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INDUCTION OF SOYBEAN RESISTANCE MECHANISMS TO ANTHRACNOSE BY BIOCONTROL AGENTS[1]

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ABSTRACT - The biological control, thinking about the integrated management, has been inserted with other management techniques to disease control, such as soybean anthracnose. The aims of this work were to verify the action of *Trichoderma* and *Bacillus* isolates in the induction of soybean resistance mechanisms to anthracnose as a function of seed treatment. The statistical design was entirely randomised, in a 5 x 2 (agent species x sampling times) factorial scheme with five replicates. Soybean seeds were treated with *Bacillus amyloliquefaciens* BV03, *B. subtilis* BV02, *Trichoderma asperellum* BV10, Carbendazim + Thiram and distilled water (control). Seven days after seedling emergence, 2 µL of 1 x 10^{-4} *Colletotrichum truncatum* spores were inoculated on the cotyledons. Catalase (CAT), peroxidase (POX), phenylalanine ammonia lyase (PAL) and glyceollin (GLY) activities before and after pathogen inoculation, as well as the diameter of the anthracnose lesion on the cotyledons, were evaluated. Data were submitted to analysis of variance and, when significant, the mean values were compared by Fisher's test ($p < 0.05$). The treatments did not influence the first sampling time before inoculation. *Trichoderma asperellum* BV10 increased POX and PAL activities up to 173%, while *B. amyloliquefaciens* BV03 increased POX activity. Glyceollin was not influenced by the treatments. The *T. asperellum* BV10 reduces the diameter of the anthracnose lesion by up to 61%. Thus, *T. asperellum* BV10 has the potential to control soybean anthracnose, improved the response defense against *C. truncatum*, when performed on seed treatment.

Keywords: *Colletotrichum truncatum*. *Glycine max.* Resistance induction.

INDUÇÃO DE MECANISMOS DE RESISTÊNCIA DE SOJA À ANTRACNOSE COM AGENTES DE BIOCONTROLE

RESUMO – O controle biológico, pensando no manejo integrado, tem sido inserido em conjunto com outras técnicas de manejo de doenças, como antracnose da cultura da soja. O objetivo deste trabalho foi verificar a ação de isolados de *Trichoderma* e *Bacillus* na indução de mecanismos de resistência da soja à antracnose. O delineamento estatístico foi inteiramente casualizado em esquema fatorial 5 x 2 (agentes de controle biológico x horários de coleta) com cinco repetições. Sementes de soja foram tratadas com *Trichoderma asperellum* BV10, *Bacillus subtilis* BV02, *B. amyloliquefaciens* BV03, Carbendazim + Thiram e água destilada (testemunha). Sete dias após a emergência foi realizada a inoculação de *Colletotrichum truncatum* sobre os cotilédones com 2 µL de suspensão esporos (5 x 104 mL^{-1}) sobre os cotilédones. A atividade de catalase (CAT), peroxidase (POX), fenilalanina amônia liase (FAL), gliceolina (GLI) foram avaliadas antes e após a inoculação do patógeno. Os dados foram submetidos à análise de variância e as médias comparadas pelo teste de Fisher ($p<0,05$). Os tratamentos não influenciaram o primeiro horário de coleta. O *T. asperellum* BV10 incrementou a atividade de POX e FAL em até 173%. *B. amyloliquefaciens* BV03 aumentou a atividade de POX. A GLI não foi influenciada pelos tratamentos em nenhum horário de coleta e ensaio. *T. asperellum* BV10 reduziu até 61% do diâmetro da lesão de antracnose. O tratamento de sementes de soja com *T. asperellum* BV10 possui potencial no controle da antracnose, aumentando a resposta de defesa de plântulas à *C. truncatum*.

Palavras chaves: *Colletotrichum truncatum*. *Glycine max*. Indução de resistência.

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INTRODUCTION

Soybean is a food source rich in proteins for both humans and animals, and is an important crop in world agribusiness, transforming Brazil into one of the two largest oilseed producers, along with the United States of America, generating jobs and income in producing regions, through the long and organized production chain (CATTELAN; DALL'AGNOL, 2018; CONAB, 2020).

Among the limiting factors of soybean production, phytopathological losses are considered important, and can be caused by viruses, bacteria, nematodes, and fungi, agents that cause diseases such as anthracnose, which together can damage up to 32% of crop production (ALMEIDA; FERREIRA; YORINORI, 2005; SAVARY; WILLOCQUET; PETHYBRIDGE, 2019).

Anthracnose, caused by the fungi *Colletotrichum truncatum*, *C. plurivorum*, *C. musicola*, and *C.* sojae (ROGÉRIO et al., 2020), is considered one of the most frequent and harmful, especially in the Brazilian Cerrado, which may cause loss of up to 100% when control measures are not adopted (DIAS, PINHEIRO; CAFÉ-FILHO, 2016; DIAS; DIAS-NETO; SANTOS, 2019; ROGÉRIO; GLADIEUX; MASSOLA JUNIOR, 2019). At high temperatures and humidity, especially in rainy years, the pathogen causes the failure of the seeds to germinate or reduces their vigor promoting seedling death, compromising the final soybean stand. It can also affect petioles and leaf ribs, in addition to stems and pods at any phenology stage of the crop, causing necrosis. Severe seed abortion, loss of pods and grain deterioration are responsible for the highest losses in culture when climatic conditions are favorable to the development of the pathogen (ALMEIDA; FERREIRA; YORINORI, 2005;; DIAS, PINHEIRO; CAFÉ-FILHO, 2016; ROGÉRIO; CIAMPI-GUILLARDI,; BARBIERI, 2016; BORAH, 2019; NATARAJ et al., 2020).

