# INDUCTION OF PHYTOALEXINS IN SORGHUM, SOYBEAN AND BEANS BY SUSPENSION OF ENDOPHYTIC FUNGI

## INDUÇÃO DE FITOALEXINAS EM SORGO, SOJA E FEIJÃO POR SUSPENSÃO DE FUNGOS ENDOFÍTICOS

**Elaine Pittner[1*], Janaina Marek[1], Douglas Bortuli[1] Adriana Knob[1], Leandro Alvarenga Santos[1], Cacilda Márcia Duarte Rios Faria[1]**

[1]State University of the Western Center, Guarapuava, Parana, Brazil

[*] Corresponding Author: E-mail: elainepittner@hotmail.com - Phone number: +55 42 3629-8323

**ABSTRACT**

O objetivo deste trabalho foi avaliar a capacidade de alguns fungos endofíticos em ativar fitoalexinas. Os fungos endofíticos foram isolados de plantas daninhas, na sequência foi realizado uma suspensão destes fungos e testados nos ensaios biológicos para verificar a indução de fitoalexinas. Em sorgo os mesocótilos das plântulas foram acondicionados em tubos contendo os tratamentos e ao final foi realizado a leitura em 480 nm. Em cotilédones de soja e nos hipocótilos de feijão após aplicação dos tratamentos nestes, foram extraídas as fitoalexinas e realizado a leitura. Todos os tratamentos foram capazes de induzir o acúmulo de fitoalexina, porém os mais eficientes foram as suspensões dos fungos D10 (*Aspergilus japonicus)* D11 (*Trichoderma tomentosum*) e, Acibenzolar-s-metil (ASM) e ácido salicílico (AS).

**Palavras-chave:** Acibenzolar-s-metil, ácido salicílico, plantas daninhas, biocida, metabólitos.

**ABSTRACT**

The objective of this work was to evaluate the ability of some endophytic fungi to activate phytoalexins. Endophytic fungi were isolated from weeds, followed by suspension of these fungi and tested in the biological assays to verify the phytoalexins induction. In sorghum the seedlings mesocotyls were packed in tubes containing the treatments and at the end reading was realized at 480 nm. In soybean cotyledons and bean hypocotyls after application of these treatments, phytoalexins were extracted and read. All treatments were able to induce the accumulation of phytoalexin, but the most efficient were suspensions of the fungi D10 (*Aspergilus japonicus*) D11 (*Trichoderma tomentosum*) and, Acibenzolar-s-metil (ASM) and salicylic acid (SA).

**Key words:** Acibenzolar-s-methyl, salicylic acid, weeds, biocide, metabolites.

## INTRODUCTION

The synthesis of phytoalexins occurs in cells adjacent to the site of infection through secondary metabolism, have low molecular mass and are mostly regulated by induction due to biotic and abiotic stress, and the molecular mechanisms underlying their cytotoxicity are largely unknown (AHUJA *et al*., 2012).

Among the metabolites that are part of phytoalexins, there is a great chemical diversity, with more than three hundred types already characterized among different classes of chemical compounds, such as coumarins, diterpenes and flavonoids, and are present in more than twenty families of superior plants (JEANDET *et al*., 2013).

Phytoalexins are biocides, can be injurious to bacteria, fungi and nematodes. The mode of action on fungi includes cytoplasmic granulation,

disorganization of cellular contents, disruption of plasmalemma and inhibition of fungal enzymes, which reflects in the inhibition of germination, germ tube growth, and mycelium (AHUJA *et al*., 2012; JEANDET *et al*., 2013). Glyceollin, soy phytoalexin is a pterocarpenoid, which is very important in the interaction of this legume with phytopathogens, in response to infection, and also by treatment with other biotic and abiotic agents. The production of phytoalexins can be induced by compounds called elicitors, which may be of microbial origin (exogenous elicitor) or of the plant itself (endogenous elicitor) (NG, 2011).

