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Strains of *Bradyrhizobium* spp. in the biological control of phytopathogens in the soybean[1]

Maira Cristina Schuster Russiano[2], Sérgio Miguel Mazaro[3], Carlos Guilherme dos Santos Russiano[4], Marco Antonio Nogueira[5], Marcieli da Silva[2]

ABSTRACT - Bacteria of genus *Bradyrhisobium* form a symbiotic relationship with legumes, promoting biological nitrogen fixation (BNF). However, their effect on the biological control of disease has not been investigated. The aim of this study was to evaluate the effect of four strains of *Bradyrhisobium* spp., namely SEMIA 5080, SEMIA 5079, SEMIA 5019 and SEMIA 587, on the *in-vitro* control of four soil phytopathogens: *Fusarium crassistipitatum*, *Macrophomina phaseolina*, *Rhisoctonia solani* and *Sclerotinia sclerotiorum*, which cause root rot in the soybean. All the strains of *Bradyrhisobium* spp. and of the phytopathogens were obtained from Embrapa Soja in Londrina, Paraná. *In-vitro* tests were conducted using the circle method (adapted), and comparing soybean seeds inoculated with the bacteria and the phytopathogens. The dishes subjected to the circle test were evaluated using a scanning electron microscope (SEM). Two tests of antibiosis were conducted using filtrates of metabolites from *Bradyrhisobium* spp.: assessment of the minimum inhibitory concentration (MIC) and of the antibiosis in Petri® dishes. The results were submitted to the Lilliefors test of normality, followed by ANOVA and regression analysis using the Genes software. The comparison showed that the SEMIA 5080 and SEMIA 5019 strains achieved the best control of the four phytopathogens, with a good performance by the SEMIA 5079 strain; however, the SEMIA 587 strain showed no control over the pathogens. There was morphological damage to the hyphae of each of the phytopathogens subjected to the circle method. There was no antibiosis from the filtered or volatile metabolites.

Key words: Biological control. Rhizobacteria. Soil pathogens. *Glycine max.*

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*Author for correspondence
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[1]Masters dissertation
[2]Postgraduate Program in Agronomy, Universidade Tecnológica Federal do Paraná, Pato Branco-PR, Brasil, maira.schuster@outlook.com (ORCID ID 0000-0001-7424-2190), marcielidasilva@hotmail.com (ORCID ID 0000-0002-3807-6727)
[3]Postgraduate Program in Agronomy, Universidade Tecnológica Federal do Paraná, Pato Branco-PR, Brasil, sergio@utfpr.edu.br (ORCID ID 0000-0003-2787-9409)
[4]Postgraduate Program in Agroecosytems, Universidade Tecnológica Federal do Paraná, Dois VizinhosPR, Brasil, gui_russiano@hotmail.com (ORCID ID 0000-0002-9629-4482)
[5]Embrapa Soja Londrina-PR, Brasil, marco.nogueira@embrapa.br (ORCID ID 0000-0001-7747-9839)

INTRODUCTION

Brazil produces one quarter of the global supply of oilseeds, of which the soybean makes up 96% of those produced in the country (USDA, 2022), in addition to being the most exported commodity. In January 2022, the Institute for Applied Economic Research (IPEA) announced that the soybean complex (grain, bran and oil) was responsible for USD 1.6 billion of the total export value (USD 3.2 billion), with China as the main destination. Compared to 2021, export values increased by 5,223.9%, 44.7% and 1,974.0%, respectively for grain, bran and oil (IPEA, 2022).

Among the nutrients needed to sustain such high productivity, nitrogen (N) is the element most required by the crop. Bacteria of genus *Bradyrhizobium* meet this demand, eliminating the use of chemical nitrogen fertilisation as long as there is effective nodule formation on the roots of the host. (HUNGRIA *et al*., 2017; HUNGRIA; ARAUJO; CAMPO, 2009; HUNGRIA; MENDES, 2015). *Bradyrhisobium* spp. is therefore used in biological nitrogen fixation (BNF) and in co-inoculation, associating *Bradyrhisobium* spp. with Rhizobium spp., and the N fixers Azospirillum spp. and Pseudomonas spp. that act in producing phytohormones, and phosphate solubilisers, or biological control agents, such as Bacillus spp. (SANTOS; NOGUEIRA; HUNGARY, 2019).

Bacteria of genus *Bradyrhisobium* are also considered as promoting plant growth, since they produce siderophores (iron chelators), solubilise phosphate, and synthesise auxins, such as indole-3-acetic acid (IAA), which contribute to plant growth (DESHWAL *et al*., 2003).

The inoculants registered in Brazil for BNF use four strains of *Bradyrhisobium* spp.: SEMIA 5019, SEMINA 5080, SEMIA 5079 and SEMIA 587. Chueire *et al*. (2003), divided the strains into two groups, where SEMIA 587 and SEMIA 5019 are considered strains of *B. elkanii*, and SEMIA 5080 and SEMIA 5079, strains of *B. japonicum*. However, Delamuta *et al*. (2013) reclassified SEMIA 5080 as *B. diazoefficiens* based on its phylogenetic characteristics.

According to Pal and Mcspadden Gardener (2006), the term biological control applies to the use of microbial antagonists, also known as biological control agents (BCA), to suppress disease. Rhizosphere microorganisms, such as *Bradyrhisobium* spp., are promising biocontrol agents, considering that the soil is a more stable environment in terms of temperature and humidity, which favours their establishment and survival. Promising examples of rhizobacteria used in the biocontrol of soil-dwelling phytopathogens are genera Bacillus, Streptomyces and Pseudomonas. Among the mechanisms of action of these rhizobacteria, those that stand out are competition for space and nutrients, and antibiosis through the release of secondary metabolites and hydrolytic enzymes that induce resistance and promote plant growth (DAVE *et al*., 2021; HASHEM; TABASSUM; ALLAH, 2021; KHALIL *et al*., 2021; YAO *et al*., 2021).

Highlighted among soil diseases that affect soybean crops are various forms of root rot, such as grey rot, caused by *Macrophomina phaseolina*; red root rot, caused by the *Fusarium* spp. complex; rhizoctoniosis causing dead patches due to *Rhizoctonia solani* (REIS, 2014); and stem and pod rot, or white mould, caused by *Sclerotinia sclerotiorum* (ANDRADE *et al*., 2015; BOLTON; THOMMA; NELSON, 2006).

The search for multifunctional microorganisms has intensified, with the aim of combining several beneficial properties in a single microorganism, such as growth promotion, nutrient availability and the inhibition of phytopathogenic agents.

MATERIAL AND METHODS

The experiments took place at the phytopathology and seed analysis laboratories of the Dois Vizinhos campus of the Federal Technological University of Paraná.

Strains of *Bradyrhisobium* spp. and phytopathogens used in the study

The strains of *Bradyrhisobium* spp. used in the study — SEMIA 5080 (*Bradyrhisobium* diazoefficiens), SEMIA 5079 (*Bradyrhisobium* japonicum), SEMIA 5019 (*Bradyrhisobium* elkanii) and SEMIA 587 (*Bradyrhisobium* elkanii) — were obtained from the Multifunctional Microorganism collection at Embrapa Soja: Diazotrophic and Plant-Growth Promoting Bacteria (WFCC Collection # 1213, WDCM Collection # 1054), in Londrina, Paraná.

