Avanços Científicos e Tecnológicos em Bioprocessos

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APRESENTAÇÃO

Avanços Científicos e Tecnológicos em Bioprocessos é uma obra que reúne vinte e três capítulos com temas em pesquisas científicas realizadas no campo da biotecnologia, e que envolve agentes biológicos e bioquímicos na geração de produtos ou processos. Nesta obra se concentram diversos avanços descritos nas metodologias e nos resultados, distribuídos em quatro tópicos principais, envolvendo: processos químicos e biotecnológicos no aproveitamento de resíduos; produção de metabólitos e enzimas; métodos analíticos e de simulação; e biotratamentos envolvidos na geração de energias. Esta obra foi escrita por jovens pesquisadores brasileiros que estão desenvolvendo suas teses e/ou dissertações em instituições nacionais. Por este motivo, os aspectos inovadores e o alcance dos resultados apresentados podem ser um grande estímulo para aqueles que visam conhecer com maior amplitude alguns dos aspectos biotecnológicos estudados em algumas das instituições de nosso país.

Alberdan Silva Santos

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CAPÍTULO 10

BIOPRODUCTS FROM *Trichoderma harzianum* AS INDUCER OF RESISTANCE TO ANTHRACNOSE IN BEANS

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RESUMO: *Trichoderma harzianum* é um organismo de controle biológico com ação em vários patossistemas. No entanto, sua eficácia é afetada por problemas na formulação dos produtos e interações com a planta ou patógeno. Diante disso, o objetivo deste estudo foi comparar a indução de resistência e controle de antracnose em feijão (*Phaseolus vulgaris*) causada por células vivas de *Trichoderma harzianum* e também pelos metabólitos produzidos na fermentação submersa. Os bioprodutos foram aplicados via tratamento de sementes e também via aplicação foliar. Avaliações realizadas em plantas apresentaram aumento significativo na atividade específica da peroxidase e β-1,3-glucanase após a inoculação do patógeno, indicando a ativação da resistência induzida. As respostas de indução não afetaram o acúmulo de plantas de massa seca. O uso de *T. harzianum* na forma de esporos vivos ou filtrado de cultura reduziu a severidade da doença e tem o potencial de controlar a antracnose do feijoeiro e/ou integrar programas de manejo de doenças.
PALAVRAS-CHAVE: *Phaseolus vulgaris,* β-1,3-glucanase, Peroxidase, Controle Biológico

ABSTRACT: *Trichoderma harzianum* is a biological control organism with action in several pathosystems. However, its effectiveness is affected by problems in the formulation of products and interactions with the plant or pathogen. Taking this into consideration, the aim of this study was to compare the control and the induction of resistance to anthracnose in beans (*Phaseolus vulgaris*) caused by living cells of *Trichoderma harzianum* and

also by the metabolites produced in submerged fermentation. The bioproducts were applied via seed treatment and also via foliar application. Evaluations carried out in plants presented significant increase in specific peroxidase and in β-1,3-glucanase activities after pathogen inoculation, indicating the activation of induced resistance. The induction responses did not affect the accumulation of dry massin plants. The use of *T. harzianum*in the form of living organisms or filtrate culture reduced the disease severity and has the potential to control the bean anthracnose and/or integrate disease management programs.
KEYWORDS: *Phaseolus vulgaris,* β-1,3-glucanase, Peroxidase, Biological control

1 I INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is an important agricultural crop characterized as asource of protein and fiber for human consumption, making it one of the most consumed legumes worldwide. The most important consumers regionally are South America (9.3 kg/per capita/year), the Caribbean (9.1 kg/per capita/year), Central America (8.8 kg/per capita/year) and Middle Africa (8.0 kg/per capita/year) (LUNA-VITAL et al., 2015).

Despite the importance of the beans for world agriculture, farmers fail to realize maximum potential yields because of several limiting factors, among them the incidence and severity of diseases. Anthracnose is one of the most common diseases in this crop, caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) Scrib. (CHIORATO et al., 2006). It is a seed-borne disease and complete crop failure can be expected under heavy infection, due to the use of infected seeds and favourable climatic conditions for the pathogen growth (PINTO et al., 2010).

