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Antimicrobial action against of *Macrophomina phaseolina* and control of the grey stem in soybean by homeopathic remedies *Nosode* and *Sulphur*

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The aims of the study were to evaluate the control of botrytis in the soybean stem, spraying homeopathic solutions of *Sulphur* and *Nosode* of *Macrophomina phaseolina* in the dynamizations 6, 12, 24, 36 and 48CH. As additional treatment, we used distilled water and hydroalcoholic solution at 30% ethanol. *In vitro* tests were performed so as to analyze the number of micro-sclerotia and mycelial growth of the fungi, and *in vivo* tests to track the progress of the botrytis stem and the size of the lesion. The experimental design was in randomized blocks, with five replications. For the area under the mycelial growth curve (AUMGC) in the first test, there was no significant effect of *Sulphur* and Nosode of *M. phaseolina*. *In vivo* studies, comparing *Sulphur* with the control treatment, showed a reduction of 14 and 15% for the dynamizations of 12 and 48 CH, respectively. For the amount of micro-sclerotia of *M. phaseolina*, in the first test, Sulphur showed a reduction of up to 50%; *Nosode* of *M. phaseolina* showed no significant reduction of micro-sclerotia. In the second test, *Nosode* was not significant while *Sulphur* caused a reduction of up to 33%. For area under the disease progress curve (AUDPC), in both trials, the drugs proved to be ineffective. These results indicate the potential of these homeopathic remedies in controlling the fungus, *M. phaseolina*.

Key words: Alternative control, *glycine max*, homeopathy.

INTRODUCTION

Brazil is the second largest producer of soybean in the world after the United States, with a production of 98,981.600 tons (Conab, 2016). Over the years and with the increase in cultivated soybean area, is the emergence of diseases that impair productivity. Botrytis stem caused by the fungus *Macrophomina phaseolina* is one of the most important disease, It affects the roots and lower part of the stem; it first causes discoloration and then a dark brown or black coloration. When the weather is favorable, that is, periods of drought, heat and very

high temperature, the disease can attack young plants. In mature plants, the infection usually occurs from the middle of the cycle (Almeida et al., 2014). Due to the degree of dangerousness of the disease, it is necessary to control it, which is based on the practice of direct planting and vegetation cover, in order to avoid water stress; chemical and physical soil management; rigorousness at the time of planting; use of recommended varieties for the region; promoting unsuitable environment for the fungus (Almeida et al., 2014), as well as alternative methods of control, such as homeopathic treatment.

Homeopathic medicine is all pharmaceutical form intended to be administered according to the principle of similarity, with preventive and therapeutic purposes, obtained by the method of dilutions followed by succussions and/or successive grindings. The succussion consists of vigorous and rhythmical agitation of dissolved drugs in an appropriate inert ingredient and the boosting results from the process of dilutions followed by succussions and/or successive grindings of the drug, with the purpose of medical power development (Farmacopéia homeopática brasileira, 2011). According to Carneiro et al. (2011), alternative methods of agriculture are modern methods, developed in sophisticated and complex system of agricultural techniques, whose main objective is not the economic exploitation short sighted and inconsequential, but the economic exploitation in the long term, maintaining stable and self-sustaining the agricultural ecosystem. Therefore, this study aimed to develop an alternative method through homeopathy to control *M. phaseolina* in soybean; check the antimicrobial activity of the homeopathic remedies *Nosode* of *M. phaseolina* and *Sepia* against *M. phaseolina*; and control stem botrytis in soybean plants by using these drugs.

