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Trichoderma as a Biocontrol Agent against *Sclerotinia* Stem Rot or White Mold on Soybeans in Brazil: Usage and Technology

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Abstract

Biological control agents are alternatives to chemical pesticides in the management of plant diseases. Currently, hundreds of bioproducts are commercially available in international market varying mainly in antagonistic microorganisms and formulation. We screened four *Trichoderma*-based products as to their efficacy in controlling *Sclerotinia* stem rot (SSR) under protected and field environments and their effect on soybean seeds' sanity and physiological qualities. We also tested application technologies through seed microbiolization and foliar spraying to deliver the microorganisms, and their compatibility with chemical fungicides. In vitro assays showed that all *Trichoderma* strains were antagonistic to *S. sclerotiorum* evidencing hyperparasitic activity. Moreover, the bioproducts reduced fungi incidence on soybean seeds, promoted faster seedling emergence and did not hamper seeds' vigor. Increases of 14 and 37% were registered for root length and shoot fresh weight over that of the untreated control indicating potential application of the bioproducts as soybean growth promoters. Thiophanate-methyl and procymidone were the most compatible, without drastically affecting spore germination or mycelium growth. Under field conditions, all *Trichoderma* strains reduced SSR incidence and increased soybean grain yield. Formulation interferes on bioproducts' viability and efficacy deserving special attention upon development.

Keywords: biological control, *Sclerotinia sclerotiorum*, *Glycine max*, hyperparasitism, physiological seed quality

1. Situation of *Sclerotinia* stem rot in Brazil

The white mold or *Sclerotinia* stem rot (*Sclerotinia sclerotiorum*) is an important disease in Brazil and in the fields is founded more than 7 million ha with the disease on soybeans in populations of the sclerotia between 1 and 500 per meter square (**Figure 1**). The evolution of agricultural practices over the years undoubtedly increased global food production [1, 2]. Those practices included the use of fertilizers, machinery, improved genetic plant materials, adoption of different cropping systems (no-tillage, crop rotation, intercropping), and intensive use of chemicals. In this scenario, pests coevolved with crops demanding new management strategies,

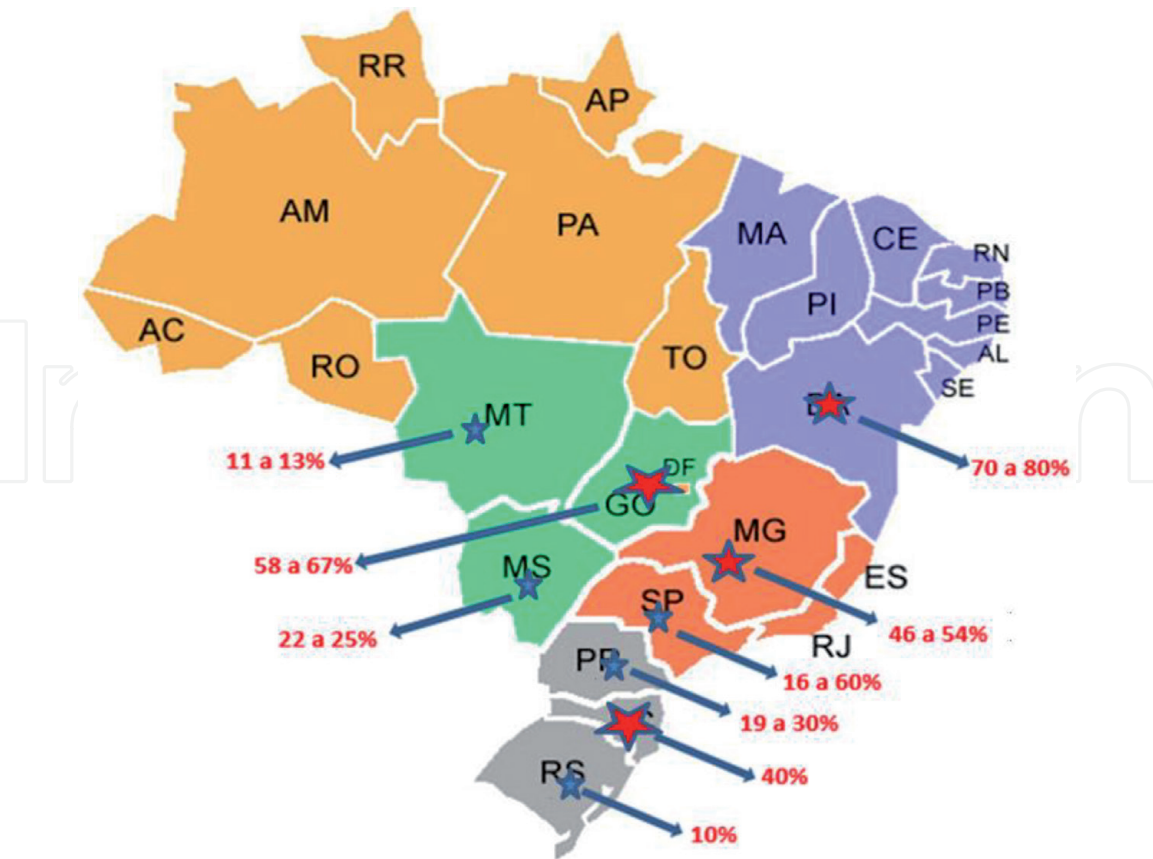


Figure 1. Estimation of the production area in Brazil (soybean, beans, and cotton), in which *S. sclerotiorum* had been detected in crops (1–40% incidence in plants) [sources: researchers from universities, foundations, rural extension agencies, cooperatives, and consultants, through personal communication]. Red star—higher levels of incidence, blue star—medium to lower levels of incidence. Sclerotinia affects an estimated area of 7.5 million ha (22.9%). Total area to soybean production in Brazil is 33.9 million ha (CONAB and IBGE—Brazil).

as diseases and insects’ outbreaks became recurrent [3–8]. Chemical pesticides have long been used in plant protection. However, they frequently increase production costs, negatively affect the environment, and are ineffective against resistant populations [9, 10]. The search for eco-friendly efficient alternatives to control plant diseases is not a recent need. One option includes the use of antagonistic microorganisms, such as *Trichoderma* spp.

Trichoderma is a genus of soil-borne fungi with well-known anti-phytopathogen activities. Mechanisms of action include competition, mycoparasitism, antibiosis, and host-induced systemic resistance [11]. *Trichoderma* species compete with pathogens mainly for nutrients and ecological niches. Besides rapid growth and abundant production of spores, some strains can synthesize siderophores that inhibit the growth of other fungi during competition [12]. Mycoparasitism traits of *Trichoderma* rely on activity of cell wall-degrading enzymes secreted by the fungus after its hyphae coil around and further penetrate the pathogen’s hyphae cells [13].

Trichoderma also produces various antimicrobial compounds that can be purified and directly used against pathogenic fungi [14–16]. The last mechanism of action triggers systemic defense responses in host plants, which interfere with pathogen’s establishment, colonization, and multiplication [14, 17]. In addition to the mentioned mechanisms, some *Trichoderma* strains promote plants protection by stimulating their development [18, 19].

