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Biological Control Associated With Plant Nutrition for *Meloidogyne javanica* and *Pratylenchus brachyurus* Management in Soybean

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Abstract

Meloidogyne javanica and *Pratylenchus brachyurus* stand out among the main nematodes in soybean crops. Research on integrated management are often conducted, due to the low efficiency of the main control methods when they are applied alone. Thus, the aim of the present study was to assess the potential of biological control and plant nutrition products to control these nematodes in soybean. The effect of each product alone on nematode hatching and mortality was also assessed. A greenhouse experiment was also carried out, evaluating five doses of the product for biological control based on *Bacillus* and *Trichoderma*, with and without the presence of the product for nutrition, inoculated with 2000 eggs and juveniles for the gall nematode or 1000 specimens for the nematode lesions. After 30 days of multiplication, the aerial part was removed and the soil was revolved to receive the new sowing of the soybean with the respective treatments mentioned above. After 60 days, the experiments were evaluated for nematological parameters. Both products reduced hatching and increased nematode mortality. Treatments with biological control were efficient in reducing *M. javanica* and *P. brachyurus*, mainly when applied at doses close to 5 and 8 kg ha^{-1}, respectively. The nutrition product negatively influences the biological control.

Keywords: alternative control, integrated management, root-knot-nematode, root-lesion nematodes

1. Introduction

Soybean (*Glycine max* (L.) Merril) cultivation in Brazil is continuously increasing, possibly due to the development of new cultivars resistant to pests and diseases, besides the expansion of new cultivated areas resulting from the developed high-technologies, from favorable weather conditions and high culture adaptability to different cultivation regions (CONAB, 2017).

Despite such improvements, some factors limit productivity. Nematodes stand out among these factors, since they cause significant growing losses. Root-knot nematodes (*Meloidogyne* spp.) and root-lesion nematodes (*Pratylenchus brachyurus* (Godfrey) Filipjev & Sch. Stekhoven) are often associated with damages to the crop. *Meloidogyne javanica* (Treub) Chitwood is responsible for 10 to 40% losses, mainly in sandy and medium-sandy textured soil (Inomoto & Silva, 2011). Cell hyperplasia and hypertrophy are characteristic symptoms of nematode-associated infections; they lead to gall formation in the root system due to root thickening in the areas affected by the sedentary females, fact that facilitates the diagnosis (Moens et al., 2009). *Pratylenchus brachyurus*, in its turn, causes root-lesions that get darker due to the injection of enzymes and toxins in the root cortex during nematode migration and feeding inside the root (Henning et al., 2014). Problems associated with this nematode have been related to successive cultivation of host plants after soybeans, including maize, millet and *Brachiaria*, as well as to the culture expansion to regions presenting sandy and low-fertility soil (Inomoto, 2011).

Managing these phytonematodes is a complex; therefore, it is necessary integrating different practices. Accordingly, the biological control has been standing out due to the introduction of antagonistic microorganisms in order to decrease damages caused to plants of economic interest (Collange et al., 2011). Rhizobacteria, mainly

bacteria belonging to genus *Bacillus*, present nematicide potential, besides their potential to promote root development and plant growth (Xiong et al., 2015; Berlitz et al., 2016). Some fungi living in the soil have also been studied in order to check their potential for nematode management. Emphasis is given to *Trichoderma*, which has varying antagonistic-action mechanisms to control nematodes, including antibiosis, parasitism, the competition for penetration sites in the roots and resistance induction (Al-Shammari et al., 2013; Kath et al., 2017).

Other alternative consist in using products focused on nutrition, which help making minerals available for plant growth. The nutrients are responsible for increasing or reducing disease severity and for inducing host-plant resistance (Santana-Gomes et al., 2013).

Based on the aforementioned, the aim of the present study was to assess the efficiency of different doses of a product based on microorganisms (Nem-Out™), associated or not with a plant nutrition product (Soil-Set®) in the *M. javanica* and *P. brachyurus* control in soybean, and their effect on nematode hatching and mortality.

2. Material and Methods

Experiment *in vitro* were conducted at the Laboratory of Nematology order to assess the effect of the products on nematode hatching and mortality. These experiments followed a completely randomized design with six treatments and seven repetitions.

The hatching experiment was carried out with *M. javanica*, whose eggs came from a pure population multiplied on tomato plants. Roots with egg masses were placed in 500 mL Erlenmeyer and added with 300 mL of 0.5% Sodium hypochlorite. The Erlenmeyer was closed with a rubber cap and stirred for two minutes to induce egg-detachment from the egg masses. The suspension was sieved (through 500-mesh sieve) and washed in water. Eggs were collected and calibrated to 600 eggs in 0.5 mL of water. The procedure was conducted in a Peters camera coupled to a light microscope.