The control of the anthracnose is achieved mainly by treating soil and plants with fungicides that present many drawbacks. On the other hand, available fungicides usually have lower efficacy when weather conditions favor the pathogen growth and therefore the disease progress. (CHALFOUN et al., 2011). One alternative is the use of microorganisms with the capacity to elicit induction of systemic resistance against plant diseases. In this way, *Trichoderma* spp. has acting in resistance induction in several pathosystems (TEIXEIRA et al., 2012; PEDRO et al., 2012).

The induction of resistance may be related to changes in protein synthesis. There is a set of proteins related to the interaction of *T. harzianum*with pathogenic fungi. Pereira et al., (2014) conducted a study of proteomics related to the presence or absence of *Rhizoctonia solani* or *Fusarium solani* on bean plants treated with *T. harzianum*, cataloguing proteins involved in the pathosystem. These authors demonstrated the existence of a differential expression pattern of genes related to plant defense system: chitinase, β-1,3-glucanase, peroxidase and lipoxygenase, with highest expression in the presence of *T. harzianum*.

The benefits obtained from the use of biological control are reiterated consecutively. However, responses are still variable and dependent on the plant x pathogen x organism interaction. In addition, there are numerous obstacles related to the use of bioproducts (MACHADO, 2012) as well as difficulties of mass production, contamination, formulations and stability, which could be circumvented with the use of more efficient bioprocesses. As the use of a living organism can induce systemic resistance, secondary metabolites produced by them in culture medium also may be used for the same purpose (VINALE et al., 2008; YOSHIOKA et al., 2012; VINALE, 2014).

However, there is few studies determining how the interaction of agricultural cultures with *R. solani* and *Trichoderma harzianum*and their secondary metabolites affect the plants growth and the level of expression of defense-related genes/proteins (SHARMA et al., 2017; PASCALE et al., 2017; MAYO et al., 2015). Based on this aspect, the aim of this study was to compare the control and the induction of resistance to anthracnose caused by living cells of *Trichoderma harzianum* and also by the metabolites produced in submerged fermentation.

2 I MATERIAL AND METHODS

2.1 Microorganisms

Trichodermil SC 1306®, a commercial product containing *Trichoderma harzianum* with $2x10^9$ viable conidia/mL as active ingredient was used as source of fungal agent. Avirulent isolate of *Colletotrichum lindemuthianum*, from the Mycology Collection of Plant Pathology Laboratory at Federal University of Santa Maria, GenBank accession number: BankIt1952792 COEJ KX845471, was used as pathogen.

2.2 Fermentations

Fermentations were carried out in Erlenmeyer flasks of 250-mL containing 50 ml of autoclaved (121°C for 20 min) liquid culture medium composed of sucrose 5 $g.L^{-1}$, corn steep liquor 15.0% (m/v) and yeast extract 10 $g.L^{-1}$ at pH 6.5. After cooling, each flask was inoculated using 1 mL of spore suspension of *T. harzianum*. Incubation was performed in a shaker (Innova 44R, New Brunswick) under constant stirring at 120 rpm, 28 ºC and with a photoperiod of 12 h for 96 h. After the end of fermentation, fermented broth was vacuum-filtered in membrane Millipore ® of 12 μm with to remove the fungal cells, and then immediately frozen for later analysis.

2.3 Biological activity of bioproducts under greenhouse conditions

In all tests were used *Phaseolus vulgaris* seeds from *Minuano* cultivar with

susceptibility to *Colletotrichum lindemuthianum*. Before the sown, the seeds were disinfested in 70% alcohol bath for 30 s, following by 1% sodium hypochlorite bath for 30 sand for three baths of 30 s each in distilled and sterile water. The seeds remained at room temperature for 2 h for drying on filter paper.

2.3.1 Induction of resistance

Four treatments were accomplished aiming to evaluate the induction to anthracnose resistance in beans. Treatments T1 and T2 consisted in the application of commercial product (Trichodermil® SC) in seeds and leaf, respectively, whereas T3 and T4 filtered culture broth without cells was applied in seeds and leaf, respectively.