The main control strategies for soybean anthracnose are the use of healthy seeds, crop rotation, limiting plant population to reduce microclimate formation, proper soil management, balanced fertilization, seed treatment, chemical treatment with chemical or biological agents (GODOY; ALMEIDA; SOARES, 2014; SILVA; SANTOS; AMARAL, 2020). Seed treatment and aerial spraying with fungicides are the main measures to control soybean anthracnose (PEREIRA; OLIVEIRA; ROSA, 2009; PESQUEIRA; BACCHI; GAVASSONI, 2016). However, isolated control measures lose efficiency in the short or long term, such as chemical control, with the recorded appearance of populations of *C. truncatum* resistant to the fungicide Carbendazim, used in seed treatment (GODOY; BUENO; GAZZIERO, 2015; POTI; MAHAWAN; CHEEWANGKOON, 2020). Despite the efficiency of chemical treatment, other tools should be included as an alternative for managing this phytopathogen for successful integrated disease management. An example is induced resistance in plants.

Innate immune resistance has been reported as an important defense mechanism against diseases and can be triggered during pathogen-host interaction and/ or when induced by eliciting agents that may be of microbial origin, such as biocontrol agents (DUBERY; SANABRIA; HUANG, 2012; KUSHALAPPA; YOGENDRA; SHAILESH, 2016; LOLLE; STEVENS; COAKER, 2020; WANG et al., 2020). During the immune response, the plant activates mechanisms that include peroxidase, catalase, polyphenoloxidase, phenylalanine ammonia lyase, and phytoalexins activity (DUBERY; SANABRIA; HUANG, 2012; YANG; CAO; RUI, 2017).

In induced resistance, the elicitor plays a key role in controlling diseases and can be classified as a biotic due to its source material, such as plant extracts, algae, bacteria, and fungi (DUBERY; SANABRIA; HUANG, 2012; THAKUR; SOHAL, 2013; XING et al., 2015; SOLINO et al., 2016; MALIK, KUMAR; NADARAJAH, 2020). When dealing with bacteria and fungi, the microbial genera *Bacillus* and *Trichoderma* have been used for plant disease biocontrol as well as to increase agricultural crop productivity (BETTIOL; MORANDI, 2009; TAHIR; GU; WU, 2017; GLICK, 2015; PENHA et al., 2020). In this context, the objective of this study was to evaluate the induction of soybean plant resistance mechanisms to anthracnose by biocontrol agents through seed treatment.

MATERIALS AND METHODS

The assays were conducted at the phytopathology laboratory of the University of Rio Verde (UNIRV). The *Colletotrichum truncatum* isolate CPA002 was supplied by the company *Campos Pesquisa Agrícola*, kept on potato dextrose agar (PDA) growth medium at 25 ± 2 °C and 12 hours light photoperiod until sporulation and used to prepare the spore suspension for inoculation. DNA extraction and SSR genotyping were previously carried out by *Campos Pesquisa Agrícola* to confirm the identification of *C. truncantum* isolates. Further, morphological structures were analyzed by microscope to confirm the purity of the material.

The bacterium *Bacillus subtilis* (type BV-02), registered as the Ministério da Agricultura e Pecuária e Abastecimento - MAPA: 43418, is a commercial product (c. p.) with a concentration suspension formulation of 3×10^9 CFU mL^{-1}. The bacterium *Bacillus amyloliquefaciens* (type BV-02), registered as MAPA: 34518, has a c. p. concentration suspension formulation of 3×10^9 CFU mL^{-1}. The fungus *Trichoderma asperellum* (type BV-10), registered as MAPA: 34018, has a c. p. concentration

suspension formulation of 1×10^{10} viable conidia mL^{-1}.

The trials were carried out in a completely randomized design, in a 5 x 2 factorial arrangement design with five replicates. Factor A was represented by three species of biocontrol agents, namely *T. asperellum* (1 mL c. p. kg $seed^{-1}$, Tricho-Turbo®), *B. subtilis* (2 mL c.p. kg $seed^{-1}$, Bio-Immune®), and *B. amyloliquefaciens* (2 mL c. p. kg $seed^{-1}$), in addition to a control treatment (distilled water) and a chemical fungicide, Carbendazim (150 g L^{-1}) + Thiram (350 g L^{-1}) at the concentration 200 mL c.p. 100 kg of $seeds^{-1}$. Factor B corresponded to the sampling times before (0 hrs) and after (24 hours) pathogen inoculation. The samples were evaluated destructively considering 25 replicates per treatment. Thereby, five replicates were used to measure glyceollin at the first sampling time (before inoculation) and five replicates for the second sampling time (after inoculation). A similar procedure was carried out for enzymatic activity quantification. The anthracnose severity was analyzed in the last 5 replicates.

The microorganisms *B. amyloliquefaciens* (BV-03) and *B. subtilis* (BV-02) were identified and characterized by Empresa Brasileira de Pesquisa Agropecuária – Embrapa and deposited in the "Diazotrophic and Plant Growth Promotion Rhizobacteria Culture Collection of Embrapa Soja", Word Federation Culture Collection #1213, and World Data Center for Microorganisms #1054, respectively. Sequence analysis of the 16S RNA region was performed by BOX-PCR. Strain BV-02 (CNPSo 3219) was classified as the *B. subtilis* operational group within the *B. subtilis* species complex and strain BV-03 (CNPSo 3418) was classified as the *B. amyloliquefaciens* operational group within the *B. subtilis* species complex.

The fungus *T. asperellum* (BV-10) was identified and characterized by the Fungus Detection and Identification Service of the Fungi Systematics and Ecology Laboratory, Department of Phytopathology DFP of the Federal University of Lavras. The filamentous fungus sample, type BV–10, was submitted to microscopic preparation and phylogenetic analysis of the RPB2 gene region (second-largest subunit of RNA polymerase), and grouped with the reference isolate of *Trichoderma asperellum* species, with a posterior probability of 100% and a bootstrap value of 72%. The isolate was deposited at the institution under the code CML 3751.

Soybean seeds were packaged in a 100 g container and coated to avoid excessive moisture uptake, using 600 mL of commercial product per 100 kg of seeds (LUDWIG; LUCCA FILHO.; BAUDET, 2011). Subsequently, the IPRO 7739 treated soybean seeds were sown in a 250-mL polyethylene container with a mixture of sand and commercial organic fertilizer, in the proportion of 1:1, respectively.