The first report on the use of *Trichoderma* as a biological control agent of phytopathogens in Brazil dates back to 1950 against tobacco mosaic virus [20]. Further research evaluated its potential controlling plant pathogenic fungi and oomycetes

that led to the development of *Trichoderma*-based commercial products [21]. Among them, bioproducts recommended for the control of the etiologic agent of stem rot disease [*Sclerotinia sclerotiorum* (Lib.) de Bary] deserve special attention [22]. *Sclerotinia* stem rot (SSR) is one of the most relevant diseases in soybean crops [*Glycine max* (L.) Merrill]. Early symptoms are water-soaked irregular spots that progress to brownish lesions and eventual necrosis, wilt of leaves, and plant death. A characteristic white cotton-like mycelium on infected tissues is diagnostically detectable. At later stage of disease development, survival structures of the fungus, named sclerotia, are formed on hosts. Seed lots contaminated with fungus mycelia or sclerotia constitute the most common source of dissemination of the pathogen. Under favorable climate conditions, the disease can impair soybean yields in up to 70% [22].

Efficient chemical control of SSR relies on prophylactic application of fungicides, since curative spraying does not revert yield losses despite being effective in reducing the inoculum potential for subsequent crops. The intensive long-term fungicide-based management strategies for the control of this disease resulted in the development of resistant *S. sclerotiorum* strains toward many active ingredients (such as carbendazim, dimetachlone, and thiophanate-methyl), demanding constant baseline sensitivity studies to monitor the field efficacy of chemicals [23–29]. The need to introduce alternative molecules to control this devastating pathogen, allied to environmental and food securities, opened the market for bioproducts. Worldwide, many entrepreneurs invest in the development of formulations and registration of bioproducts. Commercialization, however, is often hindered by farmer's mistrust on products' consistent performance under field conditions, as most research are confined to the laboratory and monitoring of quality is not regularly done [30].

We accessed the efficacy of different *Trichoderma*-based fungicides on the control of SSR and their possible effect on initial development of soybean plants. The bioproducts differed in formulation and strains and were both tested in vitro and under field conditions. We also verified the application technology, through spraying or seed treatment, and compatibility with chemical fungicides, as those are important aspects to address further applications of bioproducts in agriculture by demonstrating consortium to other plant disease management strategies.

2. Development of biological control in Brazil using *Trichoderma*

2.1 Microorganisms and growth conditions

Trichoderma strains used in this research were recovered from bioproducts available on the market (**Table 1**) after plating in PDA medium (20% potato extract, 2% dextrose, and 2% agar). All inhibition assays were against the *S. sclerotiorum* strain Jatai, characterized as highly aggressive [31]. This strain was field-isolated from a commercial soybean crop at the state of Goias, Brazil. Pathogen was recovered in PDA medium from sclerotia previously disinfested with 50% (v v⁻¹) ethanol for 30 s followed by immersion in 0.5% (w v⁻¹) sodium hypochlorite solution for 1 min. Later, sclerotia were rinsed thrice with sterile distilled water and incubated for myceliogenic germination. Microorganisms' incubation conditions were at 22 ± 2°C under 12 h photoperiod, otherwise stated, for indicated periods.

2.2 Monitoring of quality of bioproducts

Products (**Tables 1** and **2**) were serially diluted in sterile distilled water and plated in PDA medium for 5 days. *Trichoderma* titer was obtained by counting the

Bioproduct codification	Active ingredient/ microorganism	Formulation	Titer reported by the manufacturer
SF04	<i>T. asperellum</i> ^a	WG	1.0 × 10 ^{10e}
IBLF006	<i>T. harzianum</i> ^b	WP	5.0 × 10 ^{10e}
ESALQ-1306	<i>T. harzianum</i> ^c	CS	2.0 × 10 ^{9f}
Tricho	<i>Trichoderma</i> spp. ^d	WP	1.0 × 10 ^{8 e}

WG, wettable granule; WP, wettable powder; CS, concentrated suspension

^aStrain SF04

^bStrain IBLF006

^cStrain ESALQ-1306

^dNot specified on product's label

^eColony-forming units (CFU) *m L*⁻¹ or CFU *g*⁻¹

^fViable conidia *m L*⁻¹

Table 1.
Information on the bioproducts used in this study.

	SF04	IBLF006	ESALQ-1306	Tricho
Bioproduct label	1.0 × 10 ^{10a}	5.0 × 10 ^{10a}	2.0 × 10 ^{9b}	1.0 × 10 ^{8a}
Hemocytometer	8.8 × 10 ¹⁰	1.2 × 10 ¹⁰	1.9 × 10 ¹⁰	6.7 × 10 ⁹
Plate ^a	5.0 × 10 ¹⁰	3.0 × 10 ⁶	6.0 × 10 ⁹	1.0 × 10 ⁷
Bacterial contamination ^{c, d}	5.0 × 10 ⁵ A	6.0 × 10 ⁶ C	1.0 × 10 ⁶ A	3.0 × 10 ⁶ B
Viability (%) ^c	98 A	60 C	98 A	90 B

Titer of *Trichoderma* spp. was accessed through the number of viable spores counted in a hemocytometer slide and of colony-forming units recovered in PDA plates. Concentrations were compared to titers reported by the manufacturer. Bacterial contamination and spore viability also determine bioproducts' quality

^aCFU *mL*⁻¹ or CFU *g*⁻¹

^bViable conidia *mL*⁻¹

^cAverages followed by different uppercase letters are statistically different by the Tukey test (*p* < 0.05)

^dData transformed to $\sqrt{\text{_____}} \times 0.5$

Table 2.
Monitoring of quality of bioproducts.

number of colonies grown in vitro and by the number of viable spores in a hemocytometer slide visualized under light microscope (Olympus CX40). The percentage of germinated spores was used to access fungus viability 24 h after incubation. Only spores with germ tube bigger than or equivalent to spore's own size were considered viable. Bacterial contaminations were expressed in colony-forming units (CFU) per mL or per gram of each bioproduct, detected 2 days after plating diluted aliquots of bioproducts in tryptic soya agar medium (1.5% casein peptone (pancreatic), 0.5% soya peptone, 0.5% sodium chloride, 1.5% agar, pH 7.3) [32].