For the seeds treatments (T1 and T3), a dose of 1 ml of commercial product or filtered culture broth for each 200 seeds was used. Plants were grown in pots of 3 L, with commercial substrate supplemented with complete nutrient solution and kept in a heated greenhouse during the whole experiment. A suspension of *C. lindemuthianum* spores at a concentration of 3.5 x 10^5 spores per mL was sprayed with a manual sprayer in plants when they began issuance of flower buds.

For foliar treatments (T2 and T4), 200 seeds without treatment were grown in pots of 3 L, with commercial substrate supplemented with complete nutrient solution and kept in a heated greenhouse during the whole experiment. When the reproductive stage started at the products were sprayed using a manual sprayer. The dose for commercial product was 10 mL per liter, whereas for culture filtrate was 400 ml per liter. A suspension of *C. lindemuthianum* spores at a concentration of 3.5 x 10^5 spores per mL was sprayed with a manual sprayer in plants when they began issuance of flower buds.

Two control tests were accomplished (C1 and C2). In C1, the seeds without treatment were cultivated and the pathogen inoculated as described above. In the C2, the seeds without treatment and free of pathogen were cultivated as described above.

Five days after inoculation (DAI) of pathogen, when the plants began to present the foliar disease symptoms, daily evaluations were carried out based on a diagrammatic scale adapted from Dalla Pria, Amorim and Bergamin Filho (2003), with rates from 1 (no disease symptoms) to 9 (very severe infection). Based on these evaluations, the area under the disease progress curve (AUDPC) was calculated (CAMPBELL; MADDEN, 1990), according to the following equation:

$$AUDPC = \sum\left[\left(\frac{Y_1 + Y_2}{2}\right) * (t_2 - t_1)\right]$$

Y_1: evaluation grade at time t1;

Y_2: Consecutive evaluation grade at time t2;

t_2 - t_1 the time elapsed between two consecutive evaluations.

Disease severity was evaluated on the tenth day after inoculation of pathogen (DAI). The same diagrammatic scale was used as well as a converted score in the index of disease (ID) of McKinney (MCKINNEY, 1923):

$$ID\ (\%) = \frac{\Sigma(Y * X_y)}{(X_t * h)} * 100$$

Y: note;

X_y: number of plants with this note;

X_t: total number of plants;

h: maximum value of the scale.

2.3.2 Enzymes related to induction of resistance

For biochemical analysis, entire plants were removed, separating the third pair of leaves. Leaf veins were removed for analysis. Roots were also collected for the determination of IAA-oxidase, prioritizing the primary root and older roots, removing by absorbent and secondary roots. All collected tissue was weighed and immediately frozen. The samples were stored in this condition until the completion of the analyses.

Peroxidase activity

For the determination of peroxidase activity, 0.2 g of frozen tissue was macerated with approximately 1 mg of polyvinylpyrrolidone plus 20 mL of 0.05 M phosphate buffer (pH 7.0). The homogenate was filtered, centrifuged at 6,000 rpm for 30 min and the precipitate was discarded. The entire procedure was carried out at maximum temperature of 4ºC. Peroxidase activity was determined using the methodology described by Campos et al. (2004). In tubes previously immersed in ice bath, 2.5 mL of phosphate-citrate buffer pH 5.0 (0.2 M dibasic sodium phosphate and 0.1 M citric acid), 1.5 mL of enzyme extract, 0.25 mL of 0.5% guaiacol and 0.25 mL of 3% H_2O_2 were mixed. Subsequently, they were mixed in vortex for 15 seconds. This mixture was incubated in water bath at 30°C for 15 min. After that, 0.25 mL of 2% sodium metabisulfite was added. The samples were mixed in vortex again and let to sit for 10 min. As a control for the enzyme reaction, the enzyme extract was replaced by water. The absorbance was read at 450 nm, in UV PC spectrophotometer Shimadzu. One enzyme unit was defined as the amount of enzyme extract that provided absorbance increase of 0.001 unit per minute (CAMPOS et al., 2004), expressed per gram of fresh tissueor per f mg of protein (specific activity).

