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Nitrogen Fertilization and Rhizobacteria in the Control of *Meloidogyne javanica* in Common Bean Plants

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Abstract

The effects of treating seeds of the common bean with rhizobacteria before sowing associated with different nitrogen (N) doses on the control of the nematode *Meloidogyne javanica* was evaluated. The assay was carried out in a greenhouse in random blocks, with a factorial arrangement of 5 × 5 + 1: five N doses (0, 25, 50, 100, and 200 kg/ha); five isolates: *Bacillus pumilus* (1, 60 and 76), *Bacillus subtilis*.-34, *Bacillus* sp.-36, and one control treatment. The plants were inoculated with 3,000 nematode eggs/pot. After 60 days the assay was evaluated. In the absence of N, *B. pumilus*-60 promoted a reduction of more than 50% in the number of galls and egg masses compared to the control. *B. pumilus* (1 and 76) and *Bacillus* sp.-36 reduced the number of galls in combination with increased N doses. Treatment of seeds with *Bacillus* in combination with N fertilization contributed to a reduction in the number of galls and nematode egg masses. *B. pumilus* (60 and 76) reduced the number of galls and egg masses in the absence of N, indicating a nematicidal effect. The treatments had no effect on the number of second-stage juveniles, eggs, or the plant parameters.

Keywords: root-knot nematode, biological control, *Phaseolus vulgaris*

1. Introduction

The common bean (*Phaseolus vulgaris* L.) has great socio-economic importance in Brazil, it is one of the main components of the diet due to its high protein content (Soares et al., 2006). The crop is cultivated throughout Brazil because good adaptation to a range of edaphoclimatic conditions. The main producing regions are the States of Paraná, Bahia, Minas Gerais, Goiás and São Paulo (IBGE, 2014).

The nematode specie *Meloidogyne javanica* (Treub) Chitwood is one of the main causes of reductions in bean crop yields, especially in regions routinely subject to high temperatures that increase environmental stress and affect plant tolerance to the nematode (Pedrosa, 2000). The losses in common bean crops caused by parasitism by *M. incognita* (Kofoid; White) Chitwood and *M. javanica* may reach up to 90% (Agudelo, 1980).

The standard measures to control *Meloidogyne* spp. in common bean crops are crop rotation, fallow periods, and use of resistant varieties. However, the strategy of using high resistance varieties is limited, there are no available genetic materials for farmers (Pedrosa et al., 2000; Simão et al., 2005). Moreover, crop rotation and fallow periods are difficult to establish. The farmers prefer to cultivate crops during the three annual seasons in irrigated areas, which causes an increased nematode population density in these regions.

Nitrogen fertilizers are extremely important for plant development. The incorporation of some fertilizers into the soil can have a suppressive effect on nematode populations; for example, urease activity can convert urea to ammonia, which is toxic to nematodes (Sidiqqui et al., 2001).

The treatment of seeds with rhizobacteria particularly *Bacillus* species, has produced encouraging results for the control of many soil pathogens, including root-knot nematodes (*Meloidogyne* spp.) (Ludwig et al., 2013). Numerous mechanisms are involved in the nematode control by rhizobacteria, such as alteration of root exudates and the consequent limitation of nematode penetration in the roots (Lian et al., 2007), reduction of juvenile hatching (Campos et al., 2006), production of enzymes and toxic compounds, and the induction of systemic resistance in the host plant (Oostendorp & Sikora, 1990). In addition to acting as a biological control, rhizobacteria may also promote plant growth by synthesizing growth regulators such as indole acetic acid (IAA),

gibberellins, and cytokines by biological nitrogen fixation or by solubilizing inorganic phosphates present in the soil.

A few studies have shown that a combined treatment with rhizobacteria and nitrogen fertilizer can improve plant development and crop yield (Khan et al., 2013). Nevertheless, there is no information on how such combinations affect nematode populations in common bean crops. Therefore, the aim of the present work was to evaluate the combination of treating seeds with five rhizobacterial isolates and the applying different doses of a nitrogen fertilizer for the control of *M. javanica*.