MATERIALS AND METHODS

For this study, two experiments were performed *in vivo* and *in vitro*. For the *in vivo* test, the isolated *M. phaseolina* was put in rice medium and liquid medium PD (potato dextrose); and three discs of the *M. phaseolina* colony grown in Petri dish with 1 cm diameter. Containing what media was added to PDA medium (potato, dextrose and agar). For the *in vivo* test of the second experiment, the isolated *M. phaseolina* was put in petri dishes (0,1 cm diameter) with PDA medium and after the fungi present micro-sclerotia, medium with mycelial disks and micro-sclerotia were used for inoculation of the soybean plants in the pots. In both trials, the soybean seeds used in the *in vivo* experiment were from the cultivar NK 412113 (V-MAX convencional). In both trials the following homeopathic medicines were used: *Sulphur* and *Nosode* of *M. phaseolina*, the first being a homeopathic polychrest and the second starting the healing principle by the similar. The *Sulphur* was acquired in homeopathic pharmacy in the 6CH boosting and manipulated to 12, 24, 36 and 48CH as described by the Brazilian Homeopathic Pharmacopoeia (2011), diluted 1: 100 and sucussioning 100 times. The *Nosode* of *M. phaseolina* was obtained from mycelium grown on potato dextrose liquid medium (PD), which was allowed to stir in orbital shaker at 150 rpm for 7days, after filtration was carried out of the the fungus to separate all the culture medium. Three grammes (3 g) of the fungus (*M. phaseolina*) was placed in amber glass together with 27 ml of 70% grain alcohol with the glass encased by aluminum foil and left at rest for 21 days, stirring only once a day in a soft way, producing the mother tincture (Bonato, 2007) and through this, were manipulated as the Brazilian Homeopathic Pharmacopoeia (2011). The dynamizations used were the same of *Sulphur*, that is to say 6, 12, 24, 36 and 48CH.

As additional treatments, we used distilled water and hydroalcoholic solution of 30% ethanol. Water and ethanol were used because they are solvents in the preparation of homeopathic drugs.

To determine the antifungal activity, we used medicines in their due boosting, being incorporated into the culture medium (PDA) still liquid and hot in the Erlenmeyer flask. 0.005% and 5 uL of treatment were used to each 100 ml of PDA culture medium (Bonato, 2007). After, the PDA medium was poured and complete solidification was expected. It was added in the center of each petri dish a disc, removed from the fungal colony, to grow. The dishes were still incubated at room temperature in the dark. Daily measurements were initiated 24 h after the beginning of the experiment and ended when the fungal colonies reached the edges of the petri dish. Such measurements were performed according to the methodology described by Stangarlin et al. (1999) through the method of diametrically opposed measures to evaluate the diameter of the colony. At the end of the daily measurements for determining the area under the mycelial growth curve (AUMGC), we pulled out a known amount of the fungus, standardizing the pickup location and made to count the number of micro-sclerotia in magnifying glass. Through simple rule of three, the total number of micro-sclerotia in each petri dish was obtained.

For both tests *in vivo* tests, 1 liter pots containing the soil mixture were used, along wish sand and organic matter in the proportion 2: 1: 1, autoclaved at 120°C for 1 h. We used the drugs *Sulphur* and *Nosode* of the fungus *M. phaseolina*. The inoculum of *M. phaseolina* for the first test was produced using a rice medium (rice 25 g to 30 ml of water) and in each pot were added two grains of rice colonized with micro-sclerotia of the fungus *M. phaseolina*, in the center of the pot at 1 cm depth. Soybean seeds were sown around the inoculum, at a distance of 2 cm from it and subsequently held the thinning, maintaining three soybean plants per pot. In the first trial, the treatments were administered three days before sowing, in the sowing day, 3, 10 and 17 days after sowing. The application was on the soil in a proportion of 0.1% (100 uL of treatment to 100 ml of distilled water) or 1 uL per pot. We evaluated the drought of the stem giving values from one to four, and admitted one when there was an absence of drought, two in the presence of drought up to 1/3 of the stem, three for the presence of drought up to 2/3 of the stem, and four for the presence of drought in the whole stem. The values were transformed and calculated, the area under the disease progress curve (AUDPC). As for the second test, inoculum of *M. phaseolina* was produced on PDA and was added to each pot three discs containing the fungal mycelium, each of the disks placed in contact with the soybean plant root. This procedure

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Table 1. Area under the mycelial growth curve (AUMGC) of *M. phaseolina* in PDA medium with different dynamizations of the remedies *Sulphur* and *Nosode* of *M. phaseolina.* Data referring to the first test.

Boosting (CH)	*Sulphur*	Average	*Nosode* of *M. phaseolina*	Average
6	20.52 [ns]	-	20.16 [ns]	-
12	19.78	-	20.76	-
24	20.91	20.58	20.41	20.89
36	19.65	-	20.2	-
48	19.32	-	20.52	-
Average additional treatment hydroalcoholic solution at 30% ethanol				23.31
Average additional treatment distilled water				20.78
Factorial average				20.74
C.V.(%)	3.45	-	2.19	-

[ns]: Absence of significance between dynamizations of the same remedy or among remedies. Additional treatment: distilled water.

was performed when the plants issued the first trifoliate leaf. In the same day, after the inoculation the soil of the pots was covered with hay.