2.3 In vitro antagonistic activity to *S. sclerotiorum*

Antagonism of *Trichoderma* strains to the phytopathogen *S. sclerotiorum* was verified by the dual culture assay modified from [33]. Briefly, 5-mm-dia mycelial agar discs of the fungi were collected from the edge of 3-day-old colonies and simultaneously placed on opposite sides of PDA plates. Plates were incubated as described [34]. Seven days after incubation, fungi growth was scored according to a modified scale proposed by Bell et al. [35]. We used quant software [36] to develop a diagrammatic scale in which *Trichoderma* strains were classified based on the area of the plate they covered. We considered antagonist or efficient those attributed

scores up to 3.0 (at least 50% of the plate's surface). The search for efficient and environmentally safe alternatives to replace chemical pesticides routinely used in food production systems has brought to the market biological products containing antagonistic microorganisms. Among them, *Trichoderma* spp. deserves special attention. Surveys indicate the availability of more than 250 *Trichoderma*-based products in the international market and its growing adoption in Brazilian agriculture [37]. The diversity of products mainly comprises differences in fungus strains and formulations, aspects preponderant in bioproducts' efficacy, and shelf life. The quality of a biological product can vary from its manufacturing process to its application in the field. Therefore, it must be periodically checked to ensure efficiency. Although there is no standard methodology, the evaluation of the quality of a bioproduct is basically done by three criteria: concentration in plates, spore count, and viability. Verification of bacterial contamination is equally important as its presence reduces the shelf life of the product.

Higher concentrations of the antagonist in the tested bioproducts were recorded by direct quantification of the number of viable spores in a hemocytometer slide. Compared to quantification in a plate, this may occur because nearby propagules, after spread on plate's surface, visually form only one colony, underestimating the result, which does not happen in the individualized spore counting under a light microscope. From all counted spores, IBLF006 was the only bioproduct with germination percentage lower than 90, coincident to its higher contamination by bacterial cells. By accessing the concentration on the plate through the indirect counting method (serial dilutions of fungi suspensions), the bioproduct SF04 showed *Trichoderma* concentration beyond stated in the product's label, equivalent to 5.0×10^{10} CFU g⁻¹ (Table 4). All evaluated bioproducts met the minimum concentration of the antagonist recommended for commercialization, of 2×10^6 CFU mL⁻¹ or CFU g⁻¹ in culture medium, and no pathogenic contaminants belonging to the genera *Salmonella*, *Shigella*, or *Vibrio* [30]. However, they exceeded the bound for microbial contamination (1×10^4 counts per mL/g).

There is a clear need to standardize and specify on the product's label the methodology adopted for quality monitoring, due to discrepancies between antagonist concentration values measured by spores counting (direct quantification) and by the number of in vitro colonies (indirect). Besides, limitations and modifications of the methodologies interfere in the result [38–41]. The maintenance of viability, especially during storage of the bioproduct, requires studies on formulations more adequate to the stability of microorganisms. In the market, *Trichoderma* is sold on its cultivation substrate, for example, bioproducts Tricho and IBLF006, which is ground and packed. This formulation (WP) hinders field spraying due to nozzle clogging and has less fungus viability (60–90%) and increased possibility of contamination by other fungi and bacteria (up to 12× higher than in the bioproduct SF04), reducing products' efficacy in the field. Other formulations available on the market, such as pure spores, spores mixed in oil, concentrated suspension (CS), or even wettable granules (WG), facilitate the application technology. To increase shelf life, adjuvants are usually added to protect propagules [42]. Recently, some bioproducts have evolved with the emergence of antimicrobial secondary metabolite formulations. Those formulations allow greater stability of the active ingredient under room temperature storage making it easier commercialization of the bioproduct without loss of quality. In addition, they offer advantages in terms of ease of application, protection against UV radiation under field conditions, action against the target pathogen without compromising the soil microbiota, and protection of the active ingredient when mixed to other chemicals [43]. It is very important to choose a product with quality combined with a formulation that guarantees stability and efficacy in the application and control of SSR.

The antagonistic effect of the strains was first verified through simultaneous cultivation under in vitro conditions, determining the area of the plate occupied by the colonies of *Trichoderma* and of *Sclerotinia*. According to the diagrammatic scale we developed, following the one proposed in 1982 [35], the bioproducts scored 2.5. Therefore, they did not differ statistically among themselves as to the percentage of the area of the plate they covered (62.3–64.4%) and were considered antagonist to *S. sclerotiorum*. Reduction of phytopathogen growth can be attributed to competition for space and nutrients of the culture medium, in which the great environmental fitness of *Trichoderma* and its rapid radial growth in in vitro cultivation are highlighted. In vitro dual culture assays are important tools in the selection process of biocontrol agents. Those tests provide useful information on strains' efficacy and variability and on pathogens' susceptibility to evaluated agents. The tests are conducted under controlled environment conditions minimizing variable effects of temperature, humidity, light, and soil microflora [15, 35]. We did not observe differences in antagonistic potency among the *Trichoderma* strains used in this study, for which the soybean-isolated *S. sclerotiorum* was susceptible. Dual culture assays are also used to analyze antagonist-phytopathogen interactions at ultrastructural level, with the aid of light or electron microscopy [44]. SEM images showed that all *Trichoderma* strains were able to colonize *S. sclerotiorum*, by either penetrating or strangling its hyphae (**Figure 2**). It also noted the growth of parallel hyphae [45]. Both interactions observed in our study, strangulation and penetration, can be interpreted as a hyperparasitic behavior of *Trichoderma* strains present in the bioproducts [46]. During hyperparasitism, *Trichoderma* species detect hyphae of

