

ACCUMULATION OF PHYTOALEXINS IN BEANS, SOYBEANS AND SORGHUM BY FUNGAL FILTRATES¹

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ABSTRACT - The use of elicitors in plants for disease control is demonstrated by the accumulation of phytoalexins. Among these elicitors, metabolic products from fungi have been shown to be effective in the activation of mechanisms of induced resistance. Thus, this study aimed to evaluate the effects of filtrates from the saprobic fungi *Curvularia inaequalis*, *Pseudobotrytis terrestris*, *Memmoniella echinata* and *Curvularia eragrostidis* in inducing phaseolin, glyceolin and deoxyanthocyanidins. For this, fungal mycelial disks were liquid-subcultured in potato dextrose (BD) media and maintained in BOD at 25 °C and 12 h of light and darkness. After 20 days, the cultures were filtered and, the filtrates were used in the experiments. Phaseolin was measured in etiolated hypocotyls of bean. For glyceolin evaluation, cotyledons of soybean were used. The deoxyanthocyanidin accumulation was evaluated in etiolated mesocotyls of sorghum. The results were subjected to analysis of variance and compared by regression tests ($p < 0.05$). The filtrates of the saprobic fungi *C. eragrostidis*, *C. inaequalis*, *P. terrestris* and *M. echinata* promoted 9, 8, 9 and 7% greater increases in the concentration of phaseolin accumulations, respectively. The glyceolin accumulation varied with the concentration of *C. eragrostidis*, *C. inaequalis* and *P. terrestris* by adjusting the behavior to a cubic model. The deoxyanthocyanidin accumulation increased with concentration, while the filtrates of *M. echinata* and *C. eragrostidis* promoted 2 to 3 times more increases than the application of water. The filtrates of *P. terrestris*, *C. eragrostidis*, *C. inaequalis* and *M. echinata* induced accumulations of phytoalexins in soybean, beans and sorghum.

Keywords: Deoxyanthocyanidins. Glyceolin. Phaseolin.

ACÚMULO DE FITOALEXINAS EM FEIJÃO, SOJA E SORGO POR FILTRADOS DE FUNGOS

RESUMO - O uso de elicitores em plantas para o controle de doenças tem demonstrado ação no acúmulo de fitoalexinas. Dentre estes, produtos do metabolismo de fungos apresentam eficiência na ativação de mecanismos de resistência induzida das plantas. O objetivo deste trabalho foi avaliar o efeito de filtrados dos fungos sapróbios *Curvularia inaequalis*, *Pseudobotrytis terrestris*, *Memnomiella echinata* e *Curvularia eragrostidis* na indução de faseolina, gliceolina e deoxiantocianidinas. Para isso, discos de micélio dos fungos foram repicados em meio líquido BD (batata e dextrose) e mantidos em B.O.D. a 25 °C, 12h luz/escuro. Após 20 dias foi realizada filtragem e utilizado os filtrados nos experimentos. A faseolina foi determinada em hipocótilos estiolados de feijão. Para avaliação da gliceolina, foram utilizados cotilédones de plântulas de soja. O acúmulo de deoxiantocianidinas foi avaliado em mesocótilos estiolados de sorgo. Os Resultados foram submetidos à análise de variância e comparados pelo teste de regressão ($p < 0,05$). Os filtrados dos fungos sapróbios *C. eragrostis*, *C. inaequalis*, *P. terrestris* e *M. echinata* promoveram maior incremento no acúmulo de faseolina nas concentrações 9, 8, 9 e 7%, respectivamente. O acúmulo de gliceolina variou com as concentrações dos sapróbios *C. eragrostis*, *C. inaequalis* e *P. terrestris*, ajustando-se o comportamento ao modelo cúbico. O acúmulo de deoxiantocianidinas aumentou conforme a concentração, enquanto os filtrados de *M. echinata* e *C. eragrostidis* promoveram aumento de 2 e 3 vezes mais que a aplicação de água. A aplicação dos filtrados de *P. terrestris*, *C. eragrostidis*, *C. inaequalis* e *M. echinata* induziram o acúmulo de fitoalexinas em soja, feijão e sorgo.

Palavras-chave: Faseolina. Gliceolina. Deoxiantocianidinas.

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INTRODUCTION

Loss of harvest in agriculture due to pathogen attack is a serious problem worldwide. Plants are constantly under attack by many pathogens with high destructive potential. Plants respond to pathogens by the formation of reactive oxygen species (ROS), synthesis of pathogenesis-related proteins (PRs), activation of defense genes for cell wall reinforcement and production of antimicrobial compounds. Among these antimicrobial compounds are phytoalexins, which are induced during attack or exposure of the plant to an elicitor agent and constitute an important part of the defense repertoire of the plant (PEDRAS et al., 2008, 2009).

