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Compatibility and antimicrobial activity of *Trichoderma* spp. combined with diazotrophs and growth-promoting bacteria

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Abstract: The study evaluated the compatibility, *in vitro*, of diazotrophs *Bradyrhizobium* spp., and plant growth-promoting bacteria (PGPB), *Azospirillum brasilense* and *Bacillus* spp., widely used as biofertilizers and biostimulantion soybean culture combined with as biofungicides/bionematicides *Trichoderma harzianum* and *Trichoderma asperellum*. Compatibility was evaluated through antagonistic activity and the minimum inhibitory concentration (MIC), using the techniques of dual cultures and disc diffusion, respectively. The experiment was conducted at the soil microbiology laboratory out at the Embrapa Temperate Climate, Pelotas, RS, Brazil. We adopted a completely randomized design (CRD) with three replications and the data were submitted to ANOVA followed by Tukey's test ($p \leq 0.05$) for compare of the mycelial growth variables, that showed significant difference. *B. elkanii* strain SEMIA.587 was compatible with all accessions of *Trichoderma* spp. *A. brasilense* strains Ab-V5 and Ab-V6, were compatible with the *T. asperellum* accession UFRA.T09. *B. elkanii* strain SEMIA.5019, *B. japonicum* strain SEMIA.5079 and *B. diazoefficiens* strain SEMIA.5080, *B. subtilis* strain CNPMS.B2084 and *B. megaterium* strain CNPMS.B119 inhibited from 47% to 83% the growth of *Trichoderma* spp. The MIC demonstrated that diazotrophs and PGPB are not sensitive to *Trichoderma* spp. *B. elkanii* strain SEMIA.587 can be used in combination with all *Trichoderma* spp. accessions, as well as the Ab-V5 and Ab-V6 strains of *A. brasilense* with the *T. asperellum* accession UFRA.T09 in the inoculation via a seeding furrow.

Keywords: Biofungicide; Dual Cultures; MIC; PGPB; Strains.

Introduction

The use of biological agents in soybean *Glycine max* (L.) has shown relevant results in the uptake of nitrogen and phosphorus, accumulation of plant biomass, synthesis of phytohormones, and increase in nodulation (Chagas et al., 2017; Moretti et al., 2020; Duré et al., 2022; Braccini et al., 2023), in addition to mitigating the environmental impacts caused by the use of chemical inputs (Hungria et al., 2015). The diazotrophs *Bradyrhizobium* spp. are widely used in biological nitrogen fixation (BNF) (Kaschuk et al., 2016; Mirriam et al., 2022) and its effectiveness supplies the totality of the nitrogen demand necessary for the crop (Hungria et al., 2020; Mahmud et al., 2020).

Brazilian legislation contains records of four strains of *Bradyrhizobium* spp. (*B. japonicum* SEMIA.5079, *B. diazoefficiens* SEMIA.5080, *B. elkanii* SEMIA.5019 and SEMIA.587) available for soybean crops (MAPA, 2011). However, some studies have shown that the association of these microorganisms with PGPB, through the so-called co-inoculation technique, has shown benefits for plant development (Iliäœić et al., 2017; Moretti et al., 2019; Garcia et al., 2021).

The main combinations involve the co-inoculation of *Bradyrhizobium* spp. and *A. brasilense* strains Ab-V5 and Ab-V6. Currently, this technique is applied in 25% of the soybean cultivation area (Santos et al., 2021) and results in phytohormone production and increases in root growth, benefiting the performance of *Bradyrhizobium* spp. in nodulation and, consequently, in BNF (Hungria et al., 2015; Ferri et al., 2017; Barbosa et al., 2021).

The genus *Bacillus* has been successfully employed with *Bradyrhizobium* spp. for inoculation, showing improvement in nodulation, growth, and shoot and root length (Atieno et al., 2012; Sibponkrung et al., 2020; Miljaković et al., 2022). Simultaneously, there has been an increase in the use of disease biocontrol agents based on *Trichoderma* spp., which in addition to controlling root pathogens and being a more sustainable alternative (Iturralde et al., 2020; Barbosa et al., 2022; Conte et al., 2022), show promising results in promoting growth (Conte et al., 2022), synthesizing indole acetic acid and auxins (Chagas et al., 2017), and increasing tolerance to abiotic stress (Tyśkiewicz et al., 2022).

Inoculation of soybean seeds with *Trichoderma* spp. via a seeding furrow is an increasingly used practice (Meyer et al., 2022) because, in addition to being efficient biocontrol agents (Conte et al., 2022), they have potential as plant growth promoters (Chagas et al., 2017; Junior et al., 2022). Using co-inoculation technology via seeding furrows, *Trichoderma* spp., when used in conjunction with PGPB and diazotrophs, enables increased efficiency in the inoculation process (Ferri et al., 2017; Brignoli et al., 2023). When using the co-inoculation technique, it is important that no organism is inhibitory to one another or interferes too much with the growth of the other (Whipps, 2001; Thomloudi et al., 2019).

Information related to the compatibility of bacteria and fungi beneficial to the development of the soybean crop is still limited (Karuppiah et al., 2019; Mattos et al., 2020) and, for the most part, is limited to greenhouse evaluations (Ayoubi et al., 2012; Cadore et al., 2020; El-Nahrawy et al., 2020). Thus, the aim of this study was to evaluate the *in vitro* compatibility of diazotrophs (*B. japonicum*, *B. diazoefficiens* and *B. elkanii*) and PGPB (*A. brasilense*, *B. subtilis*, *B. megaterium*) with biocontrol fungi, *T. asperellum* and *T. harzianum*, by evaluating their antagonistic effects.