These were grown from freeze-dried ampoules in Yeast Mannitol (YM) culture medium (0.5 g K_2HPO_4; 0.2 g $MgSO_4.7H_2O$; 0.1 g NaCl, 5.0 g mannitol; 0. 4 g yeast extract; 10 mL Congo red solution (0.25 g Congo red in 100 mL deionised water). For the solid YMA medium, 15 g agar per litre were used. The YM medium was poured into Erlenmeyer flasks and autoclaved for 20 minutes at 121 °C.

The phytopathogens *Fusarium crassistipitatum* (CMES 24), *Macrophomina phaseolina* (CMES 1574), *Rhizoctonia solani* (CMES1861) and *Sclerotinia sclerotiorum* (CMES 2131) were obtained from the Multifunctional Microorganism collection at Embrapa Soja in Londrina, Paraná. The isolates were received in Petri® dishes, and spread onto new dishes containing PDA medium.

First experiment: direct comparison (adapted circle method)

Each experimental unit comprised one disposable Petri® dish, 8.3 cm in diameter, with 15 mL of YMA medium. *Bradyrhisobium* spp. were produced in YM medium, and after 10 days growth, were placed in the centre of the dish using autoclaved beakers measuring 4 cm in diameter, as per the circle method adapted from Mariano (1993).

The dishes were placed for 7 days in a BOD chamber at 25 °C under a photoperiod of 12 h. A 7 mm disc of mycelium from each phytopathogen was then inserted into the centre of each dish with the aid of a sterile platinum loop. The dishes were closed and sealed with PVC film and returned to the BOD chamber under the same temperature regime and photoperiod.

The control consisted of the mycelium disk of each pathogen only, and was not inoculated with *Bradyrhizobium* spp.

Mycelial growth in the phytopathogens was monitored daily using a graduated rule. Two similar lines were drawn on the outer side of the bottom of the Petri® dish to monitor the growth in diameter of the colony, whose mean value was used in the calculation. The percentage inhibition of mycelial growth was calculated based on the formula by Menten *et al*. (1976):

$$Inhibition(\%) = \left[\frac{\theta \text{ of the control} - \theta \text{ of the treatment}}{\theta \text{ of the control}} \right] * 100 \quad (1)$$

Second experiment: Antagonism via seed inoculation

Soybean seeds of the NIDERA 5909 cultivar were used, previously disinfected with a 5% sodium hypochlorite solution for five minutes, washed with deionised water as per RAS (BRAZIL, 2009), and then submitted to UV radiation for 20 minutes. Next, 3 mL of YM medium containing the growth from each strain of *Bradyrhisobium* spp., at a concentration of 5 x 109 CFU/mL, were applied to 1 kg of seeds and homogenised in plastic bags.

The soybean seeds inoculated with each isolate were placed 1 cm from the edge of the Petri® dish containing PDA (Potato, Dextrose and Agar) medium. A 7 mm mycelium disc of the phytopathogen was placed 1 cm away from the edge on the opposite side of the dish. The dishes were incubated in a BOD chamber at 25 °C under a photoperiod of 12 h.

During incubation, mycelial growth in the phytopathogens was monitored daily using a graduated rule, on two straight lines drawn on the outside of the bottom of the dishes, as described above.

Third experiment: Scanning electron microscopy (SEM)

The dishes from the adapted circle method were used for the SEM, employing a Hitachi TM 3000 microscope. The readings were standardised at 500x magnification with a viewing scale of 200 µm.

Fourth experiment: Effect of the filtered metabolites in the dishes

Under aseptic conditions, 10 mL from each Erlenmeyer flask containing each strain (SEMIA 5080, SEMIA 5079, SEMIA 5019 and SEMIA 587) with 10 days' growth was transferred to 15 mL Falcon tubes and centrifuged at 4000 rpm for 10 minutes. The supernatant was filtered through a Millex Millipore® membrane (33 mm in diameter, with a pore size of 0.22 µm) using an attached syringe. Each experimental unit included one Petri® dish containing 1.5 mL of the filtered metabolite of *Bradyrhisobium* spp., homogenised in 15 mL of melting DBA medium, to obtain a concentration of 10% metabolite in the dish. After cooling and solidifying the medium, a 7 mm mycelium disc of the respective pathogen was transferred to the centre of the dish, as adapted from Solino *et al*. (2017). The vertical and horizontal diameter of the mycelial growth of each experimental unit was measured daily using a graduated rule, until growth was complete. The control was made up of metabolite-free dishes containing a 7 mm diameter mycelium disk of each phytopathogen.

Fifth experiment: Minimum inhibitory concentration (MIC)

The method described by Vismara (2019) is based on microbial growth in 150 mL YM culture medium in 250 mL Erlenmeyer flasks, autoclaved at 121 °C for 20 minutes. The medium was inoculated with three 7 mm discs of culture medium cultivated with each strain of *Bradyrhisobium* spp. for 10 days. The glassware was closed with cotton and kraft paper to allow for gas exchange, and was incubated in a BOD chamber at 28 °C for 10 days under a photoperiod of 12 h, shaken daily for 1 h at 150 rpm to aerate the medium. After this period, 5 mL of the culture medium suspension from each treatment was filtered through a Millex Millipore® membrane with a pore diameter of 0.22 µm to obtain the working solution containing cell-free metabolites at 100% concentration.

Suspensions of propagules of the phytopathogenic fungi under test were prepared from Petri® dishes containing 10 days' growth of the phytopathogen, by pouring 10 mL of physiological solution onto the dish, and scraping the mycelium with the aid of a sterile platinum loop. The suspension was quantified in a Neubauer chamber until 10^5 spores were counted per mL.

Using a 12-channel micropipette, 100 µL of autoclaved BD culture medium was added to each well. One hundred µL of the working solution of each strain of *Bradyrhizobium* (SEMIA 5080, SEMIA 5079,

SEMIA 5019 or SEMIA 587) was added to the first well of rows A, B, C, E, F and G; row D was considered the control, containing only BD culture medium, while the first four wells of row H contained the culture medium with the relevant fungal suspension. Another fungal suspension was used in the next four wells, with the working solution in the last four wells, from which microdilution of the remaining 11 wells in the sequence was carried out. Then 20 µL of the fungal suspension of each pathogen (*F.* crassistipitatum, *M. phaseolina*, *R. solani* and *S. sclerotiorum*) was added to the wells, except for row D and the last four wells of row H, which were used as the negative control. This procedure was carried out on eight dishes. After 48 h incubation at 25 °C, 10 µL of 1% tetrazolium solution was added to each of the wells of each dish, which remained under incubation and were evaluated every 24 h. After 48 h incubation, it was possible to determine the presence or absence of mitochondrial activity by the pathogen in a well. A reddish colour indicates mitochondrial activity due to the reduction of tetrazolium to formazan by the electrons generated in the active respiratory chain where growth occurs, while in the absence of any activity the unreduced compound remains colourless (BELOTI, 1999).

Sixth experiment: Effect of the volatile metabolites

Disposable two-part Petri dishes, 8.3 cm in diameter, were used to evaluate the volatile metabolites, where YM medium with seven days' growth of each strain of *Bradyrhisobium* spp. was used on one side of the dish; on the other side of the dish, a 7mm mycelium disc of the phytopathogens to be tested was placed in PDA medium. The dishes were incubated in a BOD chamber at 25 °C for 7 days under a photoperiod of 12 h, when the mycelial growth of each phytopathogen was evaluated using a rule.

