In vitro control of *Colletotrichum lindemuthianum* by *Trichoderma* spp. and *in vivo* with Alternative Products

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Abstract— *Beans (Phaseolus vulgaris) are extremely important because it is included daily in the diet of themajority of the Brazilian population. Several factors negatively affect the productivity of this crop, especially diseases.One of the main ones is anthracnose (Colletotrichum lindemuthianum), which can cause total production damage and depreciation of the final product. The study and development of new strategies for integrated anthracnose management can reduce the cost of production and consequently reduce environmental impacts. The objective of this work was to evaluate the efficiency of Trichoderma fungi in in vitro tests (antagonism, production of volatile and non-volatile compounds) for control of C. lindemuthianum and to evaluate the efficiency of endophytic fungi, salicylic acid, copper phosphite, acibenzolar-S-methyl (ASM) and fungicide for anthracnose control in greenhouse bean plants. Anthracnose was controlled in bean plants with the use of alternative products. The endophytic fungi Trichoderma viride and Trichoderma tomentosum inhibited C. lindemuthianum mycelial growth in the three in vitro tests. In greenhouse, T. viride, T. tomentosum, salicylic acid, ASM and fungicide were effective, but copper phosphite was not efficient in controlling anthracnose.*
Keywords— *anthracnose, Phaseolus vulgaris, salicylic acid, copper phosphite, acibenzolar-S-methyl, azoxystrobin + diphenoconazole.*

I. INTRODUCTION

The bean (*Phaseolus vulgaris* L.) is a plant originating from Latin America, grown mainly in tropical and subtropical regions of the globe. Beans are an important source of protein for populations of developing countries, especially for the lower income classes. [1].

This legume adapts to different edaphoclimatic conditions. However, this wide adaptability has favored the emergence of pests and especially diseases that compromise the productivity and quality of the final product[2].

Anthracnose caused by *Colletotrichum lindemuthianum* (Sacc. &Magn.) Lams. Scribis the main fungal disease of bean crop. It is a devastating disease in regions with moderate temperatures and high relative humidity, which can cause up to 100% damage to grain yield in highly susceptible cultivars and compromising seed quality[3].

The causative agent of anthracnose can cause symptoms in all organs of the plant shoot. In the stem and petiole, the lesions are depressed and dark and may deepen into the infected tissue if environmental conditions are favorable.

The most characteristic leaf symptoms appear on the abaxial face with darkening along the ribs. Rounded lesions, initially light brown in color, evolving to depressed and dark lesions with a lighter center are observed in the pods. When conditions are favorable, a pink mass develops in the lesions center due to fungus spore production[4].

Among the main methods for controlling anthracnose in beans are chemical control and use of resistant cultivars. However, chemical control with fungicides can cause soil, environmental and human contamination, while plant resistance in cultivars can be broken by the pathogen.

New alternatives for disease control are replacing the use of fungicides and contributing to modern and more sustainable agriculture to protect plants and maintain a less pesticide-dependent disease defense system [6]. Therefore, one of the possibilities of alternative control is the biological control and induction of defense mechanisms in plants [7].

The objective of this work was to evaluate the efficacy of alternative products applied in bean plants to control

anthracnose in greenhouse and to analyze antagonistic characteristics of endophytic fungi*Trichoderma viride*(Pers.) and*Trichoderma tomentosum*(Pers.) for control of*Colletotrichum lindemuthianum in vitro*.

II.   MATERIAL AND METHODS

The experiments were carried out at State University of Ponta Grossa(UEPG), located in Ponta Grossa - Paraná (Brazil), conducted in a laboratory and greenhouse.

In vitro experiments consisted of analyzes of the antagonistic effect and production of volatile and nonvolatile compounds of *T. viride*and*T. tomentosum*on*C. Lindemuthianum* growth.

For the antagonistic effectstudy, the paired culture technique was used in Petri dishes [8]. 5 mm diameter discs of endophytic and phytopathogenic fungi colonies previously grown in potato-dextrose-agar (PDA) medium were placed on opposite sides, equidistant, in plates containing PDA culture medium. Plates containing only the pathogen were the witness.

