Evaluation of Biocontrol of the Meloidogyne Javanica with Bacillus Subtilis and Purpureocillium Lilacinus in Greenhouse with Lettuce

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Abstract: Background: The aim of this study was to evaluate the product “ICB Nutrisolo Paecilomyces GL” (Purpureocillium lilacinus) (Thom.) and Bacillus subtilis (Cohn) MTox1556-5, Bacillus subtilis CCGB-LFB-117 (LFB-FIOCRUZ-117), Bacillus subtilis CCGB-LFB-757 (LFB-FIOCRUZ-757) against eggs and juveniles (J2) of Meloidogyne javanica in greenhouse. Results: The bioassays indicated that MTox1556-5 and the product containing P. lilacinus reduced by 90% the number of eggs/J2 of M. javanica per root system. In standard B. subtilis CCGB LFB 117 (LFB-FIOCRUZ 117) and B. subtilis CCGB LFB 757 (LFB-FIOCRUZ 757) the number of eggs and J2 was reduced by 58% and 78%, respectively. The weight of shoots and roots did not differ significantly between treatments. The protein profiles of isolates showed different bands, distributed as follows: B. subtilis MTox 1556-5: 100 to 21 kDa; B. subtilis CCGB LFB 117 (LFB-FIOCRUZ 117): 119 to 23 kDa; B. subtilis CCGB LFB 757 (LFB-FIOCRUZ 757): 51 to 17 kDa. Conclusions: Data infer that the biological control agents inhibit the formation of eggs in the galls. This fact may be related to enzymes produced by P. lilacinus or the set of antibiotics, enzymes and proteases produced by B. subtilis, which acted on the fecundity and fertility of M. javanica nematodes.

Keywords: Nematoda, Meloidogynidae, Bacilaceae, Bioproducts.

I. INTRODUCTION

In the agricultural context, the losses that occur in productivity may be related to different factors, biotic and abiotic, in which pests and diseases are highlighted. The major agricultural pests generally comprise the group of insects belonging to the orders Lepidoptera, Coleoptera and Hemiptera. The major plant diseases may be caused by fungi and viruses, but also by bacteria and nematodes, corresponding to the main factors that cause loss of production.

The nematodes are among the pests of great importance in agriculture, especially in leguminous, grassy and fruit plants, whose damage caused by this pest amounted to $125 billion worldwide, corresponding losses to 50% (Talwana et al. 2015; Oka 2010). The gall-forming nematode, Meloidogyne spp. (root-knot nematode) is considered among the most economically important pests in agriculture. The life cycle of this genus corresponds to the hatching of egg masses deposited by females in the roots of host plants. The infection begins with the penetration of second-stage juveniles (J2) in roots (Abo-Hashem & Elyoussr 2011).

Within the roots the female form the mass of eggs. With this, a partial blockage in the circulation of the plant sap occurs, decreasing the capacity to absorb nutrients and water, thus reducing viability and production. The use of chemical products is restricted due damage to the environment and the main effect of pesticides is their ineffectiveness after prolonged use, which have led to bans or limits on the use of nematicides (Oka et al., 2013; Khan et al. 2011a; Xia et al. 2011; El-Hadad et al. 2010).

The use of biological control agents or compounds produced by such organisms may provide additional opportunities to manage the damage caused by the root-knot nematode (Radwan et al. 2012). Bacteria and fungi can
produce substances that affect the nematodes, such as antibiotics, siderophores, and a variety of enzymes (Xia et al. 2011; Tian et al. 2007). These microorganisms can also act as competitors of nematodes for colonization sites and nutrients (Dong & Zhang 2006).

Different species of bacteria possess nematicidal properties that affect the pest viability by producing harmful enzymes (Tian et al. 2007; Siddiqui & Mahmood 1999). The bacterium Bacillus subtilis (Cohn 1872) (Bacillaceae) produces compounds such as the antibiotics zwitermicin-A and kanosamine (Leifert et al. 1995), lipopeptides, as well as antifungal proteins of the bacinubin class (Liu et al. 2007; Pal-Bais et al. 2004). According to Todorova and Kozhuharova (2010) some of these bacterial strains can produce up to 70 kinds of antibiotics. In soil, B. subtilis interferes with the reproductive cycle of the nematode, acting in the direction toward the juvenile host plant (Sharma & Gomes 1996). Researchers in the agricultural field have identified different bacterial strains with nematicidal activity and a number of formulated products such as Stanessting® and Biocure-X® are already available for the control of M. incognita (Khalil et al. 2012; Khan et al. 2011b)