The formation of phytoalexins only occurs after the primary precursor metabolites undergo a new secondary processing route. For example, the phenylalanine substrate is divided for the synthesis of various flavonoid phytoalexins such as phenylalanine ammonia lyase (PAL), a key role enzyme in the phenylpropanoid route. However, the synthesis of most phytoalexins requires the activity of several biosynthetic enzymes and requires several highly regulated transduction signals. One of the modes of action for such high specialization with such biochemical cascades is through the use of a common DNA "cis-acting" sequence element with a promoter from each gene encoding an enzyme required for the synthesis of phytoalexin. For example, multigene or single-stranded PAL are regulated at mRNA and protein levels to act strictly in this case on the biosynthesis pathway of phytoalexins of flavonoids (ZERNOVA *et al*., 2014).

The objective of this work was to evaluate the ability of some endophytic fungi to activate phytoalexins.

## MATERIAL AND METHOD

### *ISOLATION OF ENDOPHYTIC FUNGI*

Isolation of endophytic fungi was performed according to Araújo *et al.* (2005). In the laboratory of Phytopathology, UNICENTRO, weed samples: *Brachiara plataginea, Rumex obtusiolius* L.*., Sida rhombifolia* L.*., Euphorbia heterophylla* L. The plants were submitted to the superficial disinfection method, which consists of washing abundantly in running water, immersed in 70% ethanol for one minute; immersed in 3% sodium hypochlorite for three minutes; put back immersed in 70% ethanol for 30 seconds; rinse twice in distilled and sterilized water; and cut the samples into fragments of 8-12 mm. After the fragments of the samples were transferred to Petri dishes containing PDA medium with pH 6.8, with addition of 100 μg $mL^{-1}$ of chloramphenicol and 100 μg $mL^{-1}$ of streptomycin. Five fragments were placed on each plate and the same were kept in incubator type B.O.D. at 28°C ± 1, under a 12-hour photoperiod.

From each plant 5 PDA plates were prepared (Potato 200g, Dextrose 20g, Agar 20g) from each part of the plant, that is, 5 leaf fragments plates, 5 stem fragments plates and 5 root fragments plates of all weeds, in all 15 PDA plates for each plant.

Plates were observed daily and after fungal growth, mycelial fragments were peeled onto other PDA plates. And again it was repeated for the isolation of each fungus.

After the isolation, the endophytic fungi were kept in PDA medium, sealed with plastic film in the refrigerator and repetitions were performed every 60 days.

### *OBTAINING ENDOPHYTIC FUNGI SUSPENSION*

To obtain the suspension the endophytic fungi were grown in Petri dishes containing PDA medium at 28 ° C. After 7 days of culture, two 9-mm disks from the colonies were transferred to 250 ml Erlenmeyers containing 150 ml of PD medium (potato-dextrose) with 20% potato and 2% dextrose. The fungi were grown for 7 days under constant stirring at 20 g at 28°C, under photoperiod of 12 hours. After this period, spore counting was performed and calibrated in a newbauer chamber with sterile distilled water for 1x108 conidia.$m^{-1}$ (PERELLO *et al*., 2008).

### *PHYTOALEXINS TESTS*

For the tests of phytoalexins in sorghum, soybean and beans, the experiments were carried out with 5 replicates. In the samples where the spectrophotometer readings were above 1.5 the samples were diluted and read again to make the data more accurate.

The treatments used were as follows: Suspension of endophytic fungus (EF) D1, suspension of EF D2, suspension of EF D3, suspension of EF D4, suspension of EF D5, suspension of EF D6, suspension of EF D6, suspension of EF D7, suspension of EF D8, suspension of EF D9, suspension of EF D10, suspension of EF

D11, salicylic acid (SA) 0.7 mM, Acibenzolar-s-methyl (ASM) 300 mg $L^{-1}$, fungicide (Azoxystrobin 120g/L + Tebuconazole 200 g/L) and control (sterilized distilled water). Endophytic fungi with the best results were identified by analyzing the amplified products with specific ITS1 and ITS4 primers for internal transcription (WHITE, 1990).

