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Combined Use of Green Manure and Biological Agents to Control *Meloidogyne javanica* (Treub) Chitwood in Soybean

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Abstract

Root-knot nematode management requires the adoption of integrated practices. Biological agents and cultural control practices are the most widely used, but little is known about their combined effects. This study aimed to assess the interaction effects of the biological agents *Trichoderma harzianum* + *Purpureocillium lilacinum* and different green manures on the control of *Meloidogyne javanica* in soybean under greenhouse conditions. Green manures from white oat, *Urochloa ruziziensis*, *Crotalaria spectabilis*, millet, and buckwheat were grown separately and applied onto the soil surface. Subsequently, soybean seeds were treated with the biological agent and planted. The experiment was repeated twice to confirm the results. In Trial 1 and 2, application of green manure or biological treatment alone was efficient in reducing nematode populations. In Trial 1, there was an interaction between factors on total nematode number and number of nematodes per gram of root. Combined use of biological control with white oat and millet green manure produced great results, since when associated with the biological one, the reduction in the total number of nematodes was potentiated by 55 and 49%, respectively (Trial 1). There was no interaction between green manure and biological factors for Trial 2, and the best results were observed with green manures of *C. spectabilis*, *U. ruziziensis* and white oat, with a reduction in the population density of the nematode in 60, 59 and 44%, respectively. It is concluded that green manure application and *T. harzianum* + *P. lilacinum* were effective in reducing nematode populations when applied separately. White oat and millet green manures associated with *T. harzianum* + *P. lilacinum* increase thecontrol of *M. javanica* in soybean.

Keywords: green manure, *Purpureocillium lilacinum*, root-knot nematode, *Trichoderma harzianum*

1. Introduction

Plant-parasitic nematodes can cause significant damage to soybean, particularly those of the genus *Meloidogyne*, commonly known as root-knot nematodes. Given the high frequency and widespread distribution of root-knot nematodes, it is becoming increasingly difficult to use infested fields for agricultural purposes (Ramzan et al., 2019). The management of these pathogens is complex, and isolated methods rarely provide the expected results. Novel, more efficient and sustainable methods are needed for nematode control. Root-knot nematodes are responsible for losses of up to 55% in soybeans under severe infestation (Silva et al., 2020).

A strategy that has yielded good agricultural results is the use of cover crops in rotation systems (Ferreira et al., 2020). Plants such as buckwheat (*Fagopyrum esculentum* Moench), millet (*Pennisetum glaucum* (L.) R.Br.), *Crotalaria spectabilis* Roth, *Urochloa ruziziensis* (R. German & Evrard), and white oat (*Avena sativa* L.) have been described as excellent alternatives for crop rotation, providing optimal soil cover through green manure production, promoting nutrient recycling, and enhancing the growth of beneficial soil microbiota (Garcia-Franco et al., 2015; Gonçalves et al., 2016; Forstall-Sosa et al., 2021). Furthermore, millet, *C. spectabilis*, *U. ruziziensis*, and white oat were shown to efficiently reduce populations of *Meloidogyne* spp. (Dias-Arieira et al., 2003; Miamoto et al., 2016; Carraro-Lemes et al., 2020; Chidichima et al., 2021).

Biological control is another alternative for reducing or replacing chemical nematicides, with the added advantages of being environmentally friendly and less aggressive to human health (Metwally et al., 2019). Opportunistic fungi are the most commonly used biocontrol agents for plant parasitic nematodes because, beyond the antagonistic action, these microorganisms can be easily cultured in artificial media (Pomella et al., 2009). Fungi of the genus *Trichoderma* and the fungal species *Purpureocillium lilacinum* (Thom.) Samson (= *Paecilomyces lilacinus* (Thom.) Samson) are among the most used agents for nematode control, given their ability to reduce *Meloidogyne* spp. populations by more than 70% (Kiewnick et al., 2011; Zhang et al., 2015).

Trichoderma spp. display different mechanisms of action against nematodes. Antagonistic effects are attributed to antibiosis (characterized by the production of secondary metabolites with a toxic action on nematodes), direct parasitism of nematode eggs and juveniles, and induction of plant resistance to diseases (Abdel-Azeem et al., 2020). *P. lilacinum* is able to parasitize eggs and juveniles as well as sedentary females, releasing enzymes such as proteases and chitinases to degrade the first barrier of its hosts (eggshell or cuticle of females) (Yang et al., 2015; Ahmed & Monjil, 2019).

