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Interaction between *Trichoderma asperellum* and *Bacillus* spp. in the biological control of disease in the soya bean[1]

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ABSTRACT - One of the main phytosanitary problems in major crops are phytonematodes, which are characterised as difficult to control. In view of this, biocontrol, through the use of biological agents, has become a driving force in controlling these pathogens. The aim of this study was to evaluate the compatibility of microorganisms, and detect the possibility of antagonistic effects, influence on growth, or synergistic action when microorganisms are jointly applied to crops. Microbiological analyses were carried out to observe the interaction of the fungus *Trichoderma asperellum* with the bacteria *Bacillus subtilis* and *Bacillus methylotrophicus*. It was found that the interaction between the genera *Bacillus* and *Trichoderma* did not promote any increase in the fresh or dry weight of the roots or shoots of the soya plants; however, based on the results of the microbiological analysis of the fungi and bacteria, the interaction was found to be promising, particularly in the evaluation carried out on the seventh day. Considering the number of viable spores, the interaction between *B. subtilis* + *T. asperellum* showed better results than *B. methylotrophicus* + *T. asperellum*. The Interaction between the genera *Bacillus* and *Trichoderma* showed the potential for joint use in the biological control of the soya bean.

Key words: Compatibility. Biological control. Interaction.

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INTRODUCTION

Soya is an important crop worldwide, with Brazil the largest producer, responsible for 124 million tons of soya beans in the 2020/21 season (Companhia Nacional de Abastecimento, 2022). Demand for the soya bean is expected to grow significantly in the coming years, highlighting the importance of ever more efficient production and of overcoming the obstacles posed by parasitic plant disease, which can be of bacterial, fungal or viral origin, or caused by nematodes.

Biological control is characterised by a reduced risk to human health and greater environmental sustainability (Rufino; Araújo; Nogueira, 2018), which through the use of antagonistic microorganisms, is seen as an alternative form of management (Silva *et al*., 2014). The great diversity of these microorganisms, together with their antagonistic relationships, has afforded interesting results in controlling plant disease (Braga Junior *et al*., 2017; Heling *et al*., 2017; Oliveira *et al*., 2017). Compared to chemical methods, biological control is a viable alternative, resulting in less environmental damage. Research shows that some bacteria, such as *Bacillus subtilis* and *B. thuringiensis*, show considerable potential for controlling phytonematodes (Bavaresco; Guaberto; Araujo, 2020; Machado; Costa, 2017; Mazzuchelli; Mazzuchelli; Araujo, 2020). Another example is the use of fungi from genera *Penicillium*, *Trichoderma* and *Myrothecium*, which have shown good results in controlling nematodes by producing substances that are toxic to the pathogens (Eapen; Beena; Ramana, 2005; Miao *et al.*, 2019; Mukhtar; Tariq-Khan; Aslam, 2021; Nguyen *et al*., 2018; Sikandar *et al*., 2020).

In an effort to maximise results, farmers have begun to use multiple biological products from different species of microorganisms in the same application. Understanding the compatibility of microorganisms that are used for biological control is of great importance in developing strategies for the management of phytonematodes. However, interaction analysis is complex, and results that show both compatible and synergistic behaviour are rare, making it necessary to demonstrate not only the interaction but also any antagonism between the microorganisms.

Although there are studies that evaluate the interactions of combinations of chemical and biological products used in a single application, the interactions between fungi and bacteria used for biological control require further research, considering the lack of information on the compatibility of these microorganisms. The aim of this study was to evaluate the compatibility of microorganisms, and detect the possibility of antagonistic effects, influence on growth, or synergistic action when microorganisms are jointly applied to crops.

MATERIAL AND METHODS

The study was carried out from March 2019 to October 2020 in two *in-vitro* trials and under greenhouse conditions at the Tissue Culture Laboratory (LCTV), while the microbiological analysis was performed at the Laboratory for Phytopathology and Nematology, both located on the Rio Verde Campus of the Instituto Federal Goiano (17°48'24.231" W, 50°54' 23.634" S, Altitude: 748 m).