The treatments were administered in the second test in the soil using spray three days before inoculation, on the day of inoculation, 3, 10 and 17 days after inoculation. Treatments were applied to the soil at a proportion of 0.1% or 1 uL per pot. Growth measurements were taken of the injury on the plant stem, every two days, using graduated scale. With the data obtained, we calculated the area under the disease progress curve (AUDPC). In both tests, the experimental design was a randomized block design with five repetitions, and the Tukey test at 5% probability of error was used to compare means. The factor was 2 x 5 + 2, being 2, the number of drugs (*Sulphur* and *Nosode*), 5 the number of dynamizations that were used, which were: 6, 12, 24, 36 and 48CH, and 2 corresponds to additional treatments, namely water and 30% ethanol. In the absence of a significant difference for the factorial and for the additional treatments, the data were shown in a table, otherwise, graphs were prepared.

RESULTS AND DISCUSSION

As shown in Table 1, for the area under the mycelial growth curve of the first test, there was no significant effect for *Sulphur* and *Nosode* on *M. phaseolina*. Through Figure 1, it is seen that in the second test for the area below the mycelial growth curve of *M. phaseolina* on PDA medium, both drugs showed the same behavior represented by quadratic equations, demonstrating the significance of *Sulphur* and *Nosode* of *M*. *phaseolina*. For dynamizations 12 and 48CH, *Sulphur* differed from *Nosode* of *M. phaseolina* showing greater reduction in AUMGC. Comparing *Sulphur* with the control there is a reduction of 14 and 15% in the dynamizations 12 and 48CH, respectively. In a work carried out by Toledo et al. (2015), it was found that the mycelial growth of *Alternaria solani* in the presence of *Sulphur* homeopathic preparation in 100 CH, was inhibited by 16.97% compared to the control. In another work, where Sinha and Singh (1983) observed the toxic effect of *Sulphur* on *Aspergillus parasiticus*, had 100% inhibition in mycelial growth using the boosting 200 CH, showing that *Sulphur,* subject to the boosting and on what the fungus will act, can be as effective as chemicals. Since there was no normal data for the amount of micro-sclerotia of *M. phaseolina* from the first test, it was carried out the transformation of the data into root of x+0.5. Through Figure 2, it can be observed that *Sulphur* showed a reduction of 50% in the number of micro-sclerotia, this effect being represented by a second degree equation. As for *Nosode* of *M. phaseolina,* it showed no significant reduction of micro-sclerotia.

For the amount of micro-sclerotia of the second test, as the data did not show any normality, it was carried out the transformation of that data into root of x+0.5. In Figure 3, it can be observed that *Nosode* of *M. phaseolina* did not show significance, while *Sulphur* showed a behavior represented by quadratic equation and provided a reduction of up to 33%, a very important number since the micro-sclerotia are the form of survival of the fungus on the ground, and it contributes to the spread and survival of this for long periods. The reduction of sporulation was also observed with *Sulphur* in the work conducted by Toledo et al., (2015) where it had 53% of reduction for the boosting 30 CH to 63% for boosting 6 CH. In work done by Toledo et al. (2013) seeking to evaluate the inhibition of sporulation of *A. solani* by *Ferrum sulphuricum,* it was found that the dynamizations 6, 12, 18, 21, 24, 27, 30, 48, 66, 69, 72, 75, 78, 81 and 96 CH decreased sporulation and were statistically different from the controls distilled water and hydroalcoholic solution 70%, having the inhibition of 71.6% in the dynamizations 24 and 78 CH and variation of 88.42% in the dynamizations 21 and 96 CH of spore inhibition. Toledo et al. (2015) also found little effect of using isopathy, in the case of *A. solani* both for mycelial growth and also for the number of micro-sclerotia. With regard to the first test, according to Table 2, there was no statistical difference among the treatments and, bearing this in mind, none of the remedies showed themselves