To evaluate the production of volatile metabolites, two Petri dishes containing PDA medium were used. In one was placed a culture disc of the pathogen and in another plate a culture disc of the antagonist. The plaque with the pathogen was superimposed on the plaque with the antagonist and these were wrapped in plastic wrap. The control consisted of a plate containing the pathogen overlaid with another plate containing only PDA medium[9].

The evaluation of nonvolatile metabolites production was performed by the cellophane paper method, which consists of transferring a colony disc from the antagonist to the center of Petri dishes containing PDA medium, overlaid with washed and sterilized transparent cellophane paper [9]. Seven days after of the antagonist transfer to cellophane paper surface, the adherent growth paper was removed from the plate and a pathogen colony disc was transferred to the plate'scenter. The control consisted of pathogen cultivation after cellophane removal, without previous antagonist overlap.

The three tests plates with colonies were kept in a BOD chamber at 25ºC±1. The design used was completely randomized with 3 treatments (*T. viride, T. tomentosum* and control) and 10 replicates for each test, in each Petri dish containing the colonies a repetition was considered. For all tests, daily ray measurements were performed on two diametrically opposed axes, with the aid of a millimeter ruler.

At the end of each experiment, the percentage of mycelial growth inhibition was calculated [10].

The obtained data were submitted to analysis of variance and the means compared by Tukey test at 5% probability. Data were transformed into arc sen $\sqrt{(x + 0.5) / 100}$ and the analyzes were performed with the aid of SASM-Agri statistical software[11].

The experiments were conducted in greenhouse and repeated twice. The first experiment began on October 27, 2017 and the second on April 20, 2018.

The cultivar BRS Esteio was used, with two seeds sown in each pot with capacity of 3 liters of soil with black earth soil, using MAP (mono ammonium phosphate) fertilizer, with a dose of 21 mg per pot. The experimental design used was randomized blocks with 7 treatments and 5 replications, two pots with one plant considered a repetition.

The treatments used were: suspension of *T. viride* conidia; suspension of *T. tomentosum* conidia; salicylic acid ($C_7H_6O_3$; 10 mmol L^{-1}); acibenzolar-S-methyl - ASM (Bion®; 25 g ha^{-1});azoxystrobin + diphenoconazole fungicide (Amistar Top®; 500 mL ha^{-1}); copper phosphite(N 11%; P_2O_5 22%; S 1.76%; Cu 4%; 1000 mL ha^{-1}) and control (sterile distilled water).

The treatments were sprayed when bean plants were in vegetative stage V3 (first developed trifoliate). For all treatments, 20 mL of syrup was applied to each plant with the aid of a hand sprayer.

The inoculation of the pathogen *C. lindemuthianum* conidial suspension was performed three days after the treatments application. For inoculum production, pod-like culture medium was used [12]. The plants were inoculated by spraying the conidia suspension and after the pots were placed in moist plastic transparent bags for 48 hours and kept in a greenhouse. [13].

The assessment of anthracnose severity began with the onset of the first leaf symptoms and was performed according to diagrammatic scale [14] with a five-day interval in cotyledonary leaves up to the fourth trifolium, totaling 10 evaluations. With severity data the area under the disease progress curve (AUDPC) was calculated [15]. The AUDPC values were subjected to analysis of variance and means compared by the Scott-Knott test at 5% probability, with the aid of SASM-Agri software. [11].

III.   RESULTS AND DISCUSSION

The evaluation period for mycelial growth of the fungus *Colletotrichum lindemuthianum* was seven days for the three tests, with colony size measured daily. Endophytic fungi (*T. viride* and *T. tomentosum*) affected the pathogenic fungus *C. lindemuthianum* in all tests.

For the test of antagonistic effect of endophytic fungi on the pathogenic fungus, it was found that there was

significant difference between the treatments used (Table 1). The fungus *T. tomentosum* provided higher percentage of mycelial growth inhibition of the pathogenic fungus, differing from *T. viride* and both differed from the control when evaluated daily. On average, endophytic fungi differed only from the control, with no differences between them.

It was observed that the highest inhibition percentage occurred on the third day of evaluation, with 83.25% for *T. viride* fungus and 80.0% for *T. tomentosum* fungus. However, on average there was no statistically significant difference between endophytic fungi.