### *BIOASSAY FOR THE PRODUCTION OF PHYTOALEXINS IN SORGHUM MESOCOTYLS*

For the bioassay of phytoalexins, Nutribem, sorghum seeds [*Sorghum bicolor* (L.) Moench], were disinfected in 1% sodium hypochlorite (15 min), washed in distilled water and soaked in water, at room temperature for 12 hours. After this period, they were rolled onto moist germinated paper sheets and incubated in the dark at 28 ± 2 ° C for 4 days. The seedlings formed were exposed to light for 4 hours to paralyze the elongation of mesocotyls (NICHOLSON *et al*., 1988). In this way, seedlings with uniformly elongated mesocotyls were obtained and suitable for the phytoalexins production bioassay (WULFF, 1997).

The mesocotyls were immersed in the treatments, 10 ml of each treatment, with five replicates for each treatment and the tubes were capped with cotton and remained in a humid chamber at 25 ± 2°C under fluorescent light for a period of 65 hours (WULFF, 1997). After that time, 3 mesocotyls per replicate (5 replicates per treatment) were cut and a 2.5 cm heavy portion, cut into small segments and placed in microcentrifuge tubes containing 1.4 mL of 80% acidified methanol (0.1% HCl, v/v). The mesocotyl segments were maintained at 4°C in methanol for 96 hours for pigment extraction and the absorbance was determined at 480 nm (NICHOLSON *et al.*, 1988). Data were expressed as absorbance at 480 nm per gram of fresh tissue (Abs (480 nm)/g.f.t.).

### *BIOASSAY FOR THE PRODUCTION OF PHYTOALEXINS IN SOY COTYLEDONS*

Soybeans "BRS 284" were sown in sterilized sand and kept in greenhouse in 128 cell styrofoam trays. Ten days after sowing the cotyledons were detached from the seedlings, washed in distilled water, dried and cut in an approximate 1 mm thick section and 6 mm in diameter from the lower surface. Subsequently, cut cotyledons were placed in Petri dishes containing filter paper moistened with sterile distilled water. An aliquot of 75μL of each treatment was applied on each cotyledon. The petri dishes were kept in the dark at 25°C. After 20 hours, the cotyledons were transferred to 125 mL erlenmeyers containing 15 mL of sterile distilled water and allowed to stir for 1 h to extract phytoalexin formed (BONALDO, 2004). The absorbance was determined at 285nm in a spectrophotometer (AYERS *et al*., 1976).

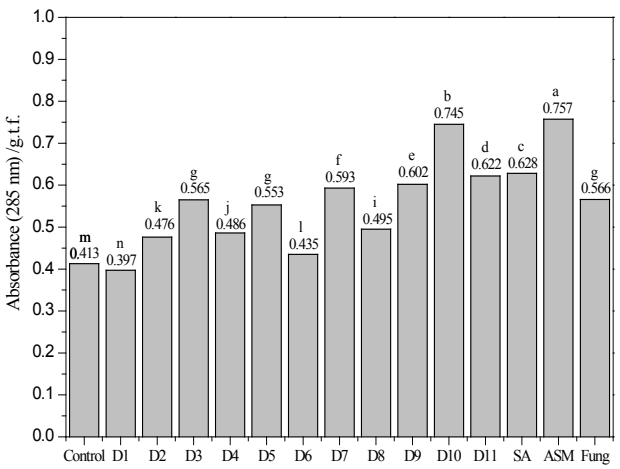
### *BIOASSAY FOR THE PRODUCTION OF PHYTOALEXINS IN BEANS*

The determination of phaseolin was performed according to the methodology proposed by Dixon *et al.* (1983), with some modifications. IPRColibri bean seeds were disinfested in 1% sodium hypochlorite solution for 5 minutes. They were then washed in sterile distilled water, sterilized by autoclaving sterilization at 120°C for one hour and kept in an air-conditioned chamber at 24°C in the dark for seven days. After this period, 5 cm of the hypocotyl segments were cut out of the seedlings, washed in sterile water and kept on absorbent paper for 30 minutes. Hypocotyl segments (1g) were transferred to Petri dishes containing filter paper moistened with sterile distilled water. 200 μL were applied to each hypocotyl, depending on the treatments. Petri dishes were kept at 25°C in the dark for 48 hours. After this period, the hypocotyls were transferred to test tubes containing 10 mL of ethanol, which were kept at 4°C for 48 hours for extraction of the phytoalexin formed, and then stirred for one hour. The content of phaseolin formed was measured in a spectrophotometer at 280 nm.