A broad spectrum of phytoalexins induced by different pathogenic agents has been described in several plant species (AHUJA et al., 2012; IRITI, FAORO, 2009). Stangarlin et al. (2011) reported that the synthesis of phytoalexins constitutes one of the fastest metabolic responses of plants to the action of external elicitors. Thus, the analysis of these substances represents an important tool in the study of resistance induction events (PEITER-BENINCA et al., 2008; VIECELLI et al., 2009).

Phaseolin, glyceolin and deoxyanthocyanidins are often reported in induction studies, indicating that they can be considered indicators of this type of analysis because they are easily measured and respond effectively to elicitors.

A variety of elicitors have been described in the literature. Among them are biotic agents such as microorganisms that can activate plant defense mechanisms (DUBERY et al., 2012). Phytopathogenic, nonpathogenic and saprobic fungal filtrates can activate plant defense systems, as they have molecules such as proteins, oligosaccharides, oligopeptides and toxins, among others, that function as recognition signatures of the microorganisms; these signatures are recognized by protein receptors present on the cell membrane of the plant cells (DUBERY et al., 2012). To identify inducing agents of plant resistance to pathogens, it is important to observe biochemical changes occurring in the plant, such as the accumulation of phytoalexins (CHOUDHARY; PRAKASH; JOHRI, 2007).

Therefore, the goal of this work was to evaluate the potential of saprobic fungal filtrates in activating defense mechanisms in plants by means of the accumulation of phytoalexins in bean hypocotyls, soybean cotyledons and stenciled mesocotyls of sorghum.

MATERIAL AND METHODS

The experiments were carried out in the Laboratory of Alternative Control and Induction of Resistance of the State University of Maringá from

January 2012 to December 2013 in Maringá, PR, Brazil.

The filtrates were obtained from repetitions of one disc (7 mm) of BDA with mycelia of the saprobic fungi *M. echinata*, *C. eragrostidis*, *C. inaequalis* and *P. terrestris* in 100 mL of PD liquid culture media sterilized at 120 °C and 1 atm for 20 min. The liquid media containing the saprobic fungi were incubated in a growth chamber at a temperature of 25 ± 2 °C and a 12-h photoperiod for 20 days and then filtered on Whatman #1 paper to separate the filtrate from the mycelial mass. The filtrates were tested for their capacity to induce the accumulation of phytoalexins in bean (phaseolin), soybean (glyceolin) and sorghum (deoxyanthocyanidins). Filtrations of saprobic fungi + BD media were used at concentrations of 0, 5, 10, 15 and 20%; acibenzolar-s-methyl (ASM) at a concentration of 5 g 100 L⁻¹ and sterilized distilled water were used as controls, totaling 26 treatments.

The determination of phaseolin was adopted from the methodology proposed by Dixon et al. (1983). Bean seeds were disinfected with 1% sodium hypochlorite for 5 min, washed in sterilized distilled water, seeded in sterilized sand and maintained in a climate-controlled chamber at 25 ± 2 °C in the dark. After 7 days, stenciled hypocotyl segments were detached from the seedlings and washed in sterilized water. Four hypocotyl segments were weighed and placed into a germination box containing filter paper moistened with distilled water. Different treatments (concentrations of the filtrates) were sprayed onto the hypocotyls, and the germination boxes were kept at 25 ± 2 °C in the dark for 48 h. After this period, the hypocotyls were transferred to test tubes containing 10 mL of ethanol and kept at 4 °C for 48 h for phytoalexin extraction. Afterward, the hypocotyls were shaken for 1 h for extraction and measurement of the formed phaseolin, the latter of which was carried out using a spectrophotometer at 280 nm. The results were expressed as units of absorbance per gram of fresh weight (ABS g fw⁻¹).

For the induction of glyceolin, soybean seeds (cultivar IAC-8) were sown in sterilized sand and maintained in the greenhouse. After 7 days, the cotyledons were removed from the seedlings, washed in sterilized distilled water, dried and cut to a thickness of approximately 1 mm and a diameter of 6 mm on the abaxial surface. Five cotyledons were weighed and placed in a Petri dish containing filter paper moistened with sterilized distilled water. A 75-µL aliquot of treatment solution (filtrate + BD media at a concentration of 0, 5, 10, 15 or 20%; ASM at 5 g 100 L⁻¹; or sterilized distilled water [as a control]) was applied to the cut of each cotyledon. The Petri dishes were kept at 25 °C in the dark. After 20 h, the cotyledons were transferred to test tubes containing 15 mL of sterilized distilled water and stirred for 1 h for phytoalexin extraction. The absorbance was determined at 285 nm (AYERS et

al., 1976; ZIEGLER, PONTZEN, 1982), and the results were expressed as absorbance units per gram of fresh weight (ABS g fw⁻¹).