Results

The results obtained in the antagonism test, through the dual culture technique, demonstrated that all evaluated bacteria, diazotrophs and PGPB, had an inhibitory effect against the isolates of *T. asperellum* and *T. harzianum* (Table 2). As an exception, *B. elkanii* strain SEMIA.587 did not form an inhibition halo with the evaluated *Trichoderma* spp. isolates (Fig.1A), nor

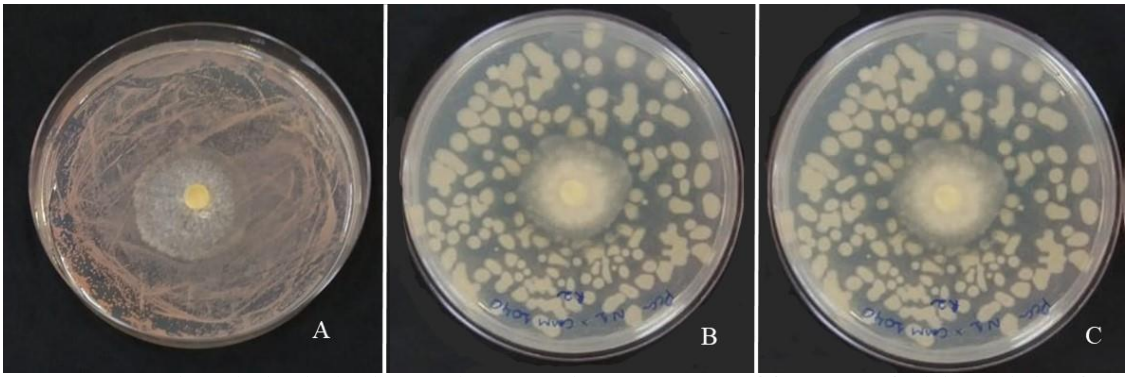

Fig 1. Antagonistic activity through the dual culture technique, without occurrence of formation of inhibition halo, after 120 hours of inoculation. *Bradyrhizobium elkanii* strain SEMIA.587 *vs Trichoderma harzianum* strain Esalq.1306 (A); *Azospirillum brasilense* strain Ab-V6 *vs Trichoderma asperellum* strain UFRA.T09 (B); and *Azospirillum brasilense* Ab-V5 *vs Trichoderma aperellum* strain UFRA.T09 (C). Photograph: Gobbi, PC.

Table 1. Selective medium and incubation conditions in a microbiological oven for the growth of different microorganisms used in the evaluation of antagonism and minimum inhibitory concentration. Embrapa Clima Temperado, Pelotas-RS, Brazil.

Microorganism	Culture medium	Incubation conditions
Azospirillum spp.	Red Congo	24 hours in a bacteriological oven at 28°C
Bacillus spp.	Casein-Peptone Dextrose Yeast Agar	24 hours in a bacteriological oven at 28°C
Bradyrhizobium spp.	Yeast Mannitol Agar	24 hours in a bacteriological oven at 28°C
Trichoderma spp.	Potato Dextrose Agar	7 days in BOD at 27°C

did *A. brasilense* strains Ab-V5 (Fig.1B) and Ab-V6 (Fig.1C) when faced with *T. asperellum* strain UFRA.T09.

When assessing PGPB compatibility of *A. brasilense* strains Ab-V5 and Ab-V6 with *Trichoderma* spp. isolates, the smallest halos of mycelial growth inhibitions occurred for *T. asperellum* strain UFRA.T09 (47.04% ($F=6.976$; $df=4$; $p=0.006$) and 62.85% ($F=6.778$; $df=4$, $p=0.007$), respectively; however, the latter did not differ significantly from *T. harzianum* strain Esalq.1306 (65.73%; Table 3).

In contrast, for PGPB, *B. megaterium* strain CNPMS.B119 and *B. subtilis* strain CNPMS.B2084, the highest percentage of inhibition occurred when confronted with *T. asperellum* strain UFRA.T09 (77.96% ($F=71.28$; $df=4$; $p<0.05$) and 68.44% ($F=30.00$; $df=4$; $P<0.05$), respectively) and did not differ for other evaluated isolates (Table 3).

Bradyrhizobium diazoefficiens strain SEMIA.5080 ($F=4.486$; $df=4$; $p=0.025$) and *B. japonicum* strain SEMIA.5079 ($F=7.177$, $df=4$; $p=0.003$) exhibited the lowest percentages of inhibition against the test agents *T. asperellum* strain UFRA.T12 (65.55% and 68.61%, respectively) and *T. harzianum* strain Esalq.1306 (74.63% and 66.67%, respectively). For *B. elkanii* strain SEMIA.5019, there were no significant differences between the evaluated *Trichoderma* spp. isolates ($F=3.00$; $df=4$; $p=0.072$; Table 3).

In the evaluation of the response of test agents to different antagonists, *B. elkanii* strain SEMIA.587 exhibited the lowest mean percentages of mycelial growth inhibition for *T. asperellum* strain UFRA.T06 (54.38%; $F=20.426$; $df=7$; $p< 0.05$), *T. asperellum* strain UFRA.T12 (47.02%; $F=7.727$; $df=7$; $p=0.0003$) and *T. asperellum* strain UFRA.T52 (49.12%; $F=21.208$; $df=7$; $p<0.05$) compared to other antagonists (Table 3). For *T. asperellum* strain UFRA.T09, the lowest suppression occurred in the interaction with *A. brasilense* strain Ab-V5 ($F=34.05$; $df=7$; $p<0.05$; Table 3).

The lowest percentage of mycelial inhibition for *T. harzianum* strain Esalq.1306 was observed with the antagonist *B. subtilis* strain CNPMS.B2084 (53.76%); however, it did not differ from *B. elkanii* strain SEMIA.587 (59.54%) or *B. megaterium* strain CNPMS.B119 (60.56%). The highest inhibition percentages for *T. harzianum* strain Esalq.1306 were verified in interactions with *B. diazoefficiens* strain SEMIA.5080 (74.63%), *B. elkanii* strain SEMIA.5019 (74.63%) and *B. japonicum* strain SEMIA.5079 (66.67%; $F=16.432$; $df=7$; $p<0.05$; Table 3).

Antagonists *B. diazoefficiens* strain SEMIA.5080, *B. japonicum* SEMIA.5079 and *B. elkanii* strain SEMIA.5019 also exhibited the highest inhibition values for *T. asperellum* strain UFRA.T09 ($F=34.05$; $df=7$; $p<0.05$) and *T. asperellum* strain UFRA.T12 ($F=7.73$; $df=7$; $p=0.0004$; Table 3). For *T. asperellum* strain UFRA.T06 and *T. asperellum* strain UFRA.T52, the highest percentage of inhibition occurred when confronted with *A. brasilense* strain Ab-V6 (80.28%; $F=20.43$; $df=7$; $p<0.05$ and 80.09%; $F=21.21$; $df=7$; $p<0.05$, respectively; Table 3).

The evaluation of microbial activity through the MIC demonstrated that diazotrophs and PGPB were not sensitive to the evaluated *Trichoderma* spp. isolates since there was no inhibition of bacterial growth at the different concentrations tested. Additionally, *B. subtilis* strain CNPMS.B2084 (Fig.2A), *B. megaterium* strain CNPMS.B119 (Fig.2B), *B. elkanii* strain SEMIA.5019 (Fig.2C), *B. japonicum* strain SEMIA.5079 (Fig.2D) and *B. diazoefficiens* strain SEMIA.5080 (Fig.2E) colonized the entire area of the plate, preventing the growth of *Trichoderma* spp. isolates at all evaluated concentrations.

