EFFECTS OF SALICYLIC ACID PRE-TREATMENT AND *Colletotrichum lindemuthianum* POST-INOCULATION OF A COWPEA CULTIVAR ON THE FUNGAL INFECTION PROCESS

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Abstract - Activation of plant defense mechanisms upon pathogen infection involves a signal cascade that is often accompanied by cell death, oxidative burst, hypersensitive reaction (HR) and induction of PR-Proteins. In addition, salicylic acid (SA) and hydrogen peroxide (H_2O_2) are compounds associated with the plant defense mechanisms and there is a body of evidence suggesting that they play important roles in the plant signaling after biotrophic pathogen attack. However, the function of these compounds in interactions involving hemibiotrophic pathogens is not well understood. In this current work we show the effects of SA pre-treatment and *C. lindemuthianum* post-inoculation of a cowpea [*Vigna unguiculata* (L.) Walp.] cultivar, Vita 3, on the number of leaf lesions, and fungus germination and penetration peg formation during the infection process. Cowpea leaves were previously treated with different concentrations of SA (5 and 10 mM) and inoculated with conidial suspension 24h after SA treatment. The number of lesions per leaf was evaluated 3, 4, 9 and 18 days after inoculation. Penetration peg formation was evaluated at 24, 48, 72, 96 and 120 hours after inoculation but on the cowpea leaves previously treated with 10 mM SA. The results obtained showed an increase in the number of lesions and penetration peg formation in SA-treated leaves when compared with control plants, suggesting that this compound could facilitate the fungal infection of cowpea leaves by *C. lindemuthianum*.

Keywords: *Vigna unguiculata*, salicylic acid, *C. lindemuthianum*

EFEITOS DO PRÉ-TRATAMENTO COM ÁCIDO SALICÍLICO E PÓS-INOCULAÇÃO DE UM CULTIVAR DE FEIJÃO-CAUPI COM *Colletotrichum lindemuthianum* NO PROCESSO DE INFECÇÃO DO FUNGO

Resumo – A ativação do mecanismo de defesa de plantas após o ataque de patógenos envolve uma cascata de sinais, normalmente acompanhada de morte celular, estresse oxidativo, resposta hipersensitiva (HR) e indução de PR-Proteínas. Em adição, SA e H_2O_2 são compostos associados com o mecanismo de defesa vegetal, existindo diversas evidências sugerindo a participação destes

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compostos no processo de sinalização da planta após o ataque de patógenos biotróficos. Entretanto, o papel destes compostos em interações envolvendo patógenos hemibiotróficos ou necrotróficos ainda não é bem compreendido. O presente trabalho apresenta os efeitos do pré-tratamento com SA e pós-inoculação de um cultivar de feijão-caupi, Vita 3, com *C. lindemuthianum* no número de lesões foliares, germinação do fungo e formação da hifa de penetração durante o processo de infecção. Folhas de feijão-caupi foram previamente tratadas com diferentes concentrações de SA (5 and 10 mM) e inoculadas com o fungo 24 h após tratamento com SA. O número de lesões por folha foi avaliado 3, 4, 9 e 18 dias após a inoculação com o fungo enquanto a formação do tudo de penetração avaliado 24, 48, 72, 96 e 120 horas após inoculação, mas em folhas previamente tratadas com SA a 10 mM. Os resultados obtidos mostraram aumento no número de lesões e na formação do tubo de penetração em folhas de plantas previamente tratadas com SA, quando comparadas com plantas controle, sugerindo que o SA pode atuar facilitando o processo de infecção das folhas de feijão-caupi pelo fungo *C. lindemuthianum*.

Palavras-chave: *Vigna unguiculata*, ácido salicílico, *C. lindemuthianum.*

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most important crops in Northeastern and North of Brazil. Although well adapted to tropical climate conditions, this crop is constantly threatened by pathogen attacks. To survive plants use a defense arsenal in response to pathogen challenges, which comprises different physical barriers, the oxidative burst, accumulation of H_2O_2 and SA, HR, and increased concentration of inducible compounds like phytoalexins and PR-Proteins as an attempt of blocking or delaying the pathogen penetration (Deverall and Dann, 1995; Dorey *et al.*, 1997; Van Loon and Van Strien, 1999; Jeandet *et al.*, 2002).

SA participates in the plant defense mechanism as a signaling molecule responsible for the activation of the systemic acquired resistance (SAR) and induction of the synthesis of PR-Proteins (Kessmann *et al.*, 1994; Martinez *et al.*, 2000; Fernandes *et al.*, 2006). However, in interactions involving hemibiotrophic or necrotrophic pathogens, the role of SA is not clear. Indeed, the fact of SA be able of inducing cell death in the site of infection by hemibiotrophic or necrotrophic pathogens can facilitate fungal penetration and development with the establishment of the ideal fungal growth conditions (Govrin and Levine, 2000; Hückelhoven and Kogel, 2003; Fernandes, 2004).