After the third day, the percentage of inhibition of endophytic fungi on the pathogen decreases for both treatments by the seventh day evaluationsend. This can be explained by the fact that fungi of the genus *Trichoderma* have very accelerated mycelial growth rate (IVCM), whereas those of the genus *Colletotrichum* have lower IVCM. With accelerated IVCM, endophytic fungi had already occupied most of the Petri dish by the experimented.

In a study by Sharma et al. [16] also observed a reduction in growth of *Colletotrichum capsici*(Syd. & P. Syd.) and *Colletotrichum truncatum* (Schwein) by *T. harzianum* when the plating pairing test was performed, with no growth of endophytic fungus on pathogens.

In tests conducted by Sundaramoorthy and Balabaskar [17], the percentage of mycelial growth inhibition of*Fusarium oxysporum*f. sp. *lycopersici*((Sacc.) Snyder & Hansen) by the antagonism of different*Trichoderma* species by the paired cultivation method was only 39.12% when using *T. viride*, contrary to the results of this work.

In the susceptibility evaluation of *C. lindemuthianum* to volatile compounds produced by endophytic fungi, it was found that there were significant differences only between endophytic and control in both daily and final average evaluations (Table 2).

The highest percentage of mycelial inhibition of both endophytic fungi was observed on the second day of evaluation, in which *T. tomentosum* resulted in 78.57% and *T. viride* in 72.36% of pathogen inhibition.

In this test there was also a decrease in the percentage of inhibition over the days, as verified in the test for the antagonistic effect. These data confirm the greater antagonistic interaction for *C. lindemuthianum* inhibition found in pairing studies previously discussed.

Isaias et al. [18] evaluated the susceptibility of *Sclerotium rolfsii*(Sacc) and *Verticillium dahliae*(Kleb) to volatile and nonvolatile metabolites secreted by *Trichoderma* isolates and found that there was variation in the percentage of mycelial growth inhibition of these pathogens. The results show that there was a reduction of growth around 60% of the fungus *S. rolfsii*when submitted to some isolates only. For *V. dahlie*, all *Trichoderma* isolates showed approximately 80% inhibitory action.

No inhibition of mycelial growth of *Sclerotiniasclerotiorum*((Lib.) DeBary) was observed when *T. tomentosum*was used [19] suggesting that the volatile metabolites produced by this species have no effect on mycelial growth of the pathogenic fungus.

When analyzing the secretion of nonvolatile compounds by *Trichoderma* species, it was concluded that there was a high inhibition of apical pathogenic fungus on the second day of evaluation (Table 3), in which *T. tomentosum* and *T. viride* were similar (89.40 and 83.87% inhibition respectively).

From the third day there was a decrease in inhibition for both fungi tested. However, there were statistical differences between the fungi tested in daily evaluations, being the fungus *T. tomentosum* with higher inhibition percentages. Overall, no statistically significant differences were observed between the two endophytic fungi.

The results obtained by Isaias et al. [18] demonstrated that there is production of volatile and nonvolatile compounds by *Trichoderma* species that inhibited mycelial growth of pathogenic microorganisms, but such microorganisms may or may not be susceptible to the compounds. In the present work, it is evident that the fungus *C. lindemuthianum* is sensitive to volatile (Table 2) and non-volatile (Table 3) compounds produced by the *Trichoderma* species tested.

Raza et al. [20] found that nonvolatile compounds produced by *T. harzianum* SQR-T037 significantly inhibited mycelial growth of *Fusarium oxysporum* f. sp. niveum (Smith).

In another experiment, Joshi et al. [21] found that nonvolatile compounds produced by 33 different *Trichoderma* species inhibited around 40 - 55% mycelial growth of the fungus *Colletotrichum falcatum* (Went), the causal agent of sugarcane red rot.

Assessments of anthracnose severity in greenhouse began with the onset of the first symptoms at three days after pathogen inoculation in the first experiment and five days after inoculation in the second experiment, where severity was lower than in the first experiment.

In both experiments, symptoms were first observed in cotyledonary leaves, progressing to trifolium as the plant developed.