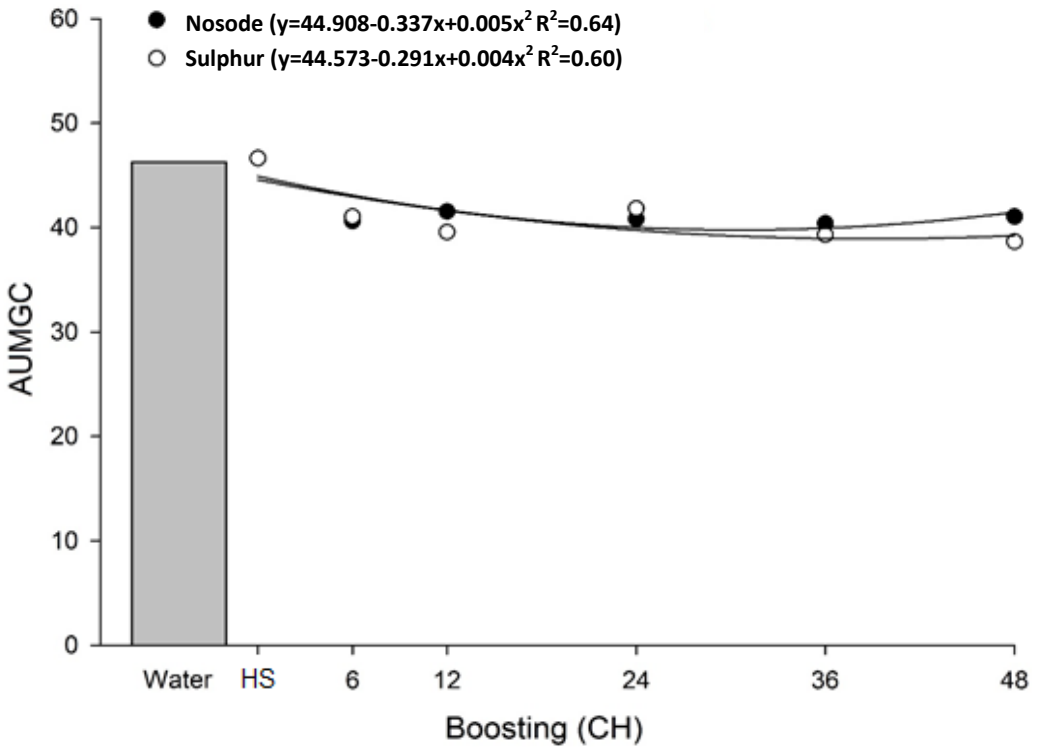

Figure 1. Area under the mycelial growth curve (AUMGC) of *M. phaseolina* in PDA medium with dynamizations of the remedies *Nosode* of *M. phaseolina* and *Sulphur*. HS: Hydroalcoholic solution (30% ethanol). Data referring to the second test.