## RESULTS AND DISCUSSION

It can be seen in Figure 1, that in addition to the ASM, the D10 isolate stood out against the other treatments, mainly when compared to the control, in which the phytoalexins level was about 50% higher than the control. The fact that a substance is able to induce the plant to accumulate phytoalexin is very important, being one of the first mechanisms activated in induced resistance, since accumulation of phytoalexins occurs at the site of infection (AHUJA *et al*., 2012).

**Figure 1.** Accumulation of phytoalexins (Phaseolin) in bean hypocotyls treated with endophytic fungi suspension (D1 to D11), salicylic acid (SA), Acibenzolar-s-methyl (ASM), fungicide (Azoxystrobin with Tebuconazole) and control (sterilized distilled water). Averages followed by the same letter do not differ from each other by the Scott-Knott test at the 5% probability level.




Accumulation of phytoalexins in plants and production of other low molecular weight antimicrobial metabolites are an integral part of phytosanitary protection. The chemical structures of phytoalexins vary between different plant families and include flavonoids and terpenoids (AHUJA *et al*., 2012). The antimicrobial properties of phytoalexins, which have been extensively studied, suggest their potential role in host defense machinery (PEDRAS *et al*., 2011).

Phytoalexins can inhibit the proliferation of phytopathogenic microorganisms, since they have mechanisms of cellular disorganization and even of rupture of the cells of these organisms, preventing the growth and proliferation of these (SCHWAN-ESTRADA *et al.*, 2008).

ASM demonstrates the ability to activate resistance in diverse cultures and against a wide spectrum of microorganisms, between bacteria and fungi (MANDAL *et al.*, 2008), so the production of phytoalexins is one of the forms of protection that ASM can produce, thus, this product is used as a positive control in the production of phytoalexins (BONALDO *et al*., 2004; PEDRAS *et al*., 2011).

Among the evaluated fungi, the D10 isolate suspension was able to induce plants, soybean, sorghum and beans to accumulate phytoalexins (Figures 1, 2 and 3). These data are in agreement with the results obtained by BOUÉ *et al.* (2000) that tested several species of *Aspergillus*, verified that all were able to accumulate phytoalexins in the evaluated plants.

LEE *et al.* (2010) observed that the fungus *Rhizopus nicrosporus var. oligosporus* induced the production of phytoalexin in different soybean varieties. KIM *et al.* (2010) observed that isolates of *Aspergillus sojae* provoked accumulation of phytoalexins in soy cotyledons and produced potent antifungal effects on *Botrytis cinerea, Fusarium oxysprum, Phytophthora capsici, e Sclerotinia sclerotiorum.*

The suspension of D11 isolate was also able to induce a considerable accumulation of phytoalexins in plants, beans, sorghum and soybeans. These results are similar to those of YEDIDIA *et al.* (2003) that in a research with *Trichoderma asperellum* also observed accumulation of phytoalexins in sorghum and soybean plants. CONTRERAS-CORNEJO *et al.* (2011) concluded that *Trichoderma* species are able to regulate multiple defense responses, such as the accumulation of phytoalexins.

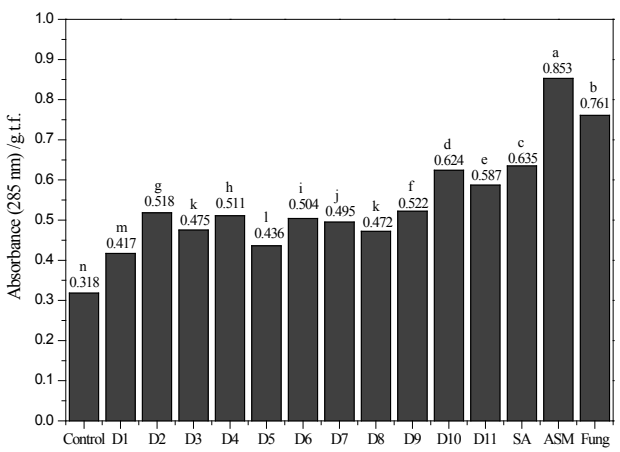
In Figure 1, 2 and 3, when the accumulation of phytoalexins in the plants, soybean, sorghum and beans in the treatment with SA is observed, this was presented in the third place. When plants are infected by pathogens or in contact with elicitors they result in the activation of defense and induce the production of phytoalexins, which are toxic substances to pathogenic fungi (HASEGAWA *et al*., 2010). As well as being an elicitor, it is also an internal marker that plays a crucial role in the growth and development of the plant and resistance induction processes (HEIL *et al.*, 2002). SA is involved in the signal of transduction systems, which stimulate specific enzymes that catalyze biosynthetic reactions to produce defense compounds (CHEN *et al*., 2009). Thus, it can provide protection for plants against pathogens. The exogenous application of SA may result in the induction of defense compounds and consequently in the resistance against the pathogens, among them the production of phytoalexins (MANDAL, 2008; CHEN *et al*., 2009). In addition, some SA derivatives and analogues act as exogenous chemical inducers of RSA (ANFOKA, 2000).