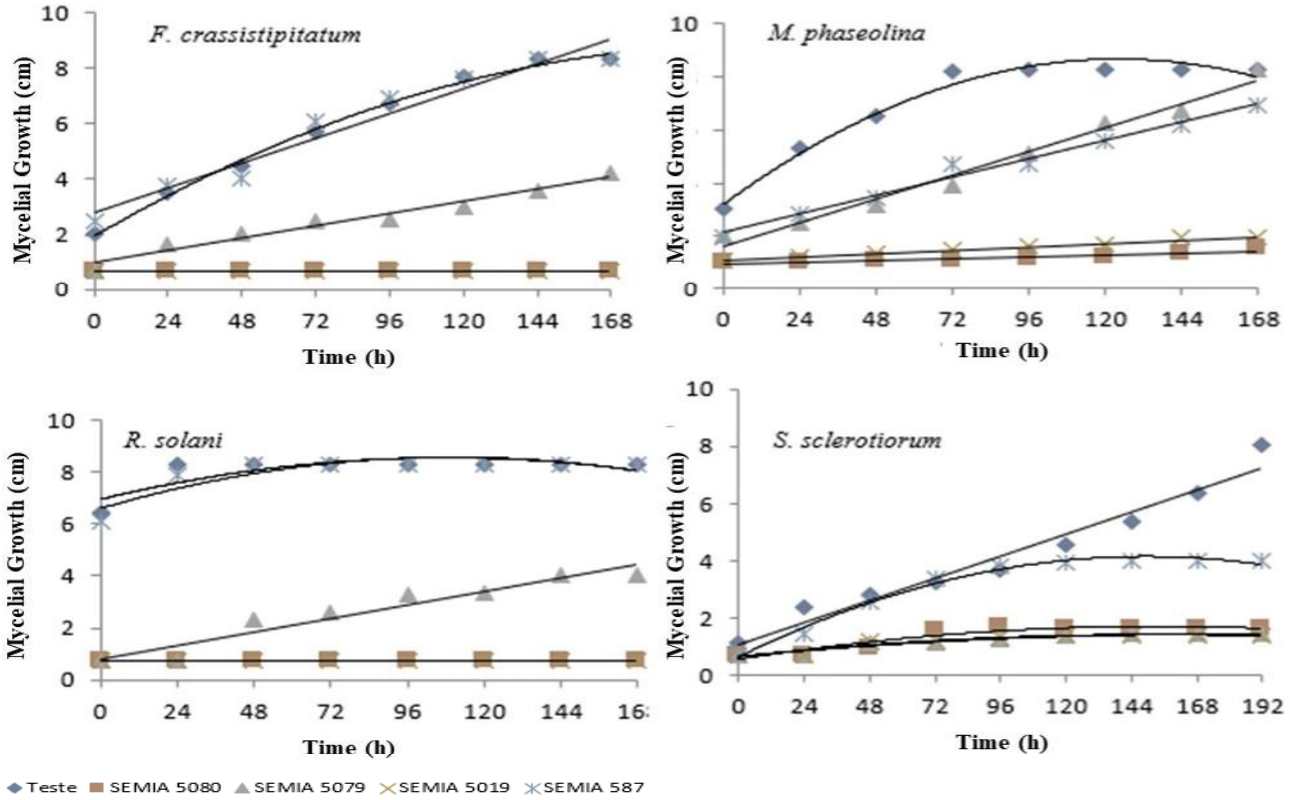
Experimental design and statistical analysis

The experimental design was completely randomised in a two-factor scheme, where one of the factors was the different strains of *Bradyrhisobium* and the other, the growth time (24h) of each phytopathogen. ANOVA was carried out for time-dependent evaluations, followed by regression analysis using the Genes software (CRUZ, 2016).

RESULTS AND DISCUSSION

The SEMIA 5080 and SEMIA 5019 strains showed antagonistic action towards all the phytopathogens in the comparison, reducing mycelial growth relative to the control (Figure 1, Figure 2 and Table 1). The SEMIA 5079

Figure 1 - Antagonistic potential of the SEMIA 5080, SEMIA 5079, SEMIA 5019 and SEMIA 587 strains of *Bradyrhisobium* spp. on the *in-vitro* mycelial growth of *F. crassistipitatum*, M. phaseolina, *R. solani* and *S. sclerotiorum* using the circle method



strain was antagonistic towards *S. sclerotiorum*, very similar to SEMIA 5080 and 5019; however for the other phytopathogens there was a less pronounced reduction in mycelial growth than in the highlighted strains. SEMIA 587 was antagonistic towards *S. sclerotiorum* only.

Figures 3 and 4, and Table 2, show the results of inoculating the soybean seeds with strains of *Bradyrhisobium* spp. It can be seen that the SEMIA 587 strain had no effect on the mycelial growth of any of the phytopathogens, with a result similar to that of the control treatment; while the SEMIA 5080, SEMIA 5079 and SEMIA 5019 strains do not differ from each other, but do differ from the control, with a 20.55% reduction in mycelial growth for *F. crassistipitatum* after 96 h evaluation. The SEMIA 5019 and SEMIA 5080 strains show a 20% reduction in mycelial growth in *M. phaseolina*, and a 17% reduction in mycelial growth in *R. solani* and *S. sclerotiorum*.

Where the soybean seeds were inoculated with strains of *Bradyrhisobium* spp., the SEMIA 5079, SEMIA 5019 and SEMIA 5080 strains showed a very similar inhibitory response in reducing the mycelial growth of *F. crassistipitatum*; however, for the remaining phytopathogens, SEMIA 5019 and SEMIA 5080, the results were better.

The comparative results between the adapted circle method and seed inoculation also show that the SEMIA 5019 and SEMIA 5080 strains stood out in reducing the mycelial growth of the phytopathogens in relation to the other strains, and prove that the SEMIA 587 strain has no antagonistic effect on the phytopathogens under test. The difference between the methods can be explained mainly by the differences inherent to each, where the antagonistic activity of SEMIA 5019 and SEMIA 5019 can be seen in the circle method, since the phytopathogens did not approach the barrier formed by *Bradyrhisobium*

Table 1 - Equations of the curves shown in Figure 1, adjusted R^2 and point of maximum (Pmax)

Treatment F. crassistipitatum	Equation	Adjusted R^2 (%)	Pmax.	
			X	Y
CONTROL	$Y = 1.9635 + 0.0642x - 0.0002x^2$	95.58	160.50	7.12
SEMIA 5080	$\bar{y} = 0.70$	-	-	-
SEMIA 5079	$Y = 0.9875 + 0.0184x$	96.12	-	-
SEMIA 5019	$\bar{y} = 0.70$	-	-	-
SEMIA 587	$Y = 2.8177 + 0.0372x$	94.01	-	-
M. phaseolina				
CONTROL	$y = 3.2005 + 0.0882x - 0.0004x^2$	96.69	110.25	8.06
SEMIA 5080	$y = 0.9229 + 0.0031x$	88.70	-	-
SEMIA 5079	$y= 1.5998 + 0.0375x$	97.84	-	-
SEMIA 5019	$y = 1.0843 + 0.0054x$	98.15	-	-
SEMIA 587	$y = 2.125 + 0.0288x$	98.14	-	-
R. solani				
CONTROL	$Y = 6.963 + 0.0295x - 0.0001x^2$	53.33	147.50	9.14
SEMIA 5080	$\bar{y} = 0.70$	-	-	-
SEMIA 5079	$Y = 0.8031 + 0.0216x$	90.17	-	-
SEMIA 5019	$\bar{y} = 0.70$	-	-	-
SEMIA 587	$Y = 6.6094 + 0.0357x - 0.0002x^2$	70.96	89.25	8.20
S. sclerotiorum				
CONTROL	$Y = 1.0868 + 0.0321x$	95.59	-	-
SEMIA 5080	$Y = 0.5453 + 0.0151x - 0.00005x^2$	87.57	151	1.68
SEMIA 5079	$Y = 0.6406 + 0.009x - 0.00005x^2$	93.12	90	1.05
SEMIA 5019	$Y = 0.648 + 0.0102x - 0.00005x^2$	88.62	102	1.17
SEMIA 587	$Y = 0.625 + 0.0471x - 0.0002x^2$	98.22	117.75	3.40

