockage of xylem vessels, compromising water and nutrient transport, and reducing plant growth and productivity (Marquez et al., 2021).

It is noteworthy that the fungus-nematode complex can result in the imbalance of different factors associated with plant and rhizosphere development, translating into irregular crop yields (Sikder and Vestergård, 2019; Jain et al., 2024). Other studies reported reductions in vegetative variables in plants affected by both fungi and nematodes (Husain et al., 2019).

Despite the numerous studies on the behavior of pathogens involved in complex diseases, little is known about the response of plants to such interactions. Pathogens may activate the plant's defense mechanisms, induced by pathogen- and damage-associated molecular patterns. Such responses can be evaluated by determining the activity of enzymes involved with induced or acquired systemic resistance. Here, polyphenol oxidase (PPO) activity was higher at 7 DAI in plants subjected to single inoculation. Accordingly, it has been shown that *Meloidogyne* spp. and *M. phaseolina* activate PPO expression as a defense response, induced by molecular signals from the pathogen (Archana et al., 2023).

The PPO is related to the biosynthesis of phenolic compounds, which can inhibit the growth of pathogenic microorganisms and reinforce the cell wall of plants, contributing to lignification by generating a physical and chemical barrier (Duangmal and Apenten, 1999; Costa et al., 2020; Archana et al., 2023). However, the enzyme was markedly inhibited (> 90%) when pathogens were inoculated together. Previous studies demonstrated that the simultaneous presence of different pathogens may affect the expression of defense enzymes, owing to antagonistic or synergistic interactions (Abdullah et al., 2017). In such cases, plants may prioritize their defense responses toward one pathogen over another.

Different from what was observed for PPO, there were no differences in glucanase (GLU) or peroxidase (POX) activities between single- and co-inoculation treatments. Plants responded in a similar way to the different pathosystems. For these enzymes, differences were only observed between evaluation periods, with the highest activities recorded at 17 DAI. The GLU activation tends to occur only after plants recognize pathogens. Thus, the initial response is likely mediated by jasmonic acid, and, if the infection persists, salicylic acid pathways may be activated, leading to late GLU expression (Costa et al., 2020). The GLU activity did not differ between co-inoculation (Mj + Mp) and single-inoculation treatments. The lack of additive effects between pathogens can be attributed to the specificities of their interactions, which modulate the response of enzymes (Rajput et al., 2021; Elsharkawy et al., 2022).

The POX participates in lignin deposition and the formation of reactive oxygen species. The enzyme is activated when the infection has been established and the plant needs to reinforce its structural barriers (Shigenaga and Argueso, 2016). The POX is associated with plant resistance against nematodes and responds to H_2O_2 accumulation (Elsharkawy et al., 2022). This mechanism is generally activated in later stages of infection because the plant initially resorts to other strategies to contain the pathogen. A persistent infection leads to changes in cellular redox balance, activation of more intense defense pathways, and increases in POX expression (Abdullah et al., 2017; Costa et al., 2020).

The phenylalanine ammonia lyase (PAL) is one of the first enzymes to be activated in secondary metabolism, rapidly triggering a series of mechanisms, such as the phenylpropanoid pathway and the synthesis of phytoalexins, lignin, benzoic acid, and salicylic acid. In particular, salicylic acid is crucial for the amplification of systemic defense responses (Rojas et al., 2014). The modulation of key enzymes, such as PAL, may also be related to the activation of other defense mechanisms not evaluated here, such as superoxide dismutase. As PAL plays a crucial role in nematode resistance (Rajput et al., 2021), the lack of significant differences in PAL activity across treatments and evaluation periods suggests that it was activated uniformly over time or not at all. There are reports that nematodes can suppress PAL activity in susceptible plants and that co-inoculation can lead to competition between pathogens, resulting in a less effective defense response (Sato et al., 2019).

The complexity of plant-pathogen and pathogen-pathogen interactions, as well as the redundancy in plant defense systems, make it challenging to identify specific responses, particularly when multiple pathogens are involved. This study underscores the complexity of interactions between *M. phaseolina* and *M. javanica* in soybean. The observed antagonistic interactions suggest that the presence of one pathogen can negatively influence the development or reproduction of the other, possibly due to competition for resources, alterations in the root system, or induction of defense mechanisms in host plants. Furthermore, it was shown that *M. phaseolina* is more responsible for crop damage than *M. javanica.* Finally, PPO activity was highest in the early stages of isolated infection (Mj and Mp) but was suppressed in co-inoculated (Mj + Mp) plants, suggesting that pathogen interactions may compromise the initial defense response of soybean plants. Glucanase and POX have a well-defined temporal dynamic, with significant increases in the most advanced stages (17 DAI), indicating a potential role in late plant defense. On the other hand, the uniformity of PAL activity across treatments and evaluation periods is indicative of its low relevance in the studied context.

CONCLUSIONS

This study found that co-inoculation of soybean plants with *Meloidogyne javanica* and *Macrophomina phaseolina* resulted in negative effects on nematode reproduction and the severity of fungal disease compared with sole inoculation. The greatest damage to plant development was caused by inoculation with the fungus alone or combined with the nematode.

Enzymatic analysis revealed an increase in polyphenol oxidase activity in treatments containing *M. javanica* or *M. phaseolina*. By contrast, glucanase and peroxidase expression did not differ between single- or co-inoculation treatments but was influenced by evaluation period, being expressed at the later stage of infection. Phenylalanine ammonia lyase expression was not influenced by treatment or evaluation period.

Author contribution

Conceptualization: L.Z.A., C.R.D-A. Methodology: L.Z.A., M.T.R.S., E.T.S., A.R.C., J.M.H.D.D., C.R.D-A. Software: L.Z.A., M.T.R.S., G.G.V. Validation: L.Z.A., M.T.R.S., G.G.V., E.T.S., K.R.F.S-E., C.R.D-A. Formal analysis: L.Z.A. Investigation: L.Z.A., C.R.D-A. Resources: L.Z.A., C.R.D-A. Data curation: L.Z.A. Writing-original draft: L.Z.A. Writing-review & editing: L.Z.A., C.R.D-A. Visualization: L.Z.A., C.R.D-A. Supervision: L.Z.A., K.R.F.S-E., C.R.D-A. Project administration: L.Z.A. Funding acquisition: L.Z.A., C.R.D-A. All co-authors reviewed the final version and approved the manuscript before submission.

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