Seven days after sowing, seedlings with the first pair of leaves and extended cotyledons were inoculated with 2 µL of a 5 x 10^4 mL^{-1} spore suspension of *C. truncatum*; subsequently, the plants were kept in a partially controlled relative humidity chamber for 72 h (adapted from WRATHER; EROLD, 1990).

The evaluated variables were anthracnose severity in soybean cotyledons, catalase, peroxidase, phenylalanine ammonia lyase activity, and glyceollin accumulation.

To assess the size of anthracnose lesions in soybean cotyledons, the lesion diameter (ALD) was measured using images of the lesions and processed by ImageJ® version 1.8.0 software, seven days after inoculation and data were expressed in cm. For enzymatic quantification, cotyledons were collected 7 days after sowing, before *C. truncatum* inoculation (first sampling time) and 24 hours after pathogen inoculation (second sampling time). The samples were weighed, immediately placed in aluminum foil envelopes and stored at -20 °C.

Enzyme extract was obtained by macerating and homogenizing plant tissue samples in 4 mL of 0.01 M sodium phosphate buffer (pH 6.0), followed by centrifugation at 6,500 *g* for 30 minutes at 4°C. The supernatant was collected and considered the enzymatic extract; it was used in the determination of total protein content and for the enzymatic activity determination of peroxidase, catalase, and phenylalanine ammonia lyase (FAN et al., 2011).

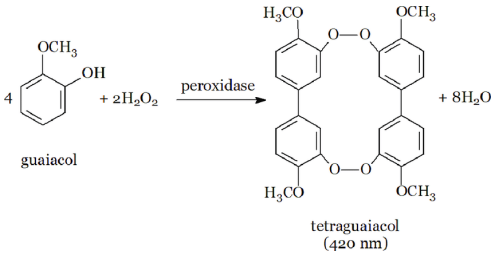
Total protein quantification was performed through the Bradford method (1976). Briefly, absorbance values were plotted on a standard curve of bovine serum albumin (BSA) concentrations, and protein concentrations were expressed in µg protein mL^{-1}.

Catalase activity (CAT) was quantified *via* Goth's method (1991), modified by Tománková et al. (2006). The determination was performed using the direct spectrophotometric method at a wavelength of 405 nm, and the results were expressed in µmol min^{-1} mg^{-1} protein. The reaction that determines CAT activity in the oxidative process is shown as Reaction (1):

$$\underset{\text{Hydrogen Peroxide}}{2\,H_2O_2} \xrightarrow{\text{Catalase}} \underset{\text{Water}}{2\,H_2O} + \underset{\text{Oxygen}}{O_2} \qquad (1)$$

Guaiacol peroxidase (POX) activity was quantified by spectrophotometry at 470 nm, and the

results were expressed in absorbance min^{-1} mg^{-1} protein (LUSSO; PASCHOLATI, 1999). The reaction that determines POX activity in oxidative process is shown as Reaction (2):



(2)

Phenylalanine ammonia lyase (PAL) activity was determined by colorimetric quantification of the trans-cinnamic acid released from the phenylalanine substrate. Absorbance was determined at 290 nm, and enzymatic activity was expressed in µg of trans-cinnamic acid h^{-1} mg^{-1} protein (UMESHA, 2006).

For glyceollin build-up determination (GLY), cotyledons were collected at 7 days after sowing, before inoculation (6 plants and 12 cotyledons), and 24 hours after inoculation (6 plants and 12 cotyledons). Cotyledons were kept in Petri® dishes on filter paper moistened with distilled water. The plates were capped and kept in the dark at 26°C. After 20 hours, the cotyledons were removed from the plates, cut in half and added into a Falcon tube containing 5 mL of distilled water and stirred for 1 hour. The extracted glyceollin was measured by spectrophotometry at 285 nm (AYERS et al., 1976). Results were expressed in unit absorbance per gram of fresh weight (ABS gfw^{-1}).

The assays were performed twice. The obtained data were subjected to analysis of variance (ANOVA), and joint analyses were performed between the assays. For significant ANOVA results, data were run in a factorial scheme and means were compared by Fisher's test ($p < 0.05$).

RESULTS AND DISCUSSION

Anthracnose severity in seedling cotyledons receiving *T. asperellum* seed treatment was 61% and 24% lower than that of the control in the first and second experimental assays, respectively. The other treatments did not differ from the control (Figure 1).

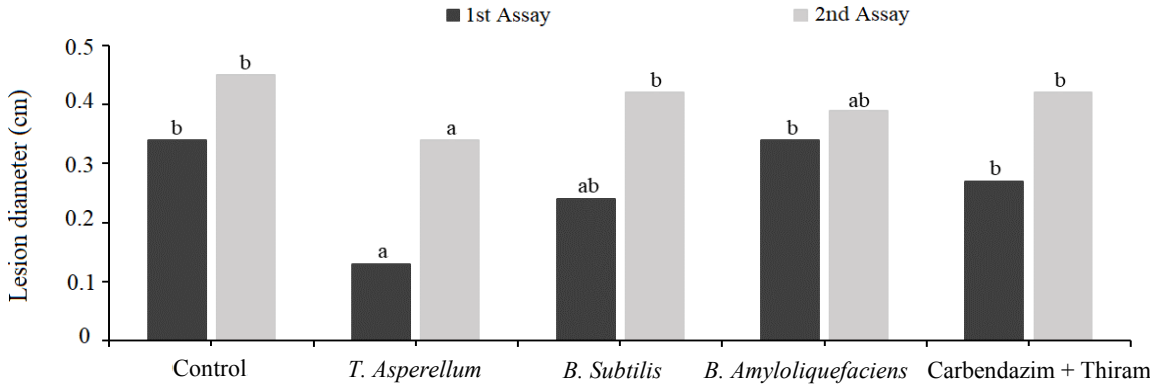


Figure 1. Diameter of anthracnose lesions in soybean cotyledons in assay I and assay II treated with biocontrol agents 7 days after *C. truncatum* inoculation. *T. asperellum* = *Trichoderma asperellum* BV10 (Tricho-Turbo®); *B. subtilis* = *Bacillus subtilis* BV02 (Bio-Immune®). Means followed by the same lowercase letter over the bar do not significantly differ from each other by the Fisher test ($p < 0.05$).