Obtaining and multiplying the biological control agents

The microorganisms were initially obtained from commercial products used in biological control: the SF 04 isolate of *Trichoderma asperellum* from the commercial product Quality® (Lallemand), the SF 267 isolate of *Bacillus methylotrophicus* from the commercial product Onix® OG (Lallemand), and the QST 2808 isolate of *Bacillus subtilis* from the commercial product Rizos® OG (Lallemand). The samples taken for microbiological analysis and for conducting the experiment came from sealed packages to ensure that the viability and composition of the products had not been altered by external conditions.

Laboratory (*in-vitro*) evaluation

The laboratory experiment was carried out twice in a completely randomised design to detect the percentage of viable and nonviable spores, and included three treatments: 1. *T. asperellum* (fungus); 2. *B. subtilis* (bacteria) + *T. asperellum* (fungus); 3. *B. methylotrophicus* (bacteria) + *T. asperellum* (fungus), with four replications, to give 24 experimental units.

For the fungal conidia germination test, the formulations of the fungus-based products were diluted into concentrations of 1.0×10^6 spores mL^{-1} and mixed with the bacterial suspensions at a concentration of 1.0×10^8 $CFU.mL^{-1}$. A 15 µL aliquot of the suspension was then removed, and four drops were added to PDA (Potato Dextrose and Agar) culture medium. The dilutions were based on the best evaluation made under an optical microscope (Bettiol *et al*., 2012).

The commercial biological products were submitted to microbiological analysis. The fungal spores that comprised the products were counted in a Neubauer chamber, plated on PDA culture medium, and kept in a BOD incubator at 25 ± 1 ºC for 20 hours to verify their viability, as per Bettiol *et al*. (2012).

Once germination had begun, a drop of lactophenol blue (8 µL) was added to each sample. The conidia were considered nonviable if unstained, and viable when swollen or germinated. The percentage of viable and nonviable conidia was determined under an optical microscope with a 40x objective (four fields per Petri dish), evaluating 100 spores per slide (Bettiol *et al*., 2012; adapted).

Table 1 - Chemical characterisation of the soil used in the trials

pH	P	K	S	H + Al	Al	Ca	Mg	K	SB	T	m	V	OM
H_2O	--------- mg dm^{-3} --------			---------- $cmol_c$ dm^{-3} ----------							------- % -------		dm^{-3}
5.84	6.6	97	4.6	2.5	0.0	5.1	1.4	0.25	6.8	9.2	0.0	73	13.1

pH in water; P = phosphorus; S = sulphur; H + Al = hydrogen + aluminium; Al = aluminium; Ca = calcium; Mg = magnesium; K = potassium; SB = Sum of Exchangeable Bases; T = effective CEC; m = Aluminium Saturation Index; V = Base Saturation Index; OM = Organic Matter. Laboratório Solotech Cerrado Ltda ME, Rio Verde, Goiás

The products comprising bacteria were examined by serial dilution with aliquot plating on nutrient agar (NA) medium, and stored in a bacteriological growth chamber at ±35 ºC for 24 hours. The colony forming units were then counted ($CFU.mL^{-1}$) (Alfenas; Mafia, 2016).

Evaluation in the greenhouse

The experiment under greenhouse conditions was conducted in a randomised block design (RBD). Six treatments were evaluated: 1. Negative control (absence of microorganism), 2. *B. subtilis* (bacteria), 3. *B. methylotrophicus* (bacteria), 4. *T. asperellum* (fungus), 5. *B. subtilis* (bacteria) + *T. asperellum* (fungus), 6. *B. methylotrophicus* (bacteria) + *T. asperellum* (fungus), in five blocks.