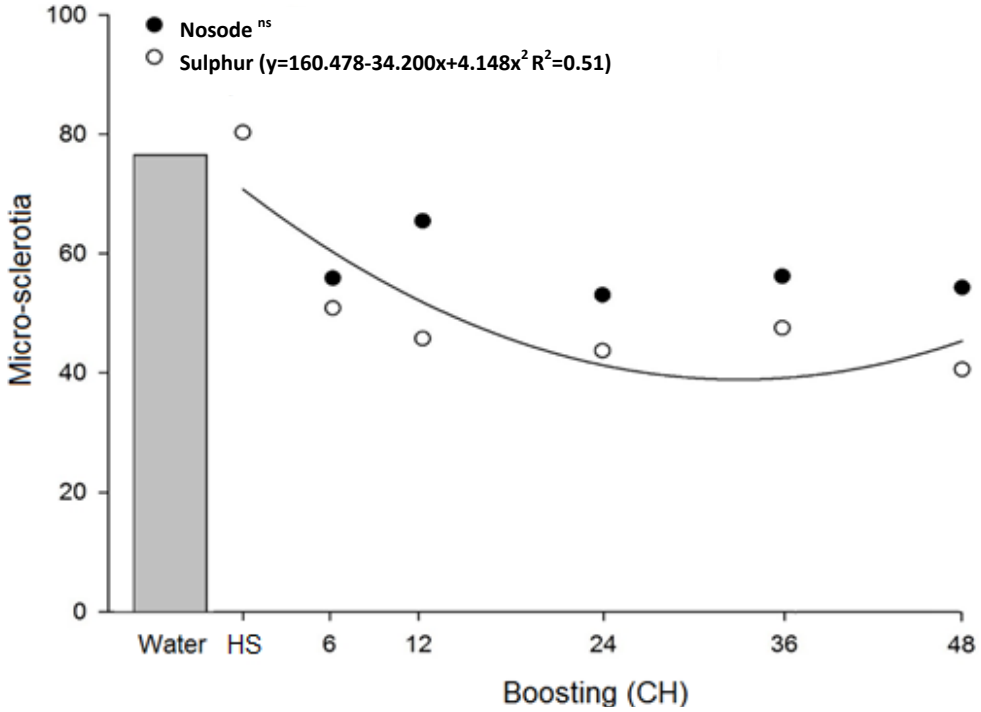

Figure 2. Quantity of micro-sclerotia of *M. phaseolina* in PDA medium with dynamizations of the remedies *Nosode* of *M. phaseolina* and *Sulphur*. Data were transformed into root of x+0,5. ^{ns}: Absence of significance between dynamizations of the same remedy or among remedies. HS: Hydroalcoholic solution (30% ethanol). Data referring to the first test.

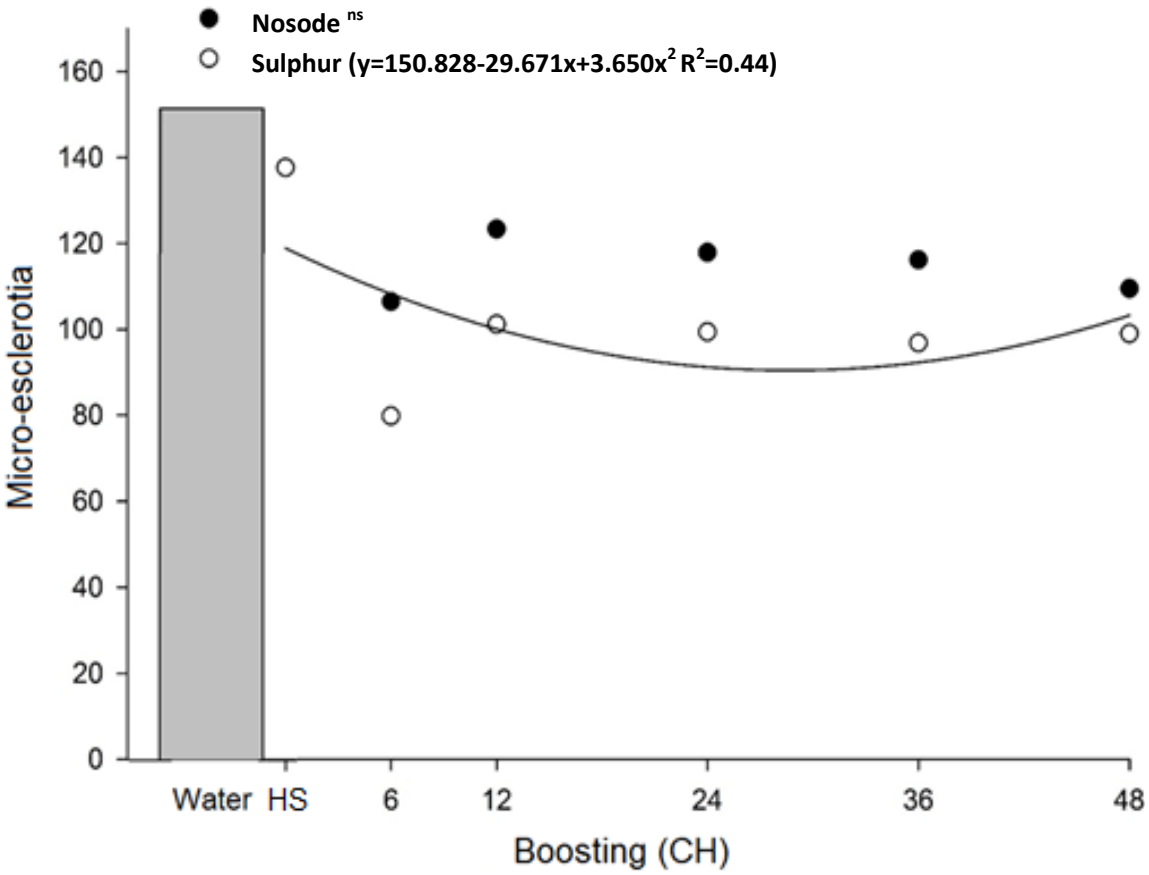

Figure 3. Quantity of micro-sclerotia of *Macrophomina phaseolina* in PDA medium with dynamizations of the remedies *Nosode* of *M. phaseolina* and *Sulphur*. Data were transformed into root of x+0,5. ns: Absence of significance between dynamizations of the same remedy or among remedies. HS: Hydroalcoholic solution (30% ethanol). Data referring to the second test.