DURANGO *et al*. (2013) tested different concentrations of SA against the production of phytoalexins in cotyledons of four bean cultivars and found that in all concentrations (3, 5, 7 and 14 mM)

and the SA was efficient in accumulating phytoalexins in beans.

In Figure 2, we have the accumulation of phytoalexins (glyceollin) in soybean cotyledons. It is possible to verify that the results for the application of the ASM and the fungicide were 50% higher than the control.

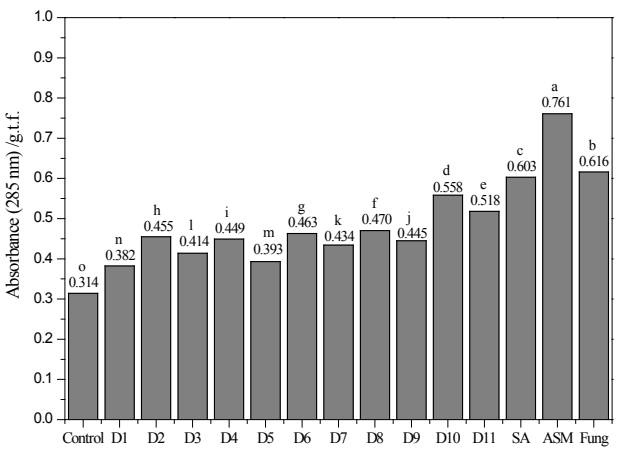
**Figure 2.** Accumulation of phytoalexins (glyceollin) in soybean cotyledons treated with endophytic fungi suspension (D1 to D11), salicylic acid (SA), Acibenzolar-s-methyl (ASM), fungicide (Azoxystrobin with Tebuconazole) and control (sterilized distilled water). Average followed by the same letter do not differ from each other by the Scott-Knott test at the 5% probability level.




Glyceollin is one of the main phytoestrogens of soybeans, there is a great diversity of elicitors that stimulates the accumulation of glyceollin. And it has great applicability in medicine and agriculture, which may inhibit the action of nematodes, bacteria and fungi (NG *et al.*, 2011). In Figure 3, the accumulation of phytoalexin was reached with levels higher than control by all treatments, but the ASM was the one that got double in relation to the control, and the fungi D10 and D11 together with the SA and fungicide were also quite efficient.

Endophytic fungi were identified by the molecular biology technique and we verified that the fungus D10 is *Aspergilus japonicus* and the fungus D11 is *Trichoderma tomentosum.*

**Figure 3.** Phytoalexins accumulation in sorghum mesocotyls treated with suspension of endophytic fungi (D1 to D11), salicylic acid (SA), Acibenzolar-s-methyl (ASM), fungicide (Azoxystrobin with Tebuconazole) and control (sterilized distilled water). Average followed by the same letter do not differ from each other by the Scott-Knott test at the 5% probability level.




## CONCLUSIONS

Suspensions of the isolates D10 (*A. japonicus*) and D11 (*T. tomentosum*) show similar results generally in the three experiments with phytoalexins, this reinforces the potential of using the suspensions of microorganisms as resistance inducers, because in these suspensions besides the presence of conidia of the fungi, probably also has metabolites resulting from its development. It is known that the use of microorganisms in biological control is dependent on the availability and effectiveness of control agents or their metabolites.

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