The suspension (0.5 mL) containing the eggs and 9.5 mL of treatments were placed in Petri dishes (8 cm diameter). The treatments consisted of a biological control product, Nem-Out™ (based on *Bacillus subtilis* (Ehrenberg) Cohn, *B. licheniformis* (Weigmann) Chester and *Trichoderma longibrachiatum* Rifai (total micro-biological counting 3.75×10^8 UFC g^{-1}), plus protease, silanase and cellulose; the amount of these proteins was not informed by the manufacturer, Improcrop® do Brasil Ltda) at doses of 4 and 8 kg ha^{-1}, and a plant nutrition product, Soil-Set® (composed of 3.7% sulfur (33.8 g L^{-1}), 3.00% (36.9 g L^{-1}) copper, 1.6% iron (19.7 g L^{-1}), 0.8% manganese (9.8 g L^{-1}), 3.2% zinc (39.4 g L^{-1}), 2.13% organic carbon, at density 1.23 g cm^{-3} and pH 2.55, Improcrop® do Brasil Ltda) at dose of 1 and 2 L ha^{-1}. Distilled water and Furadan 350SC (Carbofuran at dose 4.5 L of the commercial product per hectare) were used as control.

The plates were incubated in BOD at 27 °C and the hatched juveniles and remaining eggs were assessed eight days after incubation. The evaluations were performed in Peters camera coupled to light microscope, at 10x magnitude, and expressed in hatching percentage.

Nematodes belonging to both species were subjected to the method suggested by Coolen and D'Herde (1972) to test mortality. The suspension containing eggs and eventual juveniles was deposited in Baermann funnel (Baermann, 1917) and active nematodes were collected 24 hours later. The experiment was installed and incubated according to the procedure adopted for the hatching test. However, the evaluation was carried out 24 hours after incubation by taking into account the live and dead nematodes in the suspension. At the time of assess, in each sample was added NaOH 1N at 10% of the solution volume, in order to facilitate live-nematode identification. Results were expressed in mortality percentage.

Data were subjected to analysis of variance and the means were compared by the Scott-Knott test at 5% probability, using Sisvar statistical software (Ferreira, 2011).

Other experiment was conducted in a greenhouse, located at 23°47′28.4″ S latitude, 53°15′24.0″ W longitude, and altitude 379 meters. The study followed a completely randomized design, in a factorial 5 × 2, with five doses of biological control product (0, 2.5, 5.0, 7.5, and 10 kg ha^{-1}), with and without the plant nutrition product, and six repetitions for each treatment.

The experiment with *M. javanica* was conducted from December 2015 to March 2016 (Mj-2016), under mean minimum and maximum temperatures 16.9 and 29.3 °C, respectively, and was repeated between November 2016 and February 2017 (Mj-2017), when temperatures were 15.5 and 26.1 °C, respectively. The experiment with *P. brachyurus* was carried out simultaneously to the experiment with *M. javanica* in the 2016 year.

Nematodes were multiplied in the experimental units, aiming at simulating the field condition under which nematodes are living in the soil and in root remnants. In order to do so, soybean cv. Pintado seedlings were produced on trays filled with BioPlant® commercial substrate; 15 days after germination they were transplanted to pots filled with 3.5 kg of a mixture containing soil and sand (2:1), previously autoclaved at 120 °C for 2 hours. Seedlings were inoculated with a population of approximately 2000 eggs and eventual second-stage juveniles (J2) of *M. javanica*, or with 1000 *P. brachyurus* specimens, three days after the transplant. The inocula were distributed in 3 mL of water and deposited in two holes (2 cm deep) opened in the soil around the plant, which was closed after the inoculation. The *M. javanica* and *P. brachyurus* inocula were obtained from pure population kept on tomato plants cv. Santa Clara and on maize cv. AL Bandeirante, respectively. They were extracted according to the methodology suggested by Hussey and Barker, adapted by Boneti and Ferraz (1981), and Coolen and D'Herde (1972), respectively.

After 30 days, aerial part was eliminated, and the soil was slightly revolved for soybean sowing. The biological control product was applied on the sowing groove, at the doses mentioned, with or without the plant nutrition product, also applied on the sowing groove, at dose 1 L of commercial product (Soil-Set®) per hectare, in 1 L of water. Plant-nutrition product application was repeated on the surface thirty days after the first application in the units treated with Soil-Set®.