The mycelial growth of *T. harzianum* strain Esalq.1306 and *T. asperellum* strain UFRA.T12 was observed up to a concentration of 1×10^5 conidia mL^{-1}, while that for *T. asperellum* strain UFRA.T06, *T. asperellum* strain UFRA.T09 and *T. asperellum* strain UFRA.T52 occurred up to a concentration of 1×10^6 conidia mL^{-1} (Table 4). The evaluated *Trichoderma* spp. isolates showed reduced growth compared to the control, as shown in the antagonism test; however, for the most part, they did not differ from the control (Table 4).

Discussion

The formation of the halo of inhibition of diazotrophs and PGPB against the isolates of *T. asperellum* and *T. harzianum* did not nullify the development of the fungi after 120 hours of cultivation. However, all interactions, with and without halo formation, showed a reduction in mycelial growth in *Trichoderma* spp. isolates. Among the evaluated bacterial isolates, the diazotrophs *B. diazoefficiens* strain SEMIA.5080, *B. japonicum* strain SEMIA.5079 and *B. elkanii* strain SEMIA.5019 showed the greatest inhibitory effects against *Trichoderma* spp., and the interaction neutrality was verified for *B. elkanii* strain SEMIA.587 with *Trichoderma* spp. evaluation. Bécquer et al. (2013) found a neutral interaction between *Sinorhizobium* (Ensifer) *meliloti* and *T. harzianum* in an *in vitro* antagonism evaluation after 96 hours of cultivation, and although they did not show formation of an inhibitory halo, there was a reduction in the diameter of the *T. harzianum* colony.

Table 2. Values of inhibition halos (mm) of the antagonists, diazotrophs and plant growth-promoting bacteria, against the test agents, *Trichoderma* spp. Embrapa Clima Temperado, Pelotas-RS, Brazil.

Antagonists	*Trichoderma harzianum* Esalq.1306	*Trichoderma asperellum* UFRA.T06	*Trichoderma asperellum* UFRA.T09	*Trichoderma asperellum* UFRA.T12	*Trichoderma asperellum* UFRA.T52
Azospirillum brasilense Ab-V6	5.00	1.67	0.00	0.87	1.33
Azospirillum brasilense Ab-V5	5.33	4.00	0.00	2.17	3.67
Bacillus subtilis CNPMS.B2084	5.33	2.66	1.67	2.00	2.33
Bacillus megaterium CNPMS.B119	3.00	5.67	3.00	1.67	1.33
Bradyrhizobium elkanii SEMIA.587	0.00	0.00	0.00	0.00	0.00
Bradyrhizobium elkanii SEMIA.5019	3.00	3.33	4.00	4.00	3.67
Bradyrhizobium japonicum SEMIA.5079	2.67	1.67	1.33	2.67	1.17
Bradyrhizobium diazoefficiens SEMIA.5080	3.00	1.00	2.00	1.67	1.33
Coefficient of variation (CV%	52.57	72.33	99.39	63.08	69.30

The *in vitro* compatibility of *B. elkanii* strains SEMIA.587 and SEMIA.5019 evaluated by Mattos et al. (2020) was also shown to be the most compatible with the different isolates of *T. asperellum*. The strains belonging to *B. elkanii* produce rhizobiotoxins and present intrinsic resistance to antibiotics, unlike strains of *B. japonicum* (Minamisawa et al., 1996). The production of rhizobiotoxin limits endogenous ethylene production in soybean plants in low land soils, with poor natural drainage, favoring plant nodulation (Mattos et al., 2019). Thus, the synthesis of rhizobiotoxins can be a factor in the neutral interaction of *B. elkanii* SEMIA.587 and *Trichoderma* spp. verified in the present study.

The use of *B. elkanii* together with *T. harzianum* favored soybean production (Silva et al., 2018), and co-inoculation of *B. elkanii* and *T. asperellum* resulted in increased plant height and root dry mass production, indicating its application in cowpea, *Vigna unguiculata* (L.) Walp. (Fabaceae) (Costa et al., 2020).

Biological agents are considered compatible when they do not have a suppressive effect on each other *in vitro* co-cultivation or in rhizosphere colonization assays (Thomloudi et al., 2019). Antagonistic effects *in vitro* partially reflect the response of an interaction in culture systems (Prasadd and Babu, 2017) since microorganisms can colonize different ecological niches in the rhizosphere without interfering with each other's growth (Niu et al., 2020), making the antagonistic activity notorious when space and nutrients are limited, and perhaps this is the main effect of the antagonistic action of fungi (Carvalho et al., 2014).

The use of the consortium *B. japonicum* and *T. harzianum* in the soybean crop resulted in an increase in the shoot and root length and in the N content compared to the use of *B. japonicum* alone (El-Nahrawy et al., 2020). The co-inoculation of *B. japonicum* and *T. harzianum* increased the fresh root mass without interfering with nodulation by *B. japonicum*, with soybean plants presenting nodules with the same ultrastructure as those inoculated only with *B. japonicum*. In addition, no fungal structures were observed below the root epidermis, indicating that *T. harzianum* colonized the soybean rhizosphere and remained on the surface of the roots (Iturralde et al., 2020).

The *in vitro* antagonism of *Bradyrhizobium* spp. against *T. asperellum* was evaluated by Mattos et al. (2020), and unlike the results obtained in this study, the authors observed the antagonistic action of *B. elkanii* strain SEMIA.587, *B. elkanii* strain SEMIA.5019, *B. japonicum* strain SEMIA.5079 and *B. diazoefficiens* strain SEMIA.5080 against *T. asperellum* strain UFRA.T09 and the formation of halos ranging from 1 to 5 mm diameter, demonstrating low potential for suppression of *Bradyrhizobium* spp. This occurred similarly in the present study, with halos ranging from 1.00 to 4.00 mm (Table 2) in the evaluations of *Bradyrhizobium* spp. against *T. asperellum* and *T. harzianum* isolates.

Bacillus subtilis strain CNPMS.B2084 and *B. megaterium* strain CNPMS.B119 showed the lowest results of mycelial growth inhibition in *T. harzianum* strain Esalq.1306, when compared to other bacterial isolates, although there was formation of an inhibition halo. The antagonism of *Bacillus* spp. against phytopathogenic fungi is often related to the production of secondary metabolites with antibiotic properties and competition for nutrients and space (Harba et al., 2020; Miljaković et al., 2022); however, evaluations of the effect of these microorganisms against beneficial fungi *in vitro* are still limited.