2. Material and Methods

The work was carried out in the State University of Montes Claros/UNIMONTES-Janaúba Campus. Five rhizobacterial isolates from a bacterial library available in the Laboratory of Phytopathology were tested. The selected isolates were previously shown to be promising candidates for use in the control of *M. javanica* in banana trees (Ribeiro et al., 2012); the isolates used were: *Bacillus pumilus*-1, *B. pumilus*-60, *B. pumilus*-76, *B. subtilis*-34, and *Bacillus* sp.-36 (Ribeiro et al., 2012). The isolates were identified by the fatty acid profile test by gas chromatography (Sanhueza & Melo, 2007) in the Environmental Microbiology laboratory of the National Center for Environmental Research of the Brazilian Agricultural Research Corporation (Embrapa).

2.1 Bacterial Inoculum and Seed Treatment

Bacteria maintained in TSA (Tryptic-Soy-Agar) medium at -4 °C were transferred to fresh TSA medium and incubated at 28 °C for 48 hours. In a flow chamber, the bacteria were resuspended in sterile saline solution (0.85% NaCl), transferred to centrifuge tubes, and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and 3 mL saline solution (0.85% NaCl) were added to the pellet. The resulting suspension was agitated and calibrated in a spectrophotometer to an optical density of OD540 = 0.5 absorbance (approximately 10^8 CFU). This quantification was determined by estimating bacterial concentrations in the medium by serial dilution and counting in plates containing TSA medium.

The rhizobacterial inoculum was prepared by the addition of 15 mL suspension of each *Bacillus* isolate to polyethylene bags containing 35 g previously sterilized peat from AGROLATINO®. A quantity of 250 g inoculum per 50 kg common bean seeds were used. A 10% sucrose solution was added to the inoculum in order to improve its fixation to the seeds (Xavier et al., 2011).

Samples (100 g) of common bean type carioca cultivar 'Pérola' were sterilized with 1% sodium hypochlorite for 10 min, washed four times in sterile distilled water, and spread on absorbing paper to dry in a flow chamber for 2 hours (Chun et al., 1997). The seeds were subsequently inoculated with one of the bacterial isolates and kept at room temperature for 24 hours.

2.2 Experimental Design, Data Collection and Statistical Analysis

The *M. javanica* inoculum was prepared from a pure population of the nematode maintained in tomato plants cultivar 'Kada' grown in pots containing previously autoclaved sandy soil. *M. javanica* eggs were extracted from the roots as described by Hussey and Barker (1973) and modified by Bonetti and Ferraz (1981).

The assay was carried out in a greenhouse using the randomized block design with a factorial scheme of 5 × 5 + 1: five nitrogen doses (0, 25, 50, 100 and 200 kg/ha) in the form of urea, five rhizobacterial isolates (*B. pumilus*-1, *B. subtilis*-34, *Bacillus* sp.-36, *B. pumilus*-60 and *B. pumilus*-76) added to common bean seeds, and one control treatment (untreated seeds cultivated in soils infested with *M. javanica*). Each treatment was performed 10 replications and the experimental unit consisted of a pot containing one common bean plant.

The experiment was conducted in 4 L pots filled with sandy soil fluvisol with the following composition: 9 dag/kg clay, 88 dag/kg sand, 8 dag/kg silt, 0.2 dag/kg organic matter, pH 7.3, 53.7 mg/dm P; 70 mg/dm K; 1.6 cmolc/dm Ca; 0.5 cmolc/dm Mg; and 84% base saturation. Five common bean seeds were sown in each pot and, after germination, thinned to one plant per pot. Twenty days after sowing, 3 mL of an aqueous suspension containing 3,000 *M. javanica* eggs were added to three holes surrounding the plants.

Based on the chemical analysis of the soil, and taking into account the recommendations for common bean crops (Ribeiro et al., 1999), a 100 mg/dm^{-3} P_2O_5 with single superphosphate as the source and 0.5 mg/dm^{-3} boron with boric acid as the source when the beans were sown was applied. The nitrogen dose treatments as solutions at 15, 30, and 45 days after seed germination were applied. Irrigation was performed daily, keeping the soil constantly moist.