Figure 2 - Illustration of the circle method and effectiveness of the respective strains (Control, SEMIA 587, SEMIA 5019, SEMIA 5080 and SEMIA 5079) in controlling *M. phaseolina*

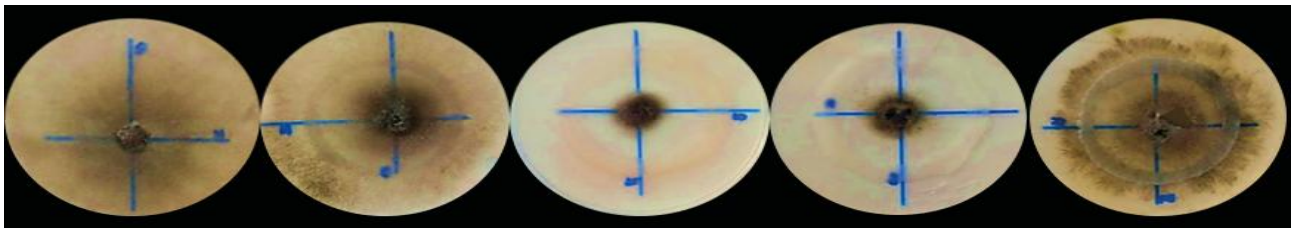


Figure 3 - Antagonistic potential of the SEMIA 5080, SEMIA 5079, SEMIA 5019 and SEMIA 587 strains of *Bradyrhisobium* spp. inoculated into soybean seeds, on the mycelial growth of *F. crassistipitatum*; *M. phaseolina*, *R. solani* and *S. sclerotiorum*

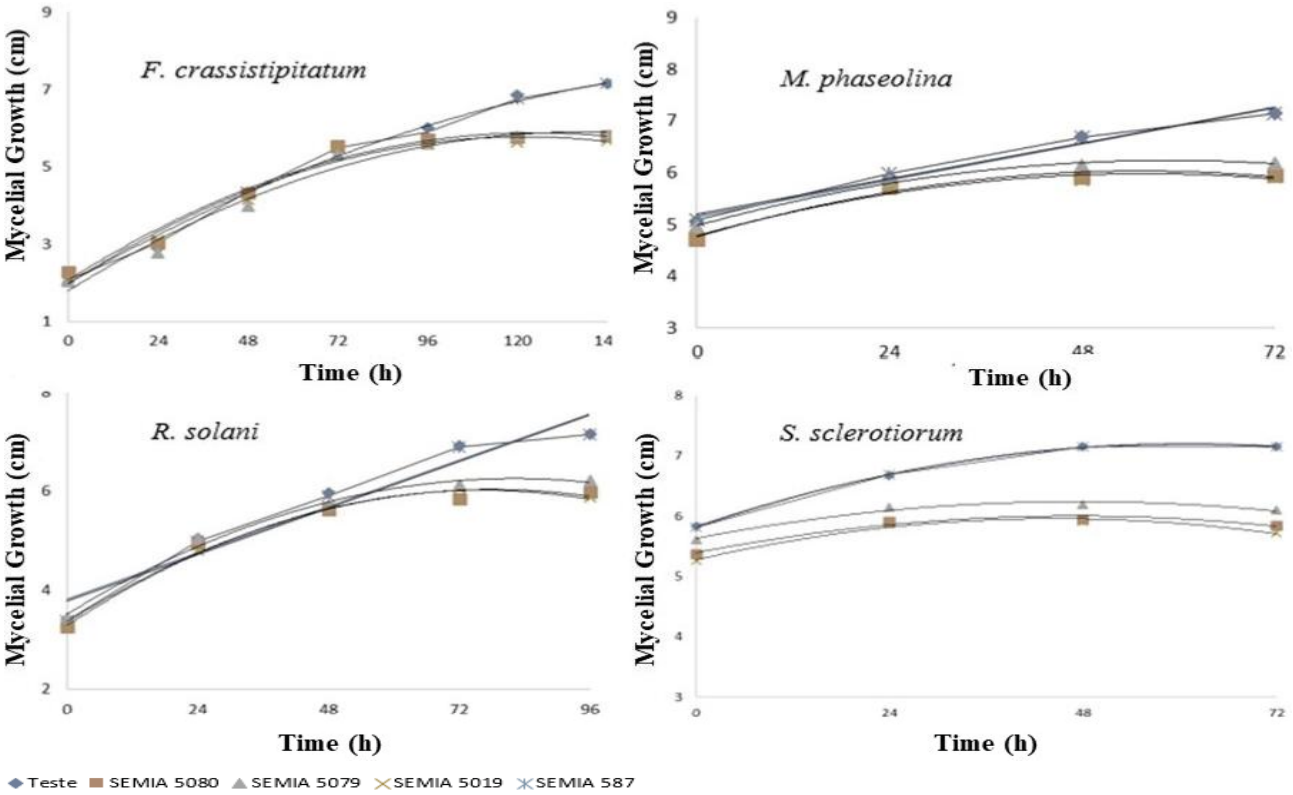


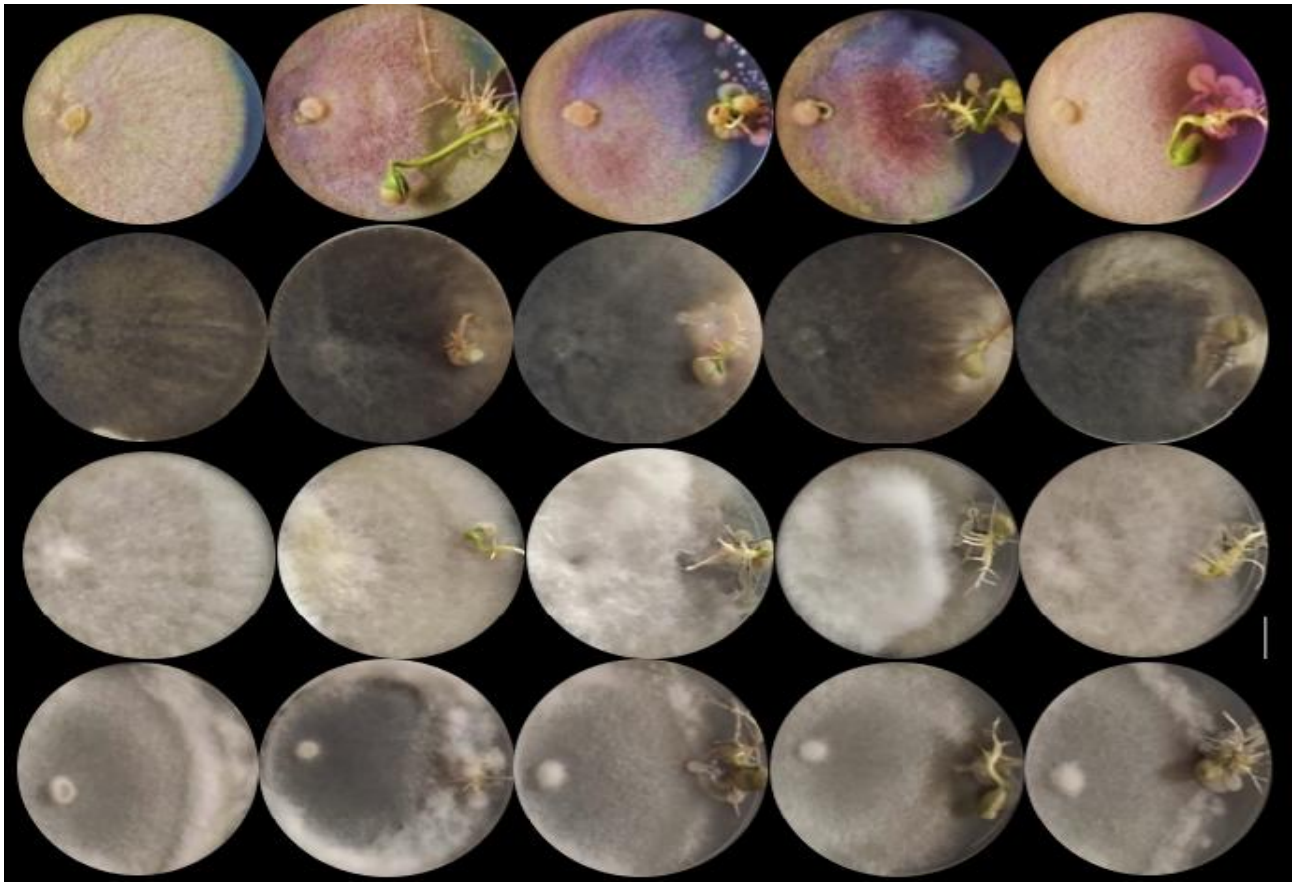
Table 2 - Equations of the curves shown in Figure 3, adjusted R^2 and point of maximum (Pmax) for the control, SEMIA 587, SEMIA 5019, SEMIA 5080 and SEMIA 5079 treatments, respectively

