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Control of leaf spot in yam with leaf extracts of bitter melon

Controle da mancha foliar em inhame com extrato foliar de melão de São Caetano

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The use of plant extracts to induce resistance against pathogens has shown to be a promising alternative in the management of plant diseases. The objective was to evaluate the potential of ethanolic extract of *Mormodica charantia* on management of the *Curvularia eragrostidis* and enzyme activation involved on defense responses in yam (*Dioscorea alata*). The isolate of *C. eragrostidis* was obtained from leaves of yam with symptoms. Mycelial growth and sporulation were evaluated. The treatments were: extract of *Momordica charantia* 10, 100, 500 and 1000 ppm, acibenzolar-S-methyl (200 $mg.L^{-1}$), fungicide (200 $g.ha^{-1}$) and control. The evaluations were done daily, until the 15th day of the application of the treatments. The severity of the disease was evaluated at the end of the experiment, by the size of lesions in each plant. For determination of β-1,3-glucanase and phenylalanine ammonia lyase, leaves were collected in periods of 192 h after treatments application. Fungitoxic action of the treatments in the control of *C. eragrostidis in vitro* was verified. The *M. charantia* extract conferred significative levels of protection in yam plants against *C. eragrostidis* reducing incidence and disease severity. Thus, it was promoted increase in enzymatic activity, preconditioning yam in induced defense mechanisms.
Keywords: alternative control, induction of resistance, pathogenesis-related enzymes.

A utilização de extratos vegetais na indução de resistência contra patógenos tem se mostrado uma alternativa promissora no manejo de doenças de plantas. Objetivou-se avaliar o extrato etanólico de *Mormodica charantia* no manejo da *Curvularia eragrostidis* e ativação enzimática envolvida nas respostas de defesa em inhame (*Dioscorea alata*). O isolado de *C. eragrostidis* foi obtido a partir de folhas de inhame com sintomas. O crescimento micelial e a esporulação foram avaliados. Os tratamentos foram: extrato de *Momordica charantia* 10, 100, 500 e 1000 ppm, acibenzolar-S-metil (200 $mg.L^{-1}$), fungicida (200 $g.ha^{-1}$) e controle. As avaliações foram feitas diariamente, até o 15º dia de aplicação dos tratamentos. A severidade da doença foi avaliada no final da experiência, pelo tamanho das lesões em cada planta. A determinação da atividade de β-1,3-glucanase e fenilalanina amônia liase foi feita 192 h após a aplicação dos tratamentos. Foi verificada a ação fungicida dos tratamentos no controle da *C. eragrostidis in vitro*. O extrato de *M. charantia* conferiu níveis significativos de proteção em plantas de inhame contra a *C. eragrostidis*, reduzindo a incidência e a gravidade da doença. Assim, foi promovido o aumento da atividade enzimática, pré-condicionando o inhame em mecanismos de defesa induzidos.
Palavras-chave: controle alternativo, indução de resistência, enzima relacionadas à patogênese.

1. INTRODUCTION

The yam, *Dioscorea alata* L. (Dioscoreaceae), is a crop cultivated widely in the tropics and subtropics [1] and, has considerable socioeconomic importance in Brazil. The yam is an agricultural option to broaden consumption in the domestic market and meet the demands of foreign markets, but phytosanitary problems hinder the cultivation of this plant. Among the main diseases that affect yams, leaf spot caused by *Curvularia eragrostidis* (Henn.) Meyer (Pleosporales: Pleosporaceae) is of major importance in northeastern Brazil due to its high incidence and severity [2].

The symptoms of this disease are necrotic dark brown spots surrounded by a yellow halo, which cause stunted plant development and can lead to the complete shedding of all leaves [3], which drastically reduces the production of tubers. There is a scarcity of resistant cultivars and

environmental considerations demand a limit on the application of pesticides. To avoid indiscriminate use of fungicides, alternative methods of control are urgently needed [4].

The defense system of plants can be induced through the activation of latent mechanisms by biotic or abiotic external agents [5]. Vegetal extracts with antimicrobial properties have also been successfully employed in the induction of the resistance of plants to pathogens [6, 7]. The *Momordica charantia* L. (Cucurbitaceae), bitter melon, has medicinal properties with antibacterial activity as well as antifungal activity against *Alternaria alternata*, *Fusarium solani*, *Macrophomina phaseolina* and *Stemphylium helianthi* [6].

As the induction of resistance is a viable alternative for the management of *C. eragrostidis*, the aim of the present study was to evaluate the effect of ethanolic extract from *M. charantia* on the management of leaf spot through the activation of enzymes related to pathogenesis in the yam.

2. MATERIAL AND METHODS

The isolate of *C. eragrostidis* was obtained from yam leaves with characteristic symptoms of leaf burn collected from commercial production areas in the municipality of Areia, Brazil. *M. charantia* leaves and stems were collected from the same municipality. The vegetal material was dried in a hothouse at 40º C for 72 h, ground into a fine powder and stored at room temperature.

