

Induction of chalcone synthase and phenylalanine ammonia-lyase by salicylic acid and *Colletotrichum lindemuthianum* in common bean

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The activities of the enzymes chalcone synthase (CHS) and phenylalanine ammonia-lyase (PAL) were measured in leaf extracts obtained from four cultivars of the common bean (AB 136, Rio Tibagi, Carioca and Macanudo). Two stages of plant development were examined: plantlets (V2) and the onset of blooming (R6). Initially, the plants were either treated with salicylic acid or inoculated with the delta race of *Colletotrichum lindemuthianum* (inductive fungus) and after three days they were evaluated for enzyme activity. Afterwards, all plants were inoculated (challenged) with the virulent pathotype 33/95 of *C. lindemuthianum* except for the water control. Five days later, the activities of PAL and CHS were evaluated. There were significant changes in the activities of both enzymes three days after treatment with salicylic acid or inductive fungus when compared to the control. Five days after inoculation with with the virulent pathotype 33/95 of *C. lindemuthianum* CHS activity in the Macanudo was similar to control plants that were not treated with salicylic acid or the inductive fungus but inoculated with 33/95 *C. lindemuthianum*. The increase in enzyme activity after challenge with 33/95 *C. lindemuthianum* was greatest for the salicylic acid treatment in the cultivar AB 136, followed by Rio Tibagi and Carioca.

Key words: anthracnose, *Phaseolus vulgaris*, systemic acquired resistance.

Indução da chalcona sintase e da fenilalanina amônia-liase por ácido salicílico e *Colletotrichum lindemuthianum* em feijão: As atividades das enzimas chalcona sintase (CHS) e fenilalanina amônia-liase (PAL) foram avaliadas em extratos de folhas de quatro cultivares de feijão (AB 136, Rio Tibagi, Carioca e Macanudo), considerando-se dois estádios de desenvolvimento das plantas: plântulas (V2) e início de floração (R6). Inicialmente, as plantas foram tratadas com ácido salicílico ou inoculadas com a raça delta de *Colletotrichum lindemuthianum* (fungo indutor) e, após três dias, avaliadas quanto à atividade das enzimas. Em seguida, as plantas foram inoculadas com o patótipo virulento 33/95 de *C. lindemuthianum*, exceto as plantas-controle. Após cinco dias, avaliaram-se as atividades de PAL e CHS, observando-se acréscimos na atividade das enzimas, tanto no tratamento com ácido salicílico quanto com o fungo indutor, quando comparados com o controle. Houve alterações significativas nas atividades das enzimas depois de três dias de exposição aos tratamentos indutivos com ácido salicílico e fungo, quando comparados com o controle não induzido. Cinco dias após a inoculação com o patótipo virulento 33/95 de *C. lindemuthianum*, a atividade de CHS em Macanudo foi semelhante às plantas-controles que não haviam sido induzidas, mas inoculadas com 33/95 *C. lindemuthianum*. O aumento das atividades depois do desafio com 33/95 *C. lindemuthianum* foi maior nas plantas tratadas com ácido salicílico no cultivar AB 136, seguido por Rio Tibagi e Carioca.

Palavras-chaves: antracnose, *Phaseolus vulgaris*, resistência sistêmica adquirida.

INTRODUCTION

Plants can develop systemic, durable, and wide-spectrum resistance induced by pathogens or some chemical products, a phenomenon known as "Systemic Acquired Resistance" (SAR). The wide spectrum of action of SAR contrasts with the resistance against specific pathogen races provided by major resistance genes used in developed cultivars and, therefore, it can be less susceptible to resistance breakdown (Moraes, 1998). Induced resistance is recognized as an important biocontrol method in the production of organic vegetables (Sequeira, 1983).