It was observed that in the first experiment (Table 4) all treatments were statistically equal and differed only from copper phosphite treatment, which presented higher AUDPC compared to the others even higher than the control.

These results contradict those obtained by Gadaga et al. [5], where the authors applied different phosphite formulations and evaluated the severity of anthracnose in greenhouse, resulting in all applied products causing disease reduction and lower AUDPC than the control.

For the second experiment, the plants that received the *T. viride* and *T. tomentosum* treatment presented the lowest severity (Table 4), being statistically different from other treatments. The treatments salicylic acid, acibenzolar-S-methyl and fungicide did not differ from each other but were statistically superior to the control and copper phosphite. Copper phosphite treatment presented higher AUDPC compared to others, as observed in the first experiment.

The ability of biocontrol agents to recognize and mediate molecular events in the presence of a potential host is of paramount importance for the deployment of their weapons against their predatory host. At the molecular level, *Trichoderma* spp. is known to exhibit different transcriptomic responses at different stages of interaction against its hosts[22].

In a greenhouse experiment to control anthracnose in beans, Dildey [23] using 21 *Trichoderma* isolates, observed that all isolates controlled the disease differing from the control. *Trichoderma strigosum* (IB 28/07) provided systemic protection of bean plants to *C. lindemuthianum* as a function of inoculum concentration used in a greenhouse. [24].

De Meyer et al. [25] applied *T. harzianum* T30 seven days before the inoculation of *Botrytis cinerea* (De Bary) Whetzel on beans and observed significant reductions in disease. The authors reported a 35% reduction in disease severity, and since they did not find the fungus on the leaves, they attributed the decrease in disease symptoms to antagonist-activated resistance induction.

The application of *T. viride* can elicit a series of defense responses in plants such as phenol accumulation, enzyme induction, lignin deposition, among others. The use of this endophytic fungus can be a promising alternative to chemical fungicides, minimizing environmental impact and ensuring plant disease control[26].

Plants have elaborate mechanisms of protection against pathogens, but when there is exogenous application of salicylic acid, these defense compounds tend to increase in order to restrict the spread of fungal, bacterial or viral infections through the hypersensitivity reaction [27]. This mechanism in turn can lead to acquired resistance, especially when provided in the early stages of culture[28]. In a study by Pittner [29], with two wheat cultivars in greenhouse, the application of *T. tomentosum*, salicylic acid and ASM resulted in a decrease in the severity of

brown spot (*Bipolarissorokiniana* (Sacc.) Shoemaker) in relation to the witness, the best result was achieved with the application of the fungicide (azoxystrobin + tebuconazole).

The ASM-based product has no fungistatic action like fungicides and develops a salicylic acid-like role in the signal transduction pathway that leads to plant-acquired systemic resistance against pathogens [30]. The decrease in disease severity due to use of this product is related to increased activity of enzymes that exert antimicrobial action and antioxidant protection[31].

In an experiment conducted in a greenhouse, Gontijo Neto et al. [32] observed that bean plants treated with ASM had only 10.30% lower severity than treatment where there was no control of anthracnose. Plants that were sprayed with fungicide (methyl thiophanate + epoxiconazole + piraclostrobin) achieved a 54.60% reduction in disease progress.

Control of common bacterial growth (*Xanthomonas axonopodis* pv. *phaseoli* (Xap.)) in beans using ASM and *Bacillus cereus* (Frank & Frank.) It was effective. The use of the chemical caused a reduction of 79% of the disease, whereas the bacteria used as biological control reduced the severity of the disease by only 37% [33].

Results achieved by Moraes; Maringoni and Lima [34] demonstrate that the application of ASM to bean plants in a greenhouse was inefficient both to induce resistance to curtobacterium wilt (*Curtobacteriumflaccumfaciens* pv. *Flaccumfaciens* (Hedges)) on susceptible cultivar (IAC Carioca), as to increase resistance levels in resistant cultivars (IAC Akytã and IAC Carioca Piatã). These results contradict those of this work, since the product applications resulted in median control of anthracnose, which was statistically equal to the best treatment (*T. viride*).