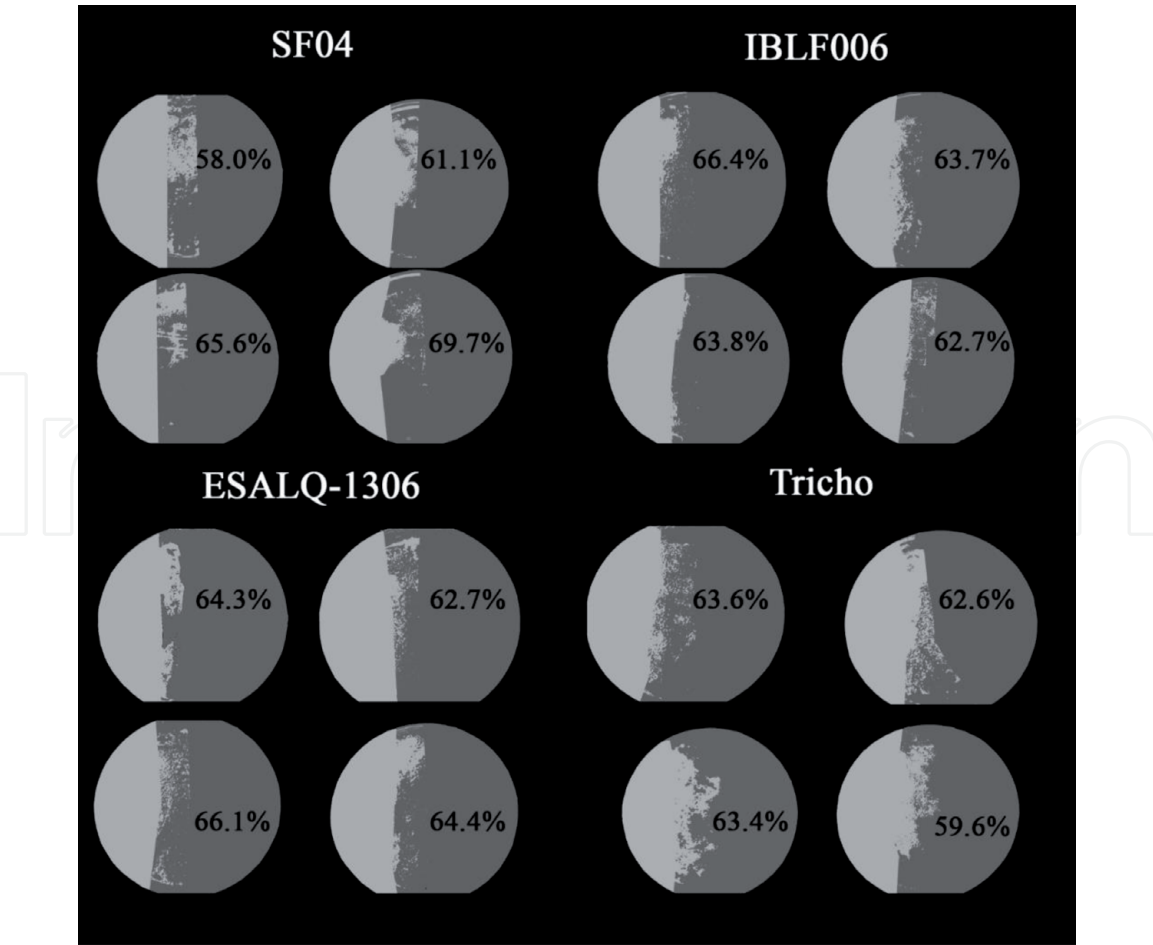


Figure 2. Scanning electron photomicrography of the interactions between *Trichoderma* spp. and *S. sclerotiorum* showing strangulation (1) and penetration (2) of antagonist hyphae in the pathogen. Bioproducts: (a) SF04, (b) IBLF006, (c) ESALQ-1306, and (d) Tricho.

susceptible fungi due to chemical stimuli produced by the host hypha itself. The antagonists form appressory structures and tightly coil around the full extent of the hypha penetrating and degrading it [47–49]. This mechanism has already been demonstrated by many researchers through interactions of *T. harzianum*, *T. viride* and *T. asperellum* with *Rhizoctonia solani*, *Pythium* spp., *Fusarium oxysporum*, *F. solani*, *S. rolfii*, *S. sclerotiorum*, *Botrytis cinerea*, *Phytophthora* spp., *Macrophomina phaseolina*, and *Alternaria solani* [45, 47–52].

The antagonistic activity of the bioproducts was also verified against the pathogen survival structures (data not shown). Percentage of non-germinated sclerotia and of sclerotia colonized by *Trichoderma* reached 55.6 and 71.25 (strain ESALQ-1306). Under simulated infested soil condition, treatments effectively controlled inoculum with a twofold reduction compared to the untreated soil. There was a negative correlation ($p < 0.01$, $r = -0.725$) between sclerotia germination and parasitism indicating that *Trichoderma* spp. colonization leads to reduction of the inoculum potential for subsequent crops. It should not be expected, however, that only direct ground spraying or seed treatment alone are effective in controlling *S. sclerotiorum*. The contact area of the antagonist with the soil is small limiting its proliferation. Besides, competition with the soil microbiota and possible adverse environmental conditions may compromise *Trichoderma* establishment. Still, direct ground spraying and seed treatment can be associated with other agricultural practices, such as mulching application of liquid compost and chemical fungicides [17, 53].

2.4 Hyperparasitism and antagonistic activity of *Trichoderma*-based products against *S. sclerotiorum* of *Trichoderma* spp. to *S. sclerotiorum*

To study the interaction between the antagonist and the pathogen, mycelia agar discs (5 mm diameter) from collation zones among both fungi colonies were collected at the seventh day of co-cultivation and further analyzed [54–55]. Discs were fixed to bristles in modified Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer 0.05 M, pH 7.2), at 4°C for 17 h, followed by four rinses with mentioned buffer. Samples were subsequently fixed with 1% (w v⁻¹) osmium tetroxide in cacodylate buffer 0.01 M (pH 7.2) during 1 hour at 4°C, rinsed thrice with distilled water, and dehydrated in graded acetone series (30, 50, 70, 90, and 100%). Samples were kept at each solution for 10 minutes, and each step was repeated three times. Drying was done with carbon dioxide using a critical point dryer (TEC-030) (Balzers, Liechtenstein). Samples were fixed to aluminum stubs and gold-coated (20 nm/180 seconds) before visualized in a scanning electron microscope model LEO 435VP (Zeiss, Oberkochen). **Figures 2 and 3** show the hyperparasitism and antagonistic activity of *Trichoderma*-based products against *S. sclerotiorum* of *Trichoderma* spp. to *S. sclerotiorum* (different commercial products) used in Brazil.

2.5 Effect of *Trichoderma* spp. on sclerotia germination

Soil parasitism of *Trichoderma* strains against *S. sclerotiorum* was evaluated. Two-mm-dia sclerotia were buried at 0.5 cm depth in acrylic boxes containing autoclaved soil, which was later sprayed with the *Trichoderma*-based products following field doses recommended by the manufactures, at a spray volume of 100 mL. Sixteen sclerotia were evenly distributed per box. Mock consisted of application of sterile distilled water. After incubation for 5 days, we transferred the sclerotia to PDA plates and let them incubate for another 10 days. The number of germinated and parasitized sclerotia was counted, and the hyperparasitism action from *Trichoderma* species was reduced until 90% the viability of sclerotia from soil in the first year of treatment.



Figure 3. Spore germination (%) of *Trichoderma* strains after treatment of soybean seeds with chemical fungicides and bioproducts. Viability was accessed right after seed treatment (ST), at 3 and 16 h after ST. Average of four replications ($n = 200$, each) \pm standard deviations are shown. Different lowercase letters are statistically different by the Tukey test at $p < 0.05$.

2.6 Compatibility of fungicides with *Trichoderma* spp. in vitro and in seed treatment

Different fungicides (Table 3), usually used in the control of *S. sclerotiorum* under field conditions, were added to autoclaved PDA medium before polymerization ($\pm 40^{\circ}\text{C}$) to five final concentrations (0.1, 1, 10, 100 and 1000 ppm).

The medium was poured into Petri dishes and inoculated with 5-mm-dia colony discs of *Trichoderma* strains (**Table 4**). Plates were then incubated for 1 week. The diameter of *Trichoderma* colonies was measured daily. At the seventh day, we calculated the growth speed index (GSI) according to [57].