Integration of two or more management techniques, such as combined use of green manure and biological control, can enhance the efficiency of nematode control (Landi et al., 2018). Fungi such as *Trichoderma* spp. and *P. lilacinum* have a saprophytic habit in soil and, in the absence of hosts, can use organic matter from green manure as substrate for growth and reproduction (Okur et al., 2016). Interactions between cover crops and biocontrol agents may not always be beneficial. Antagonistic substances released by cover crops may interfere with the development of some microorganisms, as has been reported for *Crotalaria ochroleuca* G.Don, which inhibits the ability of the fungus *Pochonia chlamydosporia* (Goddard) Zare & Gams to parasitize *Meloidogyne javanica* (Treub) Chitwood (Vilchis-Martínez et al., 2013). In consideration of these observations, this study aimed to investigate the interaction effects of the fungal agents *T. harzianum* + *P. lilacinum* and different green manures on the control of *M. javanica* in soybean.

2. Materials and Methods

Experiments were performed in a greenhouse at Umuarama, Paraná, Brazil (23°47′55″S 53°18′48″W, 430 m a.s.l). A 6 × 2 factorial completely randomized design was used, with six green manure treatments (buckwheat 'IPR 92-Altar', millet 'ADR 300', *C. spectabilis*, *U. ruziziensis*, and white oat 'Aphrodite', and an unamended control), two biological control conditions (treated and untreated), in seven replications. Trial 1 was conducted between September and December 2019. To confirm the results, the experiment was repeated between February and June 2020 (Trial 2). The mean minimum and maximum temperatures were 20.7 and 32.2 °C in Trial 1 and 18.4 and 29.3 °C in Trial 2.

First, five seeds of each cover crop were sown in polyethylene pots containing 5 L of soil. After 70 days of cultivation, plants were harvested and chopped into pieces of about 1 cm for use as green manure. Experiments were conducted in 500 mL polyethylene cups containing a 2:1 (v/v) mixture of soil and sand. The substrate was previously sterilized in a vertical autoclave at 120 °C for 2 h. Experimental units were placed in random order on a bench. The soil was corrected with 0.4 g of agricultural limestone (85% relative power of total neutralization) and fertilized with 0.24 g of NPK (14:14:14) fertilizer per experimental unit. Then, green manures were applied onto the soil surface at a rate of 5.6 and 1.1 t ha^{-1} in Trials 1 and 2, respectively. This difference in application rates was due to differences in green manure production between trials. Unamended pots were used as control.

After 20 days of green manure application, seeds of soybean (*Glycine max* (L.) Merrill) 'M6210 IPRO' were treated with biological agents *T. harzianum* (Ecotrich®, containing 1×10^{10} CFU g^{-1} product) at a dose of 150 g product 100 kg^{-1} seed and *P. lilacinum* (Nemat®, containing 7.5×10^{9} CFU g^{-1} product) at a dose of 100 g product 100 kg^{-1} seed. The spray volume was equivalent to 500 mL 100 kg^{-1} seed. After treatment, soybean seeds were sown one per pot.

Five days after sowing, plants were inoculated with 2000 eggs and eventual second-stage juveniles (J2) of *M. javanica*. The inoculum was obtained from pure populations maintained on soybean 'M6210 IPRO' in a greenhouse. Nematode extraction followed the method proposed by Hussey and Barker as adapted by Bonetti and Ferraz (1981). The nematode suspension was calibrated to 1000 nematodes mL^{-1}.

At 60 days after inoculation, plants were removed from cups and separated into shoots and roots. The roots were carefully washed, weighed, and subjected to nematode extraction by the above-mentioned method. Nematodes were counted in a Peters' slide under a light microscope. The total number of nematodes was divided by the root weight to obtain the number of nematodes per gram of root (population density). Shoots were evaluated for height, fresh weight, and dry weight. For dry weight determination, shoots were dried in a forced-air oven at 65 °C for 72 h.

Data were subjected to analysis of variance at a significance level of 5%. When necessary, raw data were transformed by $\sqrt{(x + 0.5)}$ to meet normality assumptions by the Shapiro-Wilk test. Significant means were compared by the Scott–Knott test at $p < 0.05$. Statistical analyses were performed using Sisvar software (Ferreira, 2011).