The plant cell wall also participates in resistance mechanisms, since it plays a role in active defense that may result in an accumulation of phytoalexins, which are antibiotic substances of low molecular weight synthesized and accumulated in plants after exposure to microorganisms (Braga and Dietrich, 1987; Seki *et al.*, 1999; Pelicice *et al.*, 2000). Phytoalexins and the enzymes that catalyze their synthesis appear at low concentrations or are absent from healthy plants, but they can be synthesized when the plants are exposed to certain pathogens or other abiotic stresses (i.e. wounding, extreme temperatures, etc.) (Chet, 1993). The production of phytoalexins is increased by the action of some factors, such as exogenous elicitors that are produced by pathogens, or endogenous elicitors that are produced by the plants in response to stress situations (Zarra and Revilla, 1996; Seki *et al.*, 1999). For example, one defense mechanism of legumes to mildew infection is the rapid accumulation of antimicrobial phytoalexins and isoflavonoids at the infected area (Dixon, 1986), including the production of phenylpropanoids (Dixon and Harrison, 1990).

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) and chalcone synthase (CHS; EC 2.3.1.74) are two key enzymes that are involved in phenylpropanoid biosynthesis (Smith and Banks, 1986). The first reaction of this biosynthetic pathway is the deamination of phenylalanine to cinnamic acid by PAL. In the next reaction of the metabolic pathway, CHS condenses three malonyl-CoA molecules with cinnamoyl-CoA to produce chalcone. This condensation is the main branch in the pathway for the production of flavonoids (Ryder *et al.*, 1984, 1987; Cramer *et al.*, 1989; Hahlbrock and Scheel, 1989). PAL and CHS catalyze key reactions in the biosynthesis of phytoalexin isoflavonoids in legumes. This is the first stage in the biosynthesis of secondary phenylpropanoid products by L-phenylalanine and the first reaction branch in the main flavonoid and isoflavonoid production pathway (Ellis *et al.*, 1989).

The salicylic acid concentrations needed for PAL and CHS activation and for achieving disease resistance are not toxic and they reach a peak before the onset of SAR (Chet, 1993). Studies with parsley cells in culture revealed that salicylic acid, one of the typical compounds involved in SAR expression, renders cells sensitive to the elicitor, in a process originally called conditioning (Kauss and Jeblick, 1995). This process involves cell activation mechanisms and promotes responses with small elicitor doses, while in non-treated cells there is only a weak reaction (Kauss *et al.*, 1992). Nevertheless, it has been demonstrated that although salicylic acid is involved in resistance induction, this substance is not responsible for the original signal that sensitizes plant tissues that are distant from the initial site of treatment, allowing the plant to respond to the pathogenic attack in a systemic manner.

It has been recently discovered that the enzyme salicylate synthase is a potent tool for the manipulation of salicylic acid levels in plants. By fusing the two bacterial genes *pchA* and *pchB* from *Pseudomonas aeruginosa*, which encode isochorismate synthase and isochorismate pyruvate-lyase, respectively, a novel hybrid enzyme was produced. Increases in salicylate synthase activity expressed in *Arabidopsis thaliana* were observed, while the free and conjugated salicylic acid content was increased more than 20-fold above the wild type level (Mauch *et al.*, 2001).

The objective of this work was to determine the efficiency of the delta race of the fungus *C. lindemuthianum* in the induction of PAL and CHS activities and compare it to the effect of salicylic acid with regard to the highly virulent 33/95 *C. lindemuthianum* pathotype, native of Rio Grande do Sul, on four *Phaseolus vulgaris* L. cultivars.

MATERIAL AND METHODS

This study took place at the Embrapa Temperate Climate Experimental Station (Embrapa Clima Temperado) in the State of Rio Grande do Sul, Universidade Federal do Rio Grande do Sul (UFRGS). Two experiments were carried out at two developmental stages of the bean (*Phaseolus vulgaris* L.) cultivars AB 136, Carioca, Macanudo, and Rio Tibagi. The plants were maintained in the greenhouse at a temperature of 25 ± 2 °C and relative humidity of 79 ± 5 %, with a 14 h photoperiod and $195 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ illumination. The two fungal races were obtained from Embrapa's plant pathology program.