Some strains fungi produce substances that inhibit the juveniles hatch or cause the death of their juveniles (Nitoa et al. 1999; Khan & Saxena 1997). The nematophagous fungus Purpureocillium lilacinus (=Paecilomyces lilacinus) has activity against nematodes of the genus Meloidogyne. It produces lytic enzymes like serine proteases and chitinases, which facilitate the penetration of fungal mycelium in the eggs of nematodes or the cuticle of juveniles (Khan et al. 2004). This fungus also acts on females, reducing the population density of nematodes in the soil (Khalil et al. 2012).

The aim of this study was to evaluate the efficiency of the product "ICB Nutrisolo Paecilomyces GL" consisting of Purpureocillium lilacinus that parasite eggs of nematodes (company data), B. subtilis strains, and the interaction of these microbial biocontrol agents on eggs and juveniles of Meloidogyne javanica (Nematoda: Meloidogynidae) in the greenhouse, using lettuce, Lactuca sativa (L.).

II. MATERIAL AND METHODS

Microorganisms, origin, preparation and characterization

The new isolate of B. subtilis, identified as MTox1556-5 was obtained from soil samples of the Region of Central Depression, in the State of Rio Grande do Sul, Brazil, and the species was identified by biochemical method API 50 CH (Biomerieux®). The isolates of B. subtilis CCGB LFB 117 (LFB-FIOCRUZ 117) and B. subtilis CCGB LFB 757 (LFB-FIOCRUZ 757) were obtained from FIOCRUZ (Rio de Janeiro / Brazil) - Laboratory of Bacterial Physiology.

The bacterial isolates were grown in USUAL+G medium (pH 7.4), at 180 rpm and 28°C for 48 hours (De-Barjac & Lecadet 1976). The bacterial suspension was centrifuged (10,000g), the cells were counted using Neubauer chamber and optical microscopy (400x) and the concentration was adjusted to 1.109 CFU/ml.

For bacterial characterization, according Subcommittee on the Taxonomy of the Genus Bacillus and Related Organisms of the International Committee on Systematics of Prokaryotes (ICSP) (Logan et al., 2009), the susceptibility of the isolates MTox1556-5, B. subtilis CCGB LFB 117 (LFB-FIOCRUZ 117) and B. subtilis CCGB LFB 757 (LFB-FIOCRUZ 757) to antibiotics was tested by using multidisc (Laboclin®) for Gram-positive bacteria, encompassing the following antibiotics: each strain was distributed individually in Petri dishes, inoculated in triplicate at 28°C on solid media-Himedia® for 24h. Evaluation of inhibitory halos was performed with millimeter ruler and the results compared with the table provided by Laboclin®.

Morphological characterization was performed as described by Logan et al. (2009) using differential interference contrast microscopy and the images were analyzed using Axio Vision Software (Zeiss). Enzyme tests were performed to determine the production of cellulase, xilase, amylase, lipase and protease, according to Joo et al. (2007). The protein profile of each isolate was analyzed on polyacrylamide gel (10%) according to Laemmli (1970). The band profile was compared with the Molecular Weight Marker (SDS6H2 Sigma®) using Carestream MI SE software.

The Purpureocillium lilacinus was derived from the product "ICB Nutrisolo Paecilomyces GL", from ICB BIA GRITEC LTDA (Porto Alegre, RS, Brazil).

Phytonematodes

The nematode Meloidogyne javanica was provided by ICB BIA GRITEC LTDA and the number of eggs and second stage juveniles (J2) contained in the suspension was counted using Peters chamber and microscope (400x). The final concentration was adjusted to 2,000 eggs and J2/ml.

2.3 Bioassays

Three isolates were used: B. subtilis CCGB LFB 117 (LFB-FIOCRUZ 117), B. subtilis CCGB LFB 757 (LFB-FIOCRUZ 757), B. subtilis MTox1556-5 and the fungus P. lilacinus constituting the product "ICB Nutrisolo
Paecilomyces GL”. The assays were established in greenhouse, using lettuce (Lactuca sativa cv. ’Vera’), individually placed in vials (300ml) containing sterile substrate. The substrate Plantmax® was sterilized at 121°C for 30 minutes.