When analyzing the CAT data (Table 1), it was noted that enzyme activity was not influenced by the treatment, a fact evidenced by the absence of significant differences between treatments and control at the first sampling time (prior to inoculation with *C. truncatum*) for both assays performed (I and II). Enzyme activity evaluated 24 hours after pathogen inoculation (second time) was also unchanged in assay I, but in assay II, CAT activity was lower in the *B. subtilis* BV02 treatment compared to the control. Despite the non-significant difference by Fisher's test in assay I, it is possible to observe that the value obtained before incubation with *C. truncatum* (0.57) was higher compared to the time after incubation with the pathogen (0.26), similar to assay II. The fact of not detecting significant differences is related to the higher random error obtained in assay I.

When analyzing sampling times within the treatments, it was observed that the seeds treated with *B. subtilis* promoted reduction of CAT activity at the second sampling time in assays I and II. In assay II, there was a reduction in CAT activity at the second sampling time compared to the first from the control treatment (Table 1).

POX activity did not differ as a function of seed treatment (first time) for both assays (I and II), except for the fungicide treatment in assay II, which promoted a reduction of seed enzyme activity in relation to the control (Table 2). At the second time

(after inoculation), seed treatment with *T. asperellum* BV10 promoted a 173% and 126% increase in POX activity when compared to control, in assays I and II, respectively.

There was an increase in POX activity from the second sampling time compared to the first time, in assays I and II, when treating soybean seeds with *T. asperellum* BV10 (Table 2). The *B. amyloliquefaciens* BV03 treatment promoted an increase of POX at the second sampling time compared to the first, in assay II.

Table 1. Catalase specific activity (CAT) in assay I and assay II treated with biocontrol agents 7 days after *C. truncatum* inoculation.

Treatment	Assay I			Assay II		
	1st time	2nd time	Mean	1st time	2nd time	Mean
	µmol min^{-1} mg^{-1} protein					
Control	0.55 aA	0.47 aA	0.51	4.85 aA	3.97 abB	4.41
Trichoderma asperellum	0.56 aA	0.48 aA	0.52	4.52 aA	4.48 aA	4.50
Bacillus subtilis	0.57 aA	0.26 aB	0.42	4.52 aA	3.57 bB	4.05
Bacillus amyloliquefaciens	0.56 aA	0.45 aA	0.50	4.94 aA	4.16 abA	4.55
Carbendazim + Thiram	0.55 aA	0.48 aA	0.47	4.43 aA	3.95 abA	4.19
Mean	0.56	0.41		4.65	4.03	
CV (%)	42					

Trichoderma asperellum = *Trichoderma asperellum* BV10 (Tricho-Turbo®); *Bacillus subtilis* = *Bacillus subtilis* BV02 (Bio-Immune®). Means followed by the same lowercase letter in the column and uppercase letter in the row do not significantly differ from each other by the Fisher test ($p < 0.05$).

Table 2. Specific guaiacol peroxidase (POX) activity in assay I and assay II treated with biocontrol agents 7 days after *C. truncatum* inoculation.

Treatment	Assay I			Assay II		
	1st time	2nd time	Mean	1st time	2nd time	Mean
	Absorbance min^{-1} mg^{-1} of protein					
Control	1.62 aA	1.85 bA	1.74	4.78 aA	5.56 bcA	5.17
Trichoderma asperellum	1.17 aB	3.21 aA	2.19	4.50 aB	7.03 aA	5.76
Bacillus subtilis	1.58 aA	1.89 bA	1.73	4.28 aA	4.40 cA	4.34
Bacillus amyloliquefaciens	1.39 aA	2.39 bA	1.80	4.11 abB	6.68 abA	5.40
Carbendazim + Thiram	1.27 aA	1.75 bA	1.46	2.98 bB	5.45 bcA	4.22
Mean	1.39	2.22		4.13	5.82	
CV (%)	52			19		

Trichoderma asperellum = *Trichoderma asperellum* BV10 (Tricho-Turbo®); *Bacillus subtilis* = *Bacillus subtilis* BV02 (Bio-Immune®). Means followed by the same lowercase letter in the column and uppercase letter in the line do not significantly differ from each other by the Fisher test ($p < 0.05$).

When PAL specific activity was quantified (Table 3), it was noticed that the seeds treated with *T. asperellum* BV10 showed an increase in enzyme activity in the first hour, by 65%, in relation to the control in assay I. In the second assay, PAL activity increased by 137% in response to the treatment of soybean seeds with *B. amyloliquefaciens BV03* compared to the control, not differing from *T. asperellum* BV10.

When analyzing the unfolding of sampling times within the treatments, increased PAL activity was observed in all treatments at the second sampling time when compared to the first, in assay I (Table 2). In assay II, the treatments with *B. amyloliquefaciens* BV03 and *T. asperellum* BV10 promoted an increase in PAL at the second time when compared to the first sampling time.

When quantifying glyceollin (GLY) accumulation in soybean cotyledons, phytoalexin accumulation in response to seed treatment alone was lower than in the control in response to *B. amyloliquefaciens* BV03 in the first hour of assays I

and II (Table 4). The GLY accumulation was not influenced by sampling time in assay I, that is, even after inoculation, there were no stimuli for phytoalexin synthesis. However, in assay II, there was a reduction in GLY accumulation at the second sampling time when compared to the first in all treatments, except for *B. amyloliquefaciens* BV03, when no difference was observed between the first and second sampling times (Table 4).

Table 3. Specific activity of phenylalanine ammonia lyase (PAL) in assay I and assay II treated with biocontrol agents 7 days after *C. truncatum* inoculation.