Soil for the experiment was collected from the experimental area of the Rio Verde Campus of the Instituto Federal Goiano, sieved and mixed with sand in a ratio of 2:1, and then autoclaved for 40 minutes at 120 ºC (Santos *et al*., 2019; adapted). The soil was placed in 3-litre plastic pots and fertiliser was applied based on the soil analysis (Table 1). Eight seeds from the Monsoy 7110 IPRO variety of soya bean were sown in polyethylene pots. Aqueous solutions of the biological products were then applied following the commercial recommendations of 500 $mL.ha^{-1}$ to simulate in-furrow application. The greenhouse trial was evaluated 0, 7, 30, and 45 days after planting (DAP).

The collected soil samples were submitted to microbiological analysis to quantify the number of $CFU.mL^{-1}$. Ten g of soil were weighed and added to an Erlenmeyer flask with 90 mL of autoclaved water. Serial dilution was then carried out and 100 µL of the suspension was pipetted onto NA medium (Nutrient Agar) to evaluate the bacteria. To evaluate the fungus, deep plating was carried out on PDA (Potato Dextrose Agar; Triton X-100) medium, which was conditioned for seven days in a BOD incubator at 25 ºC; the number of colonies was then counted ($CFU.mL^{-1}$).

The fresh and dry weight of the roots and shoots of the soya plants was evaluated 45 days after planting (DAP).

Statistical Analysis

The data on spore viability from the laboratory (*in-vitro*) evaluation were submitted to analysis of variance and, when significant, Tukey's test ($P < 0.05$) was carried out to compare the mean values and identify the treatments that showed a synergistic or antagonistic interaction.

The experiment in the greenhouse was organised in a randomised block design in a 6 x 4 factorial scheme (six treatments and four periods of evaluation) in five blocks. The data were subjected to analysis of variance and, when significant, were evaluated by Tukey's test ($P < 0.05$). In addition, the time factor was evaluated using regression analysis.

The fresh and dry weight data of the shoots and roots were submitted to analysis of variance based on the six treatments in four replications, and when significant, were evaluated by Tukey's test ($P < 0.05$).

The statistical analysis was carried out using the Sisvar v5.6 software.

RESULTS AND DISCUSSION

Laboratory (*in-vitro*) evaluation

There were differences between the treatments in the laboratory *in-vitro* experiment, (Table 2). In the first trial, the treatment with *T. asperellum* had the highest mean number of viable spores, with the spore count not differing from that of the treatment with *B. subtilis* + *T. asperellum*; this treatment, however, was equal to the *B. methylotrophicus* + *T. asperellum* combination, which had the lowest mean value. The count of nonviable spores was inversely proportional, i.e. the highest counts of nonviable spores were found in the treatments with *B. methylotrophicus* + *T. asperellum* and *B. subtilis* + *T. asperellum*, respectively.

In the second trial, the differences were obvious, with the treatments that included *T. asperellum* and *B. subtilis* + *T. asperellum* being statistically the best, and the treatment with *B. methylotrophicus* + *T. asperellum* the worst. In other words, the second trial shows that the

viable spore count was lower in the *B. methylotrophicus* + *T. asperellum* treatment, which, as a result, had a higher count of nonviable spores. It should be noted that for this treatment, the viable spore counts in the second trial were greater than 71%.

According to Kupper *et al*. (2013), the effectiveness of antagonists, whether *in vitro* or in a greenhouse, may often be insufficient to establish the population threshold required for biological control in the field, albeit serving as an indication of the viability of controlling phytopathogens under natural conditions of infection. In other words, the significant reduction found in the treatment with *B. methylotrophicus* + *T. asperellum* (Table 2), although presenting significantly lower values than the other treatments, does not invalidate its use in the field in biological control programs, since the data do not suggest a worrying negative interaction between the two microorganisms.