Seven days after planting, the lettuces were inoculated with the treatments: (A): B. subtilis CCGB LFB 117 (LFB-FIOCRUZ 117) with nematodes, (B): B. subtilis CCGB LFB 757 (LFB-FIOCRUZ 757) with nematodes; (C): Product “ICB Nutrisolo Paecilomyces GL” with nematodes; (D): MTox1556-5 with nematodes; (E): the interaction of the product “ICB Nutrisolo Paecilomyces GL” with the isolate MTox1556-5 and nematodes, (F): control applying only nematodes; (G): control without application of treatments. In each treatment, 5ml of bacterial or fungal inoculum (1.10⁹cells/ml) and 5 ml of nematode suspension (2,000 eggs and J2/ml) were applied in the rhizosphere region.

Each treatment consisted of five replicates with one plant/pot and the evaluations were 60 days after application. The fresh weight of shoots and roots and the number of galls per root system were evaluated. In laboratory, the eggs were extracted from the roots of plants (Hussey & Barker, 1973) and three counts (eggs and J2) were made of each sample, with Peters chamber and optical microscope (400x). To determine the nematode reproduction factor, eggs (mature and immature) and juveniles were quantified (Oostenbrink, 1966).

Recovery of soil fungi and bacteria used in bioassays

The substrate used in these experiments was placed in sterile vials and dried in an oven at 30°C for 48h. For recovery of bacterial colonies, 1g of soil was weighed, diluted to 10⁻⁴, pasteurized (80°C, 15 min) and inoculated in triplicate at 28°C on Nutrient Agar - Himedia® (NA: peptic digest of animal tissue g/l; sodium chloride 5g/l; beef extract 1g/l; yeast extract 1.5g/l; agar 15g/l; pH 7.4±0.2). The bacterial colonies were quantified 24 hours after inoculation and compared with control.

For the recovery of fungal colonies, the diluted soil was not pasteurized. Samples were inoculated on Potato Dextrose Agar - Himedia® (PDA: potatoes, infusion from 200g/l; dextrose 20g/l; agar 15g/l; pH 5.6±0.2), in triplicate, incubated at 28°C and the colonies with fungal characteristics of P. lilacinus were quantified 9 days after inoculation. For the correct quantification of the colonies, the strain of P. lilacinus, which is a component of the product, was used as a positive control.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) and differences between means were analyzed using Least Significant Difference (LSD). A significance level of α=0.05 was used in all analyses. The data were corrected for multiple comparisons using the Bonferroni method. For analyses, the values were transformed by the expression log10 (x+1). All analyses were realized with the SPSS 17.0 software.

III. RESULTS

The antibiogram showed that all strains of B. subtilis were sensitive to erythromycin, vancomycin, chloramphenicol and tetracycline, and the new isolate B. subtilis MTox 1556-5 was resistant to gentamicin, penicillin and oxacillin. The evaluated isolates of B. subtilis had positive reactions to surfactin and protease, and MTox 1556-5 showed positive reaction for amylase. All isolates were negative for lipase, cellulase and xilase (Table 1).

<table>
<thead>
<tr>
<th>Bacillus subtilis</th>
<th>C</th>
<th>Ci</th>
<th>C</th>
<th>Cl</th>
<th>Er</th>
<th>G</th>
<th>O</th>
<th>P</th>
<th>Ri</th>
<th>S</th>
<th>T</th>
<th>V</th>
<th>Sur</th>
<th>Pr</th>
<th>A</th>
<th>Li</th>
<th>C</th>
<th>Xil</th>
<th>Cel</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTox155 6-5</td>
<td>φ</td>
<td>φ</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>φ</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4,51</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CCGB LFB B 117</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>φ</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4,73</td>
</tr>
<tr>
<td>(LFB-FIOCRUZ 117)</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>φ</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CCGB LFB B 757</td>
<td>φ</td>
<td>φ</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<td>S</td>
<td>S</td>
<td>S</td>
<td>φ</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(LFB-FIOCRUZ)</td>
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<td></td>
<td></td>
<td>5,06</td>
</tr>
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</table>