In the first experiment, the seeds were sown in plastic trays (55 x 40 x 15 cm) filled with sterilized sand and irrigated with nutrient solution (Smith *et al.*, 1963). The germi-

nated seeds were left to grow for nine days, reaching the plantlet stage at which the primary leaves become expanded (V2). In the second experiment, the seeds were germinated in plastic bags containing 3 Kg of a sterilized soil mixture (soil, manure and vermiculite, 5:3:1, v/v/v). The plants grew until the onset of the blooming stage (R6). Spores (1.4×10^6 spores.ml⁻¹) of the delta race of *C. lindemuthianum* (inductive fungus) or 10 mmol.L⁻¹ salicylic acid were separately sprayed in all the leaves with a hand atomizer. After three days the plants were assessed, and challenged with the virulent isolate 33/95 of *C. lindemuthianum* (at a concentration of 2.27×10^4 spores ml⁻¹) and reevaluated five days later. Mathur's medium was used to produce the inoculum (Mathur *et al.*, 1950). Plants were sprayed with water in the control treatment. Once inoculated, the plants were kept at air temperature and relative humidity of 22 ± 2 °C and 95 %, respectively. Photosynthetic photon flux density was approximately 195 $\mu\text{E s}^{-1}.\text{m}^{-2}$ during 14 h per day.

Leaves were collected at random from the whole plants three days after the induction treatments, and five days after challenge with the virulent pathotype (Irving and Kuc, 1990). The tissue was weighed, rapidly frozen on dry ice, and stored at -80 °C for later analysis.

The CHS extraction was carried out by grinding the leaves with 300 μl 50 mmol.L⁻¹ KH₂PO₄, pH 8.0, containing 20 mmol.L⁻¹ ascorbic acid. The mixture was ultrasonicated in ice for 2 min and centrifuged (25,000 g_n, 15 min). The activity of CHS was determined according to Edwards and Kessman (1990) using 100 μl of the supernatant.

PAL activity was determined in crude leaf extracts obtained by grinding the leaves with 4 ml of 0.5 mol.L⁻¹ borate

buffer (4 °C) containing 0.05 g of polyvinylpyrrolidone in a Polytron mixer at full speed. The protein extract obtained after filtration (Whatman 1) and centrifugation (25,000 g_n, 15min) was used to assay the PAL activity (Hyodo and Yang, 1971; Hyodo *et al.*, 1978).

Protein concentration in the extracts was determined by the Lowry method (Lowry *et al.*, 1951).

The experiments were designed according to a split plot arrangement in randomized complete design with factors A (cultivars) x B (treatments) and 5 replicates. The sample analyses were done in triplicate and the enzyme specific activities were subjected to analysis of variance. The correlation coefficients were calculated according to Zonta and Machado (1984) and means were compared using the Duncan test ($\pm = 0.05$).

RESULTS AND DISCUSSION

Cultivars AB 136, Rio Tibagi and Carioca at stages V2 and R6 showed significant increase in PAL activity after treatment with salicylic acid or inoculation with the inductive fungus in relation to the control. No significant differences occurred between the treatments with salicylic acid and the inductive fungus for cultivar Macanudo after inoculation with the virulent pathotype at both developmental stages (tables 1 and 2).

After challenging with the virulent pathotype of *C. lindemuthianum* PAL activities in both growth stages continued to increase. Except for Macanudo, the other cultivars had higher activity than the control plants not induced with salicylic acid and the inductive fungus. These control plants however, also showed higher PAL activity than the absolute control plants, which were not induced or challenged.

Table 1. PAL activity of bean plantlets at the V2 stage of cultivars AB 136, Rio Tibagi, Carioca, and Macanudo three days after treatment with salicylic acid or inoculation with the delta race of *C. lindemuthianum* and five days after challenge with the virulent pathotype 33/95 of *C. lindemuthianum*.