Sixty days after nematode inoculation, the following characteristics were evaluated: root weight, plant weight, number of leaves, and fresh and dry weight of the aerial part. For dry weight determination, the plants were dried in a growth chamber under forced ventilation at 65 °C to a constant weight.

In order to evaluate damage and nematode populations, plant roots were harvested, washed, weighed, and stained with floxin B (15 mg/L for 20 min) to enable gall and egg masses counting (Taylor & Sasser, 1978). After extraction according to the method of Hussey and Barker (1973) modified by Bonetti and Ferraz (1981), the number of eggs per root were quantified using a Peters chamber under an optical microscope. The number of second-stage juveniles was determined after removal of 100 cm^3 soil from the pots; juvenile extraction was carried out using the method described by Jenkins (1964), followed by counting on a Peters chamber under an optical microscope.

The data were subjected to an analysis of variance and the means of the bacterial concentration were compared using the Scott-Knott test at a 5% significance level. Nitrogen doses were adjusted to regression models using the statistical software SISVAR (Ferreira, 2011). The means of the treatments were compared to the control using Dunnett's test at a 5% significance level using the statistical software SAS (SAS Institute, 2000).

3. Results

A significant reduction in the number of egg masses and galls per root was identified between plants subjected to the combined treatments (rhizobacteria × urea doses) compared with the control (Dunnett's test, $p < 0.05$). The largest reductions in number of egg masses in comparison to controls were observed in the *B. pumilus*-60/0 N (54.68%), *B. subtilis*-34/25 kg/ha urea (62.50%), *B. pumilus*-1/50 kg/ha urea (66.8%), *B. subtilis*-34/100 kg/ha urea (84.6%), and *B. pumilus*-1/200 kg/ha urea (83.17%). The greatest reductions in number of galls compared to the control were found for *B. pumilus*-60/0 N (57.0%) and *Bacillus* sp.-36/25 kg/ha urea (69.04%). At 50, 100, and 200 kg/ha urea, the number of egg masses and galls were lower than in the controls in treatments with each isolate (Table 1).

Table 1. Number of *Meloidogyne javanica* egg masses and galls per root of common bean plants after inoculation of the seeds with rhizobacterial isolates and the addition of nitrogen doses to the soil, local and year

Isolates	Doses kg/ha									
	Egg masses					Galls				
	0	25	50	100	200	0	25	50	100	200
B. pumilus-60	37.7 a^{yz}	38.0 a^{y}	28.3 a^{y}	35.1 a^{y}	15.4 a^{y}	7.3 a^{y}	6.3 a^{y}	6.0 a^{*}	7.0 b^{y}	5.2 a^{y}
B. pumilus-76	43.6 a^{y}	52.2 b	49.5 a	34.0 a^{y}	16.5 a^{y}	7.7 a^{y}	5.8 a^{y}	4.0 a^{*}	2.6 a^{y}	4.7 a^{y}
Bacillus sp.-36	49.2 a	78.1 b	44.6 a	34.5 a^{y}	20.0 a^{y}	9.0 a^{y}	5.2 a^{y}	8.8 a^{*}	5.2 b^{y}	4.2 a^{y}
B. subtilis-34	53.3 a	31.2 a^{y}	28.7 a^{y}	12.8 a^{y}	19.1 a^{y}	11.0 a	11.0 b	6.1 a^{y}	7.3 b^{y}	3.0 a^{y}
B. pumillus-1	69.8 a	57.6 b	27.6 a^{y}	20.3 a^{y}	14.0 a^{y}	11.8 a	11.5 b	5.5 a^{y}	3.1 a^{y}	3.3 a^{y}
VC	68.1					65.5				
Control	83.2					17.0				

Note. [y] Statistical differences to the control according to the Dunnett's test at a 5% significance level.

[z] Means followed by the same lowercase letters on the column do not significantly differ between each other according to the Scott-Knott test at a 5% significance level.

There was no significant effect of rhizobacterial inoculation or application of urea in comparison with the control regarding numbers of second stage juveniles/100 cm^3 soil, or number of *M. javanica* eggs on the roots of the plants.