3. Results

Green manure and biological agents exerted significant interaction effects on total nematode number and nematode population density only in Trial 1 (Table 1). In Trial 1, all green manure treatments, associated or not with biological agents, reduced total nematode number (with the exception of buckwheat) and population density compared with control (Table 1). The best results were obtained with *U. ruziziensis* green manure, which reduced total nematode number by 98 and 96% with and without biological treatment, respectively (Table 1).

Effects between the application of green manure and biological control were observed only in treatments with green manure of white oat and millet, since when associated with the biologicals. There was reduction in the total number of nematodes by 55 and 49% in green manure and biological control, respectively. A negative effect was observed for the use of biological agents associated with buckwheat, in which the number of nematodes increase with the association (Table 1). The effects of biological agents were more pronounced when analyzing the means of the control: reductions of 46 and 48% were observed in total nematode number and nematode population density, respectively (Table 1).

There was no interaction between green manure and biological agents factors for Trial 2. The best results were observed with green manures of *C. spectabilis*, *U. ruziziensis* and white oat, with a reduction in the population density of the nematode in 60, 59 and 44%, respectively (Table 1). The population density of *M. javanica* was also lower in treatments using biological agents, with a reduction of 41%, compared with not using biological (Table 1).

Table 1. Effect of green manure and biological agents (*Trichoderma harzianum* + *Purpureocillium lilacinum*) on total number and population density of *Meloidogyne javanica*

Treatments	Total number of *M. javanica*			*M. javanica* population density		
	With Biological	Without Biological	$\bar{x}$	With Biological	Without Biological	$\bar{x}$
Trial 1						
Without green manures	9650 aB	17743 aA		1122 aB	2144 aA	
Buckwheat	6028 aA	3586 cB		505 bA	315 cA	
C. spectabilis	4857 bA	6300 bA		421 bA	538 bA	
White oat	3300 bB	7417 bA		315 cB	815 bA	
Millet	2271 cB	4514 cA		224 cB	581 bA	
U. ruziziensis	171 dA	629 dA		29 dA	93 cA	
CV (%)	28.56			34.97		
Trial 2						
Without green manure	1467	2968	2217 a	359	644	502 a
Buckwheat	1574	2293	1933 a	328	405	367 a
C. spectabilis	1061	803	932 b	201	198	200 b
White oat	803	2168	1486 b	195	366	280 b
Millet	878	2750	1814 a	193	573	383 a
U. ruziziensis	814	1443	1128 b	158	258	208 b
$\bar{x}$	1100 B	2071 A		239 B	407 A	
CV (%)	36.32			38.83		

Note. Within each parameter, means followed by the same lowercase letter in the columns and uppercase in the rows do not differ by the Scott-Knott test at 5% probability ($p < 0.05$). Transformed original data for statistical analysis. CV = coefficient of variation.

Plant height and root fresh weight were influenced by green manure treatment only, not being affected by biological treatment. Lower plant height was observed in control soybean. Plant height was higher in soybean grown in soil amended with *C. spectabilis* or oat green manure in both trials. An increase in plant height was also observed with millet green manure amendment in Trial 1 and buckwheat and *U. ruziziensis* green manure treatments in Trial 2 (Table 2). Buckwheat and *C. spectabilis* green manure increased root weight in Trial 1. In Trial 2, there were no significant differences between treatments (Table 2).

Table 2. Plant height and root fresh weight of soybean treated with different green manures (30 t ha^{-1})

Treatments	Hight (cm)	Root fresh weight (g)
Trial 1		
Without green manure	32.94 c	9.51 b
Buckwheat	39.93 b	12.15 a
C. spectabilis	44.57 a	11.62 a
White oat	44.95 a	9.70 b
Millet	43.84 a	9.17 b
U. ruziziensis	35.69 c	6.69 c
CV (%)	13.55	24.72
Trial 2		
Without green manure	19.68 b	4.32^{ns}
Buckwheat	25.56 a	5.19
C. spectabilis	26.36 a	4.97
White oat	23.93 a	5.35
Millet	21.99 b	5.22
U. ruziziensis	27.36 a	5.07
CV (%)	15.26	23.40

Note. Means followed by the same lowercase letter in the columns and uppercase in the rows do not differ by the Scott-Knott test at 5% probability ($p < 0.05$). CV = coefficient of variation.

There were significant interaction effects of factors on shoot fresh and dry weights in Trial 1 (Table 3). Green manure application increased accumulation of shoot fresh and dry weights, except for *U. ruziziensis* green manure combined with biological agents, which did not differ from the control. In the absence of biological agents, the major shoot weight gain was observed in plants grow in soil amended with millet green manure (Table 3).