Treatments	PAL activity (nmol.h ⁻¹ .mg ⁻¹ protein) ^a							
	Three days after induction				Five days after challenge			
	AB 136	Rio Tibagi	Carioca	Macanudo	AB 136	Rio Tibagi	Carioca	Macanudo
Salicylic acid	60.43a	49.70a	36.07a	51.13a	97.60a	77.27a	40.37a	79.73a
<i>C. lindemuthianum</i> (delta race)	56.33b	47.63b	34.20b	50.07a	89.00b	71.73b	38.10b	78.50a
Control	27.80c	23.83c	20.50c	24.73b	34.30d	26.63d	22.63d	29.00c
Virulent pathotype					83.96c	55.20c	33.53c	62.13b

^a Means followed by different letters in the same column are significantly different (Duncan test at $p \leq 0.05$).

Table 2. PAL activity of bean plant at the R6 stage of cultivars AB 136, Rio Tibagi, Carioca and Macanudo three days after treatment with salicylic acid or inoculation with the delta race of *C. lindemuthianum* and five days after challenge with the virulent pathotype 33/95 of *C. lindemuthianum*.

Treatments	PAL activity (nmol.h ⁻¹ .mg ⁻¹ protein) ^a							
	Three days after induction				Five days after challenge			
	AB 136	Rio Tibagi	Carioca	Macanudo	AB 136	Rio Tibagi	Carioca	Macanudo
Salicylic acid	52.23a	37.40a	28.53a	39.56a	69.76a	57.23a	39.17a	58.43a
<i>C. lindemuthianum</i> (delta race)	46.83b	35.56b	26.40b	38.73a	67.93b	54.10b	36.06b	57.80a
Control	25.60c	19.00c	13.73c	20.30b	26.90d	20.80d	16.30d	20.36c
Virulent pathotype					64.40c	47.73c	25.46c	54.47b

^a Means followed by different letters in the same column are significantly different (Duncan test at $p \leq 0.05$).

These results are in agreement with those obtained by Cui *et al.* (1996), who observed a rapid increase in the activities of PAL and CHS in sorghum plantlets after inoculation with *Bipolaris maydis*. Orczyk *et al.* (1996) also obtained a fast induction of PAL activity as a response to the attack of this pathogen in sorghum. The increase in PAL activity has frequently been mentioned as a defense reaction of plants to pathogen attack, showing significant increases after infection by pathogens or wounding (Chet, 1993, Seki *et al.*, 1999, Cui *et al.*, 2000; Logemann *et al.*, 2000). Tepper *et al.* (1989) suggest that there are differences in the relative levels and timing of PAL and CHS mRNA which accumulate in a cultivar in response to different elicitors, and that any relationship between elicitor activity and specificity of plant-microbe interaction is highly complex. They also observed that the variability between induction patterns was demonstrated between cultivars. In the present case, it seems possible that an

additive effect occurred in cultivar Macanudo, causing an increase in the induction activity values of the enzyme by the elicitor that originated from the fungus. A similar effect was observed by Lawton and Lamb (1987) and Dixon and Harrison (1990) in a suspension of cultured bean cells.

CHS activity in beans was induced by the treatments with salicylic acid and by inoculation with the inductive fungus at both developmental stages (tables 3 and 4). No significant differences were observed between the treatments for the Carioca and Rio Tibagi cultivars at both developmental stages. These results are consistent with those observed by Tepper *et al.* (1989) and Tepper and Anderson (1990), since the different responses of the cultivars to the induction of PAL and CHS activity might be related to the specificity between the cultivar and the elicitor, therefore determining the activation of defense mechanisms.

Table 3. CHS activity of bean plantlets at the V2 stage of cultivars AB 136, Rio Tibagi, Carioca and Macanudo three days after treatment with salicylic acid or inoculation with the delta race of *C. lindemuthianum* and five days after challenge with the virulent pathotype 33/95 of *C. lindemuthianum*.