Treatment F. crassistipitatum	Equation	Adjusted R^2 (%)	Pmax.	
			x	Y
CONTROL	y = 0.0001x2 + 0.0552x + 1.9848	97.28	-	-
SEMIA 5080	y = -0.0002x2 + 0.06x + 1.7941	97.86	-	-
SEMIA 5079	y = -0.0001x2 + 0.0552x + 1.9848	99.39	-	-
SEMIA 5019	$y = 1.9707 + 0.0624x - 0.0003x^2$	97.57	104	5,21
SEMIA 587	$\bar{y} = 4.94$	-	-	-

Continuation Table 2

M. phaseolina				
CONTROL	$y = 5.8314 + 0.0446x - 0.0004x^2$	78.54	55.75	7.07
SEMIA 5080	$y = 5.3931 + 0.026x - 0.0003x^2$	84.61	43.33	5.96
SEMIA 5079	$y = 5.6309 + 0.0258x - 0.0003x^2$	94.37	43.00	6.19
SEMIA 5019	$y = 5.2774 + 0.0307x - 0.0003x2$	84.70	51.17	6.06
SEMIA 587	$y = 5.8095 + 0.046x - 0.0004x^2$	81.18	57.50	7.13
R. solani				
CONTROL	$y = 3.8244 + 0.039x$	91.75	-	-
SEMIA 5080	$y = 3.3592 + 0.0685x - 0.0004x^2$	96.38	85.63	6.29
SEMIA 5079	$y = 3.5171 + 0.0667x - 0.0004x^2$	98.49	83.38	6.30
SEMIA 5019	$y = 3.2913 + 0.0716x - 0.0005x^2$	99.09	71.60	5.85
SEMIA 587	$y = 3.7846 + 0.039x$	92.13	-	-
S. sclerotiorum				
CONTROL	$y = 5.1482 + 0.0294x$	97.40	-	-
SEMIA 5080	$y = 4.7582 + 0.0462x - 0.0004x^2$	93.41	57.75	8.76
SEMIA 5079	$y = 4.9763 + 0.0428x - 0.0004x^2$	98.73	53.50	8.41
SEMIA 5019	$y = 4.7819 + 0.043x - 0.0004x^2$	93.11	53.75	8.25
SEMIA 587	$y = 5.1944 + 0.0287x$	97.02	-	-

Figure 4 - Petri dishes containing soybean seeds inoculated with the different strains (Control, SEMIA 587, SEMIA 5019, SEMIA 5080 and SEMIA 5079) in the control of *F. crassistipitatum*, M. phaseolina, R. solani and S. sclerotiorum, respectively by row



spp. In addition to the methods being different, the medium also affords small differences, as the YM medium is specific to the growth of *Bradyrhisobium* spp., while the BDA medium used in the seed inoculation method is specific to fungi. Growth in the mycelial structure of the pathogens is therefore distinct to each culture medium, and may have been favoured in the PDA medium; however control of the strains was effective in both media.

Similar results were found by Nozaki, Camargo and Barreto (2004), showing that the lighting regime, temperature and culture media confirm the difference in mycelial growth as seen in ten isolates of *Diaporthe citri*, the causal agent of melanosis in citrus plants. Shahid *et al*. (2021) also found that the production of metabolites from *Pseudomonas* spp. varies depending on the culture medium used.

Huynh, Navi and Yang (2022) found that when inoculating *Bradyrhisobium japonicum* into soybean seeds, there was a reduction in sudden death syndrome caused by *Fusarium virguliforme*, in addition to a greater number of nodules and more root mass. The antagonistic potential of this genus has also been shown to suppress *Fusarium solani*, *Macrophomina Phaseolina*, *Rhizoctonia solani* and *Ralstonia solanacearum* (CHATTOPADHYAY; BANERJEE; HANDIQUE, 2022; DESHWAL *et al*., 2003; OMAR; ABD-ALLA, 1998).

In studies using other BNF rhizobacteria, Al-ani *et al*. (2012) saw a reduction in the *in-vitro* mycelial growth of *Fusarium solani* and Macrophomina phaseolina when exposed to *Rhizobium japonicum*. There was also a reduction in the severity of the root rot caused by these pathogens, including a higher rate of emergence and the promotion of plant growth under both field and greenhouse conditions when inoculated with the bacterium. The authors explain this control as due to the antagonist being an efficient symbiont in the soybean that, in addition to fixing nitrogen, produces substances that promote plant growth, such as auxin (AIA), gibberellin (GA3) and cytokinin (Zeatin) (CASSÁN *et al*., 2009), and can indirectly suppress the pathogen by means of metabolites that protect the roots through antibiosis, inhibit spore germination and induce plant resistance.

The samples of hyphae taken from the comparison experiment using the adapted circle method and examined under the scanning electron microscope show the effectiveness of the control exerted by the strains of *Bradyrhisobium* spp., where it is possible to observe the morphological damage to the hyphae of the phytopathogens under study (Figure 5). For *F. crassistipitatum* and *R. solani*, only images of the control and the treatment with SEMIA 587 were captured. This is because the hyphae in the dishes of the other treatments were so damaged that it was impossible to collect any material to take the reading.

Figures 5A and 5B show the presence of spores of *F. crassistipitatum*. Figures 5D to 5G show damage to the hyphae of *M. phaseolina* indicated by the red arrows, including narrowing accompanied by rupture of the hyphae caused by SEMIA 5080, SEMIA 5079, SEMIA 5019 and SEMIA 587, respectively.