β-1,3-glucanase activity

Determination of β-1,3-glucanase activity was performed by the method of Abeles;

Forrence (1970), modified by Campos et al. (2009). Frozen leaves were macerated at a temperature of 2°C in 0.1 M sodium citrate buffer (pH 5.4), containing 0.1% (v/v) β-mercaptoethanol and 0.1% (w/v) L-Ascorbic acid.The homogenate was centrifuged at 15,000 g for 30 min at a temperature from 0 to 4° C. The precipitate was discarded and 0.5 mL of supernatant was treated with 1 of mL chilled acetone at -20° C for 90 min. The suspension was centrifuged at 15,000 g for 30 min between -4 and 0° c. The sedimented precipitates were vacuum-dried for 48 h at room temperature, and resuspended in 62.5 μ L of 0.05 M sodium acetate buffer (pH 5.2). 0.1 mL of laminarin at 4% was added to this suspension, which was incubated at 40° C for 10 min. The reaction was stopped by the addition of μL 375 of 3,5-dinitrosalicylic acidto the mixture, followed by heating in boiling water bath for 5 min. Resulting solution was diluted with 4.5 mL of water and agitated in vortex. The absorbance readings were made at 500 nm in UV PC spectrophotometer Shimadzu. β-1,3-glucanase activity was expressed in nmol per mg of fresh tissue to observe the reaction speed, and specific activity was expressed in nmol per mg of protein.

IAA-oxidase activity

The preparation of enzyme extract to determine the IAA-oxidase activity, the methodology used was the one proposed by Saleh (1981) with modifications. To obtain the extract, 1 g of frozen root was macerated with the use of liquid nitrogen plus 2.5 mL of distilled water. The extract obtained was centrifuged at 30,000 g for 30 min. The entire process was carried out at a maximum temperature of 4ºC. Supernatant was used to determine the enzyme and the discarded precipitate. To determine IAA-oxidase activity, 0.5 mL of the extract prepared was supplemented with 0.5 mL of a solution containing 1 m*M* of IAA, 0.25 mL of 2.4 dichlorophenol (1 mM), 0.25 mL MnCl2 (1 Mm), and 1 mL of distilled water at 25°C. The enzyme-substrate was incubated for 60 min at a temperature of 25ºC. The reaction was interrupted with the addition of 2 mL of the mixture containing 1 ml of 0.5 m $FeCl_3$ in 50 mL of 35% $HClO_4$ and the reading was made in spectrophotometer (Shimadzu, model UV 1601) at 530 nm using standard curve (GORDON; WEBER, 1951). The enzyme activity was expressed as μmol of IAA degraded by g of fresh tissue during 60 min.

Total protein

Determination of protein was performed by the method of Bradford (BRADFORD, 1976) in the vegetable extract, which was prepared for peroxidase activity reading, using albumin as standard.

2.4. Statistical analysis

For each variable analyzed, the average was calculated and the data normality was verified through Kolmogorov-Smirnov test. Subsequently, the analysis of variance was performed through F-test at 95% probability level and the differences between the averages were compared through Scott-Knott test at 95% probability level, using the software SISVAR (FERREIRA, 2011).

3 I RESULTS AND DISCUSSION

Table 1 presents the results referring to induction of resistance to anthracnose in bean by the bioproducts of *Trichoderma harzianum*. Independent of bioproduct (living organism or culture filtrate) or form of application (leaf or seeds) induced the resistance to anthracnose. The disease severity and the disease progress didn't present significant difference ($p<0.05$) compared with control free of pathogen (C2). By other hand, control inoculated with pathogen (C1) increased the disease severity and progress, being statistically different from C2 and treatments T1-T4. The effects of the treatments were observed in the activity of peroxidase, β-1,3-glucanase,IAA-oxidase as well as in the protein levels before and after inoculation.