In this present work we evaluate the role of SA in the interaction of cowpea, cv. Vita 3, with the hemibiotrophic fungus *C. lindemuthianum*, the causal agent of anthracnose. Microscopic evaluations to determine the number of lesions per leaf and penetration peg formation ratio was carried out.

Materials and Methods

Seed germination: Seeds of the Brazilian cowpea genotype Vita 3 were provided by cowpea seed bank of Escola de Agronomia (Universidade Federal do Ceará, Brazil). They were surface-sterilized with sodium hypochlorite solution, washed thoroughly with autoclaved water and soaked in distilled water for 20 minutes (Fernandes *et al.*, 2006). The imbibed seeds were then planted in glass conical jars containing a vermiculite bed watered with distilled water. The jars were maintained in a growth chamber (26 - 30 °C, 52 - 86% relative humidity, 12 h photoperiod (0 - 500 μmol m^{-2} s^{-1}). After emerging, seedlings were irrigated every two days with a modified (Silveira *et al.*, 1998) Hoagland and Arnon nutrient solution (Hoagland and Arnon, 1950).

Seedling treatments: Fully expanded primary leaves of 10-day-old Vita 3 cultivar were treated with different concentrations of SA (5 and 10 mM), whereas those of control plants were treated with distilled water (Fernandes *et al.*, 2006). Treatments were done by brushing the SA solution or distilled water on the adaxial surface of the primary leaves in the absence of surfactant. 24 h after SA treatment, cowpea leaves were sprayed with a *C. lindemuthianum* conidial suspension (10^4 spores/mL) and the plantlets kept in plastic boxes maintained in a growth chamber at approximately 80% RH until harvested. In order to evaluate the penetration peg formation ratio, leaves were treated with 10 mM SA and after 24 h inoculated with the conidial suspension, as describe above. Control plants were treated with distilled water before fungal inoculation.

***C. lindemuthianum*:** The fungus was maintained in agar potato-dextrose medium (PDA) prepared according to the manufacture instructions. The conidial suspension was obtained from fresh cultures (15 day-old cultures) which were washed with 5 mL autoclaved distilled water to release conidia. After filtration through one layer muslin cloth, suspension was adjusted to 10^4 spores mL^{-1} final concentration in autoclaved distilled water.

Macroscopic and Microscopic analyses: The number of lesions in SA-treated cowpea primary leaves was evaluated at naked eyes 3, 4, 9 and 18 days after inoculation. Fungal germination and development were assessed by evaluating the formation of penetration peg on the adaxial surface of cowpea leaves treated with 10 mM SA and subsequently inoculated with *C. lindemuthianum*, at 24, 48, 72, 96 and 120 h after inoculation. To visualize hyphal penetration into epidermal cells leaves were treated with calcofluor (Rohringer *et al.*, 1977). Briefly, cowpea primary leaves were cut off and decolorized in TCA (1.5 g TCA, 750 mL ethanol and 250 mL chloroform) overnight. Calcofluor staining was carried out by washing the pigment-free leaves with 50% ethanol (2 x 15 min), 50 mM sodium hydroxide (2 x 15 min), Milli-Q grade water (3 x 10 min) followed by incubation in 0.1 M Tris-HCl buffer, pH 8.5, for 30 minutes. After this, leaves were incubated in 0.3% calcofluor solution for 5 minutes and excess stain washed out with Milli-Q grade water (4 x 10 min).

Results and Discussion

The primary leaves of cowpea, cv. Vita 3, presented many necrotic lesions after fungal inoculation both in SA-pretreated and untreated (control) plantlets (Fig. 1). However, the number of

lesion was significantly higher ($p \leq 0.01$) in leaves previously treated with 10 mM SA when compared with the untreated controls. Interestingly, increase in the number of lesions per leaf was correlated with the increase in SA concentration used in the treatment. Indeed higher numbers of necrotic lesions were found in plants treated with 10 mM SA 18 days after inoculation (27.11 lesions/leaf) as compared with plants treated with 5 mM (18.16 lesions/leaf) and untreated plants (13.07 lesions/leaf) (R^2 = 0.9876, data not showed) (Fig. 1). These data suggest that treatment with SA before inoculation might have facilitated penetration of the hemibiotrophic fungus *C. lindemuthianum* in the cowpea leaf epidermal cells possibly by enhancement of the HR which in turn led to cell death, ideal condition for fungal development during its necrotrophic stage of nutrition. Similar results were obtained by Fernandes (2004) who suggested that the differential accumulation of SA and H_2O_2 observed only in the susceptible cowpea genotype (Gurguéia), but not in the resistant one (Mulato), might have a bearing upon the establishment of ideal conditions for fungal development and hence the success of fungal infection. Apparently such resistance mechanism involves the regulation of enzymes of the oxidative burst cycle and the SA and H_2O_2 biosynthesis pathways (APX, CAT, SOD and PAL) (Fernandes, 2004) toward the scavenge of the reactive oxygen species (ROS) and down-regulation of SA.