Table 2. Area under the disease progress curve (AUDPG) of *M. phaseolina* in soy plants cultivated in a greenhouse and being treated with different dynamizations of the remedies *Nosode* of *M. phaseolina* and *Sulphur.* Data referring to the first test.

Boosting (CH)	*Sulphur*	Average	*Nosode* of *M. phaseolina*	Average
6	727.92^{ns}		1081.6^{ns}	
12	706.88		938.34	
24	1511.6	940.01	1151.4	1025.5
36	970.65		796.44	
48	745.62		1207.4	
Average additional treatment hydroalcoholic solution at 30% ethanol				977.88
Average additional treatment distilled water				2648.30
Factorial average				982.79
C.V. (%)	77.94		77.03	

^{ns}: Absence of significance between dynamizations of the same remedies or among remedies. Additional treatment: distilled water.

efficient to reduce the area under the progress curve for botrytis of the soybean stem in the dynamizations tested.

In Table 3, for the area under the disease progress curve (AUDPC) caused by *M. phaseolina* in soybean plants, in the second test there was also not significance of the treatments. In a study conducted by Toledo et al. (2015), with tomato plants that had black spot, there was a reduction of 34.97% of AUDPC for *Sulphur* medicine in boosting 12CH and reduction of 16.79% for the same drug in boosting 30 CH, this compared to the control of distilled water.

By applying homeopathic solutions in vine for rust

Table 3. Area under the disease progress curve (AUDPG) of *M. phaseolina* in soy plants treated with dynamizations of the remedies *Nosode* of *M. phaseolina* and *Sulphur.* Data referring to the second test.

Boosting (CH)	*Sulphur*	Average	*Nosode* of *M. phaseolina*	Average
6	60.85[ns]	-	52.88[ns]	-
12	45.75	-	63.44	-
24	46.71	54.94	45.06	57.85
36	47.51	-	55.17	-
48	48.56	-	50.28	-
Average additional treatment hydroalcoholic solution at 30% ethanol				80.29
Average additional treatment distilled water				52.91
Factorial average				56.39
C.V. (%)	31.73	-	41.53	-

[ns]: Absence of significance between dynamizations of the same remedies or among remedies. Additional treatment: distilled water.

control, Souza et al. (2006) found that homeopathic treatments have reduced substantially the rust attack (*Phakopsora euvitis*) when compared to the control. Homeopathic solutions Silicea 30 CH, Isophatic 6 CH, 12 CH and 30 CH showed only 7, 17, 9 and 18% of the severity degree presented by the control treatment, which was 100%. Carneiro et al. (2007) found that biotherapic of *A. solani* in the dynamizations 26, 27 and 28 CH reduced the severity of the black spots disease on tomato plants grown in greenhouse. Toledo et al. (2015) evaluated the effect of *Sulphur* and *F. sulphuricum* in the control of the black spots disease on tomato plants, and the results showed that *Sulphur* in 12 and 30 CH minimized the severity of the disease at 10 days after inoculation of the pathogen, and 14 days after inoculation for 6 and 30 CH. Bonato and Silva (2003) confirm that different effects can be seen frequently in homeopathy, depending on the variable analyzed and/or boosting, inhibiting or stimulating the plant. No one knows for sure why this happens, but one possible explanation would be the fact that in nature there are rhythmic movements or the principle of similarity between the organism and the drug (Bonato, 2004).

Conclusion

Although, the homeopathic medicines *Sulphur* and *Nosode* of *M. phaseolina*, in the tested dynamizations have been able to inhibit the mycelial growth and the formation of micro-sclerotia in vitro of *M. phaseolina*, they were not effective to control botrytis in the soybean stem.

Conflict of interest

The authors have not declared any conflict of interest.

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