Seed treatment was done with soybean cultivar NK7074RR, considered susceptible to SSR [58]. Chemical and biological fungicides were applied at doses recommended by the manufactures (**Table 2**). Seeds were first treated with the chemical pesticides followed by application of the bioproducts. Positive controls consisted of seeds treated only with the *Trichoderma*-based products. Samples were collected at three different exposure times (0, 3, and 16 h after seed treatment). At each time, ten seeds were randomly sampled and mixed with 2 mL of sterile water

Fungicide codification	Active ingredient (ai)	Concentration of the ai (g L ⁻¹ or g kg ⁻¹)	Dose ^a
Tm	Thiophanate-methyl	500	100 ^b
TmF	Thiophanate-methyl + fluazinam	350 + 52.5	180 ^b
C	Carbendazim	500	100 ^b
F	Fluazinam	500	200 ^c
FldMM	Fludioxonil + metalaxyl-M	25 + 10	100 ^b
FiTmPy	Fipronil + t. methyl + pyraclostrobin	250 + 225 + 25	200 ^b
Pro	Procymidone	500	200 ^c

^amL of commercial product 100 kg⁻¹ of seeds
^bDose recommended for seed treatment
^cExperimental dose

Table 3.
Chemical fungicides used in the compatibility test.

Treatment	Active ingredient (ai)	Application timing ^e				Dose (L/kg ai ha ⁻¹)
		1st	2nd	3rd	4th	
Control	—	—	—	—	—	—
SF04	<i>T. asperellum</i> ^a	V ₄	V ₆	—	—	2.0 × 10 ⁹
IBLF006	<i>T. harzianum</i> ^b	V ₄	V ₆	—	—	2.0 × 10 ⁸
ESALQ-1306	<i>T. harzianum</i> ^c	V ₄	V ₆	—	—	2.0 × 10 ⁹
Tricho	<i>Trichoderma</i> spp. ^d	V ₄	V ₆	—	—	2.0 × 10 ⁶
ESALQ-1306 + Tm	<i>T. harzianum</i> ^c + thiophanate-methyl	V ₄	V ₆			2.0 × 10 ⁹
				R ₁	R ₂	0.5
Tm	Thiophanate-methyl	—	—	R ₁	R ₂	0.5
F	Fluazinam	—	—	R ₁	R ₂	0.5

^aStrain SF04
^bStrain IBLF006
^cStrain ESALQ-1306
^dNot specified on product's label
^eAccording to soybean phenological stage proposed by [56]

Table 4.
Description of active ingredients/microorganisms, application timing, and doses of products in the control of *Sclerotinia* stem rot in soybean crop.

for 1 min. Aliquots of 100 μL were plated in PDA acidified medium (1 mL lactic acid L^{-1} medium) and incubated at 25°C for 20 h. Subsequently, spore germination of *Trichoderma* strains was inhibited with lactophenol-blue cotton solution. Coverslips were placed onto two regions of each plate and 100 spores counted at each one of them under light microscope (Olympus CX40). The result was expressed as the percentage of germinated spores. Despite the pressing need to reduce the use of pesticides in food production systems, extensive monocropping areas are still chemical dependents. In this scenario, effectiveness of biological control agents requires knowledge on their compatibility with the most common pesticides used in crops. Specifically, we accessed the viability of four different *Trichoderma*-based products toward active ingredients (ai) recommended to the control of SSR on soybean crop. First, we performed an in vitro baseline sensitivity study to monitor the inhibition of mycelium growth and proceed with a seed treatment assay of successive application of chemical and biological products to determine killing effect on antagonist's spores.

We used diameter data measured from colonies during a 7-day incubation period to calculate the growth speed index (GSI) of the *Trichoderma* strains in culture medium containing different concentrations of chemical fungicides. Significant effects ($p < 0.001$, CV = 14.55%) were found for the triple interaction of fungicides, concentrations, and bioproducts. Among tested fungicides, thiophanate-methyl (Tm) was the only one with a logistic behavior (all the others adjusted to exponential regressions), requiring doses above 1.36 ppm to affect growth speed of the strains by 50% (**Table 5**). Coefficients of exponential equation of ai carbendazim (C) indicate greater impact of this fungicide on mycelial growth of *Trichoderma* spp., as observed for lower values of coefficient a and higher values of coefficient b compared to all bioproducts. Compatibility responses varied among bioproducts, fungicides, and concentrations, following a trend of the higher the concentration, the lower the development of the antagonist. In general, the fungicide carbendazim (C) was less compatible considering all tested concentrations, opposing to fungicides thiophanate-methyl (Tm) and procymidone (Pro) (**Table 5**). However, procymidone reduced the GSI from 13.6 to 83%, demanding caution in the recommendation of its use associated with bioproducts in foliar sprays, application in the soil, or seed treatment.

To check the compatibility between chemical fungicides and bioproducts in seed treatment, a common agricultural practice, we evaluated the viability of the spores of the antagonists after exposure to the active ingredients. Like the plating assay, in the soybean seed treatment, significant effects ($p < 0.001$, CV = 15.69%) were observed for the triple interaction (fungicides \times bioproducts \times exposure time). Excluding the association of thiophanate-methyl (Tm) with IBLF006 for a 3-h incubation, all fungicides reduced the germination potential of the spores (**Figure 3**). In general, fungicides that led to smaller reductions in germination were thiophanate-methyl (Tm), procymidone (Pro), and fipronil + t.-methyl + pyraclostrobin (FiTmPy) (germination rates of 87.3, 64.5, and 63.8%, respectively). These results indicate potentiality in their use combined to the bioproducts in the treatment of soybean seeds. The ai fluazinam (alone or associated to thiophanate-methyl) and carbendazim (C) drastically reduced viability of *Trichoderma* strains (up to complete inhibition of spore germination). In vitro assays report insensitivity of *T. harzianum*, *T. stromaticum*, and *T. atroviride* to procymidone [59–61] suggesting its simultaneous use with *Trichoderma* species for the control of *S. sclerotiorum* in tomato [62] and lettuce [63]. Other research demonstrate that thiophanate-methyl is compatible to many strains of *Trichoderma* spp., whereas carbendazim and fluazinam are extremely toxic [64–65], supporting our findings. The insensitiveness to dicarboximide fungicides (such as procymidone) may probably be due to higher transcriptional levels of histidine kinase genes [28]. Interestingly, the *Trichoderma* strains we