The plants were kept in greenhouse for 60 days and were daily irrigated. They were collected after this period and the root system was separated from the aerial part. The root systems were carefully washed and placed on absorbent paper to eliminate water excess; subsequently, the root fresh weight was determined. They were subjected to the aforementioned extraction methods, the total number of nematodes per root system was assessed under a Peters camera coupled to a light microscope. The recorded value was divided by the root weight to find the number of nematodes per root gram. The height, and the dry and fresh weights, of the aerial part was determined. The dry weight was found through drying in forced air circulation oven at 65 °C until reaching the constant weight.

Data were subjected to analysis of variance at 5% probability; in case of significant results, the doses were evaluated in the nutrition factor. These doses were assessed through regression analysis. If the nutrition factor was significant, the means were compared through the Bonferroni T test in the Sisvar statistical software (Ferreira, 2011).

3. Results

Except for Nem-Out 4 kg ha^{-1}, all other treatments reduced the hatching of *M. javanica* juveniles; and all treatments, including Nem-Out 4 kg ha^{-1}, led to nematode mortality and did not differ from Furadan 350SC (Table 1). With regard to *P. brachyurus*, although the treatments have caused nematode mortality, in comparison to the controls, the percentages were lower than that of Furadan 350SC.

Table 1. Hatching and mortality percentage of *Meloidogyne javanica* (Mj), and mortality percentage of *Pratylenchus brachyurus* (Pb) subjected to different treatments assessed 8 days after inoculation (DAI) in the hatching test, and 24 hours for mortality

Treatments	Hatching of Mj (%)	Mortality of Mj (%)	Mortality of Pb (%)
Control	42 a	0 b	0 c
Furadan 350SC	27 b	100 a	95 a
Nem-Out™ 4 kg ha^{-1}	41 a	100 a	74 a
Nem-Out™ 8 kg ha^{-1}	16 c	100 a	33 b
Soil-Set® 1 L ha^{-1}	26 b	100 a	35 b
Soil-Set® 2 L ha^{-1}	12 c	100 a	19 b
CV (%)	22.31	5.42	16.47

Note. Means followed by the same letter in the column did not differ from each other in the Scott-Knott test at 5% probability. CV = coefficient of variation.

There was no interaction between the biological control and nutrition factors in experiments conducted in 2016 with *M. javanica* (Mj-2016). Total number of eggs + J2 was influenced by the treatment with biological control, which recorded maximum reduction in the dose 4.06 kg ha^{-1} of the product (Figure 1A).

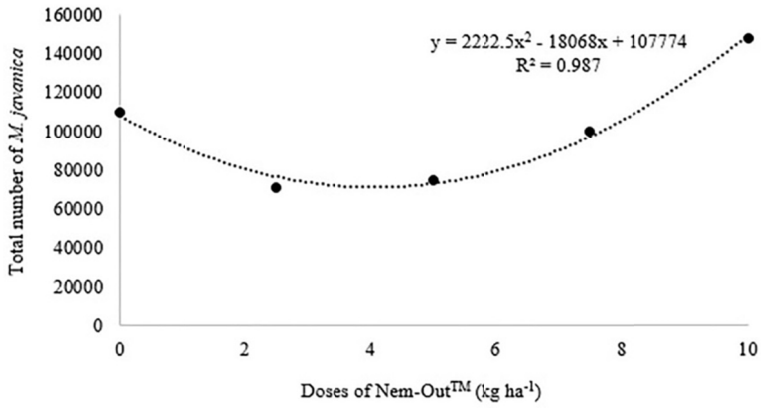

Figure 1A. Total number of *Meloidogyne javanica* in soybean roots subjected to treatment with increasing doses of the biological control product (Nem-Out™) 60 days after treatments. Mj-2016

On the other hand, the nutrition product negatively affected the biological control; general means were equal to 64825 and 147647 eggs + J2 per root system in the treatments with and without plant nutrition, respectively. There was interaction between the factors in Mj-2017; results corroborate those from 2016, since the nutrition product led to increase in the total number of *M. javanica* in the root system (Figure 1B), whereas its absence reduced nematode multiplication. In this case, the application of 5.31 kg ha^{-1} of biological control product was the best control, since it reduced by approximately 55% the total of eggs + J2 per root system.

