Antibodies against Fungal Conidia and Antibiotics Inhibit Phenylalanine Ammonia-Lyase Activation in *Citrus*

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Summary

Antibodies raised against *Trichoderma harzianum* conidia prevented accumulation of Phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5.) in *Citrus*. The observed effect of the antibodies is produced by a delay in the formation of the fungal germination tube. An increase of PAL activity of ca. 7 fold in *C. limon* and *C. sinensis* seedlings, and of ca. 2 fold in *C. paradisii* seedlings was found, as an hypersensitive response to inoculation with *T. harzianum* conidia. This increase in activity was due to «*de novo*» synthesis of the enzyme as demonstrated by the effect of protein-synthesis inhibitors and by immunotitration.

Key word index: Rutaceae, Citrus, PAL, enzyme induction, antibiotics, Trichoderma harzianum, phytopathology.

Abbreviations: BSA = Bovine Serum Albumine; PAL = Phenylalanine ammonia-lyase; PDA = Potato Dextrose Agar; PMSF = Phenyl-methyl-sulphonyl fluoride.

Introduction

Phenylalanine ammonia-lyase (PAL[1]) (E.C. 4.3.1.5.) catalyzes the conversion of L-phenylalanine to trans-cinnamic acid, with the liberation of ammonia. This reaction is a key branch point in the biosynthesis of a wide variety of natural products based on the phenylpropane skeleton (Jones, 1984). PAL levels can increase upon different changes in environmental conditions such as exposure to UV light (Schröder et al., 1976), or upon microbial infection leading to the synthesis of compounds of the phenylpropanoid pathway (Dixon et al., 1981; Hahlbrock and Scheel, 1989; Loschke and Hadwiger, 1981). This increase can also be induced by elicitors from fungal cell walls or through the action of fungal enzymes on plant cell walls (Dixon et al., 1981; Hahlbrock et al., 1981; West, 1981; Darwill and Albersheim, 1984).

Changes in PAL synthesis have been described in several systems, as a response to elicitor treatment or direct fungal infection, where the increase in PAL from low basal levels reflects an increase in transcription and translation (Lawton et al., 1983; Ebel et al., 1984; Cramer et al., 1985; Edwards et al., 1985). Therefore, the accumulation of PAL could be used as a molecular sensor to measure if fungal infection has occurred, before visual symptoms appear.

Trichoderma harzianum has been isolated from sooty molds that infect *Citrus* trees, and its ability to excrete plant cell wall hydrolyzing enzymes has been previously described (Pérez et al., 1991).

In this report we demonstrate that *T. harzianum* conidia can induce a hypersensitive response in *Citrus* seedlings that is detected through PAL induction. We also report the ability of antibodies raised against *T. harzianum* conidia to delay fungal fruiting of «*in vitro*» cultures, and to suppress the in-

crease of PAL activity in *Citrus* seedlings when added simultaneously with fungal conidia.

Materials and Methods

All reagents were analytical grade and were purchased from Sigma and Merck.

Plant material

Citrus sinensis (orange), *C. limon* (lemon) and *C. paradisii* (grapefruit) seeds were directly obtained from ripe fruits collected from selected trees from one farm in Central Chile (Huertos de Betania, Mallarauco). They were surface sterilized with 10% NaClO for 30 min, thoroughly washed with sterile water, heated at 50 °C for 10 min, cooled with sterile water to room temperature, and placed on sterile clean pleated filter paper in complete darkness at 28 °C and 100% humidity. Once radicles reached 6 cm length, the seedlings were transferred to an illuminated chamber (128 W m^{-2}) and cultivated for two weeks. These seedlings were used for all experiments.

Source of fungus

Trichoderma harzianum was isolated directly from fumagine-infected *Citrus* trees on PDA DIFCO supplemented with chloramphenicol (250 mg L^{-1}), and reisolated on PDA several times until pure cultures were obtained, as previously described (Pérez et al., 1991).