Significant interactions between the treatment with rhizobacterial isolates and nitrogen doses with regard to the number of egg masses and the number of galls ($p < 0.05$) were observed. At 25 kg/ha urea, the isolates *B. pumilus*-60 and *B. subtilis*-34 were the most effective at decreasing the number of *M. javanica* egg masses (Table 1). At 25 kg/ha urea, the greatest reduction in gall number was promoted by isolates *B. pumilus*-60, *B. pumilus*-76, and *Bacillus* sp.-36. At 100 kg/ha urea, the most effective isolates for control of *M. javanica* were *B. pumilus*-76 and *B. pumilus*-1. None urea application and the doses of 50 kg/ha and 200 kg/ha urea, isolates showed no differences for the tested endpoints (Table 1).

Regression analyses revealed that increasing the N dose, together with application of isolates *B. pumilus*-1, *B. subtilis*-34, *Bacillus* sp.-36, or *B. pumilus*-76 caused a linear reduction in the number of *M. javanica* egg masses (Figure 1). The same effect was observed for isolates *B. pumilus*-1, *Bacillus* sp.-36, and *B. pumilus*-76 with regard with the number of galls per root (Figure 2).

The treatments (bacterial isolates and urea) had no significant effect on the tested variables when applied individually; or when they were applied together with respect to the numbers of *M. javanica* eggs, second stage juveniles, or vegetative characteristics such as height, fresh and dry weight of the aerial parts, root weight and leaf number (data not shown).