In assessing the effects of biological treatment within each green manure group, it was observed that soybean growth was negatively affected by millet and *U. ruziziensis* treatments combined with fungal biocontrol agents (Table 3). In Trial 2, only green manure significantly influenced shoot fresh weight, with higher means in plants grown under buckwheat, *C. spectabilis*, and *U. ruziziensis* green manure-amended soil (Table 3). No differences in shoot dry weight were observed in Trial 2.

Table 3. Effect of green manure and biological agents (*Trichoderma harzianum* + *Purpureocillium lilacinum*) on shoot fresh and dry weights of soybean

Treatments	Shoot fresh weight (g)		Shoot dry weight (g)	
	With Biological	Without Biological	With Biological	Without Biological
Trial 1				
Without green manure	13.18 bA	11.56 cA	3.52 bA	3.07 cA
Buckwheat	20.07 aA	19.90 bA	4.60 aA	4.61 bA
C. spectabilis	18.42 aA	17.28 bA	4.49 aA	4.26 bA
White oat	22.51 aA	20.12 bA	5.18 aA	4.88 bA
Millet	19.10 aB	28.03 aA	4.43 aB	7.15 aA
U. ruziziensis	10.80 bB	16.54 bA	2.55 bB	4.02 bA
CV (%)	28.56		34.97	
Trial 2				
Without green manure	7.03 b		1.73^{ns}	
Buckwheat	9.05 a		2.16	
C. spectabilis	9.54 a		2.13	
White oat	7.21 b		1.81	
Millet	7.31 b		1.64	
U. ruziziensis	9.13 a		2.02	
CV (%)	24.79		35.35	

Note. Means followed by the same lowercase letter in the columns and uppercase in the rows do not differ by the Scott-Knott test at 5% probability ($p < 0.05$). CV = coefficient of variation.

4. Discussion

The findings showed that green manure and biological agents can reduce the population of *M. javanica* when applied alone, and may have the greatest effect when combined, as is the case with white oat (Trial 1 and Trial 2) and millet (Trial 1) associated with *T. harzianum* + *P. lilacinum*. It was observed reductions of 44 to 73% in total nematode number in soybean treated with biological agents and grown in soil amended with *U. ruziziensis* green manure, as compared with untreated plants; however, no significant differences were observed between means under the tested experimental conditions.

The differences in results between Trial 1 and Trial 2 can be explained by the variation in temperatures observed in both experiments, which were carried out at different times. It was possible to observe the dynamics of the interaction between green manure and biological agents in different environmental conditions, confirming the positive results.

The beneficial association of oat and millet green manures with biological control agents might be related to the fact that these crop residues have high C/N ratios, resulting in slow residue degradation and increased concentration of humic substances and organic matter in soil (Souza et al., 2019). These factors contribute to the development of saprophytic microorganisms, such as *T. harzianum* and *P. lilacinum*, by increasing the ability of fungi to colonize soil (Okur et al., 2016). On the other hand, rapid degradation of residues from other green manure crops may release a high amount of chemicals into the soil, the effect of which on fungal hyphae is not yet known and might be deleterious.

Different from the interaction between biological agents and green manure from grass species, the association of biological treatment with *C. spectabilis* and buckwheat green manures did not exert result synergistic or antagonistic effects on *M. javanica* control. It is hypothesized that fungi were not benefited by this type of green manure or that residue decomposition was too fast, precluding the use for saprophytic activity. Because *C. spectabilis* and buckwheat green manures have low C/N ratios, it can be assumed that part of the organic matter underwent mineralization during the 20 days between soil amendment and soybean sowing (when biological treatment was applied).

Most plant species studied here have previously shown potential to reduce *Meloidogyne* populations, including *C. spectabilis* (Miamoto et al., 2016), *U. ruziziensis* (Dias-Arieira et al., 2009), millet (Nascimento et al., 2020; Chidichima et al., 2021), and oat (Marini et al., 2016; Carraro-Lemes et al., 2020). The referred studies evaluated the efficiency of these plant species as cover crops, with or without green manure deposition, and, often, the effects of crops were attributed to their being antagonists or bad hosts to nematodes.

This study demonstrated that soil amendment with green manure can assist in the control of *M. javanica*, particularly by decreasing population density. This finding is due to the fact that decomposing plant residues can provide several benefits to soil by enhancing nutrient recycling and particle aggregation, which favor plant growth (Ferreira et al., 2015; Debiasi et al., 2016). Furthermore, in non-autoclaved soils, the presence of green manure increases organic matter levels, and its decomposition can release substances that stimulate the growth of organisms that are beneficial to plants (Quist et al., 2019).