The cold extraction method was used to obtain the ethanol extract. For such, 150 g of ground vegetal material was immersed in a recipient containing 500 mL of the solvent (ethyl alcohol) for 72 h at a temperature of 25 ± 2º C. The solution was then passed through a paper filter and the solvent was extracted using a rotary evaporator for 2 h at 78º C for the obtainment of the crude extract, which was diluted to concentrations of 10, 100, 500 and 1000 ppm.

After autoclaving, the extract was added to potato-dextrose-agar medium at the different concentrations. Acibenzolar-S-methyl (ASM), 200 $mg.L^{-1}$, and the fungicide Mancozeb® (200 $g.ha^{-1}$) were used as controls. Discs measuring 7 mm in diameter containing a fungal colony were transferred to the center of Petri dishes and maintained for seven days at 25 ± 2 °C with a 12 h photoperiod.

Mycelial growth was determined with daily diametrically opposed measurements of the diameter of the colonies with the aid of digital calipers. Sporulation was evaluated from a spore suspension obtained by the addition of 10 mL of sterilized distilled water in the dishes containing the *C. eragrostidis* colonies. The colony was then scraped with a soft-bristle brush and filtered through two layers of paper filter. The number of spores was determined in a Neubauer chamber and the results were expressed as number of spores per cm^2 of colony.

In the in vivo experiment, yam plants were obtained from a nursery and used with 45 days of age. The plants were placed in 10-L pots with vegetal earth, washed sand and bovine manure (proportion: 2:1:1, respectively) as substrate. The pots were kept in a green house at 30 ± 5º C.
3. Spraying with the treatments was performed with a 192-hour interval prior to inoculation with the fungal isolate. Inoculation with the fungal spore suspension was performed at a concentration of 1 x 10^5 conidia mL^{-1}. After inoculation, the plants were kept in a greenhouse at 30 ± 5º C for 15 days. The incidence and severity of leaf burn was measured up to the fifteenth day after inoculation using the grading scale proposed by Michereff et al. (2008) [8].

Leaf samples were collected after the evaluation period for the determination of phenylalanine ammonia-lyase and β-1,3-glucanase. In the laboratory, the leaf samples were weighed and macerated in liquid nitrogen, followed by the addition of 5 mL of sodium acetate buffer (0.1 M, pH 5.0). The extracts were transferred to microtubes and centrifuged at 14000 g for 25 minutes at 4 °C. After centrifugation, the supernatant was transferred to new microtubes. For the quantification of phenylalanine ammonia-lyase, 250 µL of each sample was used, with the addition of 1.0 mL of tris (hydroxymethyl) aminomethane/ethylene diaminetetra acetic acid buffer solution (0.5 M, pH 8.5) and 250 µL of L-phenylalanine solution as substrate at a concentration of 300 µL mL^{-1}. The reaction was incubated for 1 h at 40 °C in a water bath and

was stopped in an ice bath. Absorbance was read at 290 nm in a spectrophotometer. The results were expressed as g/mL of trans-cinnamic acid [2].

For the quantification of β-1,3-glucanase, 150 µL of the enzymatic extract and 150 µL of sodium acetate buffer (0.1 M, pH 5.0) were transferred to microtubes. The substrate was 150 µL of laminarin at a concentration of 4.0 mg mL^{-1}. The reaction was incubated for 1 h at 40 °C in a water bath and stopped with 200 µL of a 5% phenol solution and 100 µL of concentrated sulfuric acid. Readings were made in a spectrophotometer at 480 nm and compared to glucose standards. The standard glucose curve consisted of concentrations of 0, 5, 10, 20, 40, 80 and 160 µg mL^{-1} [9].

The entirely randomized experimental design was composed of seven treatments with five replications: four concentrations (10, 100, 500 and 1000 ppm) of the ethanolic extract, ASM, the fungicide Mancozeb® and the negative control (sterilized distilled water). Each working plot was composed of two plants per pot with five repetitions. The enzymatic analysis consisted of three repetitions in triplicate. The data were submitted to analysis of variance. Means were compared using the Scott-Knott test. The ASSISTAT 7.5 was used for all analyses, with the level of significance set to 5% [10].

3. RESULT AND DISCUSSION

The ethanolic extract from *M. charantia* (except at the lowest concentration), ASM and the fungicide were effective at inhibiting the mycelial growth and sporulation of *C. eragostridis in vitro*, with significant differences in comparison to the negative control (Table 1). The concentrations of 100 and 1000 ppm achieved greater inhibition of mycelial growth and sporulation.

Table 1 - Inhibition of mycelial growth and sporulation of Curvularia eragrostidis *in function of treatment with etanolic extract of* Momordica charantia, *acibenzolar-S-methyl and fungicide.*

Treatments	**Inhibition (%)**	
	Mycelial growth	Sporulation
Mc 10 ppm	5,4 c	11,02 b
Mc 100 ppm	16,0 a	44,33 a
Mc 500 ppm	12,0 b	22,02 b
Mc 1000 ppm	16,0 a	46,64 a
ASM	10,2 b	22,75 b
Fungicide	9,0 b	30,65 b
Control (SDW)	0,0 c	12,48 b
Means	9,8	27,41
CV (%)	4,78	6,56

*Means followed by different letters in the columns differ from each other by the Scott-Knott test at 5% probability (P<0,05). Mc – Ethanol extract of Momordica charantia (concentrations of 10, 100, 500 e 1000 ppm, respectively), ASM – acibenzolar-S-methyl, fungicide Maconzeb® e control – sterilized distilled water (SDW).