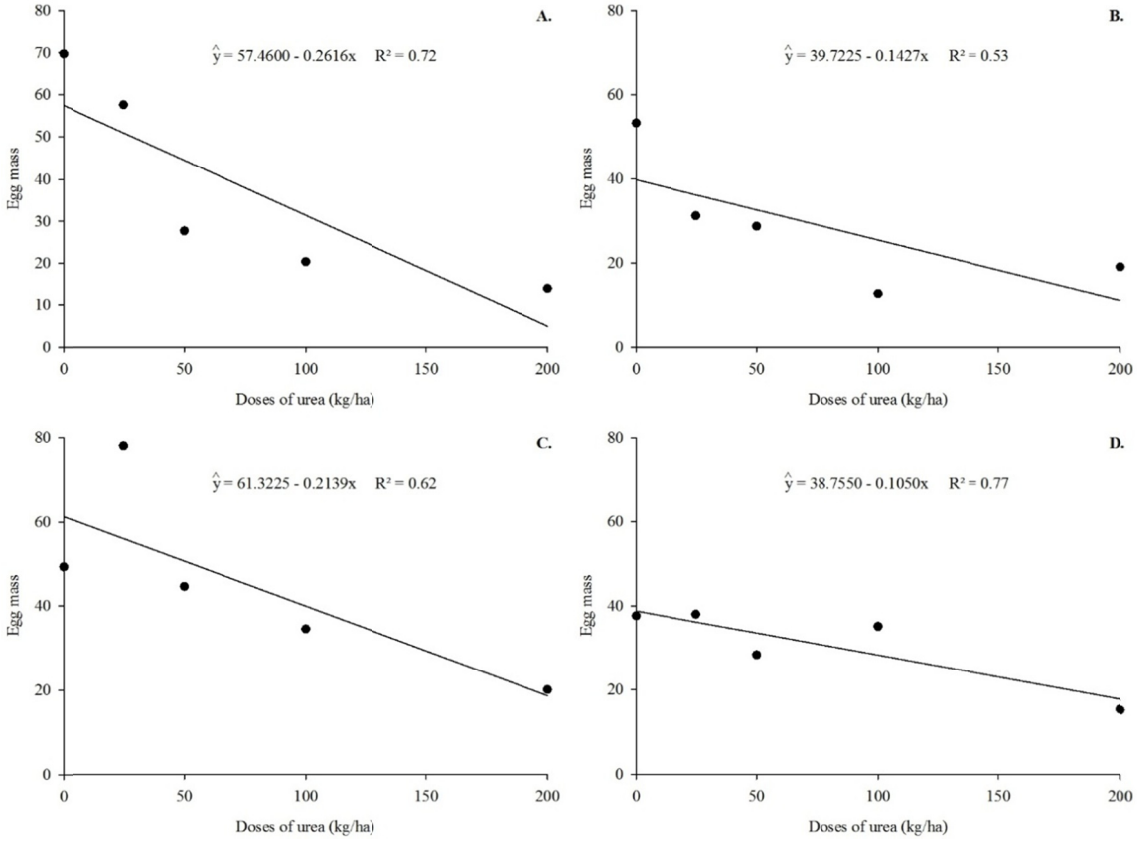

Figure 1. Number of *Meloidogyne javanica* egg masses per root of common bean plants after inoculation of the seeds with rhizobacterial isolates and the addition of nitrogen doses to the soil, Janaúba, 2018. (A) *Bacillus pumilus*-1; (B) *Bacillus subtilis*-34; (C) *Bacillus* sp-36; (D) *Bacillus pumilus*-76

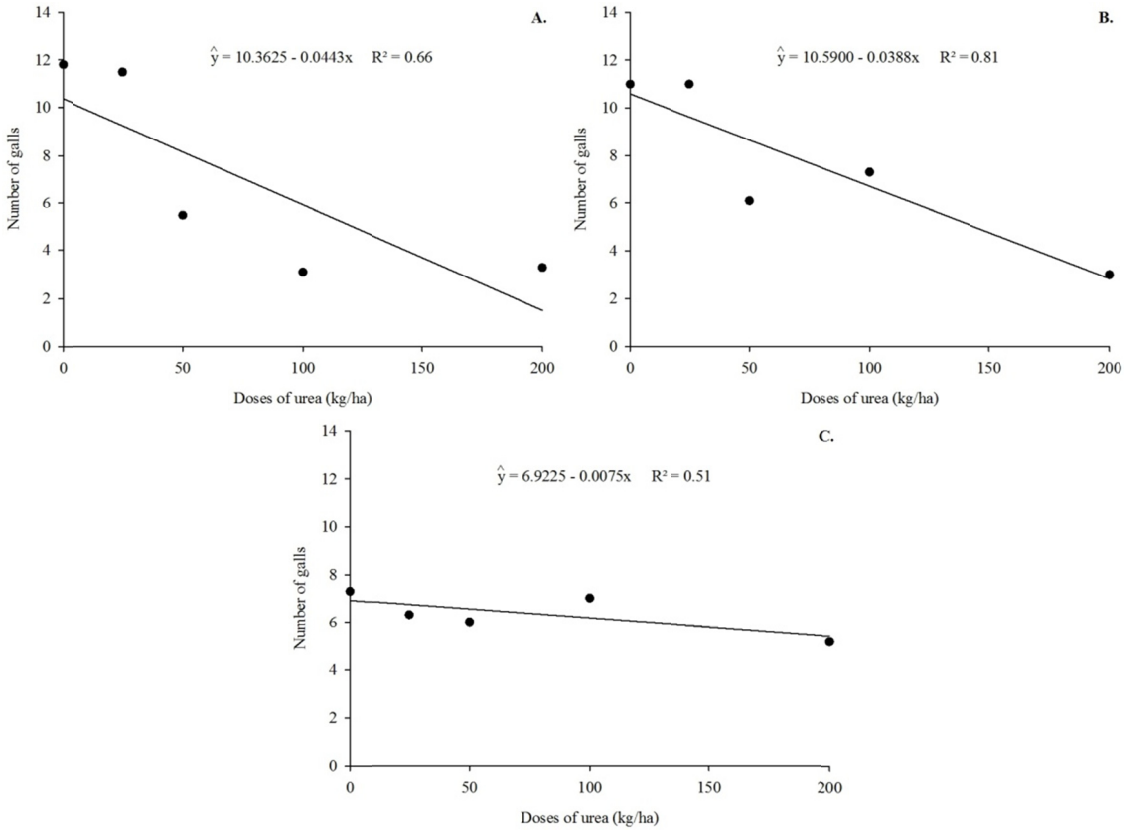

Figure 2. Number of *Meloidogyne javanica* egg masses and galls per root of common bean plants after inoculation of the seeds with rhizobacterial isolates and the addition of nitrogen doses to the soil, Janaúba, 2018. (A) *Bacillus pumilus*-1; (B) *Bacillus* sp-36; (C) *Bacillus pumilus*-76