Fungicide	SF04	IBLF006	ESALQ-1306	Tricho	SF04	IBLF006	ESALQ-1306	Tricho
0.1 ppm					1 ppm			
Tm	12.0 Ab	12.6 Aa	12.6 Aa	13.0 Aa	6.7 Bc	12.0 Aa	10.1 Ab	12.2 Aa
TmF	12.1 Aa	8.7 Db	5.9 Dd	7.9 Dc	5.3 Ca	4.0 Db	3.6 Db	3.7 Db
C	1.3 Da	1.2 Fa	1.1 Ga	1.7 Ga	0.4 Ea	1.0 Ga	0.5 Fa	1.0 Fa
F	4.6 Ca	3.0 Eb	4.4 Ea	4.8 Ea	4.7 Ca	3.3 Eb	3.8 Db	3.9 Db
FldMM	1.9 Db	2.0 Gb	2.3 Fb	3.9 Fa0	1.4 Db	2.3 Fa	2.5 Ea	2.7 Ea
FiTmPy	8.6 Bb	9.7 Ca	7.3 Cc	8.8 Cb	5.4 Ca	6.1 Ca	4.7 Cb	5.6 Ca
Pro	8.0 Bb	10.8 Ba	11.2 Ba	11.5 Ba	12.2 Aa	9.3 Bb	9.2 Bb	9.7 Bb
10 ppm					100 ppm			
Tm	0.3 Cb	1.5 Ba	1.7 Ca	0.9 Bb	0.1 Ca	0.1 Da	0.1 Da	0.2 Da
TmF	0.8 Ca	0.3 Ca	0.5 Da	0.2 Ba	0.1 Ca	0.0 Da	0.1 Da	0.1 Da
C	0.0 Ca	0.3 Ca	0.2 Da	0.2 Ba	0.1 Ca	0.5 Da	0.2 Da	0.2 Da
F	4.6 Aa	2.1 Ab	2.7 Bb	2.6 Ab	3.6 Aa	1.5 Bc	2.3 Bb	2.3 Bb
FldMM	1.8 Ba	2.3 Aa	1.9 Ca	1.9 Aa	1.1 Ba	1.1 Ca	1.0 Ca	1.1 Ca
FiTmPy	0.4 Ca	0.4 Ca	0.3 Da	0.3 Ba	0.4 Ca	0.1 Da	0.2 Da	0.1 Da
Pro	2.1 Bb	2.2 Ab	3.5 Aa	2.0 Ab	3.7 Aa	3.0 Aa	3.6 Aa	3.2 Aa
1000 ppm					0 ppm			
Tm	0.0 Ca	0.0 Ba	0.0 Ba	0.0 Ba	14.12	12.81	13.61	11.96
TmF	0.0 Ca	0.0 Ba	0.0 Ba	0.0 Ba				
C	0.0 Ca	0.3 Ba	0.1 Ba	0.0 Ba				
F	2.1 Ba	0.5 Ba	0.8 Ba	0.7 Ba				
FldMM	0.3 Ca	0.5 Ba	0.5 Ba	0.3 Ba				
FiTmPy	0.0 Ca	0.0 Ba	0.1 Ba	0.0 Ba				
Pro	3.9 Aa	2.4 Aa	3.5 Aa	3.2 Aa				

Colonies were plated in PDA medium supplemented with chemical fungicides at concentrations ranging from 0 to 1000 ppm
Averages followed by the same uppercase letters, in columns, and lowercase letters, in lines, do not differ significantly by the Tukey test ($p \leq 0.05$)

Table 5.
Growth speed index (GSI) calculated based on *Trichoderma* mycelium diameter measured daily.

studied showed completely divergent behavior regarding the benzimidazole fungicides: compatible to thiophanate-methyl but sensitive to carbendazim. Differences on tubulin-binding site or degradation/detoxification of the active ingredients could explain selectivity (however, these aspects are yet to be demonstrated).

The simultaneous use of biocontrol agents and pesticides in disease management may allow reduction of recommended doses of chemicals [66]. This possibility could mitigate compatibility problems, as the fungicides applied in low concentrations did not visibly affect *Trichoderma* growth and viability. On the other hand, such phytosanitary strategy often results in synergistic or additive effects in the control of soil-borne diseases. In seed treatment, it is recommended that sowing be done as soon as possible, since spore germination tends to decrease as exposure to chemicals is prolonged. In vitro studies have the advantage of exposing the microorganism as much as possible to the action of the chemical, a fact that does not occur in field conditions where many

factors constitute obstacles to this exposure, thus protecting the biological agent. Therefore, there is a possibility of good selectivity of thiophanate-methyl and procymidone under field conditions considering results obtained in the laboratory.

2.7 Sanity of soybean seeds treated with *Trichoderma* spp.

The efficacy of the antagonist in the control of seed pathogens was verified through the blotter test. Four hundred soybean seeds cv. NK7074RR, artificially inoculated or not with *S. sclerotiorum*, were divided in 16 pseudo-samples and distributed in transparent acrylic boxes filled with three sterile sheets of filter paper moistened with distilled water. Boxes were previously disinfected with 70% (v v⁻¹) ethanol and 2% (w v⁻¹) sodium hypochlorite solution. Before test installation, soybean seeds were subjected to -20°C for 24 h to retard germination. Artificial inoculation with *S. sclerotiorum* consisted of placing the seeds in Petri dishes completely colonized by the pathogen. Seeds were kept in contact with the fungus until visual detection of the white mycelium on them. Later, the seeds were treated with the bioproducts at the commercial dose recommended by the manufactures and incubated during 1 week at 20 ± 2°C under 12 h photoperiod and then at 12 ± 2°C for equal period with additional wetting of the paper substrate [67]. Seeds were individually checked using a stereomicroscope at the resolution of 30–80× for the identification of typical fungi-fruited bodies. Using a light microscope, we confirmed the identity of the fungi at species level when necessary.

Seeds can be source of inoculum introducing pathogens to new cultivation areas and increasing diseases in the field. Besides, physiological seed quality can be compromised by deteriorating action of fungi during storage. Alternatively, chemical seed treatment microbiolization ensures seed health by using living microorganisms. In the sanity test, soybean seeds were treated with the bioproducts. We observed predominance, but not exclusivity, of *Fusarium semitectum* (up to 31% of contaminated seeds), *Nigrospora* spp. (12%), and *Aspergillus* spp. (5%). There was also incidence of other fungi such as *Colletotrichum dematium* var. *truncata*, *Phomopsis phaseoli*, *Penicillium* spp., *Periconia* spp., *Rhizopus stolonifer*, and *Cladosporium* spp. (data not shown). All bioproducts reduced the incidence of *Penicillium* spp., *Aspergillus* spp., *R. stolonifer*, *P. phaseoli*, *Cladosporium* spp., and *Periconia* spp. compared to the control. Regarding *Phomopsis* spp., its occurrence is closely dependent on environmental conditions during seed formation and maturation [68], and its presence in the seed lot makes it unfeasible for use. Microbiolization efficacy, examined to all *Trichoderma* strains tested, can ensure sowing of seed lots obtained from fields in which maturation and harvesting coincided with high temperatures and high relative air humidity. Besides, these results suggest not only the known use of *Trichoderma* spp. in the protection of seeds against soil-borne pathogens [37] but against storage fungi, recommending its application at post-harvest. It is likely that microbiolization will promote disease control and favor seed germination, seedling emergence, and seedling early development in the field as it protects the seeds from fungi attack during storage. Moreover, strains IBLF006 and ESALQ-1306 were efficient in reducing incidence of *F. semitectum* (50 and 100%, respectively).

Now considering the soybean seeds artificially inoculated, *S. sclerotiorum* suppressed the development of the microorganisms, as well as the colonization capability of Tricho and IBLF006 strains. On the other hand, ESALQ-1306 showed higher competitiveness against *S. sclerotiorum* reducing its occurrence by 40%. This effect, combined with that found in the soil parasitism test, reiterates the potential of the bioproduct ESALQ-1306 in the containment of the dissemination of SSR through seeds. Taking into consideration soybean seeds may be contaminated by either

sclerotia or *S. sclerotiorum* mycelium, the strain ESALQ-1306 was effective in the control of both contamination forms.