Lutz *et al*. (2004) point out that testing mixtures of different effective strains is essential for achieving maximum control potential, selecting strains that complement rather than act antagonistically to each other. Shanmugan, Gupta and Dohroo (2013), in a study with ginger, found that a mixture of strains (*Burkholderia cepacia* or *Bacillus subtilis* with *Trichoderma harzianum*) showed better control, resulting in a lower incidence of ginger rhizome rot, demonstrating the viability of the interaction between the microorganisms.

Greenhouse evaluation

According to the summary of the analysis of variance, the interaction between treatments and evaluation times was significant (Table 3).

The microbiological counts of the fungi and bacteria (Table 4) showed a factorial interaction in both cases that depended on the treatments under evaluation and the days since the start of the experiment.

Table 2 - Percentage (%) of viable and nonviable spores determined in two laboratory trials for the interaction between the fungus *T. asperellum* and the bacteria *B. subtilis* and *B. methylotrophicus*

First trial		
Treatment	Viable	Nonviable
Control	78.91 a	21.09 b
B. subtilis + *T. asperellum*	64.36 ba	35.63 ba
B. methylotrophicus + *T. asperellum*	50.29 b	49.71 a
Coefficient of Variation	17.14	31.17
Summary of ANOVA: MS	819.17*	
Second trial		
Treatment	Viable	Nonviable
Control	84.06 a	15.93 b
B. subtilis + *T. asperellum*	81.31 a	18.68 b
B. methylotrophicus + *T. asperellum*	71.66 b	28.33 a
Coefficient of Variation	4.92	18.52
Summary of ANOVA: MS	169.48**	

*Significant at 5% by F-test; **Significant at 1% by F-test; The same letters in a column do not differ by Tukey's test ($P < 0.05$)

Table 3 - Summary of the analysis of variance relative to the microbiological count of fungi (CFUF) and bacteria (CFUB) for the different treatments and evaluation times

SV	DF	CFUF	CFUB
Treatment	5	27.21**	3.02^{ns}
Time	3	27.84**	53.28**
Treatment x Time	15	5.65**	6.63**
CV (%)	53.76	124.48	
Overall Mean (g)	1.51	1.23	

**Significant at 1% by F-test; ^{ns}Not significant by F-test

Table 4 - Microbiological count of fungi and bacteria (log10 CFU ml^{-1}) as a function of the treatments under evaluation

Fungi				
Treatment	Days			
	0	7	30	45
Control	0.54 Aa	0.18 Ac	0.18 Ab	0.18 Ac
B. subtilis	0.48 Aa	0.18 Ac	0.18 Ab	0.18 Ac
B. methylotrophicus	0.18 Ba	0.18 Bc	1.28 ABb	1.55 Abc
T. asperellum	0.18 Ba	1.50 Bbc	4.24 Aa	4.55 Aa
B. subtilis + *T. asperellum*	0.18 Ba	3.14 Aa	4.06 Aa	4.38 Aa
B. methylotrophicus + *T. asperellum*	0.18 Ca	2.09 Bab	3.96 Aa	1.88 Bb
Bacteria				
Treatment	Days			
	0	7	30	45
Control	0.18 Aa	0.18 Ac	2.70 Aa	0.18 Aa
B. subtilis	0.18 Ba	3.78 Aab	1.46 ABa	0.18 Ba
B. methylotrophicus	0.18 Aa	1.77 Abc	2.54 Aa	0.18 Aa
T. asperellum	0.18 Aa	2.65 Aabc	0.18 Aa	0.18 Aa
B. subtilis + *T. asperellum*	0.18 Ba	4.95 Aa	0.18 Ba	0.18 Ba
B. methylotrophicus + *T. asperellum*	0.18 Ba	4.44 Aab	2.45 ABa	0.18 Ba

Mean values followed by the same letter do not differ by Tukey's test ($P < 0.05$). Uppercase letters on a row compare the same treatment for the different times, lowercase letters in a column compare different treatments for the same time

There was no difference between the treatments in the first microbiological count of fungi and bacteria on day 0 (Table 4). On day 7, the treatments differed, with *B. subtilis* + *T. asperellum* showing the highest mean values; however, this treatment proved to be statistically equal to *B. methylotrophicus* + *T. asperellum* in terms of both the bacterial and fungal count. For the treatments using only the *B. subtilis* and *B. methylotrophicus* strains, those with *B. subtilis* + *T. asperellum* and *B. methylotrophicus* + *T. asperellum* did not differ from the treatments with *T. asperellum* in terms of the number of bacteria.