Aiming at assessing the development stages and colonization of cowpea leaves, cv. Vita, by *C. lindemuthianum*, microscopic analyses were carried out. The fungus showed uniform growth, as conidia germinated and produced germ tube and melanized appressorium (Fig. 2). Formation of penetration peg increased in plants treated with SA, from 72 hours after inoculation onward. (Fig. 3). Thus, the fungus established its biotrophic phase within 48 - 72 hai, initiating the colonization process. Often the necrotrophic phase of nutrition for most *Colletotrichum* fungi generally occurs after 48 h initial penetration (72-96 hai) (Perfect *et al.*, 1999).

The results reported in this present work suggest that SA may play an important role in resistance/susceptibility of cowpea toward the hemibiothrophic fungus *C. lindemuthianum*. This compound might act facilitating fungal penetration and colonization in cowpea tissue through enhancement of hypersensitive reaction and cell death.

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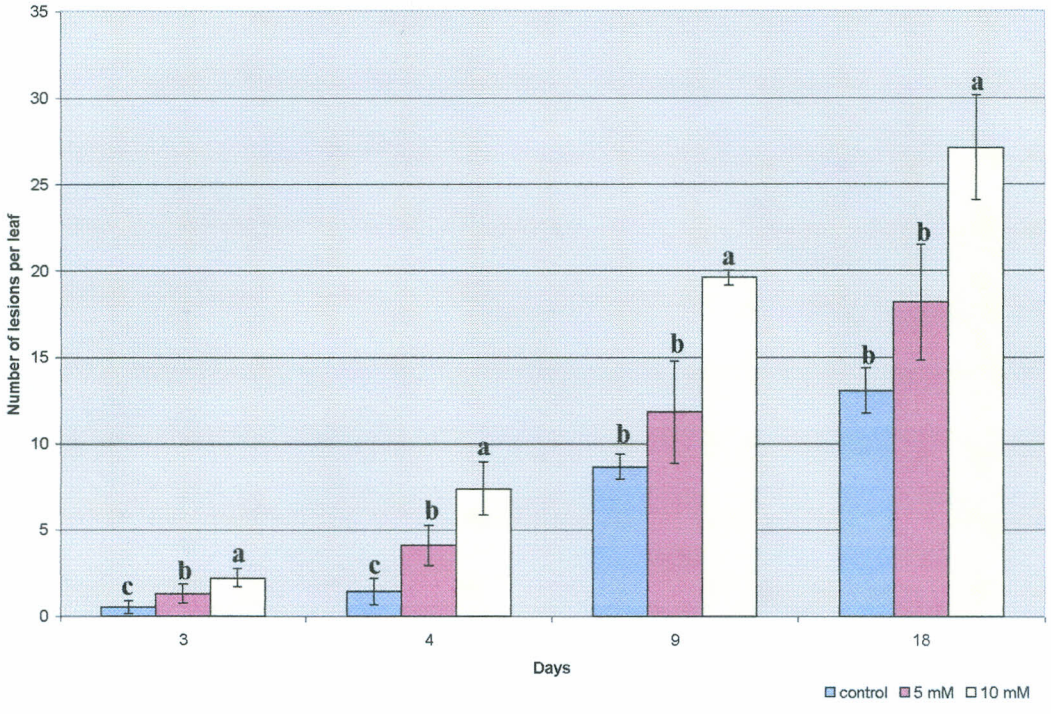

Figure 1 – Number of lesions per leaf in cowpea primary leaves, cv. Vita 3, treated with salicylic acid and challenged 24 h after treatment with the hemibiotrophic fungus *Colletotrichum lindemuthianum*. Bars indicate mean ± standard error of three repetitions. Letters indicates significance between treatments in each day according to Student's t-test.