Figures 5H and 5I show the hyphae of *R. solani*, the control and SEMIA 587; this phytopathogen was difficult to visualise using scanning electron microscopy. Figures 5K to 5M clearly show the damage caused by narrowing, accompanied by rupture of the *S. sclerotiorum* hyphae, compared to the control (Figure 5J). The strains of *Bradyrhizobium* spp. proved to be harmful to *S. sclerotiorum*, hence the damage visible in the images; however, the SEMIA 587 strain (Figure 5N) showed little damage compared to the control (Figure 5J).

For the metabolites filtered from a 10% concentration of *Bradyrhisobium* spp., there was no significant difference between the treatments with the filtrates and the controls after 72 h of evaluation; so much so, that for *F. crassistipitatum* and *M. phaseolina* the five treatments show the same curve (Figure 6 and Table 3).

The minimum inhibitory concentration showed that microdilution of the dish metabolites from the four strains of *Bradyrhisobium* spp. had no inhibitory effect on the four phytopathogens under study, as can be seen in Figure 7.

In the experiment with volatile metabolites using two-part Petri dishes, there was no significant difference between the treatments on the mycelial growth of the phytopathogenic fungi, indicating that no volatile compounds with an inhibitory effect were produced.

As noted, in addition to the volatile effect, metabolites filtered from *Bradyrhisobium* spp. in the Petri-dish and MIC tests, had no inhibitory effect on the growth of the phytopathogens under study. However, Tewari and Sharma (2020) found antibiosis in the IC-4059 strain of *Bradyrhisobium* sp. that reduced the *in-vitro* growth of F*usarium udum*; there is a 52.6% reduction in mycelial growth in the phytopathogen when exposed to the strain, 20.5% reduction when in contact with the exopolysaccharides, and 48.1% when in contact with the filtrates, showing that *Bradyrhisobium* spp. displays antibiosis, in addition to direct control.

Studies show the potential of genus Bacillus, another rhizospheric bacteria well-established on the biologics market. Leathers *et al*. (2020), using the minimum inhibitory concentration method, observed that *Bacillus nakamurai* at a concentration of 5%-20% (vol/vol) was effective in controlling *Erwinia amylovora*, a phytopathogen that affects apple and pear trees. Zhang *et al*. (2020) found that volatile organic compounds

Figure 5 - Scanning electron microscopy under 500x magnification, showing the hyphae of phytopathogens when exposed to strains of *Bradyrhisobium* spp. after nine days of evaluation. A: Control *F. crassistipitatum*, B: SEMIA 587 and hyphae of *F. crassistipitatum*, C: Control *M. phaseolina*, D: SEMIA 5080 and *M. phaseolina*, E: SEMIA 5079 and *M. phaseolina*, F: SEMIA 5019 and *M. phaseolina*, G: SEMIA 587 and *M. phaseolina*, H: Control *R. solani*, I: SEMIA 587 and *R. solani*, J: Control *S. sclerotiorum*, K: SEMIA 5080 and *S. sclerotiorum*, L: SEMIA 5079 and *S. sclerotiorum*, M: SEMIA 5019 and *S. sclerotiorum*, N: SEMIA 587 and *S. sclerotiorum*

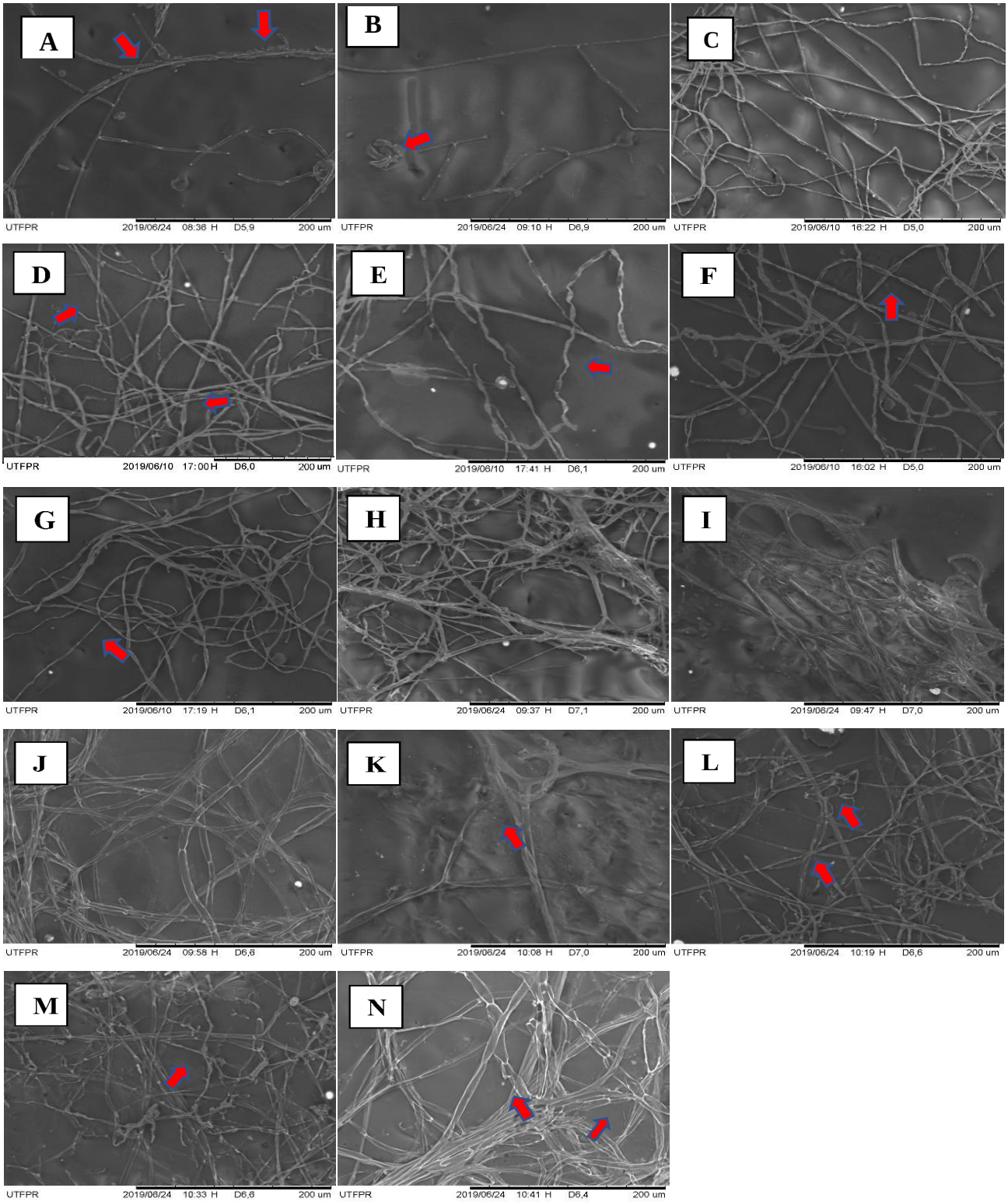


Figure 6 - Effect of the filtrates (metabolites) of the SEMIA 5080, SEMIA 5079, SEMIA 5019 and SEMIA 587 strains of *Bradyrhisobium* spp. at a concentration of 10% on the *in-vitro* growth of *F. crassistipitatum*, *M. phaseolina*, *R. solani* and *S. sclerotiorum*