Sorghum (cultivar Brandes) seeds were used to analyze the induction of deoxyanthocyanidins. Sorghum seeds were disinfected with sodium hypochlorite (1%) for 15 min, washed in sterilized water, wrapped in sheets of germination paper moistened with water and incubated in the dark at 25 ± 2 °C for 4 days. Seven days after germination, the seedlings were exposed to light for 4 h to paralyze the elongation of the mesocotyls (NICHOLSON et al., 1988). The obtained mesocotyls were excised 0.5 cm above the scutellar node, placed in test tubes containing 1 mL of each treatment (filtrate + BD media at a concentration of 5, 10, 15 or 20%; ASM at 5 g 100 L⁻¹; or sterilized distilled water [as a control]) and kept in a moist chamber at 25 °C under fluorescent light (WULFF, PASCHOLATI, 1998). After 60 h, the mesocotyls were removed from the tubes, and the 5-mm portion of the basal end of each mesocotyl was excised and discarded. The upper portion was weighed, cut into small segments and placed in microcentrifuge tubes containing 1.4 mL of 80% acidified methanol (0.1% HCl; v/v). The cut mesocotyls were maintained at 4 °C for 96 h for phytoalexin extraction, and the absorbance was determined at 480 nm (NICHOLSON et al., 1987, 1988). The results were expressed as units of absorbance per gram of fresh weight (ABS g fw⁻¹).

The bioassays of phytoalexin induction were conducted in a completely randomized design with four repetitions. The data were subjected to the Shapiro–Wilk normality test and, assuming the assumption for the analysis of variance was met, when significant were subjected to regression

analysis (p < 0.05), with a control and additional control treatment.

RESULTS AND DISCUSSION

By analyzing the influence of the concentrations of saprobic fungi on phytoalexin induction, an increase in the accumulation of phytoalexins was observed in all treatments. Based on the regression analysis, concentrations of *C. eragrostidis*, *C. inaequalis*, *P. terrestris* and *M. echinata* (9, 8, 9 and 7%, respectively) promoted higher accumulations of phaseolin in bean hypocotyls, with increases of 8, 7, 10 and 9 fold, respectively, compared with those from the water treatment. When comparing ASM, the filtrates of *C. eragrostidis*, *C. inaequalis*, *P. terrestris* and *M. echinata* promoted higher accumulations of phaseolin, with increases of 1.7, 1.2, 2.1 and 2.1 fold, respectively (Figure 1).

When studying the fractionation of *Colletotrichum lindemuthianum*, *C. trifoli* and *C. destructivum*, Anderson (1977) observed that the application of 100 µL promoted phytoalexin induction in bean cotyledons and that the glucan was essential in the activation of defense responses in the seedlings. The author also verified the presence of glucose, mannose and rhamnose, which may be involved in the cotyledon defense response. Although fractionation of the filtrates was not performed in this work, as was used by Anderson (1977), it can be inferred that the saprobic filtrates have molecules capable of increasing the accumulation of phaseolin in bean seedlings.