Treatment	Assay I			Assay II		
	1st time	2nd time	Mean	1st time	2nd time	Mean
	μg of trans-cinnamic acid h^{-1} mg^{-1} of protein					
Control	0.44 aB	5.12 bA	2.78	0.45 aA	0.61 bA	0.53
Trichoderma asperellum	0.28 aB	8.49 aA	4.38	0.39 aB	0.75 abA	0.57
Bacillus subtilis	0.29 aB	3.56 bA	1.92	0.0 aA	0.34 bA	0.37
Bacillus amyloliquefaciens	0.26 aB	5.10 bA	2.68	0.45 aB	0.84 aA	0.65
Carbendazim + Thiram	0.20 aB	4.71 bA	2.46	0.47 aA	0.61 bA	0.54
Mean	0.29	5.40		0.43	0.63	

Trichoderma asperellum = *Trichoderma asperellum* BV10 (Tricho-Turbo®); *Bacillus subtilis* = *Bacillus subtilis* BV02 (Bio-Immune®). Means followed by the same lowercase letter in the column and uppercase letter in the line do not significantly differ from each other by the Fisher test ($p < 0.05$).

Table 4. Glyceollin (GLY) accumulation in assay I and assay II treated with biocontrol agents 7 days after *C. truncatum* inoculation.

Treatment	Assay I			Assay II		
	1st time	2nd time	Mean	1st time	2nd time	Mean
	ABS g fw^{-1}					
Control	1.92 abA	1.68 aA	1.80	2.03 aA	0.90 aB	1.46
Trichoderma asperellum	2.11 abA	1.68 aA	1.90	2.24 aA	1.29 aB	1.76
Bacillus subtilis	2.29 aA	1.88 aA	2.08	1.95 aA	1.20 aB	1.58
Bacillus amyloliquefaciens	1.61 bA	1.67 aA	1.64	1.56 bA	1.41 aA	1.48
Carbendazim + Thiram	1.99 abA	1.57 aA	1.78	2.13 aA	1.27 aB	1.70
Mean	1.98	1.70		1.98	1.21	
CV (%)	24			17		

Trichoderma asperellum = *Trichoderma asperellum* BV10 (Tricho-Turbo®); *Bacillus subtilis* = *Bacillus subtilis* BV02 (Bio-Immune®). Means followed by the same lowercase letter in the column and uppercase letter in the line do not significantly differ from each other by the Fisher test ($p < 0.05$).

Table 5 shows, in green, the results that are higher than the control. Notably, *T. asperellum* BV10 was the treatment that stood out regarding the induction of resistance mechanisms and anthracnose control, as it promoted an increase in POX activity at the second sampling time (24 hours after *C. truncatum* inoculation) in assays I and II. Also, *T. asperellum* BV10 promoted an increase in PAL at the second sampling time in the assay, in addition to reducing the size of the anthracnose lesion diameter.

Treatment with *T. asperellum* BV10, *B. subtilis* BV02, *B. amyloliquefaciens* BV03 and Carbendazim + Thiram did not influence the activity of antioxidant enzymes, CAT and POX, and a key enzyme pf the metabolism of phenylpropanoids, PAL in seedling cotyledons 7 days after germination, not yet inoculated with *C. truncatum* (first sampling time), indicating that they do not continuously or systemically activate defense mechanisms in soybean seedling cotyledons when seed treatment is performed, which favors the use of biocontrol agents, since from the point of view of inducing resistance , the continuous activation of defense mechanisms generates an energy cost to the plant (MATYSSEK, et al., 2012).

Table 5. Response of the variables catalase (CAT), guaiacol peroxidase (POX), phenylalanine ammonia lyase (PAL), glyceollin (GLY), and anthracnose lesion diameter (ALD) compared to the control in assay I and assay II treated with biocontrol agents 7 days after *C. truncatum* inoculation.

Treatment	Assay I								
	Analyzed variables								
	CAT		POX		PAL		GLY		ALD
	1st T	2nd T	1st T	2nd T	1st T	2nd T	1st T	2nd T	
Trichoderma asperellum									
Bacillus subtilis									
Bacillus amyloliquefaciens									
Carbendazim + Thiram									

Treatment	Assay II								
	Analyzed variables								
	CAT		POX		PAL		GLY		ALD
	1st T	2nd T	1st T	2nd T	1st T	2nd T	1st T	2nd T	
Trichoderma asperellum									
Bacillus subtilis									
Bacillus amyloliquefaciens									
Carbendazim + Thiram									

Trichoderma asperellum = *Trichoderma asperellum* BV10 (Tricho-Turbo®); *Bacillus subtilis* = *Bacillus subtilis* BV02 (Bio-Immune®); yellow color = did not differ from the control; red color = results were lower than the control; green color = results were higher than the control.

In the second sampling time, 24 hours after the inoculation of *C. truncatum*, an increase of 173% and 126% of the POX enzyme was observed, in assays I and II, respectively, in addition to the 65% increase in FAL, after inoculation of the pathogen, in assay I, when applying the treatment of soybean seeds with *T. asperellum* BV10. The enzyme activity increase after inoculation of the pathogen did not stop the infection by *C. truncatum*, however, it suggests a containment of the fungal disease, because there was a reduction of 61% and 24% in the diameter of the anthracnose lesion in assays I and II, respectively.

The genus *Trichoderma* has been described as a potential biocontrol agent for plant pathogens in agriculture, such as the genus *Colletotrichum* (BEGUM et al., 2008; JAGTAPA; GAVATEA; DEYA, 2012; KUCHLAN; KUCHLAN; ANSARI, 2019). This acts through mechanisms of competition for space and nutrients, antibiosis, parasitism, and induction of the defense mechanisms of the plant against pathogens (CONTRERAS-CORNEJO et al., 2020; SOOD et al., 2020; VINALE; SIVASITHAMPARAM, 2020).