The investigation of the *in vitro* compatibility of several isolates of *Bacillus* spp. and *Trichoderma* spp. showed strong antagonistic activity, making prior analysis a necessary step for application in mixtures (Fuga et al., 2016). The combined application of *B. subtilis* and *T. harzianum* proves to be compatible, providing protection against *Rhizoctonia solani* Kühn; however, the best results were obtained when applied separately (Abeysinghe, 2009). According to Li et al. (2005), *B. subtilis* exerts antifungal activity against the growth of *T. harzianum*, inducing the formation of chlamydospores.

The compatibility of *A. brasilense* strains Ab-V5 and Ab-V6 varied among the evaluated *Trichoderma* spp. isolates, being more inhibitory against *T. asperillum* strains UFRA.T6 and UFRA.T12 and more compatible with *T. asperillum* UFRA.T09 without the occurrence of the formation of an inhibition halo. Co-inoculation with *T. harzianum* and *A. brasilense* in beans, *Phaseolus vulgaris* L. (Fabaceae), decreased the number of nodules compared to inoculation with *T. harzianum*; however, there was an increase in the weight of nodules (Öğüt et al., 2005) and increased concentrations of micronutrients in grains (Öğüt et al., 2006).

The assessment of MIC demonstrated that diazotrophs and PGPB are not sensitive to the tested *Trichoderma* spp. isolates since they did not show reduced growth compromised in the presence of the fungus. The inhibition potential of a microorganism may be related to competition for resources available in the substrate, speed of multiplication and colonization capacity, which defines efficiency as an antagonist of *Trichoderma* spp. isolates (Benítez et al., 2004) or even to an evaluation method.

Scorzoni et al. (2007) when comparing assessment methods of MIC of an antimicrobial, observed that the agar diffusion technique was less sensitive than the microdilution technique, concluding that the effectiveness of a method may not always be adequate for an antimicrobial due to differences in physical, volatile and diffusion properties. According to Muniz et al. (2018),

Table 3. Mean percentage of inhibition of mycelial growth (±SE) of the antagonists, diazotrophs and plant growth-promoting bacteria, against the test agents, *Trichoderma* spp. Embrapa Clima Temperado, Pelotas-RS, Brazil.

`1	*Trichoderma harzianum* Esalq.1306	*Trichoderma asperellum* UFRA.T06	*Trichoderma asperellum* UFRA.T09	*Trichoderma asperellum* UFRA.T12	*Trichoderma asperellum* UFRA.T52
Azospirillum brasilense Ab-V6	65.73±5.12 bB	80.28±2.05 aA	62.85± 3.41 bC	80.09±5.12 aA	72.15± 1.65 abABC
Azospirillum brasilense Ab-V5	63.47±1.63 aB	67.61±0.81 aBC	47.04 ±2.30 bD	64.61±3.07 aAB	64.38±5.48 aBCD
Bacillus subtilis CNPMS.B2084	53.76±1.54 cC	56.81±1.24 bcCD	68.44±0.48 aBC	61.87±1.39 bBC	53.42±0.79 cDE
Bacillus megaterium CNPMS.B119	60.56±0.81 bBC	60.09±0.81 bCD	77.96±1.07 aA	59.36±1.21 bBC	62.10±0.91 bCD
Bradyrhizobium elkanii SEMIA.587	59.54±0.74 aBC	54.38±0.23 bD	61.67±1.50 aC	47.02±0.54 cC	49.12±1.09 cE
Bradyrhizobium elkanii SEMIA.5019ns	74.63±1.49 A	62.74±4.97 CD	73.66±2.34 AB	68.46±4.63 AB	78.79±3.15 A
Bradyrhizobium japonicum SEMIA.5079	66.67±2.77 cAB	78.92±2.73 abAB	82.26±0.93 aA	68.61±3.42 bcAB	76.51±1.91 abcAB
Bradyrhizobium diazoefficiens SEMIA.5080	74.63±2.28 abA	79.71±2.79 aA	76.88±1.94 abAB	65.55±4.31 bAB	79.29±1.34 aA

Significant effect by ANOVA ($p<0.05$; ns, $p>0.05$). Means within a row followed by the same lower-case letter, or within a column followed by the same capital letter, do not differ significantly (Tukey test: $p\leq0.05$).

Table 4. Mean mycelial growth (±SE) and minimum inhibitory concentration (MIC) of five accessions of *Trichoderma* spp. against eight bacterial accessions using the disk diffusion technique. Embrapa Clima Temperado, Pelotas-RS, Brazil.

Fungi	Concetration	Control		*Azospirillum brasilense* Ab-V6		MIC	*Azospirillum brasilense* Ab-V5		MIC	*Bradyrhizobium elkanii* SEMIA.587		MIC	p-value
Trichoderma harzianum Esalq.1306	$1x10^8$	13.72 ± 0.61	a	9.59 ± 1.57	b	NA[(1)]	14.89 ± 0.87	a	NA	12.97 ± 0.50	a	NA	0.000
	$1x10^7$	13.49 ± 0.50	a	9.26 ± 1.10	b	NA	11.05 ± 1.04	b	NA	10.25 ± 0.38	b	NA	0.000
	$1x10^6$	11.59 ± 0.51	a	9.10 ± 0.33	b	NA	7.65 ± 0.33	b	NA	8.93 ± 0.50	b	NA	0.000
	$1x10^5$ns	8.93 ± 0.27		8.44 ± 0.83		NA	8.85 ± 0.96		NA	7.38 ± 0.65		NA	0.368
Trichoderma asperellum UFRA.T06	$1x10^8$	14.94 ± 0.59	a	11.08 ± 0.87	b	NA	15.80 ± 0.69	a	NA	13.52 ± 1.05	ab	NA	0.008
	$1x10^7$	13.07 ± 0.40	a	9.70 ± 1.24	b	NA	14.99 ± 0.76	a	NA	13.68 ± 0.50	a	NA	0.004
	$1x10^6$	11.51 ± 0.74	ab	12.89 ± 0.68	a	NA	9.67 ± 0.97	b	NA	12.50 ± 0.30	ab	NA	0.033
Trichoderma asperellum UFRA.T09	$1x10^8$ns	8.49 ± 0.44		8.92 ± 0.40		NA	10.49 ± 1.38		NA	9.54 ± 0.36		NA	0.333
	$1x10^7$	8.80 ± 0.47	a	8.89 ± 0.39	a	NA	9.81 ± 0.39	a	NA	10.29 ± 0.19	a	NA	0.043
	$1x10^6$	9.87 ± 0.57	a	6,53 ± 0.22	a	NA	8.84 ± 0.55	a	NA	6.53 ± 0.22	b	NA	0.001
Trichoderma asperellum UFRA.T12	$1x10^8$	11.47 ± 0.10	a	8.34 ± 0.44	b	NA	8.26 ± 0.69	b	NA	9.11 ± 0.28	b	NA	0.000
	$1x10^7$ns	9.67 ± 0.23		8.75 ± 0.26		NA	9.77 ± 0.99		NA	8.94 ± 0.80		NA	0.626
	$1x10^6$ns	6.87 ± 2.31		1.83 ± 1.83		NA	8.46 ± 0.79		NA	4.42 ± 2.73		NA	0.168
	$1x10^5$ns	7.40 ± 0.18		6.93 ± 0.14		NA	6.62 ± 0.45		NA	7.06 ± 0.21		NA	0.298
Trichoderma asperellum UFRA.T52	$1x10^8$	16.50 ± 0.29	a	15.25 ± 0.25	ab	NA	13.19 ± 0.94	b	NA	13.75 ± 0.63	b	NA	0.008
	$1x10^7$ns	12.85 ± 0.89		13.25 ± 0.63		NA	13.00 ± 0.41		NA	10.25 ± 1.03		NA	0.060
	$1x10^6$ns	10.61 ± 1.06		6.89 ± 2.35		NA	6.22 ± 2.10		NA	4.57 ± 2.75		NA	0.290