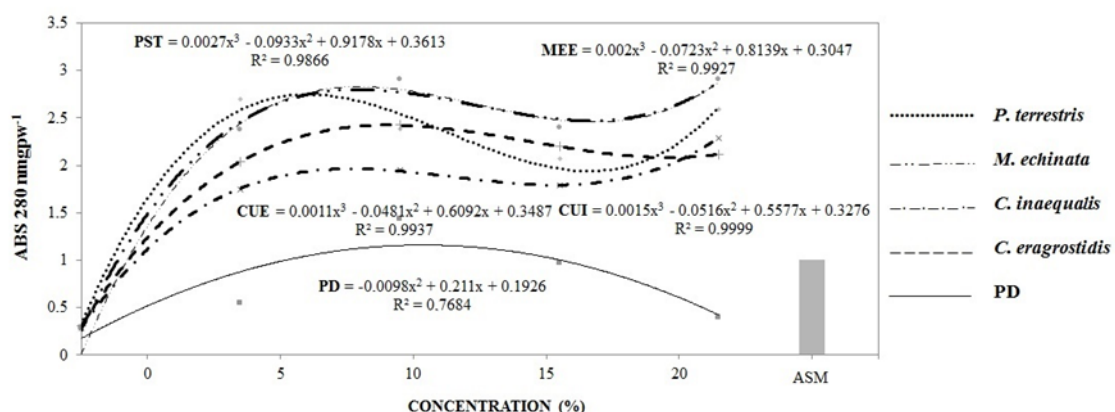


Figure 1. Accumulation of phaseolin in bean hypocotyls as a function of the application of filtrates of the saprobic fungi *Memnoniella echinata*, *Curvularia eragrostidis*, *C. inaequalis* and *Pseudobotrytis terrestris* at different concentrations. Potato dextrose (BD). Acibenzolar-S-methyl (ASM).

The glyceolin accumulation varied significantly (P < 0.05) with the concentration of the saprobes *C. inaequalis*, *C. eragrostidis* and *P. terrestris* after adjusting the fit to the cubic model, with the exception of the concentrations of

M. echinata that did not present significance. The filtrates of *C. inaequalis* at a concentration of 5.4% promoted 3 and 5 times more accumulation of phaseolin in bean seedlings than the water- and ASM-treated hypocotyls, respectively. As for

C. eragrostidis, 3 times more phaseolin was observed with the application of the filtrate in bean hypocotyls compared with the control treatments (water and ASM) (Figure 2). Similar results were found by Simões et al. (2005), who verified five- and

six-fold increases in the phytoalexin accumulation in soybean when applying preparations from β -glucan isolated from *Phytophthora sojae* and from spores of *Mucor ramosissimus*, respectively.

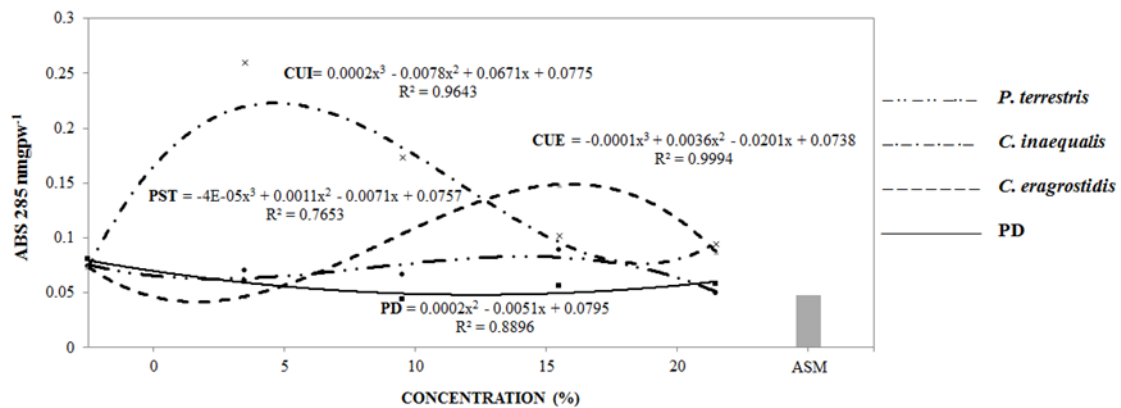


Figure 2. Accumulation of phytoalexins in soybean cotyledons as a function of the filtrate applications of the saprobic fungi *Memnoniella echinata*, *Curvularia eragrostidis*, *C. inaequalis* and *Pseudobotrytis terrestris* at different concentrations. Potato dextrose (BD). Acibenzolar-S-methyl (ASM).

As in this work, Arruda et al. (2012) evaluated the effects of different concentrations (1, 2, 4 and 10%) of aqueous extracts of *Agaricus blazei*, *Lentinula edodes* and *Pycnoporus sanguineus* on the induction of glyceolin and observed a higher accumulation of phytoalexins with higher concentrations of the aqueous extracts. According to Mazaró et al. (2008), alcoholic extracts, decoction, infusion and maceration of pitangueira induce the phytoalexin glyceolin in cotyledons of soybean. The induction effect was characterized by the increase of the concentrations of the preparations and among the evaluated concentrations; those that presented greater responses were the alcoholic extract at concentrations of 10 and 40%, infusion at 40% concentration, decoction at 40% concentration and maceration at concentrations of 10 and 40%. Mazaró et al. (2013) observed variation in the line behavior, such as that with the quadratic and cubic models in this work, and observed that preparations of *Calendula officinalis* also induced glyceolin at a concentration of 10%, whereas the alcoholic extract showed higher glyceolin accumulation at a concentration of 5%.