The ethanolic extract from bitter melon is a promising alternative for controlling phytopathogenic fungi. The use of vegetal extracts to enhance the protection of plants against diseases has demonstrated potential due to direct fungitoxic action, demonstrated by the inhibition of mycelial growth and sporulation, as well as the capacity to induce resistance [11].

The results in the treated plants inoculated with the pathogen indicate pre-immunization as a component of induced resistance in response to the application of the *M. charantia* extract and acibenzolar-S-methyl. This effect is associated with an increase in the capacity of the plant regarding the activation of cell defense responses, which are induced only after contact with a challenging pathogen, resulting in different levels of control [12].

The yam plants sprayed with the treatments demonstrated differences in the incidence and severity of leaf burn in comparison to the negative control during the evaluation period (Table

2). The bitter melon extract at a concentration of 1000 ppm led to the least severity of the disease among all treatments, with an effect equal to that achieved with the fungicide (Table 2).

Table 2 - Incidence and severity of Curvularia eragrostidis *in* Dioscorea alata.

Treatments	Incidence (%) Evaluation days					Severity
	3º	6º	9º	12º	15º	
Mc 10 ppm	25a*	45a	59a	73 b	87 b	55b
Mc 100 ppm	23a	36b	51b	64 c	79 c	50c
Mc 500 ppm	18b	29c	40d	53d	68d	45d
Mc 1000 ppm	15b	25c	37d	49d	62d	41e
ASM	16b	35b	45c	57d	70d	44d
Mancozeb®	20b	36b	51b	65c	77c	39e
Control (SDW)	24a	44a	61a	86a	100a	59a

*Means followed by the same letter in the columns do not differ by the Skott-Knott test (5%). Mc – Ethanol extract of Momordica charantia (concentrations of 10, 100, 500 e 1000 ppm, respectively), ASM – acibenzolar-S-methyl, fungicide Maconzeb® e control – sterilized distilled water (SDW).

The influence of the application interval of ASM and *M. charantia* extract was demonstrated by the reduction in the area under the disease progression curve. The period between the application of inducers (192 h) and the inoculation of the pathogen is essential to the induction of defense mechanisms [13]. The application of resistance inducers enables the early warning of the defense system in susceptible plants, which is then optimized after an attack of a pathogen [5], as occurred with the application of ASM and the bitter melon extract in the present investigation.

Momordica charantia has potential that should be explored in the search for new active ingredients aimed at controlling leaf pathogens, such as *C. eragrostidis*, in yam plants. The induction of resistance is not immediate, and the expression of the plant response can take several days. The enzymes phenylalanine ammonia-lyase and β-1,3-glucanase are indicators of the activation of defense responses in plants [14]. Therefore, the present findings demonstrate that the enzymes involved in the plant defense metabolism were activated, as the induction of resistance is characterized by the activation of biochemical mechanisms that may involve biosynthesis and an increase in certain enzymes [15].

Different degrees of pre-conditioning were found among the yam plants in response to the treatments, as reflected in the expression of the enzymes evaluated. Phenylalanine ammonia-lyase and β-1,3-glucanase were influenced by the concentrations of the *M. charantia* extract and subsequent inoculation of the pathogen. With increasing doses of extract there was an increase in enzyme activity. Plants treated with the ethanolic extract at a concentration of 1000 ppm demonstrated greater phenylalanine ammonia-lyase activity.

The *M. charantia* extract had a linear effect on phenylalanine ammonia-lyase and β-1,3-glucanase, as an increase in the activity of these enzymes was found with the increase in the concentration of the extract. The enzyme β-1,3-glucanase is activated quickly when the fungi interact with the plant to lyse the fungi cell wall. PAL is induced during the first stages of the plant-pathogen interaction. This enzyme is involved in the first step of the synthesis of phytoalexins, resulting in the production of compounds such lignin, giving to the plant cell walls greater resistance to the penetration of pathogens [16]. According to Jha (2019) [14] the increase in enzyme activity is related to the capacity of the elicitors to activate plant defense mechanisms, anticipating biochemical defense reactions that would only be activated in the presence of a pathogen.

4. CONCLUSION

Under the conditions in which the studies were conducted, it can be concluded that:

- The fungitoxic effect of the ethanolic extract from *M. charantia* and ASM was evaluated;
- The bitter melon extract conferred significant levels of protection of the yam plants against infection by *C. eragrostidis*;
- Increase in the activity of the enzymes β-1,3-glucanase and phenylalanine ammonia-lyase, thereby pre-conditioning the yam plants with induced defense mechanisms.

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