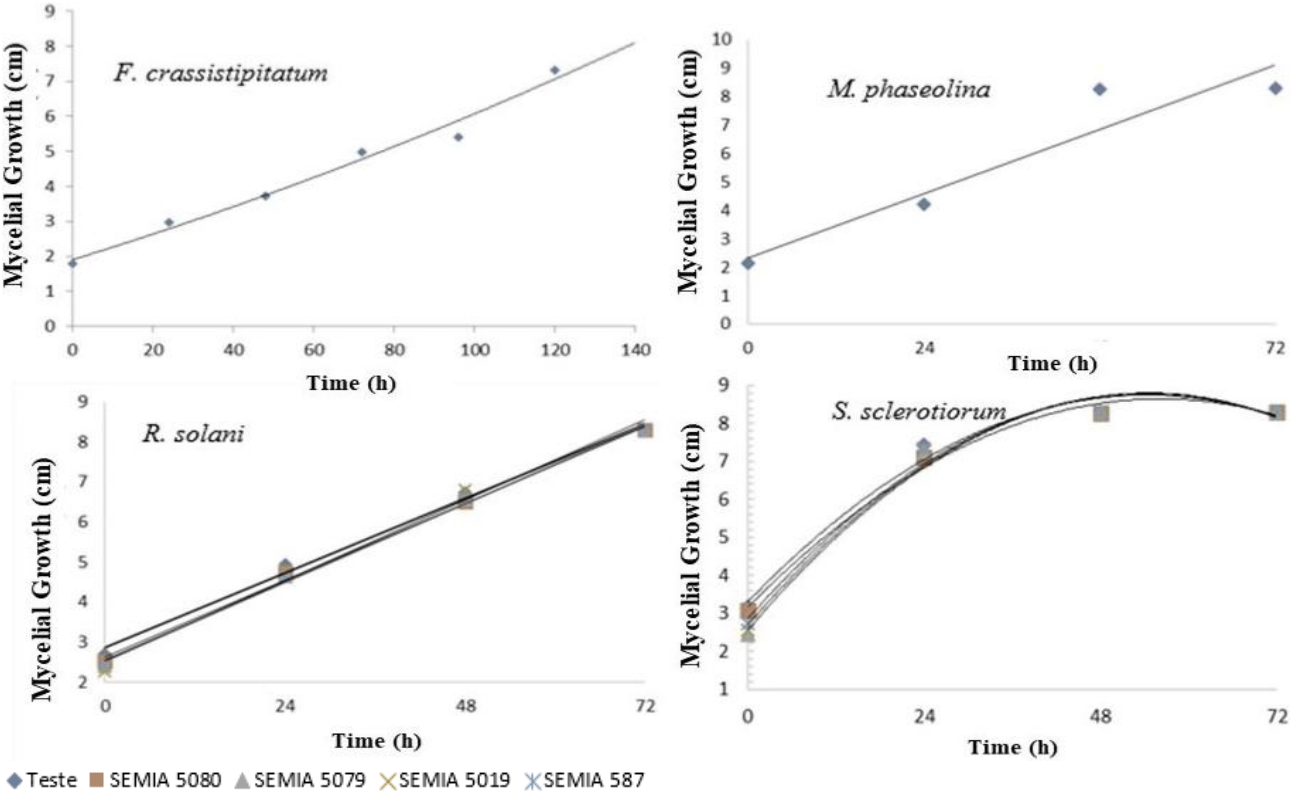
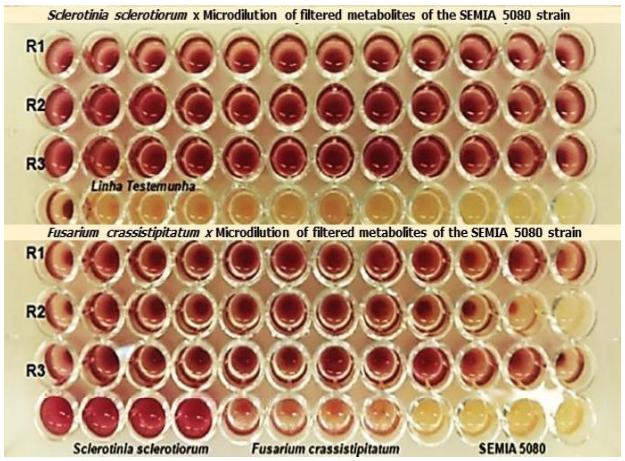


Table 3 - Equations of the curves shown in Figure 6, adjusted R^2 and point of maximum (Pmax) for the control, SEMIA 587, SEMIA 5019, SEMIA 5080 and SEMIA 5079 treatments, respectively

Treatment F. crassistipitatum	Equation	Adjusted R^2 (%)	Pmax,	
			x	Y
ALL	y.=.1.7189.+.0.0445x	98.47	-	-
M. phaseolina				
ALL	Y = 2.3513 + 0.0939x	90.10	-	-
R. solani				
CONTROL	y = 2.8682 + 0.0776x	98.99	-	-
SEMIA 5080	y = 2.6396 + 0.0798x	99.56	-	-
SEMIA 5079	y = 2.5207 + 0.0819x	99.50	-	-
SEMIA 5019	y = 2.5234 + 0.0838x	98.03	-	-
SEMIA 587	y = 2.5498 + 0.0815x	99.53	-	-
S. sclerotiorum				
CONTROL	$y = 3.3038 + 0.2004x - 0.0018x^2$	94.48	55.67	8.88
SEMIA 5080	$y = 3.1638 + 0.1951x - 0.0017x^2$	97.42	57.38	8.76
SEMIA 5079	y = 2.5613 + 0.2286x – 0.0021x2	95.29	54.43	8.78
SEMIA 5019	$y = 2.697 + 0.2238x - 0.0021x^2$	94.27	53.29	8.66
SEMIA 587	$y = 2.8613 + 0.2182x - 0.002x^2$	94.78	54.55	8.81

Figure 7 - Microdilution method (MIC) on ELISA plates to determine the minimum inhibitory concentration of metabolites from *Bradyrhisobium* spp. The filtrate of the SEMIA 5080 strain did not inhibit *S. sclerotiorum* or *F. crassistipitatum*, as shown by the tetrazolium reaction in the wells



from a strain of *Bacillus subtilis* caused morphological damage to the hyphae of *Alternaria solani*, in addition to reducing the size of the colony and inhibiting conidial germination.

CONCLUSION

The SEMIA 5080 and SEMIA 5019 strains of *Bradyrhisobium* spp. show the greatest potential for controlling the phytopathogens *F. crassistipitatum*, *M. phaseolina*, *R. solani* and *S. sclerotiorum*, followed by the SEMIA 5079 strain, while SEMIA 587 had no effect. The most common morphological changes in the fungal hyphae, caused by direct exposure of the phytopathogens to the strains of *Bradyrhisobium* spp., were rupture and narrowing of the hyphae. There was no antibiosis from the filtered or volatile metabolites in the strains of Bradyrhisobium spp. under study.

REFERENCES

AL-ANI, R. A. *et al. Rhizobium japonicum* as a biocontrol agent of soybean root rot disease caused by Fusarium solani and Macrophomina phaseolina. **Plant Protect Science**, v. 48, n. 4, p. 149-155, 2012.

ANDRADE, C. M. *et al*. HOST-induced gene silencing in the necrotrophic fungal pathogen Sclerotinia sclerotiorum. **Plant Pathology**, 2015. DOI: https://doi.org/10.1111/ppa.12447.

BELOTI, V. Frequency of 2,3,5-triphenyltetrazolium chloride (TTC) non-reducing bacteria in pasteurized milk. **Revista de Microbiologia**, v. 30, p. 137-140, 1999.

BOLTON, M. D.; THOMMA, B. H. J.; NELSON, B. D. *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. **Molecular Plant Pathology**, v. 7, p. 1-16, 2006.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. **Regras para análise de sementes**. Brasília, DF, 2009.