When triggering the plant defense response against pathogens, the genus *Trichoderma*, acts by preventing the formation of the infection or slowing its growth, through the accumulation of reactive oxygen species (ROS), phenols, phytoalexins, pathogeneses related to enzymes, oxidative cycle enzymes, phenylpropanoids cycle enzymes, increased thickness, density, and incorporation of secondary compounds, such as phenols in the cell wall, or programmed death of tissue adjacent to infection caused by pathogens (CONTRERAS-CORNEJO et al., 2020; SOOD et al., 2020; VINALE; SIVASITHAMPARAM, 2020). This response can be intensified as this biocontrol agent can act by altering carbohydrate metabolism and photosynthesis activities, physiological activities in enzymes related to plant growth and development (PÉREZ-GARCÍA; VICENTE, 2011; JAHAN et al., 2015; PASCHOLATI; DALIO, 2018; MILJAKOVI´C; MARINKOVI´C; BALEŠEVI´C-TUBI´C, 2020).

Among the biochemical reactions observed when treating soybean seeds with *T. asperellum* BV10 and later inoculating with *C. truncatum*, there is an increase in the activity of POX, which is an important biochemical mechanism in disease control (NASERINASAB; SAHEBANI; ETEBARIAN, 2011; SINGH et al., 2011; YOUSSEF; TARTOURA; ABDELRAOUF, 2016; MOHAPATRA; MITTRA, 2017). This enzyme is involved in the hydrogen peroxide (H_2O_2) dismutation reaction in water and oxygen, in this way, reducing the excess of reactive oxygen species (ROS), formed during oxidative stress caused by the pathogen is recognize of the plant (KUSHALAPPA; YOGENDRA; SHAILESH, 2016; YANG; CAO; RUI, 2017). Thus, the increase of POX, as observed in the treatment of soybean seeds with *T. asperellum*

BV10, in this work, suggests that it might cause an indirect increase in H_2O_2, since this is the main substrate of the enzyme.

The accumulation of ROS's, such as H_2O_2, are reported in reactions in incompatible interactions between plants-pathogen, that is, resistance of the plant, when it accumulates at sites of infection, activating localized cell death and induction of defense genes in adjacent cells (LEVINE et al., 1994; MEHDY, 1994). In addition, peroxidase is also involved in catalyzing the oxidation of various substrates such as phenolic compounds and lignin precursors, along with hydrogen peroxide reduction (COSIO; DUNAND, 2009).

The role of peroxidases in lignin polymerization is the most widely studied function of this class of enzymes leading to lignin formation and subsequent cell wall deposition, which will later act in the defense of the plant against pathogens (VANHOLME et al., 2010; THAKUR; SOHAL, 2013; KUSHALAPPA; YOGENDRA; SHAILESH, 2016; BEGOVIĆ et al., 2017), which may explain the delay in the development of anthracnose damage when using seed treatment with *T. asperellum* BV10, in this work.

The increase in phenylalanine ammonia lyase activity observed after *C. truncatum* inoculation (second time of assay I and the trend on the second time of assay II) and potentiated in seedlings that received *T. asperellum* BV10 seed treatment may also be involved in containing the growth of the pathogen lesion. The transient increase of the activity of phenylalanine ammonia lyase is related to acquired and induced resistance (MAUCH-MANI; SLUSARENKO, 1996; SMITH-BECKER et al., 1998; SHINE et al., 2016). The enzyme is a precursor to the phenylpropanoid pathway, originating several other compounds from benzoic acid, coumarins, precursors of lignin, ammonia, and others, used by plants for their defense (LORENZETTI et al., 2021).

Considering the increase in POX and PAL observed after the pathogen inoculation in seedlings from seeds treated with *T. asperellum* BV10 observed in this work, the consequent reduction in the diameter of anthracnose lesion in soybean cotyledons may have occurred in the first response due to the accumulation of ROS's and later deposition of lignin and phenols on the cell wall, mainly on the secondary walls, which become thicker and denser, giving resistance to pathogen attack (MIEDES et al., 2014; PASCHOLATI; DALIO, 2018).

CONCLUSIONS

Treatment of soybean seeds with *Trichoderma asperellum* BV10 promoted a reduction of anthracnose lesion diameter under controlled conditions.

The application of *T. asperellum* BV10 activated the latent defense mechanisms, promoting increased guaiacol peroxidase and phenylalanine ammonia lyase activities after inoculation with *C. truncatum*.

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REFERENCES

ALMEIDA, A. M. R.; FERREIRA, L. P.; YORINORI, J. T. et al. Doenças da soja (*Glycine max).* In: KIMATI, H.; AMORIM, L.; REZENDE, J. A. M. (Eds.). **Manual de Fitopatologia**: doenças das plantas cultivadas. São Paulo, SP: Agronômica Ceres, 2005. v. 1, cap.64, p. 569-588.

AYERS, A. R. et al. Host-pathogen interactions. IX. Quantitative assays of elicitor activity and characterization of the elicitor present in the extracellular medium of cultures of *Phytophthora megasperma* var. *sojae*. **Plant Physiology**, 57: 751-759, 1976.

BEGOVIĆ, L. et al. Involvement of peroxidases in structural changes of barley stem. **Bragantia**, 76: 352-359, 2017.

BEGUM, M. M. et al. Antagonistic Potential of Selected Fungal and Bacterial Biocontrol Agents against *Colletotrichum truncatum* of Soybean Seeds. **Pertanika Journal of Tropical Agricultural Science**, 31: 45-53, 2008.

BETTIOL, W.; MORANDI, F. A. B. **Biocontrole de doenças de plantas**: uso e perspectivas. Jaguariúna, SP: Embrapa Meio Ambiente, 2009. 341 p.

BORAH, M. Identification of soybean diseases in Assam. **International Journal of Recent Scientific Research,** 10: 34154-34159, 2019.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, 72: 248-254, 1976.

CATTELAN, A. J.; DALL'AGNOL, A. The rapid soybean growth in Brazil. **OCL**, 25: 1-12, 2018.

CONAB - Companhia Nacional De Abastecimento. **Acompanhamento da safra brasileira de grãos -**

safra 2019/20, Brasília, DF: Conab, 2020. 73 p.

CONTRERAS-CORNEJO, H. A. et al. Interactions of *Trichoderma* with plants, insects, and plant pathogen microorganism: chemical and molecular bases. In: MÉRILLION, J. M.; RAMAWAT, K. G. (Eds.) **Co-evolution of secondary metabolites**. Cham, Zug: Springer Nature Switzarland AG, 2020. p. 263-290.