Table 1. Antibiogram, morphological and enzymatic profile of the new Bacillus subtilis isolate, compared with the standards (CCGB LFB – FIOCRUZ - RJ).
Cef: Cefepime (30mcg); Cip: Ciprofloxacin (5mcg), Chl: Chloramphenicol (30mcg), Cli: Clindamycin (2mcg), Ery: Erythromycin (15mcg), Gen: Gentamicin (10mcg), Oxa: Oxacillin (1mcg), Pen: Penicillin G (10mcg), Rif: Rifampicin (5mcg), Sulf: Sulphazotrim (25mcg), Tet: Tetracycline (30mcg); Van: Vancomycin (30mcg); Sur: Surfactin; Pre: Protease; Amy: Amylase, Lip: Lipase; Cell: Cellulase; Xil: Xilase. Cell size in µm. * (S) sensitive; (R) Resistant; (ɸ) showed no inhibition zone ** (+) positive reaction.; (-) negative reaction.

The protein profiles of isolates showed different bands, distributed as follows: B. subtilis MTox 1556-5: 100 to 21 kDa; B. subtilis CCGB LFB 117 (LFB-FIOCRUZ 117): 119 to 23 kDa; B. subtilis CCGB LFB 757 (LFB-FIOCRUZ 757): 51 to 17 kDa (data not shown).

For bioassays, Table 2, the individualized application of the B. subtilis strain MTox1556-5 and the formulated product "ICB Nutrisolo Paecilomyces GL" (P. lilacinus) reduced by 90% of the number of eggs and J2 of M. javanica per root system, compared to the control. The results of the simultaneous use of the same biological control agents have shown that the level of control remained similar to the independent treatments. Considering the standard B. subtilis CCGB LFB 117 (LFB-FIOCRUZ 117) and B. subtilis CCGB LFB 757 (LFB-FIOCRUZ 757), the number of eggs and J2 was reduced by 58% and 78%, respectively. Treatment with B. subtilis CCGB LFB 117 (LFB-FIOCRUZ 117) was not statistically different from the treatment with B. subtilis CCGB LFB 757 (LFB-FIOCRUZ 757), but it differed significantly from the other treatments. Data from the weight of the plants shoots and roots treated with the tested microorganisms did not differ significantly from each other but differed from control.

Table 2. Suppression effect of bacterial and fungal treatments against Meloidogyne javanica, applied in Lactuca sativa and evaluated 60 days after treatments.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dosage CFU/ml</th>
<th>Weight(g) shoot</th>
<th>Weight(g) root</th>
<th>Average eggs+J2/root</th>
<th>Decrease in eggs+J2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis CCGB LFB 117 (LFB-FIOCRUZ 117)</td>
<td>1.10⁹</td>
<td>47,33⁻</td>
<td>17,70⁹</td>
<td>531,66⁹</td>
<td>58,12</td>
</tr>
<tr>
<td>B. subtilis CCGB LFB 757 (LFB-FIOCRUZ 757)</td>
<td>1.10⁹</td>
<td>48,66⁹</td>
<td>16,45⁹</td>
<td>270,00⁹</td>
<td>78,76</td>
</tr>
<tr>
<td>ICB Nutrisolo</td>
<td>1.10⁹</td>
<td>54,00⁹</td>
<td>17,39⁹</td>
<td>121,66⁹</td>
<td>90,43</td>
</tr>
<tr>
<td>ICB Nutrisolo + MTox1556-5</td>
<td>1.10⁹</td>
<td>57,00¹</td>
<td>13,04¹</td>
<td>115,00¹</td>
<td>90,95</td>
</tr>
<tr>
<td>B. subtilis MTox1556-5</td>
<td>1.10⁹</td>
<td>37,00⁹</td>
<td>10,87¹</td>
<td>125,00¹</td>
<td>90,17</td>
</tr>
<tr>
<td>Untreated inoculated control</td>
<td></td>
<td>40,66³</td>
<td>15,19³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-inoculated control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CCGB LFB and MTox: B. subtilis; ICB Nutrisolo: P. lilacinus; *1.10⁹ CFU/ml of ICB Nutrisolo Paecilomyces GL + 1.10⁹ CFU/ml of MTox1556-5. Values with same letter are not significantly different between treatments according to LSD test (P = 0.05).