At 30 days, the treatments with *T. asperellum*, *B. subtilis* + *T. asperellum* and *B. methylotrophicus* + *T. asperellum* showed the highest microbiological count for fungi, while on day 45, the best treatments included *T. asperellum* and *B. subtilis* + *T. asperellum* (Table 2). At 30 and 45 days, there was no difference in the microbiological bacterial count between the treatments.

The individual effects of bacteria of genus *Bacillus* and fungi of genus *Trichoderma* are well known (Abdullah; Ali; Suleman, 2008; Chowdappa *et al*., 2013). The results of the present study regarding the interaction of these two microorganisms corroborate those of Izquierdo-Garcia *et al*. (2020), Maketon, Apisitsantikul and Siriraweekul (2008), Morsy, Abdel-Kawi and Khalil (2009) and Yobo, Laing and Hunter (2011), where the authors found a positive interaction in the combined use of *Bacillus* and *Trichoderma.*

In a study with the common bean, Yobo, Laing and Hunter (2011) highlighted the potential for using a mixture of *Trichoderma* and *Bacillus* to improve plant growth and the control of *Rhizoctonia solani*. Furthermore, the results of Izquierdo-García *et al*. (2020) confirm the compatibility of *T. virens* Gl006 and *B. velezensis* Bs006 in the control of Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *physali* (Foph), in the golden berry (*Physalis peruviana*). The interaction between *B. subtilis* and *T. asperellum* is thought to have been positive.

Regarding the behaviour of the treatments in relation to the periods of evaluation (Table 4), it can be seen that for the fungi, the treatments with *T. asperellum* and *B. methylotrophicus* + *T. asperellum* showed higher counts from 30 days onwards, while *B. subtilis* + *T. asperellum* showed higher counts from day 7, with similar values on the days following the evaluation.