[(1)]No antifungal activity; Significant effect by ANOVA ($p<0.05$; ns, $p>0.05$); Means within a row followed by the same lower-case letter, do not differ significantly (Tukey test: $p<0.05$).

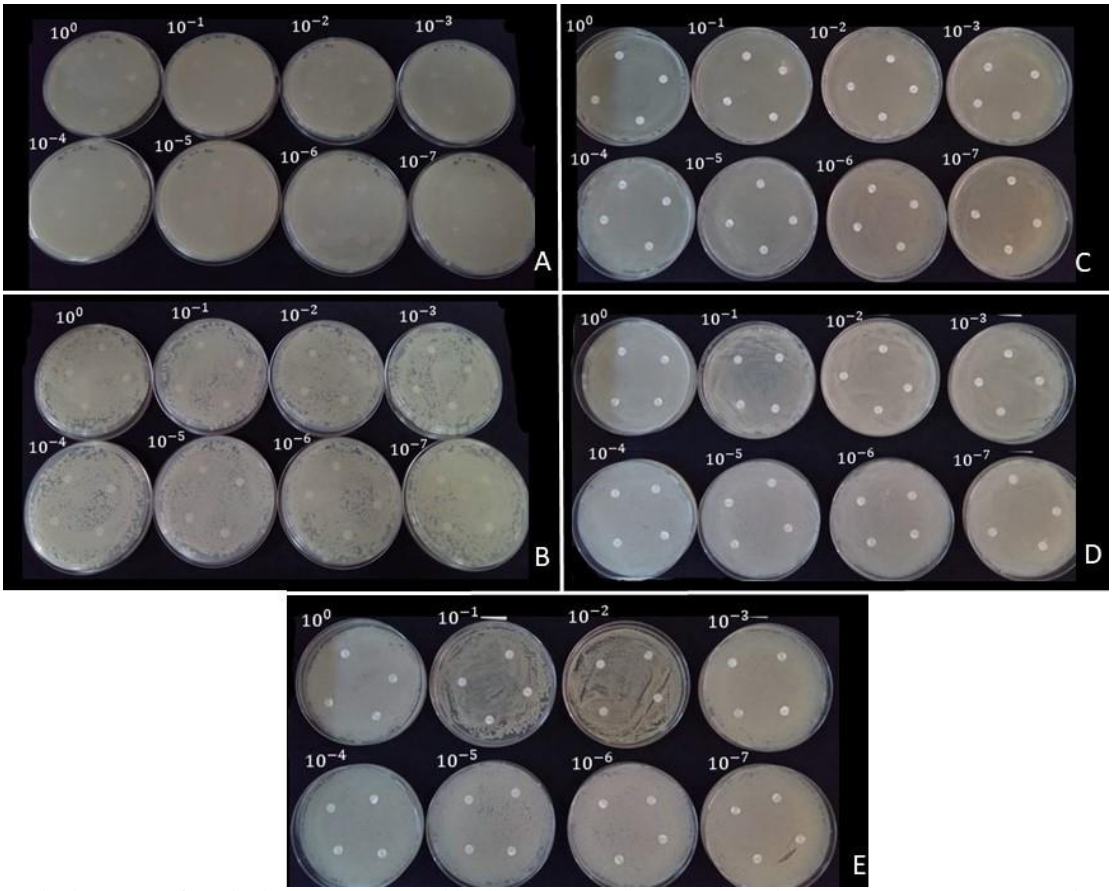

Fig 2. Antimicrobial activity of *Trichoderma* spp. assessed by determining the minimum inhibitory concentration using the disk diffusion sensitivity test, without occurrence of inhibition of bacterial growth at the different concentrations tested. Exemplarys of *Trichoderma asperellum* strain UFRA.T06 evaluated with, *Bacillus subtilis* strain CNPMS.B2084 (A); *Bacillus megaterium* strain CNPMS.B119 (B); *Bradyrhizobium elkanii* strain SEMIA.5019 (C); *Bradyrhizobium japonicum* strain SEMIA.5079 (D); *Bradyrhizobium diazoefficiens* strain SEMIA.5080 (E). Photograph: Gobbi PC.

T. harzianum has the capacity for superficial colonization in a culture medium rich in sucrose, reaching 50% of growth after 48 hours. However, the standard means of *Clinical and Laboratory Standards Institute* (NCCLS, 2003), for disc diffusion sensitivity analyses, Müeller–Hinton medium, does not contain a source of sugar, which could have limited the multiplication and colonization of fungi in this study.

Materials and methods

The experiment was conducted at the soil microbiology laboratory at Embrapa Clima Temperado, Pelotas, RS, Brazil (31°42' South and 52°24' West). Eight bacterial isolates were used: *A. brasilense* strains Ab-V5 and Ab-V6; *B. subtilis* strain CNPMS.B2084; *B. megaterium* strain CNPMS.B119; *B. elkanii* strains SEMIA.587 and SEMIA.5019; *B. japonicum* strain SEMIA.5079; and *B. diazoefficiens* strain SEMIA.5080. Five isolates of *Trichoderma* spp. were evaluated, with four isolates of *T. asperellum* (strains UFRA.T06, UFRA.T09, UFRA.T12 and UFRA.T52) and an isolate of *T. harzianum*, strain Esalq.1306, preserved in Coleção de Microrganismos Multifuncionais de Clima Temperado (CMMCT).