Treatments	CHS activity (pmol g ⁻¹ protein) ^a							
	Three days after induction				Five days after challenge			
	AB 136	Rio Tibagi	Carioca	Macanudo	AB 136	Rio Tibagi	Carioca	Macanudo
Salicylic acid	9.0a	5.2a	4.8a	6.9a	9.3a	5.3a	4.7a	6.3a
<i>C. lindemuthianum</i> (delta race)	7.8b	4.9a	4.7a	6.8a	8.3b	4.9a	4.6a	6.1a
Control	1.4c	1.0b	0.9b	2.3b	1.6d	0.9c	1.0c	1.1b
Virulent pathotype					7.7c	4.2b	3.3b	5.9a

^a Means followed by different letters in the same column are significantly different (Duncan test at $p \leq 0.05$).

Table 4. CHS activity of bean plant at the R6 stage of cultivars AB 136, Rio Tibagi, Carioca and Macanudo three days after treatment with salicylic acid or inoculation with the delta race of *C. lindemuthianum* and five days after challenge with the virulent pathotype (33/95) of *C. lindemuthianum*.

Treatments	CHS activity (pmol g ⁻¹ protein) ^a							
	Three days after induction				Five days after challenge			
	AB 136	Rio Tibagi	Carioca	Macanudo	AB 136	Rio Tibagi	Carioca	Macanudo
Salicylic acid	9.1a	5.2a	4.8a	6.9a	9.0a	5.2a	4.7a	6.9a
<i>C. lindemuthianum</i> (delta race)	7.9b	4.9a	4.8a	6.1b	7.8c	4.9a	4.6a	6.2b
Control	1.4c	1.0b	0.9b	2.3b	1.6d	1.0c	1.0c	1.1c
Virulent pathotype					8.4b	4.3b	3.5b	5.9b

^a Means followed by different letters in the same column are significantly different (Duncan test at $p \leq 0.05$).

After challenging, induced plants did not show an increase of CHS (tables 3 and 4). However, except Macanudo, the other cultivars showed higher CHS activity than the control plants also inoculated with the virulent pathotype.

In our study, the defenses of the AB 136 cultivar were probably triggered faster than in the other cultivars, who observed that this cultivar was resistant to several races of *C. lindemuthianum*, as demonstrated by Alzate-Marin (1997). A similar fact was confirmed by Cui et al. (2000) while working with cotton, in which the authors found that the induction of genes for the synthesis of terpenoid compounds in plants inoculated with *Verticillium dahliae* occurred earlier in resistant than in susceptible cultivars. They also observed that among the genes that encode the phenylpropanoid defense metabolism, mRNA of phenylalanine ammonia-lyase was expressed and mRNA coding for chalcone synthase was transcribed. These enzymes are needed for flavonoid biosynthesis. Caffeic acid *O*-methyltransferase, a key enzyme in lignin synthesis, was present at very high levels in the inoculated plants when compared to the water-treated controls.

Lower CHS and PAL enzymatic activities were detected in the water control treatments. However, after induction with salicylic acid, the inductive fungus, or the virulent pathotype we observed significant increases in the activities of these enzymes. However, it is interesting to observe that cultivar Carioca, which is considered susceptible, had lower enzyme activity in all developmental stages.

According to our results, the induction of the activity of the enzymes PAL (cv. Macanudo) and CHS (cvs. Rio Tibagi and Carioca) by *C. lindemuthianum* was equivalent to the induction by salicylic acid.

A complex response that involves the production of phytoalexin isoflavonoids may play a role in the different responses of the cultivars to the treatments, with the induction of enzymes CHS and PAL at different levels (Tepper et al., 1989) and the accumulation of phytoalexins (Hartleb, et al., 1997). The amount of enzyme mRNA accumulation would directly affect the biosynthesis of phenols and, possibly, CHS and PAL are involved in the increase of these compounds during the infection process (Hartleb et al., 1997).

In agreement with Nicholson and Hammerschmidt (1992), an increase in the activity of PAL can be considered as a biochemical marker for resistance, given that this enzyme is key for the necessary synthesis of phenols associated with resistance. Nevertheless, even if this hypothesis is correct, a specific interaction between the cultivar and the pathogen may occur.

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