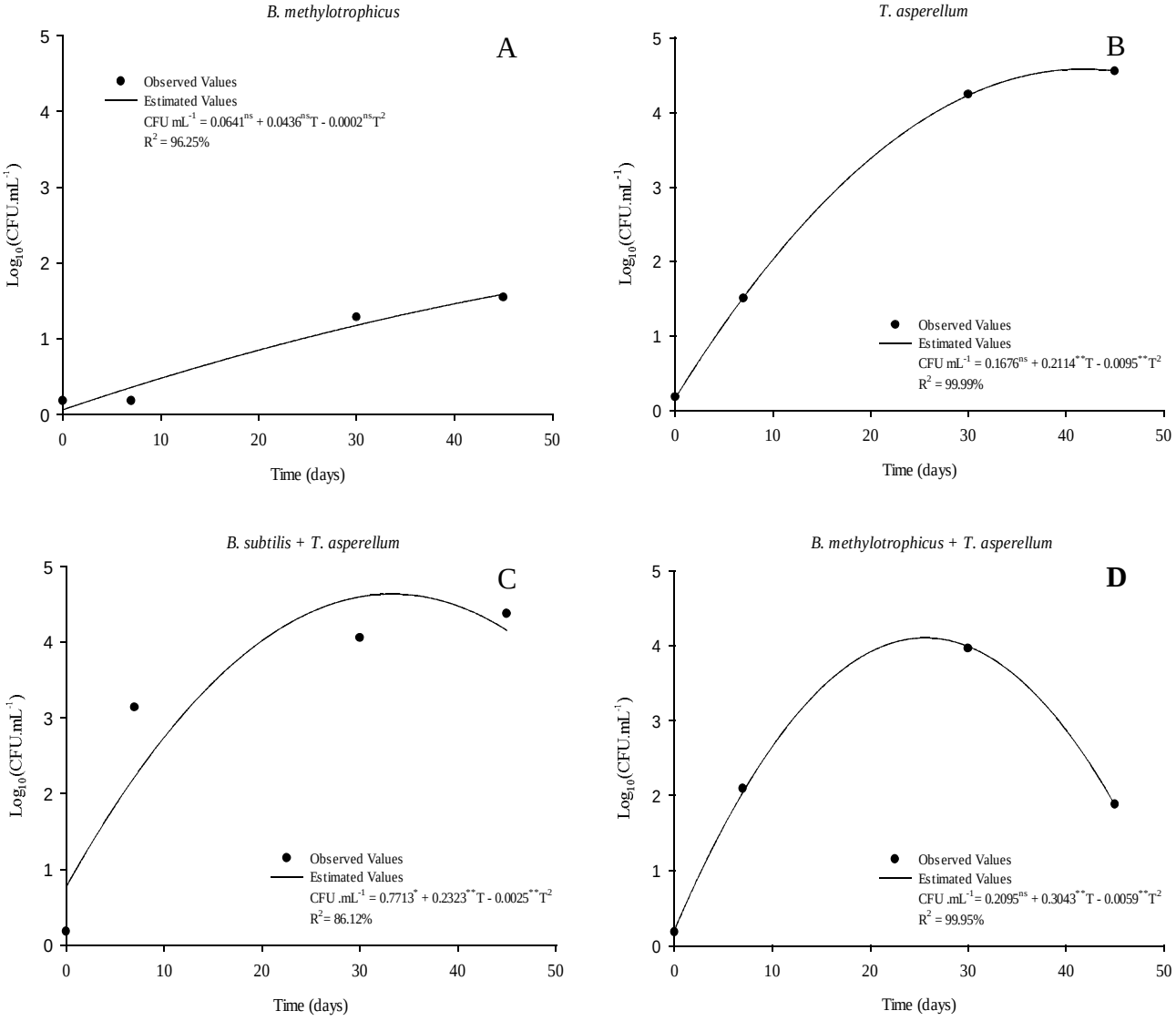
An analysis of the bacterial count data in Table 4 shows that all the treatments had significantly higher bacterial counts at time 0 from day seven onwards. Regression analysis was carried out to describe the behaviour of the treatments in relation to the counts of microorganisms throughout the evaluation.

The treatments with *B. methylotrophicus*, *T. asperellum*, *B. subtilis* + *T. asperellum* and *B. methylotrophicus* + *T. asperellum* showed quadratic behaviour with respect to the microbiological analysis of the fungi (Figure 1), while the other treatments did not show this behaviour, i.e. it was not possible to describe their behaviour by means of an equation. The experimental mean values for the control treatment and *B. subtilis*, which also did not show this behaviour, are shown in Table 4.

An increase of 96% can be seen in the treatment with *T. asperellum* at 45 days in relation to time 0. The treatment with *B. methylotrophicus* + *T. asperellum* showed an increase of 94.74% at 30 days compared to time 0; however, at 45 days, there was a reduction of 46.91% compared to the value at 30 days (Figure 1).

Among the various metabolites produced by *Bacillus* spp., those principally responsible for the antagonistic action are peptide antibiotics that control fungi and bacteria (Abo-Eldahab; El-Goorani, 1964). Studies with *B. subtilis* show *in-vitro* activity against different types of pathogens for several cultivated species through the production of antibiotics such as iturin A and suffactin, which are able to inhibit mycelial growth in fungi (Asaka; Shoda, 1996).