The compatibility of the microorganisms was evaluated through the antagonistic activity and the antimicrobial activity in the MIC using dual culture (Brito et al., 2018) and disc diffusion techniques (NCCLS, 2003), respectively.

Recovery and cultivation of biological isolates

Bacterial and fungal isolates were recovered and cultivated in Petri dishes (90 mm diameter) containing 25 mL of selective medium according to the characteristics of each species (Table 1). In a laminar flow chamber, smear loops of bacterial colonies, previously incubated in a microbiological oven, were removed (Table 1), obtaining a concentration of 1–2×10^8 CFU mL^{-1} according to tube n°0.5 of the MacFarland scale, through spectrophotometer reading (Logen Scientific SF325NM) at a wavelength of 625 nm (absorbance: 0.8-0.13).

Antagonism of diazotrophs and PGPB against Trichoderma spp.

For the *in vitro* evaluation of antagonism, 100 µL aliquots of the bacterial suspension were seeded using the *Drigalski* loop spreading method in Petri dishes (90 mm diameter) containing 25 mL of medium Casein-Peptone Dextrose Yeast Agar (PCA). In this evaluation, PCA was used for all bacterial isolates due to better visualization of mycelial growth and the halo of inhibition. A 6 mm diameter mycelium disc of an isolate previously grown in a Petri dish containing medium Potato-Dextrose Agar (PDA) (Table 1) was removed and deposited in the center of a Petri dish seeded with the antagonist (bacterial suspension), incubated inverted in a microbiological oven at 28°C, and evaluated after 120 hours of incubation.

The antagonistic activity was evaluated by measuring the mycelium growth diameter, including the inhibition halo, in the presence and absence of the antagonist, with the aid of a digital pachymeter. From these data, the mean percentage values of inhibition of the mycelial growth (ZI) of the test agent (Brito et al., 2018) were calculated using the following equation:

$$ZI = \frac{R1 - R2}{R1} \times 100$$

where:

R1- Diameter of the mycelium in the absence of the antagonist

R2- Diameter of the mycelium in the presence of the antagonist

The experiment was conducted in a CRD with three replications and a total of 40 interactions between fungi and bacteria.

Antimicrobial activity of Trichoderma spp. on bacterial isolates

The antimicrobial activity of *Trichoderma* spp. was evaluated by determining the MIC using a disc diffusion sensitivity test (NCCLS, 2003). The inoculum suspension of each accession was prepared from colonies incubated in selective medium at 28°C, 24 hours (Table 1). Each bacterium was suspended in 0.85% saline solution (autoclaved at 120°C, 20 min), and the cell concentration was adjusted to 1-2×10^8 CFU mL^{-1}, according to tube n°0.5 of the MacFarland scale. Subsequently, 100 µL of each bacterial suspension was seeded in a Petri dish (90 mm diameter) containing 25 mL of Mueller-Hinton medium using the *Drigalski* loop spreading method. Then, glass microfiber filter paper discs 934-AH™ (Whatman®) of 6 mm diameter that were previously autoclaved (120°C, 20 min) were placed on the surface of the agar using sterile tweezers and then inoculated with 10 µL (Rabanal et al., 2002) of the test agent (*Trichoderma* spp.) at different concentrations. For this, the fungi were cultivated in Petri dishes, as shown in Table 1, and the initial concentration of 1×10^8 conidia mL^{-1} was determined by counting conidia in a Neubauer chamber. Serial dilutions were then performed (10^0 to 10^{-7}) in a 0.85% saline solution. The plates were incubated inverted at 35°C, 48 hours. A positive control of the test agent (*Trichoderma* spp.) was performed at the different tested concentrations. The data were evaluated after the incubation period and the MIC was determined as the lowest concentration of the test agent responsible for inhibiting the total and partial growth of bacterial isolates. The experiment was conducted in a CRD with four repetitions.

Data analysis

The normality and homoscedasticity of the ZI and mycelial growth data in the different MICs were analyzed using Shapiro-Wilk and Bartlett tests, respectively. As the statistical assumptions were met, an analysis of variance (ANOVA) was performed, followed by the Tukey test ($p<0.05$), to compare the variables that showed significance using R v.3.4.1 software (R Development Core Team, 2022).

Conclusions

Compatibility and antimicrobial activity responses obtained in the present study confirm the importance of diazotrophs and PGPB that compose inoculants in the action of biocontrol of fungal agents. There was a neutral interaction of *B. elkanii* strain SEMIA.587 with all *Trichoderma* spp. *Bacillus subtilis* strain CNPMS.2084 and *Bacillus megaterium* strain CNPMS.B119 have the potential to inhibit the growth of *Trichoderma* spp. In contrast, *A. brasilense* strains Ab-V5 and Ab-V6 did not have suppressive mechanisms affecting *T. asperellum* UFRA.T09. Future research is needed to investigate the mechanisms of *B. elkanii* strain SEMIA.587, a producer of rhizobiotoxin, which limits endogenous ethylene production in low land soils with poor natural drainage, for greater compatibility with *Trichoderma* spp.

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References

Abeysinghe S (2009) Effect of combined use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 on biological control of Rhizoctonia solani on Solanum melongena and Capsicum annuum. Plant Pathol. J. 8: 9-16.

Ayoubi N, Zafari, D, Mirabolfathy, M (2012) Combination of *Trichoderma* species and *Bradyrhizobium japonicum* in control of *Phytophthora sojae* and soybean growth. J. Crop Prot. 1: 67-79.

Atieno M, Herrmann L, Okalebo R, Lesueur D (2012) Efficiency of different formulations of *Bradyrhizobium japonicum* and effect of co-inoculation of *Bacillus subtilis* with two different strains of *Bradyrhizobium japonicum*. World J. Microbiol. Biotechnol. 28(7): 2541-2550.

Barbosa JZ, Hungria M, Prior SA, Moura MC, Poggere G, Motta, ACV (2022). Improving yield and health of legume crops via co-inoculation with rhizobia and *Trichoderma*: A global meta-analysis. Appl. Soil Ecol. 176: 104493.

Barbosa JZ, Hungria M, Sena JVS, Poggere G, Reis AR, Corrêa RS (2021) Meta-analysis reveals benefits of co-inoculation of soybean with *Azospirillum brasilense and Bradyrhizobium* spp. in Brazil. Appl. Soil Ecol. 163: 103913.