Seed microbiolization represents a useful and promising method for the control of seed pathogens (infecting or contaminating the seed lot) and of soil-borne pathogens (as *Fusarium* spp.). It is considered an important method of application of biocontrol agents since it requires a small amount of biological material compared to the quantity needed for soil application. Furthermore, it is an efficient and low-environmental-impact strategy compared to chemical fungicides and may increase the concentration of the antagonist in the soil in medium/long terms turning it suppressive to various pathogens. *Trichoderma*-based products are commercialized as biofungicides, biostimulants, biofertilizers, and even plant growth promoters [37]. We confirmed the in vitro antagonistic activity of the bioproducts against *S. sclerotiorum* and their potential enhancement of seed health and proceeded to test the hypothesis of their effect to promote initial development of soybean seedlings. Seed treatment with the bioproducts did not favor germination though neither impaired the development of normal plantlets. Upon seed challenge with *S. sclerotiorum*, the occurrence of abnormalities varied depending on the bioproduct applied: IBLF006 presented the lowest (3.5%) and SF04 the highest incidence (22%) of abnormal seedlings. Pathogen inoculation reduced germination rate by 8.4%. Despite the greater occurrence of abnormal seedlings, the SF04 strain was detected colonizing the soybean seedlings, as did the strain ESALQ-1306, suggesting competitiveness against *S. sclerotiorum*. In the absence of the phytopathogen, the treatments did not differ among them, with average colonization of developed seedlings of 60.7%.

All bioproducts accelerated emergence speed index on sand seedbed test. The index practically doubled compared to the untreated control. This result indicates improvement in the physiological quality of soybean seeds inoculated with *Trichoderma* spp. On the other hand, the faster soybean seedlings develop, the less the seeds will be exposed to soil-borne pests. The bioproducts did not change the analyzed biometric variables, except for root length and shoot fresh weight. Under *S. sclerotiorum* infection, seedlings showed higher root growth when treated with strain ESALQ-1306 (8.26 cm), followed by SF04 and Tricho. Strain IBLF006 was like the control (6.83 cm). ESALQ-1306 promoted root growth also in uninfected seeds (8.30 cm). *Trichoderma* spp. treatment increased in up to 36.6% aerial fresh weight of artificially inoculated soybean (equivalent to 8.96 g) and reduced SSR incidence in 2.5 folds (strains ESALQ-1306 and SF04). Beneficial effects of *Trichoderma* spp. on plant development are reported in rice [76], melon [69], sorghum [70], and wheat [71, 72]. In soybean crop, growth promotion is related to the synthesis of 1-aminocyclopropane-1-carboxylate deaminase, indole acetic acid (IAA), siderophores, and biological nitrogen fixation [73, 74]. The greater root development found in our study is possibly associated with the synthesis of IAA and reduction of ethylene levels in plants. Consequently, plantlets uptake more nutrients [75] including iron and nitrogen and increase aboveground vegetative growth. This auxin-ethylene cross talk induces mitogen-activated protein kinases and proteins involved in carbohydrate metabolism and photosynthesis [77]. Likewise, signaling of growth regulators may have favored the rapid emergence of seedlings, a fundamental feature for successful crop establishment especially facing biotic and abiotic stresses.

2.8 Effect of *Trichoderma* spp. on soybean germination and early development and effect of *Trichoderma* spp. on physiological quality of soybean seeds

The standardization of the seed germination and seedling emergence on sand seedbed tests [78] to access possible effects of the antagonist on physiological

quality of soybean seeds is an important procedure. Germination test consisted of four replicates of 50 seeds each placed in filter paper rolls as recommended [78]. Soybean seeds were artificially inoculated with *S. sclerotiorum* as described (item 2.7), treated with the bioproducts, and further incubated in a growth chamber under 12 h photoperiod and $25 \pm 2^\circ\text{C}$. Eight days post-incubation, we evaluated the number of normal, abnormal, and infected plantlets with SSR or *Trichoderma* spp. Fungi presence was confirmed with a stereomicroscope. We considered abnormal those plantlets without the main root or displaying hypocotyl abnormalities, such as its absence, damages, or rotting [79]. Seedling emergence test was carried out in a greenhouse. Soybean seeds were placed in plastic trays filled with sterile sand. The substrate was uniformly moistened according to the calculus of its water retention capacity [78]. The number of emerged plantlets was checked daily since the day of the first normal seedling emergence. Counting continued until the 13th day. The emergence speed index was calculated according to Maguire [80]:

$$\text{ESI} = E_1/N_1 + E_2/N_2 + \dots + E_n/N_n \quad (1)$$

where ESI = emergence speed index; E_1, E_2, \dots, E_n = number of normal seedlings obtained at the first, second, and at the nth counting; and N_1, N_2, \dots, N_n = number of days from sowing to the first, second, and nth counting.

The register of the number of plantlets with abnormalities, with necrotic cotyledons, and infected with SSR, as well as shoot and root lengths (cm) and fresh and dry weights (g), is very important in this case or evaluation. Standard germination test followed a randomized design with four replications of 50 seeds each, whereas the seedling emergence on sand test was carried out in a completely randomized block design with four replicates of 200 seeds each. The treatments consisted of the four biological products and a control (without the antagonist) inoculated or not with *S. sclerotiorum*.

2.9 In vivo biocontrol of *S. sclerotiorum* and biological control of soybean SSR under field conditions

After laboratory and greenhouse experiments, we conducted a field study at a commercial soybean crop geo-referenced at $19^\circ 12' 54''\text{S}$ and $47^\circ 56' 58''\text{W}$, 947 m of altitude, during the summer season (from December/2009 to April/2010). Climatological data [maximum and minimum temperatures ($^\circ\text{C}$), relative air humidity (%), and pluvial precipitation (mm)] were obtained from the weather station located at the farm. Soil was classified as a ferralsol, and the field had previous report of SSR occurrence. Sowing was done with 15 seeds per linear meter using soybean cultivar BRS Valiosa RR (susceptible to SSR) at a final stand of 10 plants m^{-1} . Crop conduction was according to Embrapa [81]. Experimental design was in random blocks with seven treatments and a control (**Table 3**), with four replications. Each plot consisted of six rows of 5 m length and 0.5 m apart totalizing an area of 480 m^2 . The four central rows despising 0.5 m from both edges were considered as the useful plot. Spraying was done with a CO_2 pressurized costal sprayer equipped with XR110.02 nozzles at a volume of 200 L ha^{-1} . Environmental conditions were constantly monitored during application of the (bio)products ranging from 27.2 to 34.3°C , 47 to 65% of relative air humidity, and winds of 0 to 5 km h^{-1} . The titer of the *Trichoderma*-based products was calibrated by the viability test in PDA medium (viable conidia mL^{-1}) after incubation at $25 \pm 2^\circ\text{C}$ for 5 days. SSR incidence and severity on soybean plants were evaluated at phenological stages R_4 , $R_{5,2}$, and $R_{5,5}$ [56]. The data were used to calculate the disease index (% incidence

× % severity) [82] and the area under the disease progress curve (AUDPC) [83]. The severity of SSR was estimated by a visual scale [82] assigning percentages of 5–90% of the symptoms of the disease in individualized soybean plants. Manual harvesting of the useful plots was carried out at the R₈ stage. The productivity was obtained after mechanical track of pods, followed by correction of the moisture content of grains to 12% and extrapolation of the data to kg ha⁻¹. After harvest, the sclerotia were separated from the grains with the aid of sieves and their weight (g) determined per hectare.