Figure 1 shows the data of each replicate during the experiment. The number of eggs and J2 found in the treatment with B. subtilis CCGB LFB 117 (LFB-FIOCRUZ 117) (Figure 1A) was superior to other treatments in all repetitions, with no statistical differences only in the treatment with B. subtilis CCGB LFB 757 (LFB-FIOCRUZ 757) (Figure 1B). The best result on average was through the interaction of the treatments with fungus and bacteria (Figure 1D), where the log10 of the number of eggs and J2 remained below the value of 2.5 units/repetition. In all treatments the number of galls found per root system remained between 0 and 1 (log10), with no statistical difference between treatments with the tested microorganisms, except in the control group (Figure 1F). The weight of the shoots and weight of the roots of the plants showed no significant difference between treatments.
Figure 1. Effect of *Bacillus subtilis* and *Paecilomyces lilacinus* used in the treatment of lettuce plants infected with *Meloidogyne javanica*, in greenhouse (The bars represent the standard error).

The reproduction factor (RF) shown in all treatments differed from the control. The lowest RF observed in this study was in treatments with the interaction of the product that contains the fungus *P. lilacinus*, “ICB Nutrisolo Paecilomyces GL”, and the new isolate of *B. subtilis*, MTox1556-5. Although the number of galls did not differ significantly between treatments the interaction of fungus and bacteria showed a reduced number (Table 3).

Table 3. Number of galls and reproduction factor of *Meloidogyne javanica* treated with *Bacillus subtilis* and *Paecilomyces lilacinus* in lettuce.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage CFU/ml</th>
<th>Galls number root system</th>
<th>Reproduction factor</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em> CCGB LFB 117 (LFB-FIOCRUZ 117)</td>
<td>$1.10^9$</td>
<td>5.66ª</td>
<td>0.2658ª</td>
</tr>
<tr>
<td><em>B. subtilis</em> CCGB LFB 757 (LFB-FIOCRUZ 757)</td>
<td>$1.10^9$</td>
<td>5.00ª</td>
<td>0.0818ª</td>
</tr>
<tr>
<td>ICB Nutrisolo</td>
<td>$1.10^9$</td>
<td>5.33ª</td>
<td>0.0245ª</td>
</tr>
<tr>
<td>ICB Nutrisolo + MTox1556-5</td>
<td>$1.10^9^*$</td>
<td>3.66ª</td>
<td>0.0222ª</td>
</tr>
<tr>
<td><em>B. subtilis</em> MTox1556-5</td>
<td>$1.10^9$</td>
<td>5.33ª</td>
<td>0.0463ª</td>
</tr>
<tr>
<td>Untreated inoculated control</td>
<td></td>
<td></td>
<td>0.6358ª</td>
</tr>
</tbody>
</table>

*1.10^9* CFU/ml of ICB Nutrisolo Paecilomyces GL + 1.10^9 CFU/ml of MTox1556-5. Values with same letter are not significantly different between treatments according to LSD test (P = 0.05).

The data of recovery of fungal and bacterial colonies demonstrate that the treatment with *B. subtilis* CCGB LFB 117 (LFB-FIOCRUZ 117) obtained the largest number of *B. subtilis* colonies recovered after 60 days of experiment. The simultaneous treatment with the product “ICB Nutrisolo Paecilomyces GL” and the *B. subtilis* isolate MTox 1556-5 had the highest number of fungal colonies recovered. The number of bacterial colonies isolated...
from soil samples was higher than the number of fungal colonies recovered demonstrating that the bacterial spores were more persistent in soil (Figure 2).

Figure 2. Number of bacterial (B) and fungal (F) colonies, isolated from soil samples treated with *P. lilacinus* (ICB) and *B. subtilis* CCGB LFB 117 (LFB-FIOCRUZ 117), *B. subtilis* CCGB LFB 757 (LFB-FIOCRUZ 757) and *B. subtilis* MTox 1556-5. Bars indicate standard error. Values with same letter are not significantly different between treatments according to LSD test (P = 0.05).

IV. DISCUSSION

According to Stein (2005), between 4% and 5% of the *B. subtilis* genome is devoted to the production of antibiotics and peptide antibiotics have been recognized for more than 50 years. Regarding the enzymatic activity, Joo et al. (2007) demonstrated that different *B. subtilis* isolates showed a positive reaction to xilase, cellulase, amylase, protease and lipase. In this case, *B. subtilis* MTox 1556-5 showed activity for protease, amylase and surfactin. The authors Rahanandeh et al. (2012) also characterized two *B. subtilis* isolates positive for protease activity that caused mortality of *Pratylenchus loosi* juveniles between 62% and 86% in laboratory.