IV. CONCLUSION

Alternative productscontrolled anthracnose in bean plants.

The endophytic fungi *T. viride* and *T. tomentosum* inhibited mycelial growth of *C. lindemuthianum* in the three *in vitro* tests.

In a greenhouse, *T. viride, T. tomentosum*, salicylic acid, acibenzolar-S-methyl and fungicide treatments were effective, but copper phosphite was not efficient in controlling anthracnose.

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Table 1 - Inhibition (%) of mycelial growth on the antagonism of fungi Trichoderma viride and Trichoderma tomentosum exerted on the fungus Colletotrichum lindemuthianum.

Treatments	Evaluation Days						
	2nd	3rd	4th	5th	6th	7th	Mean
*C. lindemuthianum*x *T. tomentosum*	73,47 a[1]	80,00 a	78,71 a	75,61 a	67,42 a	61,94 a	72,86 a
*C. lindemuthianum*x *T. viride*	65,89 b	83,25 a	71,37 b	63,35 b	58,30 b	54,68 b	66,14 a
Control	0,00 c	0,00 b	0,00 c	0,00 c	0,00 c	0,00 c	0,00 b
C.V. (%)[2]	11,20	16,65	8,89	6,54	6,23	7,14	12,00

(1) Means with the same letter in the column do not differ significantly by Tukey (*p*>0.05).

(2) Coefficientofvariation.

Table 2 - Inhibition (%) of mycelial growth on the production of volatile compounds by the fungi Trichoderma viride and Trichoderma tomentosum exerted on the fungus Colletotrichum lindemuthianum.

Treatments	Evaluation Days						
	2nd	3º	2nd	5º	2nd	7º	2nd
*C. lindemuthianum*x *T. tomentosum*	78,57 a[1]	70,14 a	54,18 a	47,91 a	40,71 a	39,71 a	55,20 a
*C. lindemuthianum*x *T. viride*	72,36 a	65,11 a	52,55 a	47,40 a	39,95 a	39,00 a	52,73 a
Control	0,00 b	0,00 b	0,00 b	0,00 b	0,00 b	0,00 b	0,00 b
C.V. (%)[2]	15,69	21,81	20,01	20,89	20,09	20,37	22,66

(1) Means with the same letter in the column do not differ significantly by Tukey (*p*>0.05).

(2) Coefficientofvariation.

Table 3 - Inhibition (%) of mycelial growth on the production of non-volatile compounds by the fungi Trichoderma viride and Trichoderma tomentosum exerted on the fungus Colletotrichum lindemuthianum.

Treatments	Evaluation Days						
	2nd	3º	2nd	5º	2nd	7º	2nd
C. lindemuthianum x *T. tomentosum*	89,40 a[1]	89,42 a	87,20 a	79,13 a	72,31 a	67,70 a	80,86 a
C. lindemuthianum x *T. viride*	83,87 a	81,42 b	81,30 b	74,27 b	69,50 b	65,29 b	75,94 a
Control	0,00 b	0,00 c	0,00 c	0,00 c	0,00 c	0,00 c	0,00 b
C.V. (%)[2]	20,33	12,23	4,15	2,85	2,36	2,60	11,64

(1) Means with the same letter in the column do not differ significantly by Scott-Knott ($p>0.05$).

(2) Coefficientofvariation.

Table 4 –Area under the progression curve of Colletotrichum lindemuthianum in bean plants (Phaseolus vulgaris) under greenhouse conditions, first and second experiments.

Treatment	1st Experiment	2nd Experiment
Trichoderma viride	166,56 b[1]	23,63 d
Trichoderma tomentosum	193,53 b	30,25 d
Salicylic Acid	229,63 b	41,20 c
Acibenzolar-S-methyl (Bion®)	212,66 b	38,85 c
Azoxystrobin + difenoconazole (Amistar Top®)	267,01 b	40,23 c
Copper phosphite (Strong®)	389,68 a	81,28 a
Control	200,89 b	53,13 b
C.V. (%)[2]	20,59	17,82

(1) Means with the same letter in the column do not differ significantly by Scott-Knott ($p>0.05$).

(2) Coefficientofvariation.