Figure 1 - Fungal count behaviour in treatments evaluated over different periods. A: *B. methylotrophicus*, B: *T. asperellum*, C: *B. subtilis* + *T. asperellum* and D: *B. methylotrophicus* + *T. asperellum*



*Significant at 5% by t-test; **Significant at 1% by t-test; nsNot significant by t-test

Maketon, Apisitsantikul and Siriraweekul (2008) evaluated *B. subtilis* and *T. harzianum*, both individually and in combination, for the control of three diseases in tobacco: bacterial wilt, caused by *Ralstonia solanacearum*; root and collar rot, caused by *Pythium aphanidermatum*; and frogeye leaf spot, caused by *Cercospora nicotiana*. The results show that a combination of *B. subtilis* species and fungal species such as *T. asperellum* contributes directly or indirectly, thereby increasing productivity.

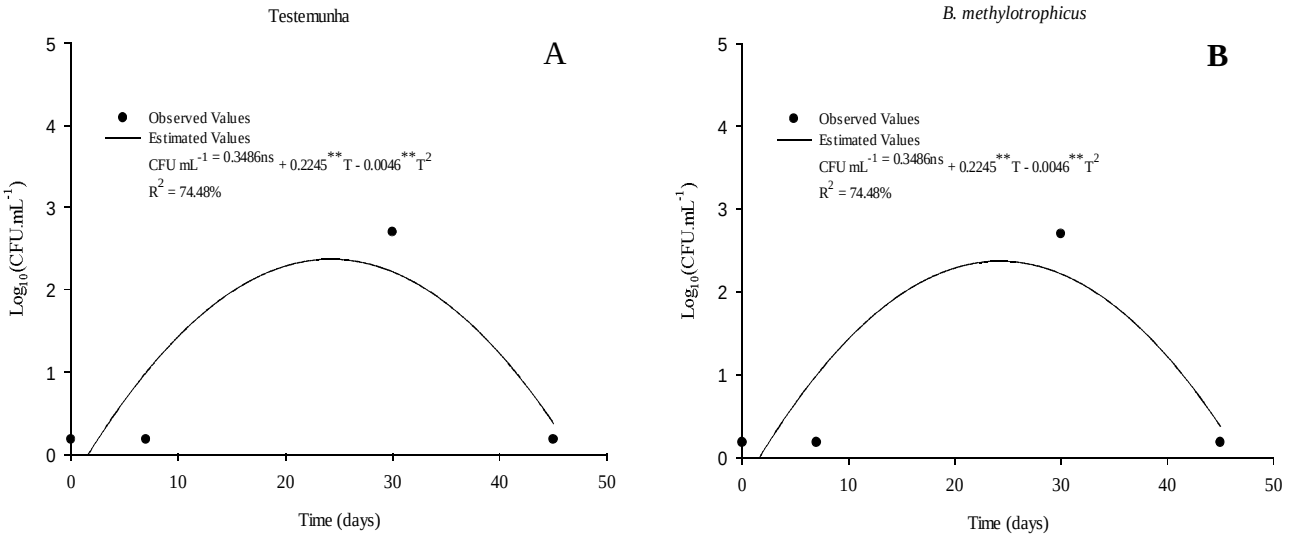
The treatments that included *B. subtilis*, *T. asperellum*, *B. subtilis* + *T. asperellum* and *B. methylotrophicus* + *T. asperellum* did not show quadratic behaviour in terms of the microbiological bacterial analysis, while the control treatment and *B. methylotrophicus* did show such behaviour (Figure 2). Despite the treatment with *B. methylotrophicus* showing quadratic behaviour throughout the period of evaluation, there was no difference in the bacterial counts (Table 4).