The *B. subtilis* isolates tested by Kavitha et al. (2012) reduced the hatching of *M. incognita* eggs, causing 92% mortality of juveniles. In the present study, treatment with *B. subtilis* MTox 1556-5 reduced the number of *M. javanica* eggs and J2 by 90%, in greenhouse.

In published studies about the mode of action of *P. lilacinus*, Kiewnick and Sikora (2006) identified the production of proteases and chitinases as the enzymes responsible for the control mechanism of the isolate *P. lilacinus* PL251 in *M. incognita*. This species reduced the nematode population between 54 and 78%, colonizing the eggs and juveniles.

Considering the formulated products found in the bibliography, *B. subtilis* (Stanessting®) and *P. lilacinus* (Bio-Nematon®) evaluated by Khalil et al. (2012) against another species of plant parasitic nematode (*M. incognita*) in tomato grown under greenhouse conditions, showed that after 62 days of experiment, the fungus reduced by 88% and 76% the number of galls and egg mass, respectively. On the other hand, the bacterial product reduced by 62% the number of galls and by 71% the egg mass. In this study, microbial agents (*B. subtilis* strain MTox 1556-5 and...
ICB Nutrisolo Paecilomyces GL, showed higher levels of control eggs and juveniles, considering the genus Meloidogyne.

In the current study, the evaluation was done 60 days after application of the treatments, which corresponds to the commercial stage of the lettuce plant (Suinaga et al., 2013) in which approximately 90% of eggs and juveniles of the target pathogen was successfully controlled. In microbial control, most of the research is related to mortality of pests.

The data presented in this study regarding the number of eggs and J2 may infer that despite the occurrence of gallng in both the treated plants with fungi or bacteria, these microbial control agents inhibit the formation of the egg masses inside the galls. This fact may be related to enzymes produced by P. lilacinus or the set of antibiotics, enzymes and proteases produced by B. subtilis, which acted on the fecundity and fertility of M. javanica nematodes. Similar results were found by Araújo and Marchesi (2009) who identified a reduction in reproduction of M. incognita on tomato plants treated with B. subtilis. Ashraf and Khan (2010) used the fungus P. lilacinus and Cladosporium oxysporum in greenhouse tests against M. javanica. After 60 days, the evaluations showed that P. lilacinus was more effective in controlling nematodes, including reduction in the number of galls, number of nematodes and a lower RF between treatments, confirming the data found in this work.

Niyaz and Hisamuddin (2009) performed various evaluations with P. lilacinus and identified that the fungus applied in the soil one week before inoculation with nematodes was more effective once the inoculum colonized the soil in this period. The results of these authors reveal, in histological sections, that fungal conidiophores were visualized around the females of M. incognita and the hyphae pierced and destroyed the eggs and egg masses.

The species of the genus Bacillus are commonly found and isolated from soil samples (Fritz et al. 2010) because they remain in the form of spores when under adverse conditions until the environment becomes favorable of its development. Thus, the results found in this study corroborate with some research studies about isolation and identification of Bacillus species in soil samples, especially in cultivated areas.

The data set presented in this study demonstrated the efficiency of B. subtilis and P. lilacinus to control M. javanica in greenhouse conditions. The company ICB Bioagritec is interested in evaluating these results in field conditions for the entry of new strains of B. subtilis in achieving a bioproduct to control nematodes.

As to the mode of action, the data suggest that the action of the microorganisms under study occur in the formation of egg mass and soil fertility, since the treatments demonstrated a lower concentration of eggs and adults of M. javanica. In addition, phytoregulators compounds produced by B. subtilis can also act directly in plant cells, triggering hypersensitivity reactions, and preventing the female J2 from obtaining enough energy to produce eggs or degrading the gelatinous mass surrounding the eggs, hampering their formation due to dehydration (Araújo et al. 2012).

In this research, enzymatic activity of B. subtilis was identified to protease, surfactin and amylase, which may be related to the control of M. javanica. Other mechanisms suppressive to nematodes are indicated by Oka (2010), for example, the release and/or production of nematicidal compounds in the soil, such as ammonia and fatty acids; increasing and/or adding antagonistic microorganisms such as those evaluated in this study.

V. ACKNOWLEDGEMENTS
This work was supported by the FAPERSG and CNPq.

VI. REFERENCES


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