4. Discussion

The present work revealed that treatment of common bean seeds with rhizobacterial isolates could be effective in reducing the number of galls and egg masses in the presence of N. The results varied among rhizobacterial isolates and nitrogen doses applied (Table 1). Previous studies have similarly shown that treatment of seeds can reduce infestation with *Meloidogyne* species (Corrêa et al., 2012).

Many reports have been demonstrated that organisms inoculated directly on the seeds are better able to colonize the plant roots, an important factor for a successful biological control (Dawar et al., 2008; Naue et al., 2014). The processes of seed germination and seedling growth are accompanied by the release of root exudates, which are used by bacteria as a source of nutrients, helping their fixation on the roots and providing a selective advantage in the bacterial colonization and survival (Kloepper, 1985).The application of rhizobacteria for nematode control by treating seeds with bacterial suspensions presents high potential to improve growth and biological control.

Despite higher doses of urea did not affect plant fresh weight, the direct effect of N application may have promoted a negative effect on to the nematodes. The adverse effect of urea on phytonematodes has been attributed to the release of ammonia in the soil following urease activity. According to Silva et al. (2006), the ammonia (NH_3) released from the transformation of ammonium (NH_4) in the soil may selectively affect nematode populations. Spiegel et al. (1987) reported that ammonia acts as a plasmolyzing agent in second-stage juveniles of *Meloidogyne*.

Sudirman and Webster (1995) indicated that some concentrations of ammonia decrease the rate of juvenile hatching, acting directly as a nematode control through its toxic properties. According to the authors high doses of nitrogen are required for a satisfactory control of nematodes. However, the accumulation of nitrate and ammonia nitrogen in the soil may per se also be phytotoxic (Huebner et al., 1983).

In the present work, any beneficial effect of rhizobacterial inoculation, application of urea or a combination of both on the number of *M. javanica* eggs and juveniles or on the development of the plants were not observed. This lack of effect may be related to the fact that the bacteria were isolated from banana trees rhizospheres

(Ribeiro et al., 2012). There is evidence of specificity between rhizobacterial isolates and host plant species (Coelho et al., 2007). Variations in the chemical composition of root exudates from different species, as well as from different cultivars and genotypes, have been reported (Baldani & Dbereiner, 1981).

There have been no previous studies on the effect of a combination of nitrogen fertilizers and rhizobacteria for nematode control in common bean. However, such studies have been carried out in other crops. A study performed by Sidiqqi et al. (2001) showed that *P. fluorescens* isolates in combination with diammonium phosphate and urea promoted significant reductions in the numbers of *M. incognita* galls. Sharifi (2012) observed increased growth, dry weight, and seed yield in safflower (*Carthamus tinctorius*) when seeds were inoculated before planting with *Pseudomonas* strain 186.

Factors such as pH, humidity, and NH_4^+ concentration in the soil solution are directly associated with NH_3 losses by volatilization. In the present study, the soil pH of 7.3 may have contributed to an accelerated volatilization process. In alkaline soils, NH_4^+ ions present in the soil solution bind to OH^-, resulting in the formation of NH_3, which is highly volatile, especially when applied to sandy soils with low cation exchange capacity and high pH (Mattos-Junior et al., 2002).

The application of 50 kg/ha N for the common bean crop associated with *B. pumilus*-60, *B. subtilis*-34, and *B. pumilus*-1 isolates gave promising results regarding the reduction in number of *M. javanica* egg masses and galls, and could potentially be used for nematode control. This suppression effect was also observed at higher N doses; however, the increased production costs involved could render the use of such doses economically impracticable.

Finally, our study shows that the combined use of seed treatment with rhizobacteria and application of nitrogen fertilizers to the soil could potentially be used for the biological control of nematodes in common bean varieties showing low or no resistance to *M. javanica*, and taking into account the limitations associated with the use of chemical controls.

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