Trichoderma species are potential biocontrol agents against a range of plant fungal pathogens. Some examples include *R. solani* [84], *F. oxysporum* f. sp. *melonis* [69], *Puccinia triticina* [85], *C. sublineolum* [70], and *P. graminis* [85]. Though plant-pathogen-*Trichoderma* interactions have been extensively studied even at the molecular level [86–88], most of the research are conducted under laboratory or greenhouse conditions. To evaluate the suppressive effect of *Trichoderma*-based products on SSR providing a means of controlling the disease, we applied the bioproducts in a commercial soybean field. The efficacy of the biological products was compared to chemical fungicides frequently used in soybean crop. Chemicals were evaluated alone or associated to *Trichoderma* spp. AUDPC values were lower in treatments with the fungicide fluazinam and with thiophanate-methyl applied in sequence to the use of strain ESALQ-1306 (Table 6). A possible synergistic effect is suggested upon association of biological and chemical products once their spraying alone led to averages statistically equal to the control (without spraying). In this association, application of the antagonist first weakens the pathogen, and the fungicide kills it. This strategy allows rotation of active ingredients with different mechanisms of action in the field with potential reduction of resistant populations of *S. sclerotiorum*. The low control efficacy observed in the application of the chemical fungicide thiophanate-methyl alone requires attention to possible loss of sensitivity of the pathogen. A resistant *S. sclerotiorum* strain was first reported in common bean crop in Brazil in 2015 [23]. Resistance was conferred by a point mutation of a leucine by a phenylalanine at position 240 of the β -tubulin gene.

The use of *Trichoderma* spp. as exclusive control method was not enough to reduce the severity of SSR (Table 6). Regarding soybean grain yield, however, strains ESALQ-1306 and SF04 maintained productivity at high levels and increased

Treatments	AUDPC	INCID. (%)	AUDI	SCLE. (g)	TGW (g)	YIELD (kg ha ⁻¹)
Control	909.4 B	25.5 B	20130.0 B	6.6 A	127.6 B	1942.5 C
SF04	685.0 B	9.2 A	5296.9 A	2.4 A	147.2 A	2890.0 A
IBLF006	766.9 B	10.8 A	7319.4 A	5.8 A	136.4 B	2523.3 B
ESALQ-1306	651.2 B	9.8 A	5005.0 A	2.2 A	145.8 A	2897.5 A
Tricho	656.9 B	12.2 A	5877.5 A	4.6 A	144.5 A	2452.5 B
ESALQ-1306 + Tm	532.5 A	10.5 A	4258.8 A	2.5 A	146.1 A	2765.0 A
Tm	658.1 B	12.8 A	6690.6 A	3.4 A	143.3 A	2945.0 A
F	440.6 A	7.5 A	2620.6 A	1.9 A	151.5 A	3015.0 A

AUDPC, area under the disease progress curve; INCID, disease incidence; AUDI, area under the disease index (% incidence × % severity); SCLE, sclerotia weight; TGW, a thousand grain weight averages followed by different uppercase letters, in columns, are statistically different by the Scott-Knott test ($p \leq 0.05$).

Table 6.
Control of *Sclerotinia* stem rot in soybean crop due to foliar spraying of *Trichoderma*-based products and chemical fungicides.

by 1.5-fold over that of the untreated control. The same result was not accomplished by Tricho or IBLF006, which presented intermediate yields, partially justified by inconsistencies in spraying due to their formulation (WP). The mixture of solid in aqueous medium is not stable and demands constant agitation to remain homogeneous, which may have been compromised by the application equipment we adopted. Biocontrol agents and chemical fungicides or their association were not able to completely prevent recurrence of the disease in the field, since they did not completely reduce the formation of sclerotia in only one year of field management. Results from our in vitro assays, though, suggest that the sclerotia were not viable in the plots treated with the bioproducts. This assumption may be supported by sclerotia weight which was one- to threefold lower compared to untreated control. Those values represent 1.9–4.9 kg of sclerotia ha⁻¹ contrasting to 5.5 kg ha⁻¹ observed in the control.

Disease symptoms were not attenuated in plants treated with the bioproducts; however, they showed significant reduction in SSR incidence and lower disease index. Incidence is the most important parameter when it comes to SSR field evaluation. Disease index estimates the damage to the plant both by the number of diseased plants (incidence) and by the lesion length (severity) [82]. Application of bioproducts alone reduced the index by 64–75%. It is important to mention that biological control does not promote total eradication of phytopathogens but the maintenance of the population at levels enough not to cause economic damages to the crop. In our study, this was reflected by the productivity increase of up to 35 bushels ha⁻¹ in relation to the untreated control, an income of US\$297 ha⁻¹.

3. Conclusions

In conclusion, we report the use of *Trichoderma* as a soybean seedling growth promoter and as a biological control agent of SSR acting synergistically to thiophanate-methyl. We found strains with in vitro hyperparasitic activity and capable of killing sclerotia. After foliar application on soybean crop, *Trichoderma* strains start soil parasitism preventing ejection of ascocarps and ascospores, as observed in vitro through sclerotia germination and colonization, leading to reduced disease incidence under field conditions. As a result, higher grain yield is achieved. Chemical fungicides may be used simultaneously with bioproducts upon dose reduction (this aspect is yet to be demonstrated). Formulation should be approached with caution during bioproducts' development as it interferes in viability and efficacy. For example, the strain ESALQ-130 showed in vitro antagonism similar to that of other strains tested. However, its formulation (CS) may have favored seeds and field applications. Despite problems on technology application of WP, suspensions are not always stable during storage, as particles may sediment and form a two-phase system that no longer resuspend. Many environmental factors interact with *Trichoderma*-based products affecting their efficacy under field conditions. We encourage that laboratory and greenhouse studies proceed to the field to confirm disease control. Quality of biological products is a threshold to efficacy and must be constantly monitored. There are still gaps in obtaining new formulations, selecting potent strains, evaluating adequate application technologies, and accessing *Trichoderma* spp. performance in cold soils (temperatures lower than 15°C). Further research should be conducted aiming at improving *Trichoderma* recommendation in agriculture, regarding dosage for different crops, intervals, and application timing.

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Conflict of interest


There is no conflict of interest in this paper.

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