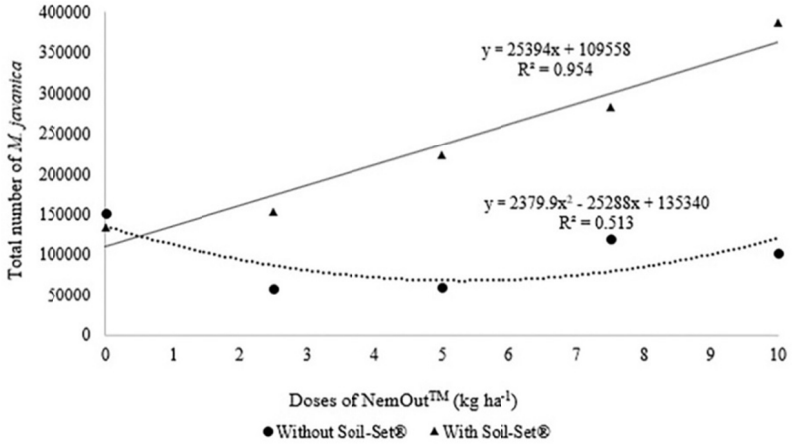

Figure 1B. Total number of *Meloidogyne javanica* in soybean roots, submitted to treatment with increasing doses of the biological control product (Nem-Out™), without or with nutrition product (Soil-Set®), 60 days after treatments. Mj-2017

Data about the total of eggs + J2 parameter directly reflected the number of eggs + J2 g^{-1} of root. Therefore, there was interaction between factors in both experiments; once more, the nutrition factor negatively influenced the biological control. The use of biological control without the nutrition factor in Mj-2016 recorded maximum reduction when 4.69 kg ha^{-1} of the product was applied; the minimum number of eggs + J2 was equal to 2936. On the other hand, the same number was 21362 (number resulting from the derivative and from the integral of the equations) at the best dose (2.84 kg ha^{-1}) with the nutrition factor (Figure 2A).

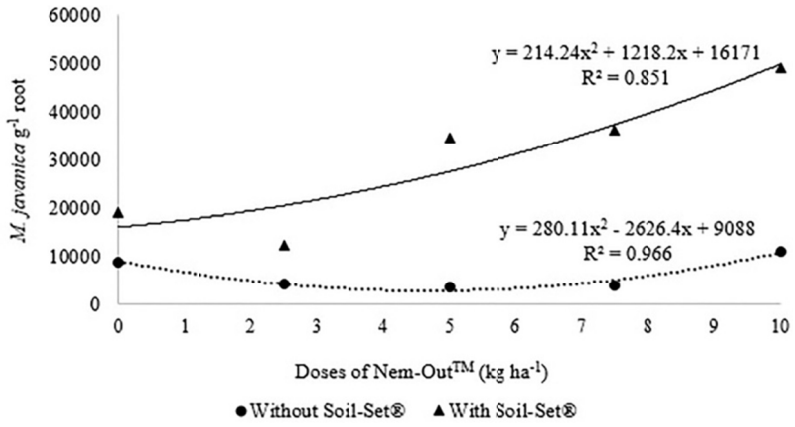

Figure 2A. Total number of *Meloidogyne javanica* in soybean roots, submitted to treatment with increasing doses of the biological control product (Nem-Out™), without or with nutrition product (Soil-Set®), 60 days after treatments. Mj-2016

Yet in this experiment, the values of eggs + J2 g^{-1} of root recorded for the controls with and without the nutrition factor were 8892 and 19094, respectively. The biological control applied without nutrition in 2017 recorded maximum *M. javanica* reduction (1600 eggs + J2) at dose 5.62 kg ha^{-1}, whereas the increase in the number of eggs + J2 was directly proportional to the dose increase within the nutrition factor (Figure 2B).

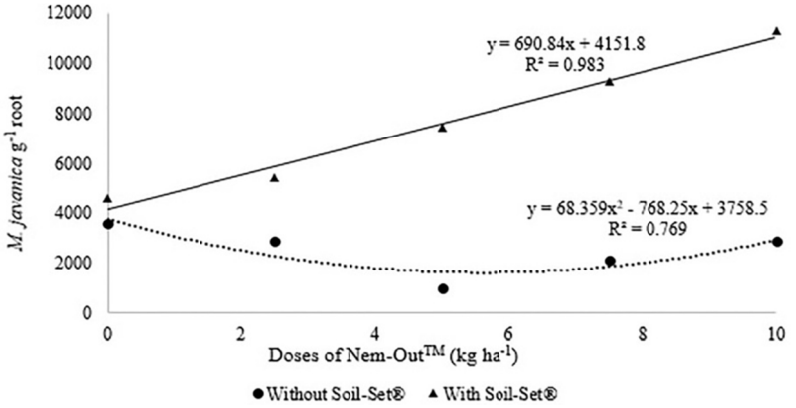

Figure 2B. *Meloidogyne javanica* g^{-1} of soybean roots, submitted to treatment with increasing doses of the biological control product (Nem-Out™), without or with nutrition product (Soil-Set®), 60 days after treatments. Mj-2017