CASSÁN, F. *et al. Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). **European Journal of Soil Biology**, v. 45, n. 1, p. 28-35, 2009.

CHATTOPADHYAY, P.; BANERJEE, G.; HANDIQUE, P. J. Use of an abscisic acid-producing *Bradyrhizobium japonicum* isolate as biocontrol agent against bacterial wilt disease caused by *Ralstonia solanacearum*. **Journal of Plant Diseases and Protection**, 2022. DOI: https://doi.org/10.1007/s41348-022-00604-9.

CHUEIRE, L. M. O. *et al*. Classificação taxonômica das estirpes de rizóbio recomendadas para as culturas da soja e do feijoeiro baseada no seqüenciamento do gene 16S rRNA. **Revista Brasileira de Ciência do Solo**, v. 27, n. 5, p. 833-840, 2003. DOI: https://dx.doi.org/10.1590/S0100-06832003000500007.

CRUZ, C. D. Genes Software – extended and integrated with the R, Matlab and Selegen. **Acta Scientiarum**, v. 38, n. 4, p. 547-552, 2016.

DAVE, K. *et al*. Facetes of rhizospheric microflora in biocontrol of phytopathogen *Macrophomina phaseolina* in oil crop soybean. **Archives of Microbiology**, 2021. DOI: 10.1007/s00203-020-02046-z.

DELAMUTA, J. R. M. *et al*. Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens* sp. nov. **International Journal of Systematic and Evolutionary Microbiology**, v. 63, pt. 9, p. 3342-3351, Sep. 2013. DOI: 10.1099/ijs.0.049130-0.

DESHWAL, V. K. *et al.* Rhizobia as a biological control agent against soil borne plant pathogenic fungi. **Indian Journal of Experimental Biology**, v. 41, n. 10, p. 1160-1164, 2003.

HASHEM, A. *et al*. *Bacillus subtilis*: a plant-growth promoting rhizobacterium that also impacts biotic stress. **Saudi Journal of Biological Sciences**, v. 26, n. 6, p. 1291-1297, 2019. DOI: 10.1016/j.sjbs.2019.05.004.

HUNGRIA, M. *et al*. Inoculum rate effects on the soybean symbiosis in new or old fields under tropical conditions. **Soil Fertility & Crop Nutrition. Agronomy Journal**, 2017.

HUNGRIA, M.; ARAUJO, R. S.; CAMPO, R. J. Biological nitrogen fixation as a key component of N nutrition for the soybean crop in Brazil. *In*: WORLD SOYBEAN CONFERENCE, 8., 2009, Beijing. **Proceedings** […]. Beijing: Chinese Academy of Agricultural Sciences/Crop Science Society of China, 2009.

HUNGRIA, M.; MENDES, I. C. Nitrogen fixation with soybean: the perfect symbiosis? *In*: DE BRUIJN, F. J. (ed.). **Biological nitrogen fixation**. Hoboken, New Jersey: John Wiley & Sons, 2015. p. 1009-1023.

HUYNH, T.; NAVI, S. S.; YANG, X. B. Biocontrol potential of *Bradyrhizobium japonicum* against soybean sudden death syndrome in irrigated and non-irrigated fields. *In*: RAJPAL, V. R.; SINGH, I.; NAVI, S. S. (ed.). **Fungal diversity, ecology and control management**. Singapore: Springer, 2022. DOI: https://doi.org/10.1007/978-981-16-8877-5_21.

IPEA. **Agronegócio brasileiro começa 2022 com superávit de US$ 7,7 bilhões**. 2022. Disponível em: https://www.ipea.gov.br/portal/index.php?option=com_content&view=article&id=38952. Acesso em: 9 jun. 2022.

KHALIL, M. R. *et al*. Rhizospheric bacteria as potential biocontrol agents against Fusarium wilt and crown and root rot diseases in tomato. **Saudi Journal of Biological Sciences**, v. 28, n. 12, p. 7460-7471, 2021.

LEATHERS, T. D. *et al.* Inhibition of *Erwinia amylovora* by *Bacillus nakamurai.* **Current Microbiology**, v. 77, n. 5, p. 875-881, 2020. DOI: 10.1007/s00284-019-01845-y.

MARIANO, R. L. R. Métodos de seleção *in vitro* para o controle microbiológico de patógeno de plantas. **Revisão Anual de Patologia de Plantas**, v. 1, p. 369-409, 1993.

MENTEN, J. O. M. *et al*. Efeito de alguns fungicidas no crescimento micelial de *Macrophomina phaseolina* (Tass.) Goid. "*in vitro*". **Fitopatologia Brasileira**, v. 1, n. 2, p. 57-66, 1976.

NOZAKI, M. H.; CAMARGO, M.; BARRETO, M. Caracterização de *Diaporthe citri* em diferentes meios de cultura, condições de temperatura e luminosidade. **Revista Fitopatologia Brasileira**, v. 29, n. 4, 2004.

OMAR, S. A.; ABD-ALLA, M. H. Biocontrol of fungal root rot diseases of crop plants by the use of rhizobia and bradyrhizobia. **Folia Microbiológica**, v. 43, p. 431-437, 1998. DOI: https://doi.org/10.1007/BF02818587.

PAL, K. K.; MCSPADDEN GARDENER, B. Biological control of plant pathogens. **The Plant Health Instructor**, 2006. DOI: 10.1094/PHI-A-2006-1117-02.

REIS, E. M. Efeitos da rotação de culturas na incidência de podridões radiciais e na produtividade da soja. **Summa Phytopathologica**, v. 40, n. 1, 2014. DOI: https://doi.org/10.1590/S0100-54052014000100001.

SANTOS, M. S.; NOGUEIRA, M. A.; HUNGRIA, M. Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. **AMB Express**, v. 9, n. 205, 2019. DOI: https://doi.org/10.1186/s13568-019-0932-0.

SHAHID, I. *et al*. Profiling of antimicrobial metabolites of plant growth promoting *Pseudomonas* spp. isolated from different plant hosts. **3 Biotech**, v. 11, n. 2, p. 48, 2021. DOI: 10.1007/s13205-020-02585-8.

SOLINO, A. J. S. *et al.* Potencial antagonista e controle in vitro de *Alternaria solani* por fungos sapróbios. **Summa Phytopathology**, v. 43, n. 3, p. 199-204. 2017.

TEWARI, S.; SHARMA, S. Rhizobial-metabolite based biocontrol of fusarium wilt in pigeon pea. **Microbial Pathogenesis**, v. 147, 104278, 2020. DOI: 10.1016/j.micpath.2020.104278.

UNITED STATES DEPARTAMENT OF AGRICULTURE. **Oilseeds and products annual**. 29 April, 2022.

VISMARA, L. S. **Óleos essenciais na indução de resistência em morangos ao mofo cinzento, à *Botrytis cinerea* in vitro e ação toxicológica**. Tese (Doutorado em Agronomia) - Universidade Tecnológica Federal do Paraná, Programa de Pós-Graduação em Agronomia, Pato Branco, 2019.

YAO, X. *et al*. Candicidin isomer production is essential for biocontrol of cucumber rhizoctonia rot by *Streptomyces albidoflavus* W68. **Applied Environmental Microbiology**, 2021. DOI: 10.1128/AEM.03078-20.

ZHANG, D. *et al.* Antifungal effects of volatiles produced by Bacillus subtilis against Alternaria solani in potato. **Frontiers in Microbiology**, 2020. DOI: 10.3389/fmicb.2020.01196.