Bécquer CJ, Lazarovits G, Lalin I (2013). In vitro interaction between *Trichoderma harzianum* and plant growth promoter rhizosphere bacteria. Cuba. J. Agric Sci. 47: 97-102.

Benítez T, Rincón AM, Limón MC, Codón AC (2004) Biocontrol mechanisms of *Trichoderma* strains. Int. Microbiol. 77(4): 249-260.

Braccini AL, Milléo MR, Alleoni B, Rondina ABL, Paulitsch F, Martins LM, Gravina F, Yoshida TH, Gravina F, Ávila VT, Figueiredo FMRS, Almeida R (2023) Influence of seed co-inoculation with B*radyrhizobium* species and *Azospirillum brasilense* on soybean development in Southern and Southeastern Brazil. Afr. J. of Agric. Res. 19: 81-90.

Brignoli FM, Zampar EJO, Junior JHVA, Cassim BMAR, Inoue TT, Batista MA (2023) Effect of different methods of inoculation and co-inoculation of *Bradyrhizobium* spp. and *Azospirillum brasilense* on soybean agronomic performance in fields with a history of inoculation. Arch. Agron. Soil Sci. 69(14): 2925-2937.

Brito TS, Lima WF, Pan R, Porfirio MG, Canello KT Chaves EID (2018) Antagonism of diazotrophic bacteria isolated from maize plants in the biocontrol of phytopathogens. Revista cultivando o saber. 11: 78-88.

Cadore LS, Vey RT, Fresinghelli JCF, Dotto L, Mendes FB, Silva ACF (2020) *Trichoderma* and *Bradyrhizobium japonicum* bioformulates on soy initial growth. Ciência e Natura. 42: e22.

Carvalho DDC, Lobo Junior M, Martins I, Inglis PW, Mello SCM (2014). Biological control of *Fusarium oxysporum* f. sp. *phaseoli* by *Trichoderma harzianum* and its use for common bean seed treatment. Trop. Plant Pathol. 39(5): 384-391.

Chagas LFB, Junior AFC, Soares LP, Fidelis RR (2017) *Trichoderma* in promoting plant growth. ver. de Agric. Neotrop. 4(3): 97-102.

Conte ED, Magro TD, Dal Bem LC, Dalmina JC, Matté JA, Schenkel VO, Schwambach j (2022). Use of *Trichoderma* spp. in no-tillage system: Effect on soil and soybean crop. Biol. Control. 171: 104941.

Costa GS, Costa, EM, Rocha LB, Silva JKP, Santos JF (2020). Nodulation and phytomass production of cowpea under co-inoculation of *Bradyrhizobium elkanii* and doses of *Trichoderma asperellum*. *In:* Anais do XI Congresso Brasileiro de Agroecologia, São Cristóvão, Sergipe.

Duré, LMM, Galeano RMS, Viana TFC, Roque CG, Matias R, Paggi GM, Corrêa BO, Brasil MS 2022. *Bacillus* strains with potential for growth promotion and control of white mold in soybean. Biol. 77: 3305-3317.

El-Nahrawy S, Elbagory M, Omara AE (2020) Biocompatibility Effect of *Bradyrhizobium japonicum* and *Trichoderma* strains on growth, nodulation and physiological traits of Soybean (*Glycine max* L.) under water deficit conditions. J. Adv. Microbiol. 20(11): 52-66.

Ferri GC, Braccini AL, Anghinoni FBG, Pereira LC (2017) Effects of associated co-inoculation of *Bradyrhizobium japonicum* with *Azospirillum brasilense* on soybean yield and growth. Afr. J. of Agric. Res. 12: 6-11.

Fuga CAG, Vieira BS, Cunha WV (2016) Efficiency and compatibility of *Trichoderma* spp. and *Bacillus* spp. isolates on the inhibition of *Sclerotium cepivorum*. Científica. 44(4): 526–531.

Garcia MVC, Nogueira MA, Hungria M (2021) Combining microorganisms in inoculants is agronomically important but industrially challenging: case study of a composite inoculant containing *Bradyrhizobium* and *Azospirillum* for the soybean crop. AMB Express. 11(71):1-13.

Harba M, Jawhar M, Arabi MIE (2020) Antagonistic comparative efficacy of *Bacillus* species against different soilborne fungal pathogens. J. Agroaliment. Processes Technol. 26(3): 111-116.

Hungria M, Nogueira MA, Araujo RS (2015) Soybean seed co-inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense*: A new biotechnological tool to improve yield and sustainability. Am. J. Plant Sci. 6: 811-817.

Hungria M, Nogueira MA, Campos LJM, Menna P, Brandi F, Ramos YG (2020) Seed pre-inoculation with *Bradyrhizobium* as time-optimizing option for large-scale soybean cropping systems, Agron. J. 112(6), 5222-5236.

Iliäœić RM, Pivić RN, Dinić ZS, Latković DS, Vlajić AS, Joå Ić DL (2017). The Enhancement of Soybean Growth and Yield in a Field Trial through Introduction of Mixtures of *Bradyrhizobium japonicum*, *Bacillus* sp. and *Pseudomonas chlororaphis*. Not. Sci. Biol. 9(2): 274–279.

Iturralde ET, Stocco MC, Faura A, Mónaco CI, Cordo C, Pérez-Giménez J, Lodeiro AR (2020) Coinoculation of soybean plants with *Bradyrhizobium japonicum* and *Trichoderma harzianum*: Coexistence of both microbes and relief of nitrate inhibition of nodulation. Biotechnol. Rep. 26: e00461.

Junior AFC, Chagas LFB, Colonia BSO, Martins ALL (2022) *Trichoderma asperellum* (Samuels, Lieckf & Nirenberg) as a promoter of vegetative growth in soybeans. Ver. de Cienc. Agr. 39(E): 50-68.

Karuppiah V, Vallikkannu M, Li T, Chen J (2019) Simultaneous and sequential based co-fermentations of *Trichoderma asperellum* GDFS1009 and *Bacillus amyloliquefaciens* 1841: a strategy to enhance the gene expression and metabolites to improve the bio-control and plant growth promoting activity. Microb. Cell Factories. 18(185): 1-16.

Kaschuk G, Nogueira MA, Luca MJ, Hungria M (2016) Response of determinate and indeterminate soybean cultivars to basal and topdressing N fertilization compared to sole inoculation with Bradyrhizobium. Field Crops Res. 195: 21-27.

Li L, Qu Q, Tian B, Zhang KQ (2005) Induction of Chlamydospores in Trichoderma harzianum and Gliocladium roseum by antifungal compounds produced by Bacillus subtilis C2. J. Phytopathol. 153: 686-693.