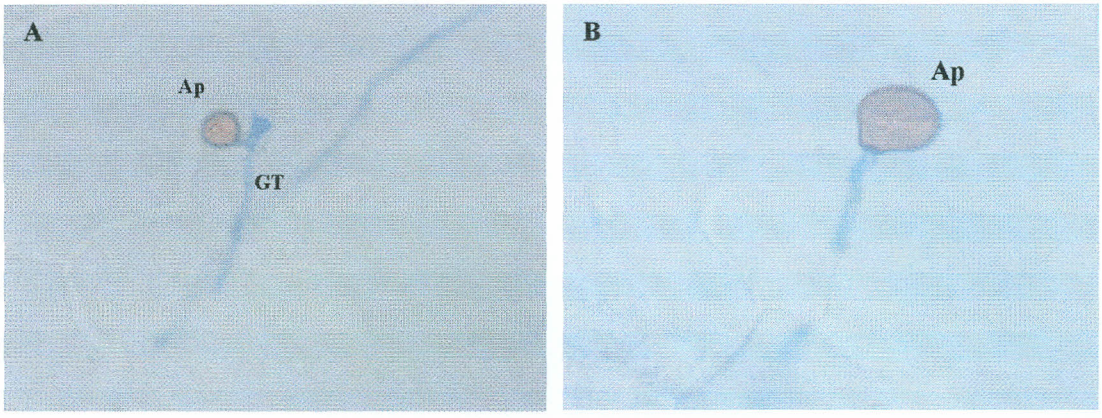

Figure 2 – Microphotographs of cytological analysis of the interaction of cowpea, cv. Vita 3, with the fungus *C. lindemuthianum*. **A** and **B**, germ tube (GT) and melanized appressorium (Ap) of *C. lindemuthianum* 48 hours after inoculation in cowpea primary leaves, previously treated with 10 mM salicylic acid.

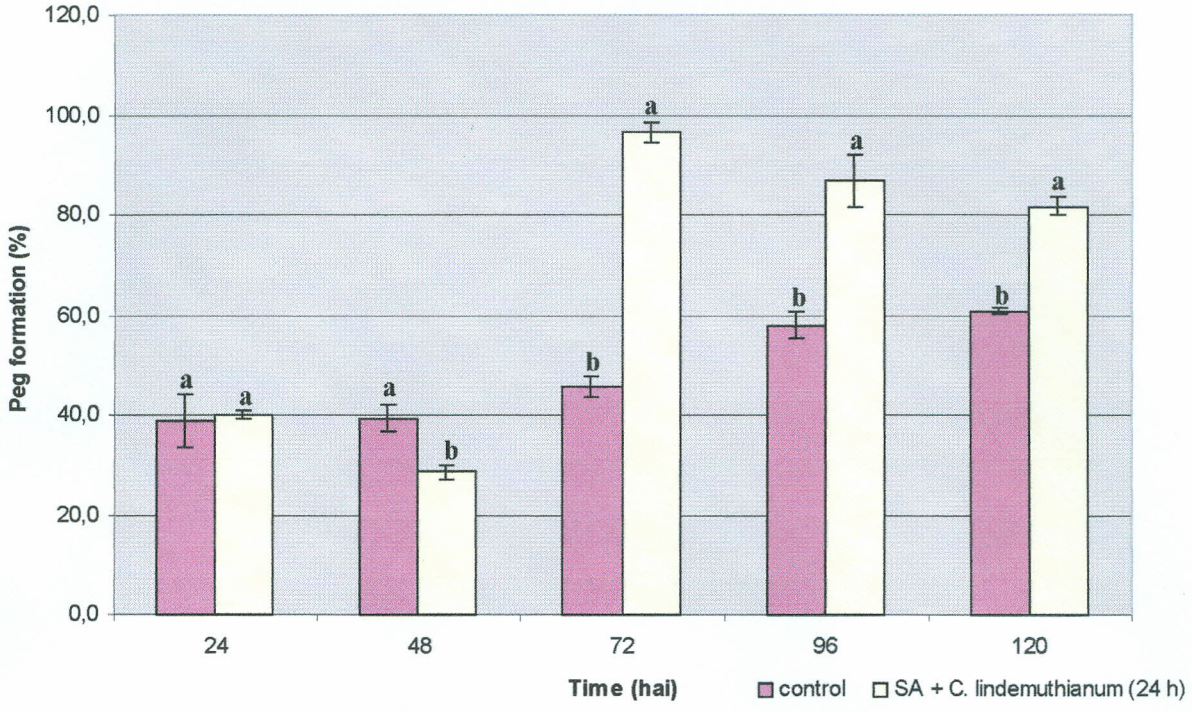

Figure 3 – Formation of penetration peg on cowpea primary leaves, cv. Vita 3, treated with 10 mM salicylic acid and challenged 24 hours after treatment with the hemibiotrophic fungus *Colletotrichum lindemuthianum*. For each individual experiment, 50 interactions sites was evaluated. Penetration peg formation (%) was calculated as the number of appressorium showing penetration peg formation divided by the number of appressorium analyzed (50) multiplied by 100. Bars indicate mean ± standard error of three repetitions. Letters indicates significance between treatments in each day according to Student's t-test.