Application of bioproducts induces defense responses in bean plants by the increase of the peroxidase activity after the application of pathogen. Peroxidase is an important enzyme of plants and it is involved in many reactions, polysaccharides, oxidation of indole-3-acetic acid, monomers, lignification, wound healing, phenol oxidation, pathogen defense, regulation of cell elongation and others (ALMAGRO et al., 2009).

β-1,3-glucanase acts directly on the glucans in the cell wall of phytopathogenic fungi, inhibiting their development. The activity of β-1,3-glucanase was similar in all treatments and comparable to C2 (healthy plant). However, the levels of β-1,3-glucanase in the plants without any treatment (C1) reduced about 50%. Assessing capacity of response of the plants, determining β-1,3-glucanase activity, it is observed that even before the presence of the pathogen, the foliar application of bioproducts of *Trichoderma harzianum* increased the levels of this enzyme, and this remains even after inoculation of the pathogen, when plants have the highest levels. The application of the live microorganism via seed didn't yield a satisfactory response, while the culture filtrate applied via seed showed response after inoculation of the pathogen, evidencing the systemic and localized action in the induction of resistance.

Correlation analysis between AUDPC and specific activity of β-1,3-glucanase prior to pathogen inoculation was negative and statistically significant ($R = 0.75$), as well as the correlation between the disease index and specific activity of β-1.3-glucanase ($R = 0.87$). These results can be attributed to the progress reduction and disease severity by the stimulation of resistance induction by the fungus, thus inducing the activity of this

isoenzyme, hampering penetration and establishment of the pathogen.

The enzyme IAA-oxidase is responsible for IAA hormone oxidation, decreasing the levels of growth hormone in the root. The activity of this enzyme increased after pathogen inoculation, but only control C1 was statistically different from other treatments. This demonstrates that the treatments were effective to prevent the disease progress caused by *C. lindemuthianum*.

Protein levels increased, but no statistical difference was verified before and after pathogen inoculation. After a pathogen attack, the plant produces numerous defense responses, which include protein synthesis and relocation, often leading to energy expenditure which is able to retard plants growth and development (HEIL; BALDWIN, 2002). In a general way, a decrease in the fresh and dry mass of plants occur, due to great energetic requirements for the synthesis of proteins and enzymes. However, in this study, fresh and dry mass of plants at the beginning of infection was not affected (data not shown), even with the increase of protein levels and enzyme activities. One of the reasons may the fact that species of *Trichoderma* act as growth promoter increasing the fresh and dry mass of plants (CHACÓN et al., 2007) and seedlings (JUNGES, et al., 2015).

The use of culture filtrate of *T. harzianum* is considered a tool in the integrated management of diseases. This organism produces a great quantity and variety of compounds. These bioproducts may still be used in association with chemical fungicides and as resistance management tool (GONZALEZ et al., 2002), or they may replace them, considering the environmental and economic advantages that they provide (LIU et al., 2008).

In this study, a joint investigation of sequential applications in seeds and leaf was not carried out. However, based on the breadth of responses observed, it is expected that the effects may be cumulative and synergistic, indicating the benefits of management programs for anthracnose in bean based on organic products produced from isolates of *Trichoderma harzianum*.

4 I CONCLUSIONS

The resistance induction responses observed in this study showed that both living cells of *Trichoderma harzianum* and culture filtrate containing secondary metabolites were efficient in the induction of resistance to anthracnose in bean plants. The severity and disease progress were controlled with the bioproducts of *Trichoderma harzianum.* The levels of enzymes associated with the anthracnose induction as peroxidase and β-1,3-glucanase increased after the inoculation of pathogen. The induction of resistance to anthracnose in beans by living cells of *Trichoderma harzianum* is not a new, since this is well documented in literature. However, the main contribution of this study was demonstrate the positive effect of culture filtrate for this purpose. These results open

new possibilities in the development of natural products for modern agriculture.