Compared to the control, the microbiolisation of soya seeds with *B. methylotrophicus* and *B. subtilis* afforded a significant reduction in the number of eggs and the reproductive factor of *Meloidogyne javanica*, an important nematode that causes damage to the soya bean (Alcebíades *et al.*, 2019). In a study with two coffee genotypes (Mundo Novo IAC 376-4 and IPR-100), three biological nematicides (*B. methylotrophicus*, *B. subtilis* and *T. asperellum*) and one treatment that included a mixture of the three nematicides, the biological mixture was efficient in controlling *Meloidogyne exigua* (Tolardo *et al*., 2019).

Table 5 shows a summary of the analysis of variance of the morphometric data under analysis.

As can be seen, there were no significant differences between the treatments in relation to the dry and fresh weight of the roots and shoots of the soya plants. Table 6 shows the mean values of the morphometric data for the different treatments.

Figure 2 - Behaviour of the bacterial count in treatments evaluated over different periods. A: Control treatment; B: *B. methylotrophicus*



**Significant at 1% by t-test; nsNot significant by t-test

Table 5 - Summary of the analysis of variance of the fresh weight (FW) and dry weight (DW) in grams of the roots and shoots of soya plants evaluated under greenhouse conditions with the fungus *T. asperellum* and the bacteria *B. subtilis* and *B. methylotrophicus*, individually and their interaction

SV	DF	Root FW	Root DW	Shoot FW	Shoot DW
Treatment	5	188.23^{ns}	18.13^{ns}	35.41^{ns}	1.70^{ns}
Overall mean (g)		30.87	10.51	23.82	10.52
CV (%)		25.06	28.08	27.22	13.86

nsNot significant by F-test. SV: Source of variation; DF: Degree of freedom; CV: Coefficient of variation

Table 6 - Fresh weight (FW) and dry weight (DM) in grams of the roots and shoots of soya plants evaluated under greenhouse conditions with the fungus *T. asperellum* and the bacteria *B. subtilis* and *B. methylotrophicus*, individually and their interaction

Treatment	Roots		Shoots	
	FW	DW	FW	DW
Control	38.00	12.81	21.36	10.27
B. subtilis	30.24	11.79	27.16	10.65
B. methylotrophicus	37.93	11.82	26.05	11.34
T. asperellum	24.59	8.62	21.97	10.31
B. subtilis + *T. asperellum*	23.97	8.18	21.06	9.65
B. methylotrophicus + *T. asperellum*	30.51	9.81	25.32	10.91

Corroborating a part of the results, Santos *et al*. (2019) found that the biological control treatments, such as *Trichoderma*, had no significant effect on increasing shoot dry weight in soya plants. However, such treatments did affect the increase in root fresh weight. It is assumed that the use of biocontrol agents in pathogen management will only have a beneficial effect on the plants if the isolate is capable of interacting with the host (Kerry; Bourne, 2002).

In a study by Costa *et al*. (2019) evaluating the effect of different concentrations of *B. subtilis*-based inoculants on the initial development of the M8210 and TMG132 commercial varieties of the soya bean, the TMG132 variety showed an increase in shoot fresh weight 30 days after sowing, and in root volume 45 days after sowing, following seed inoculation with *B. subtilis*, while the M8210 variety afforded no increase in any of the variables under analysis, corroborating the results of the present study.

In this study, the interaction between genera *Bacillus* and *Trichoderma*, despite not promoting any increase in the dry and fresh weight of the roots and shoots of the soya plants, proved to be promising due to the results of the microbiological fungal and bacterial analyses, especially in the evaluation carried out from the seventh day onwards. Considering the number of viable spores, the interaction between *B. subtilis* and *T. asperellum* afforded better results than that between *B. methylotrophicus* and *T. asperellum*.

CONCLUSIONS

1. The interaction between *Bacillus subtilis* and *Trichoderma asperellum* showed the potential for joint use in the biological control of disease in the soya bean, including a potential synergistic effect;
2. The interaction between *Bacillus methylotrophicus* and *Trichoderma asperellum* showed a reduction in viable spores, which indicates a possible antagonistic effect in the joint use of these microorganisms.

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