It is reported that that, compounds with nematicidal effects may be released during decomposition of plant matter (Chitwood, 2002). *Crotalaria spectabilis* is known to produce pyrrolizidine alkaloids, which are nematicidal (Chitwood, 2002; Colegate et al., 2012). Oat extract was shown to have a direct effect on plant-parasitic nematodes by producing flavone-*C*-glycosides (Soriano et al., 2004). Several compounds are produced and released by buckwheat, millet, and *U. ruziziensis*, such as phenolic compounds (Nambiar et al., 2005; Ahmed et al., 2014; Toledo et al., 2019). Phenolic compounds comprise a variety of secondary metabolites produced by plants, such as terpenoids, phenylpropanoids, flavonoids, isoflavonoids, and tannins. It is known that these oxidative compounds have high biological activity against phytopathogens, acting as a mechanism of plant resistance (Ohri & Pannu, 2010; Selim et al., 2014).

As biological control agents, *Trichoderma* spp. have proven effective against root-knot nematodes because of their antagonistic behavior. These fungi inhibit nematode development directly, through hyperparasitism, antibiosis, and production of toxic secondary metabolites, such as chitinases, which degrade egg cell walls, thereby minimizing the hatching of *Meloidogyne* spp. (Zhang et al., 2015, 2017). The fungi can also act indirectly, by promoting plant growth, increasing plant resistance to biotic and abiotic stresses. Moreover, *Trichoderma* spp. can induce resistance in plants by activating defense mechanisms and stimulating the synthesis of antimicrobial compounds and reactive oxygen species (Martínez-Medina et al., 2013; Abdel-Azeem et al., 2020). The *T. harzianum* strain composing the biological product used in this study is not registered for the control of nematodes but rather for the control of soil fungi. Previous studies have reported its effectiveness in controlling *M. javanica* (Miamoto et al., 2021) and *Pratylenchus brachyurus* (Godfrey) Filipjev & Sch. Stekhoven (Dias-Arieira et al., 2018).

P. lilacinum, on the other hand, is a chitinolytic organism; that is, it directly parasitizes nematode eggs and sedentary females (Khan et al., 2004, 2006). The fungus was shown to be effective in the control of *Meloidogyne* spp. The biological agent acts by producing chitinases and proteases that degrade the cell wall of nematode structures and by inducing resistance in plants (Yang et al., 2015; Ahmed & Monjil, 2019). In addition to controlling root-knot nematodes (Miamoto et al., 2021), *P. lilacinum* + *T. harzianum* efficiently reduced populations of lesion nematodes (*P. brachyurus*) in soybean grown under greenhouse and field conditions (Dias-Arieira et al., 2018).

The variation in total nematode number and nematode population density between trials can be attributed to the amount of green manure used and the temperature during experimental periods. The higher temperature observed in Trial 1 might have increased residue degradation rates, leading to greater release of chemicals into the soil. Moreover, biological agents might have had lower activity under lower temperature conditions.

Significant in vegetative parameters between treatments, it was found that green manure application enhanced soybean development, regardless of biological treatment. This finding may be attributed to the beneficial effect of organic matter on soil, improving physical, chemical, and nutrient conditions (Leite et al., 2010; Debiasi et al., 2016). In Trial 1, biological treatment increased shoot fresh and dry weights compared with the control. Such a beneficial effect of *T. harzianum* and *P. lilacinum* on plant development might be associated with the endophytic behavior of these fungi. These organisms are considered plant growth promoters, improving vigor and tolerance to stress, as well as assisting in nutrient absorption (Kumar, 2013; Ahmed & Monjil, 2019).

The interaction between decomposing organic matter, biological agents, nematodes, and plants can be very complex and is still little understood. Furthermore, these relationships may differ according to soil type and climatic conditions. Thus, further research is needed to better understand this phytopathosystem, especially at the field level.

5. Conclusions

The results of both trials allow us to conclude that green manure application and *T. harzianum* + *P. lilacinum* were effective in reducing nematode populations when applied separately. White oat and millet green manures had the best interaction with the biological control product based on *T. harzianum* + *P. lilacinum* against *M. javanica* in soybean. Biocontrol and green manure treatments enhanced soybean development.

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