COSIO, C.; DUNAND, C. Specific functions of individual class III peroxidase genes. **Journal of Experimental Botany**, 60: 391-408, 2009.

DIAS, M. D.; DIAS-NETO, J. J.; SANTOS, M. D. M. et al. Current status of soybean anthracnose associated with *Colletotrichum truncatum* in Brazil and Argentina. **Plants,** 8: 1-19, 2019.

DIAS, M. D.; PINHEIRO, V. F.; CAFÉ-FILHO, A. C. Impact of anthracnose on the yield of soybean subjected to chemical control in the north region of Brazil. **Summa Phytopathologica**, 42: 18-23, 2016.

DUBERY, I. A.; SANABRIA, N. M.; HUANG. J. C. Nonself perception in plant innate immunity. In: LOPES-LARREA, C. (Ed.). **Self and nonself**. New York, NY: Springer-Verlag New York Inc, 2012. Cap. 6, p. 79-107.

FAN, H. et al. Antiviral activity and mechanism of action of novel thiourea containing chiral phosphonate on tobacco mosaic virus. **International Journal of Molecular Sciences**, 12: 4522-4535, 2011.

GLICK, B. R. **Beneficial plant-bacterial interactions**. Cham, Zug: Springer Nature Switzerland, 2015. 243 p.

GODOY, C. V.; ALMEIDA, A. M. R.; SOARES, R. M. et al. **Doenças da Soja**. Londrina, PR: Embrapa Soja, 2014. 32 p.

GODOY, C. V.; BUENO, A. F.; GAZZIERO, D. L. P. Brazilian Soybean Pest Management and Threats to its Sustainability. **Outlooks on Pest Management**, 26: 113-117, 2015.

GOTH, L. A simple method for determination of serum catalase activity and revision of reference range. **Clinica Chimica Acta**, 196: 143-151, 1991.

JAGTAPA, G. P.; GAVATEA, D. S.; DEYA, U. Control of *Colletotrichum truncatum* causing anthracnose/pod blight of soybean by aqueous leaf extracts, biocontrol agents and fungicides**. Scientific Journal of Agriculture**, 1: 39-52, 2012.

JAHAN, A. F. et al. Biological control of anthracnose of soybean. **Research in Agriculture Livestock and Fisheries**, 2: 419-426, 2015.

KUCHLAN, P.; KUCHLAN, M. K.; ANSARI, M. M. Efficient application of *Trichoderma viride* on soybean [*Glycine max* (L.) Merrill] seed using thin layer polymer coating**. Legume Research - An International Journal**, 42: 260-264, 2019.

KUSHALAPPA, A. C.; YOGENDRA, K. N.; SHAILESH, K. Plant Innate Immune Response: qualitative and quantitative resistance. **Critical Reviews in Plant Sciences**, 35: 38-55, 2016.

LEVINE, A. et al. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. **Cell**, 79: 583-593, 1994.

LOLLE, D. S.; STEVENS, D.; COAKER, G. Plant NLR-triggered immunity: from receptor activation to downstream signaling Signe. **Current Opinion in Immunology**, 62: 99-105, 2020.

LORENZETTI, E. et al. Indução de fitoalexinas gliceolina e proteínas relacionadas à defesa em cotilédones de soja tratado com leveruras. **Acta Iguazu**, 10: 48-57, 2021.

LUDWIG, M. P.; LUCCA FILHO, O. A.; BAUDET, L. Qualidade de sementes de soja armazenadas após recobrimento com aminoácido, polímero, fungicida e inseticida. **Revista Brasileira de Sementes**, 33: 395-406, 2011.

LUSSO, M. F. G.; PASCHOLATI, S. F. Activity and isoenzymatic pattern of soluble peroxidases in maize tissues after mechanical injury or fungal inoculation. **Summa Phytopathologica**, 25: 244-249, 1999.

MALIK, N. A. A.; KUMAR, I. S.; NADARAJAH, K. Elicitor and receptor molecules: orchestrators of plant defense and immunity. **International Journal Molecular Sciences**, 21: 1-34, 2020.

MATYSSEK, R. et al. **Growth and defense in plants**: resource allocation and multiple scales. Berlin, GER: Springer, 2012. 757 p.

MAUCH-MANI, B.; SLUSARENKO, A. Production of salicylic acid precursors is a major function of phenylalanine ammonia lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. **Plant Cell**, 8: 203-212, 1996.

MEHDY, M. C. Active oxygen species in plant defense against pathogens. **Plant Physiology**, 105: 467-472, 1994.

MIEDES, E. et al. The role of the secondary cell

wall in plant resistance to pathogens. **Frontiers in Plant Science**, 5: 1-13, 2014.

MILJAKOVI´C, D.; MARINKOVI´C, J.; BALEŠEVI´C-TUBI´C, S. The significance of bacillus spp. in disease suppression and growth promotion of field and vegetable crops. **Microorganisms**, 8: 1-19, 2020.

MOHAPATRA, S.; MITTRA, B. Alleviation of *Fusarium oxysporum* induced oxidative stress in wheat by *Trichoderma viride*. **Archives of Phytopathology and Plant Protection**, 50: 84-96, 2017.

NASERINASAB, F.; SAHEBANI, N.; ETEBARIAN, H. R. Biological control of *Meloidogyne javanica* by *Trichoderma harzianum* BI and salicylic acid on tomato. **African Journal of Food Science**, 5: 276-280, 2011.

NATARAJ, V. et al. Genetic inheritance and identification of germplasm sources for anthracnose resistance in soybean [*Glycine max* (L.) Merr.]. **Genetic Resources and Crop Evolution,** 67: 1449-1456, 2020.

PASCHOLATI, S. F.; DALIO, R. J. D. Fisiologia do parasitismo: como os patógenos atacam as plantas. In: BEGAMIN FILHO, A.; AMORIM, L.; REZENDE, J. A. M. (Eds.). **Manual de fitopatologia: princípios e conceitos**. Ouro Fino, MG: Agronômica Ceres, v. 2, 5. ed. 2018. cap. 34, p. p. 424-450.