The accumulation of deoxyanthocyanidins in sorghum mesocotyls increased according to the concentration of the filtrates of *C. inaequalis* and *P. terrestris*. The filtrate of *C. inaequalis* promoted the accumulation of deoxyanthocyanidins by 3 and 0.7 fold in relation to the control and the ASM at

20% concentration, respectively. The filtrates of *M. echinata* and *C. eragrostidis* promoted 2- and 3-fold increases in deoxyanthocyanidins at concentrations of 6 and 14% compared with those of the control, respectively, and 0.5- and 1-fold increases compared with those of ASM, respectively (Figure 3). Other studies have also reported the action of fungal filtrates on the induction of deoxyanthocyanidins. Stangarlin et al. (2010) observed inducing potential of deoxyanthocyanidins and glyceolin when studying the induction of phytoalexins by the yeast *Saccharomyces boulardii* cultivated in liquid media at concentrations of 0.005, 0.05, 0.5, 5, 15 and 25 mg mL⁻¹.

The filtrates of the saprobes *C. eragrostidis*, *C. inaequalis* and *P. terrestris* can induce resistance by activating the secondary defense mechanisms in bean hypocotyls, soybean cotyledons and sorghum mesocotyls, as shown in Figures 1, 2 and 3.

Phytoalexins occur naturally in many plant species. Phytoalexins are often found during phytopathogenic screening programs (GRAY, KOKOBUN, 2001). Therefore, by analyzing phaseolin, glyceolin and deoxyanthocyanidins as indicators of resistance activation by the filtrates of *C. eragrostidis*, *C. inaequalis*, *P. terrestris* and *M. echinata*, it can be inferred that these saprobic fungi have the potential to elicit defense responses in plants such as beans, soybeans and sorghum.

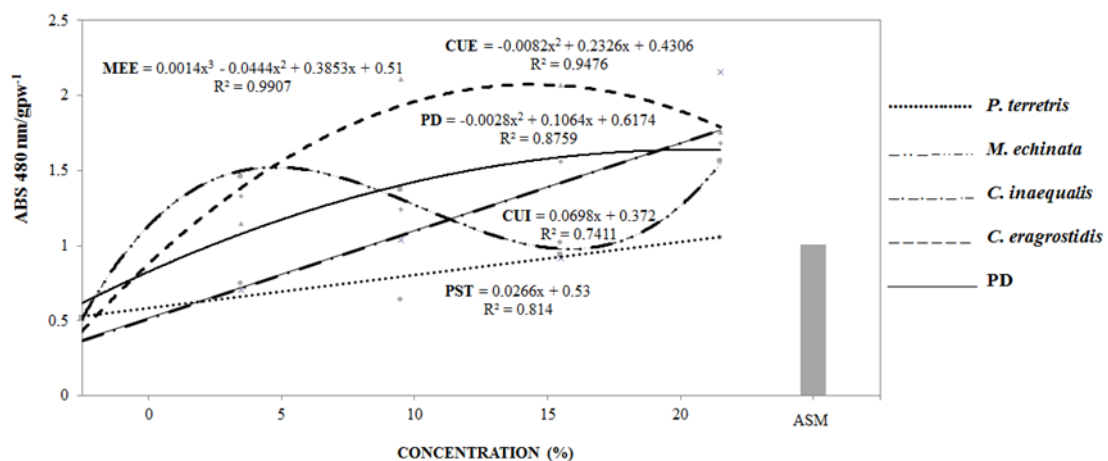


Figure 3. Accumulation of phytoalexins (deoxyanthocyanidins) in sorghum mesocotyls as a function of the application of the saprobic fungal filtrates of *Memnoniella echinata*, *Curvularia eragrostidis*, *C. inaequalis* and *Pseudobotrytis terrestris* at different concentrations. Potato dextrose (BD). Acibenzolar-S-methyl (ASM).

CONCLUSIONS

The filtrates of the saprobes *C. eragrostis*, *C. inaequalis*, *P. terrestris* and *M. echinata* promoted an increase in the accumulation of phaseolin.

The accumulation of deoxyanthocyanidins in sorghum mesocotyls increased with the application of the filtrates of *C. eragrostidis*, *C. inaequalis*, *P. terrestris* and *M. echinata*.

The filtrates of the saprobes *C. eragrostis*, *C. inaequalis* and *P. terrestris* promoted an increase in the accumulation of glyceolin.

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