The negative effect of the nutrition product was once more observed in the experiment with *P. brachyurus*. In this case, the analysis was only significant for the number of nematodes g^{-1} of root, since there was interaction between factors and best nematode control was recorded for the use of biological control alone at dose 8.24 kg ha^{-1} of the product (Figure 3).

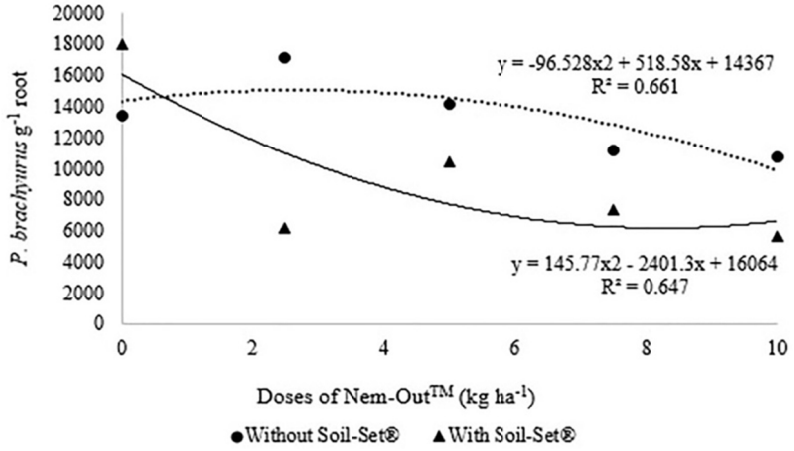

Figure 3. Number of *Pratylenchus brachyurus* g^{-1} soybean root, submitted to treatment with increasing doses of the biological control product 60 days after treatments. Year 2016

With regard to the vegetative parameters of the experiment with *M. javanica* conducted in 2016 (Mj-2016), the different doses of the biological control product, as well as the interaction between biological control and nutrition, did not influence plant height, although the nutrition factor was significant, since the means were 27.61 and 35.12 cm in treatments with and without the nutrition factor, respectively. Similar result was observed in Mj-2017, when plant height was negatively influenced by nutrition.

Fresh and dry weight of the aerial part in the experiment with *M. javanica* in 2016 recorded 2.95 and 6.43 g, and 0.98 and 1.97 g in treatments with and without nutrition, respectively. The root was also influenced by nutrition, since it recorded the respective means 6.73 and 12.48 g. In 2017, the fresh and dry weight of the aerial part recorded 30.49 and 45.98 g, and 9.07 and 12.19 g in treatments with and without this product, respectively. Yet in Mj-2016, the root weight was not affected by the treatments or by the interaction between them.

The experiment with *P. brachyurus* showed that the factors, as well as the interaction between them, did not influence plant height and the dry weight of the aerial part. The nutrition factor was significant for the dry weight of the aerial part, since it negatively influenced it; means were 11.3 and 13.4 g plants treated, or not, with the product. Similar results were recorded for the root weight, whose respective means were 19.6 and 22.9 g.

4. Discussion

The two assessed products promote reduced *M. javanica* juvenile hatching and nematode mortality (Table 1). The action of Nem-Out™ could have happen through antibiosis, since the microorganisms can produce nematicide substances capable of inhibit hatching and increase nematode mortality (Szábo et al., 2012). Soil-Set®, in its turn, is composed of nutrients (sulfur, copper, iron, manganese and zinc) that can have straight action on nematodes. Iron was efficient in reducing the *M. incognita* population when nanoparticles of it were used in okra, either in tests *in vitro* or *in vivo* (Sharma et al., 2017). Song et al. (2014) assessed the cooper effect on *Caenorhabditis elegans* Maupas, in electronic microscope and found that the nutrient causes changes in nematode cuticle, as well as on some nematode biological functions. However, it was observed that zinc did not have effect on *M. incognita* juvenile hatching (Couto et al., 2016).