PENHA, R. O. et al. *Bacillus* lipopeptides as powerful pest control agents for a more sustainable and healthy agriculture: recent studies and innovations. **Planta**, 251: 1-15, 2020.

PEREIRA, C. E.; OLIVEIRA, J. A.; ROSA, M. C. M., et al. Tratamento fungicida de sementes de soja inoculadas com *Colletotrichum truncatum*. **Ciência Rural**, 39: 2390-2395, 2009.

PÉREZ-GARCÍA, D. R.; VICENTE, A. Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. **Current Opinion in Biotechnology**, 22: 187-193, 2011.

PESQUEIRA, A. S.; BACCHI, L. M. A.; GAVASSONI, W. L. Associação de fungicidas no controle da antracnose da soja no Mato Grosso do Sul. **Ciência Agronômica**, 47: 203-202, 2016.

POTI, T.; MAHAWAN, K.; CHEEWANGKOON, R. et al. Detection and molecular characterization of carbendazim-resistant *Colletotrichum truncatum* Isolates causing anthracnose of soybean in Thailand. **Journal of Phytopathology**, 168: 267-278, 2020.

ROGÉRIO, F. et al. Genome sequence resources of *Colletotrichum truncatum*, *C. plurivorum*, *C. musicola*, and *C. sojae*: four species pathogenic to soybean (*Glycine max*). **Phytopathology**, 110: 1497-1499, 2020.

ROGÉRIO, F.; CIAMPI-GUILLARDI, M.; BARBIERI, M. C. G. Phylogeny and variability of *Colletotrichum truncatum* associated with soybean anthracnose in Brazil. **Journal of Applied Microbiology**, 122: 402-415, 2016.

ROGÉRIO, F.; GLADIEUX, P.; MASSOLA JUNIOR, N. S. et al. Multiple introductions without admixture of *Colletotrichum truncatum* associated with soybean anthracnose in Brazil. **Phytopathology,** 109: 681-689, 2019.

SAVARY, S.; WILLOCQUET, L.; PETHYBRIDGE, S. J. The global burden of pathogens and pests on major food crops. **Nature Ecology & Evolution**, 1: 1-10, 2019.

SHINE, M. B. et al. Cooperative functioning between phenylalanine ammonia lyase and isochorismate synthase activities contributes to salicylic acid biosynthesis in soybean. **New Phytologist**, 212: 627-636, 2016.

SILVA, H. F.; SANTOS, A. M. G.; AMARAL, A. C. T. et al. Bioprospection of *Trichoderma* spp. originating from a Cerrado-Caatinga ecotone on *Colletotrichum truncatum*, in soybean. **Revista Brasileira Ciência Agrária**, 15: 1-7, 2020.

SINGH, B. N. et al. *Trichoderma harzianum*-mediated reprogramming of oxidative stress response in root apoplast of sunflower enhances defense against *Rhizoctonia solani*. **European Journal of Plant Pathology**, 131: 121-134, 2011.

SMITH-BECKER, J. et al. Accumulation of salicylic acid and 4-hydroxybenzoic acid in phloem fluids of cucumber during systemic acquired resistance is preceded by a transient increase in phenylalanine ammonia-lyase activity in petioles and stems. **Plant Physiology**, 116: 231-238, 1998.

SOLINO, A. J. S. S. et al. Induction of defense mechanisms from filtrates of saprophytic fungi against early blight disease in tomato. **African Journal of Microbiology Research**, 10: 1849-1859, 2016.

SOOD, M. et al. *Trichoderma*: the "secrets" of a multitalented biocontrol agent. **Plants**, 9: 1-25, 2020.

TAHIR, H. A. S.; GU, L.; WU, H. Effect of volatile

compounds produced by *Ralstonia solanacearum* on plant growth promoting and systemic resistance inducing potential of *Bacillus* volatiles. **BMC Plant Biology**, 17: 1-16, 2017.

THAKUR, M.; SOHAL, B. S. Role of elicitors in inducing resistance in plants against pathogen infection: a review. **ISRN Biochemistry**, 1: 1-10, 2013.

TOMÁNKOVÁ, K. et al. Biochemical aspects of reactive oxygen species formation in the interaction between *Lycopersicon* spp. and *Oidium neolycopersici*. **Physiological and Molecular Plant Pathology,** 68: 22-32, 2006.

UMESHA, S. Phenylalanine ammonia lyase activity in tomato seedlings and its relationship to bacterial canker disease resistance. **Phytoparasitica**, 34: 68-71, 2006.

VANHOLME, R. et al. Lignin Biosynthesis and Structure. **Plant Physiology**, 153: 895-905, 2010.

VINALE, F.; SIVASITHAMPARAM, K. Beneficial effects of *Trichoderma* secondary metabolites on crops. **Phytotherapy Research**, 1: 1-8, 2020.

WANG, W. et al. Plant immune signaling: Advancing on two frontiers. **Journal of Integrative Plant Biology**, 62: 2-24, 2020.

WRATHER, J. A.; ELROD, J. M. Apperente systemic effect of *Colletotrichum truncatum* and *C. lagenarium* on the interaction between soybean and *C. truncatum*. **Phytopathology**, 80: 472-474, 1990.

XING, K. et al. Chitosan antimicrobial and eliciting properties for pest control in agriculture: a review. **Agronomy for Sustainable Development**, 35: 569-588, 2015.

YANG, J.; CAO, W.; RUI, Y. Interactions between nanoparticles and plants: phytotoxicity and defense mechanisms, **Journal of Plant Interactions**, 12: 158 -169, 2017.

YOUSSEF, S. A.; TARTOURA, K. A.; ABDELRAOUF, G. A. Evaluation of *Trichoderma harzianum* and *Serratia proteamaculans* effect on disease suppression, stimulation of ROS-scavenging enzymes and improving tomato growth infected by *Rhizoctonia solani*. **Biological Control**, 100: 79-86, 2016.