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Treatment	AUDPC	Disease Index (%)	Protein		IAA oxidase		Peroxidase		β-1,3-glucanase	
			BI ($mg.g^{-1}$)	AI ($mg.g^{-1}$)	BI ($U.g^{-1}$)	AI ($U.g^{-1}$)	BI ($U.g^{-1}$)	AI ($U.g^{-1}$)	BI ($U.g^{-1}$)	AI ($U.g^{-1}$)
T1	4.1^{B}	15.2^{B}	14.24^{Aa}	11.99^{Ba}	0.46^{Ab}	1.05^{Ba}	58.44^{Ab}	113.01^{Ba}	6.21^{Aa}	8.83^{Aa}
T2	4.5^{B}	16.7^{B}	14.96^{Aa}	11.72^{Ba}	0.46^{Ab}	1.00^{Ba}	59.54^{Aa}	94.07^{Ba}	6.27^{Ab}	9.91^{Aa}
T3	4.0^{B}	14.0^{B}	15.64^{Aa}	18.6^{Aa}	0.48^{Aa}	0.69^{Ba}	81.35^{Ab}	153.3^{Aa}	8.61^{Aa}	9.87^{Aa}
T4	3.5^{B}	11.0^{B}	13.31^{Aa}	16.85^{Aa}	0.96^{Ab}	1.27^{Ba}	80.95^{Ab}	130.79^{Aa}	8.63^{Aa}	8.56^{Aa}
C1	7.9^{A}	30.2^{A}	13.23^{Ab}	19.13^{Aa}	0.78^{Ab}	1.83^{Aa}	75.67^{Aa}	72.4^{Ba}	7.99^{Aa}	4.39^{Bb}
C2	3.5^{B}	11.0^{B}	14.22^{Aa}	14.15^{Ba}	0.4^{Aa}	1.00^{Ba}	67.75^{Aa}	92.84^{Ba}	7.03^{Aa}	7.48^{Aa}

Table 1 - Evaluation of area below the disease progress curve (AUDPC), McKinney Disease Index (%) and profile of protein and excreted enzymes in bean treated with *Trichoderma harzianum* or secondary metabolites by seed or by foliar application.

BI and AI – Before and after inoculation of the pathogen *Colletotrichum lindemuthianum*, respectively;

Means followed by the same upper case letter in the column do not differ by the Scott Knott test at 5% probability;

Means followed by the same lower case letter in the room do not differ before and after inoculation of pathogen by the Scott Knott test at 5% probability;

SOBRE O ORGANIZADOR

ALBERDAN SILVA SANTOS é Professor associado das faculdades de Química e Biotecnologia da UFPA; É Engenheiro Químico graduado pela UFPA; É Mestre em Química e Biotecnologia pelo Instituto de Química e Biotecnologia da UFAL; É Doutor em Bioquímica (Biotransformações com ênfase em oxidações microbiológicas) pelo Instituto de Química da UFRJ. Realizou Estágio pós-doutoral no Departamento de Biotecnologia do Instituto de Agroquímica e Tecnologia de Alimentos - IATA de Valencia, na Espanha. Atua no ensino de graduação e Pós-graduação no qual orienta Mestrandos e Doutorandos. Coordena projetos de cunho acadêmico-científico nos Laboratórios de Investigação Sistemática em Biotecnologia e Biodiversidade Molecular da UFPA, em áreas estratégicas como: Biotransformações; produção de enzimas; desenvolvimento de processos biotecnológicos no aproveitamento de resíduos agroindustriais para a produção de biomoléculas de interesse médico, cosméticas e farmacêutica; produção de biomoléculas a partir de cultivo de micro-organismos e cultivo de células vegetais. Aplica técnicas avançadas de Metabolômica e Lipidômica (CG/EM, LC/MS) na investigação metabólica de plantas e micro-organismos. Contribuiu na criação do curso de graduação e do programa de pós-graduação em Biotecnologia da UFPA. Foi o 1º Diretor da Faculdade de Biotecnologia da UFPA no período de 2009-2011. Atuou como vice-coordenador protempore do Programa de Pós-graduação em Biotecnologia da UFPA. Possui diversas publicações nas áreas da Química e Biotecnologia, assim como patentes. Recebeu a primeira Carta Patente na UFPA em dezembro de 2013. É pioneiro na otimização de processo de produção de metabólitos secundários e enzimas em cultura de células vegetais e de micro-organismos na Região Norte do Brasil.

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