The present study showed that, regardless of the dose, biological control application reduced the multiplication of both nematodes (Figures 1 to 5). The efficiency of this product (Nem-Out™) was previously proved by the *M. javanica* and *P. brachyurus* population reduction in soybean (Miamoto et al., 2017) and *Meloidogyne incognita* (Kofoid & White) Chitwood in tomato (Silva et al., 2017). Likely, the product effect regards the microorganisms composing it; thus, *B. subtilis* has been one of the most studied bacteria to nematodes biological control. Its efficiency was previously observed in the reduction of nematodes of soybean-cyst nematodes (*Heterodera glycines* Ichinohe) (Araújo et al., 2002). It was also efficient in reducing *P. brachyurus* and *Rotylenchulus reniformis* Linford & Oliveira in cotton plants, whose control was similar to the use of chemical nematicide (Higaki & Araújo, 2012), besides the results recorded for *Meloidogyne* spp. management in tomato plants and in soybean with the same bacterium (Araújo et al., 2012).

Genus *Bacillus* has different mechanisms capable of help controlling nematodes, including the production of toxins, enzymes and other metabolic products that can change nematode orientation towards the plant roots

(Araújo et al., 2002). Such bacteria are also known by their efficiency in degrading root exudates responsible for stimulating recently-hatched juveniles migration; thus impairing root location by the nematodes (Araújo et al., 2002). Moreover, there are reports about bacteria belonging to genus *Bacillus* that are capable of inducing plant resistance to many pathogens, including nematodes (Sikora et al., 2007; Adam et al., 2014; Zhang et al., 2016).

The presence of fungus *Trichoderma* likely contributed to the results, since many species and isolates of this fungus are efficient to nematode biological control in many pathosystems, including *T. longibrachiatum*. It was the biocontrol agent against *M. javanica* and *M. incognita* in tomato plants (Al-Shammari et al., 2013; Silva et al., 2017), and *Heterodera avenae* Wollenweber in wheat (Zhang et al., 2014, 2017). Although the action-mechanism used by genus *Trichoderma* acting in nematode management is not completely elucidated, but some authors report the direct parasitism of eggs and juveniles (Suárez et al., 2004; Al-Shammari et al., 2013). Besides this, these fungi can produce enzymes, including chitinase, protease and other lytic enzymes that, when secreted, can penetrate the J2-cuticle and egg gelatinous-matrix, thus severely changing their physical integrity (Al-Shammari et al., 2013; Zhang et al., 2014, 2017), which may also explain the reduction in hatching and increase in mortality observed in this study (Table 1).

Trichoderma spp. has also been cited as plant systemic resistance inductor (Contreras-Cornejo et al., 2009; Al-Shammari et al., 2013; Zhang et al., 2014; Kath et al., 2017); the study with *T. longibrachiatum*-wheat-*H. avenae* showed significant enzymatic activity increase (peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase). These enzymes are related to nematode resistance induction, and this mechanism is the key to suppress pathogens development (Zhang et al., 2014).

With regard to the current study, the use of Soil-Set® as vegetal nutrition product negatively influenced the *M. javanica* and *P. brachyurus* control (Figures 2, 3, and 4). Likely, such result derives from the presence of substances such as copper and sulfur, which may have had deleterious effect on the antagonistic microorganisms composing the biological control product, since there are reports about copper capacity to reduce the lifetime of bacterium *Bacillus thuringiensis* Berliner in greenhouse and in the field (Haddad et al., 2011). However, this product has the potential to nurture the plant and to manage nematodes, besides its possible potential to induce resistance when it is applied on the aerial parts (Miamoto et al., 2017), without any close contact with the microorganisms.

Treatments used in the present study did not promote vegetative enhancement, and it corroborated results found in other studies that used the same product (Nem-Out™) to control *M. incognita* in tomato plants (Silva et al., 2017), and *M. javanica* and *P. brachyurus* in soybean (Miamoto et al., 2017). However, research point out the potential of both genera to promote plant growth (Baños et al., 2010; Souza & Debastiani, 2015), since their permanence time, as well as the edaphic-climatic factors, can be responsible for the difference in the results.

In conclusion, the product based on *Bacillus* and *Trichoderma* is presented as effective alternative in the management of *M. javanica* and *P. brachyurus* in soybean, mainly when applied at doses close to 5 kg ha^{-1} and 8 kg ha^{-1}, respectively. The plant nutrition product (Soil-Set®) had an antagonistic effect on the biological control, not being recommended its association with the biological products.

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