Mahmud K, Makaju S, Ibrahim R, Missaoui A (2020) Current Progress in Nitrogen Fixing Plants and Microbiome Research. Plants. 9: 97.

MAPA (2011). Instrução Normativa SDA nº 13, de 24 de março de 2011. Available in: https://www.gov.br/agricultura/pt-br/assuntos/sustentabilidade/organicos/arquivos-organicos/in-13-de-28-05-2015-cporg-e-stporg.pdf

Mattos MLT, Cocco, KLT, Oliveira ACB de, Valgas RA, Scivittaro WB, Hungria M (2019) para o incremento da produtividade de genótipos de soja em terras baixas. Embrapa, Pelotas-RS (Boletim de Pesquisa e Desenvolvimento, 330) Available in: https://www.embrapa.br/busca-de-publicacoes/-/publicacao/1117798/novas-estirpes-de-bradyrizobium-para-o-incremento-da-produtividade-de-genotipos-de-soja-em-terras-baixas

Mattos MLT, Martins JFS, Fillipi MCC (2020) Efeito de agentes de biocontrole sobre bactérias degradadoras de resíduos de agrotóxicos e fixadoras de nitrogênio (Comunicado técnico 376). Embrapa, Pelotas-RS. Available in: https://ainfo.cnptia.embrapa.br/digital/bitstream/item/218217/1/COMUNICADO-TECNICO-376.pdf

Meyer MC, Mazaro SM, Silva JC (2022) Trichoderma: su uso en la agricultura. 1 ed. Embrapa, Brasília, DF.

Miljaković D, Marinković J, Tamindžić G, Đorđević V, Tintor B, Milošević D, Ignjatov M, Nikolić Z (2022) Bio-Priming of Soybean with Bradyrhizobium japonicum and *Bacillus megaterium*: Strategy to Improve Seed Germination and the Initial Seedling Growth. Plants. 11(15): 1927.

Minamisawa K, Ogawa K-I, Fukuhara H, Koga J (1996) Indolepyruvate Pathway for Indole-3-Acetic Acid Biosynthesis in *Bradyrhizobium elkanii*. Plant Cell Physiol. 37(4): 449-453.

Mirriam A, Mugwe J, Raza MA, Seleiman MF, Maitra S, Gitari HH (2022) Aggrandizing soybean yield, phosphorus use efficiency and economic returns under phosphatic fertilizer application and inoculation with Bradyrhizobium. J. Soil Sci. Plant Nutr. 22(4): 5086-5098.

Moretti LG, Crusciol CAC, Bossolani JW, Momesso L, Garcia A, Kuramae EE, Hungria M (2020) Bacterial consortium and microbial metabolites increase grain quality and soybean yield. J. Soil Sci. Plant Nutr. 20: 1923-1934.

Moretti LG, Crusciol CAC, Kuramae EE, Bossolani JW, Moreira A, Costa NR, Alves CJ, Pascoaloto IM, Rondina ABL, Hungria M (2019) Effects of growth-promoting bacteria on soybean root activity, plant development, and yield. Agron. J. 112: 418-428.

Muniz PHPC, Peixoto GHS, Teixeira MPM, Mello SCM, Carvalho DDC (2018) Production of conidia on solid substrate and surface colonization by *Trichoderma harzianum*. Rev. Agric. Neotrop. 5(4): 40-44.

NCCLS (2003) Performance standards for antimicrobial disk susceptibility tests; approved standard – 8 ed. Wayne, Pennsylvania, USA.

Niu B, Wang W, Yuan Z, Sederoff RR, Sederoff H, Chiang VL, Borriss R (2020) Microbial Interactions Within Multiple-Strain Biological Control Agents Impact Soil-Borne Plant Disease. Front. Microbiol. 11:585404.

Öğüt M, Akdağ C, Düzdemir O, Sakin MA (2005) Single and double inoculation with Azospirillum/Trichoderma: The effects on dry bean and wheat. Biol. Fertil. Soils. 41(4): 262-272.

Öğüt M, Er F (2006). Micronutrient composition of field-grown dry bean and wheat inoculated with *Azospirillum* and *Trichoderma*. J. Plant Nutr. Soil Sci. 169(5): 699-703.

Prasad AA, Babu S (2017) Compatibility of *Azospirillum brasilense* and *Pseudomonas fluorescens* in growth promotion of groundnut (*Arachis hypogea* L.). An. Acad. Bras. Cienc. 89(2): 1027-1040.

R Development Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rabanal RM, Arias A, Prado B, Hernández-Pérez M, Sánchez-Mateo CC (2002) Antimicrobial studies on three species of *Hypericum* from the Canary Islands. J. Ethnopharmacol. 81: 287-292.

Santos MS, Rodrigues TF, Nogueira MA, Hungria M (2021) The Challenge of Combining High Yields with Environmentally Friendly Bioproducts: A Review on the Compatibility of Pesticides with Microbial Inoculants. Agronomy. 11(5): 870.

Scorzoni L, Benaducci T, Almeida AMF, Silva DHS, Bolzani VS, Mendes-Giannini, MJS (2007) Comparative study of disk diffusion and microdilution methods for evaluation of antifungal activity of natural compounds against medical yeasts *Candida* spp. and *Cryptococcus* sp. Rev. Ciênc. Farm. Básica Apl. 28: 25-34.

Silva IWD, Goergen PCH, Viau LVM, Fernandes SBV, Silva JAGD, Bianchi CAM (2018) Growth Promoting Microorganisms for Treatment of Soybean Seeds. J.Agric. Sci. 10(6): 198.

Sibponkrung S, Kondo T, Tanaka K, Tittabutr P, Boonkerd N, Yoshida K-I, Teaumroong N (2020) Co-inoculation of Bacillus velezensis strain S141 and Bradyrhizobium strains promotes nodule growth and nitrogen fixation. Microorganisms. 8(5): 678.

Thomloudi EE, Tsalgatidou PC, Douka D, Spantidos T-N, Dimou M, Venieraki A, Katinakis P (2019) Multistrain versus single-strain plant growth promoting microbial inoculants - The compatibility issue. Hell. Plant Prot. J. 12(2): 61-77.

Tyśkiewicz R, Nowak A, Ozimek E, Jaroszuk-Ściseł J (2022) *Trichoderma*: The current status of its application in agriculture for the biocontrol of fungal phytopathogens and stimulation of plant growth. Int. J. Mol. Sci. 23(4): 2329.

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52: 487–511.