Source of induction stimuli

Conidia from *T. harzianum* were produced on PDA, collected from 3 to 4-d cultures, and suspended in sterile NaCl (0.85% w/v) as described in AOAC (A.O.A.C., 1980). Conidia were counted and diluted to a final concentration of 1×10^{10} conidia L^{-1}, to be used as the standard inoculum for plant infection.

Infection of Citrus seedlings

Citrus seedlings were surface sterilized with 10% commercial NaClO and washed several times with sterile water. Three seedlings were placed per dish which contained 10 mL of Sabouraud broth DIFCO preinoculated with 1 mL suspension of conidia. They were incubated at 28 °C from 0 to 72 h to run kinetic experiments. Each experimental value corresponded to the mean of four separate dishes. All experiments were run in triplicate. Controls were preinoculated either with 1 mL sterile NaCl (0.85% w/v) or 1 mL of heat inactivated conidia. To run this latter control the NaCl conidia suspension was autoclaved at 121 °C for 20 min. The absence of surviving conidia was tested on PDA plates.

The effect of replication (10 µM Ethidium bromide), transcription (2 mg L^{-1} α-Amanitin and 50 mg L^{-1} Actinomycin D) and translation (2 mg L^{-1} Cycloheximide) inhibitors was tested in similar conditions. Plates containing 10 mL of Sabouraud broth were inoculated with the different inhibitors along with 1-mL conidia suspension, before placing the three surface-sterilized seedlings. Plates were incubated for 32 h at 28 °C. The growth of *T. harzianum* was not affected by any of the inhibitors at the concentrations used in the experiments.

Experiments performed with anti-conidia antibodies were run in similar conditions. Different dilutions of the antibody were prepared in 10 mM sodium phosphate, 150 mM sodium chloride pH 7.2 and added to the Sabouraud broth. After adding conidia as in the previous experiments, surface sterilized seedlings were placed in the plates and incubated for 32 h at 28 °C.

Additional controls were performed to measure a direct effect of the inhibitors and antibodies on the activity of PAL enzyme. Three surface-sterilized seedlings were incubated in 10 mL Sabouraud broth containing the antibiotic or antibody to be tested, but in the absence of fungal conidia. Plates were incubated for 32 hours at 28 °C.

Results are expressed as relative activity (ratio of PAL activity in treated seedlings ± additions)/PAL activity in control seedlings ± additions), and correspond to the mean of at least three different experiments, run in quadruplicate.

Enzyme activity

Homogenates of seedlings with different treatments were obtained after chopping the seedlings in 0.1 M sodium borate pH 8.8, 1 mM PMSF, and 10 mM β-mercapto ethanol, using 1 L kg^{-1} wet seedling mass. PAL was assayed spectrophotometrically (Zucker, 1965) in supernatants obtained after centrifugation of homogenates at 12,100 × g for 15 min. As a positive control, a commercial enzyme (Sigma) was used daily to monitor changes during the assay. Negative controls were performed with boiled enzyme from the corresponding treatment. The direct effect of replication, transcription and translation inhibitors, as well as the direct effect of antibodies on PAL activity was tested with homogenates from control seedlings, or with commercial PAL. The enzyme unit was defined as the amount of enzyme necessary to produce 1 picomole of cinnamic acid from L-Phe per second (1 pkat). Proteins were measured by the Coomassie blue dye method (Bradford, 1976).

Antibodies

The production of antisera was done as described by Bailey (1984). Blood samples were obtained from all rabbits before inoculation with antigens, to obtain preimmune sera.

Antibodies against conidia from *T. harzianum* (anti-conidia antibodies) were raised by inoculating 1×10^3 conidia at the following times: day 1 (subcutaneous, with complete Freund's coadjuvant); day 7 (subcutaneous, with incomplete Freund's coadjuvant) and day 15 (as in day 7). The animal was bled at day 28 and the serum titrated. The titre of the anti-conidia serum was estimated to be 1/400 by immunoaggregation.

PAL antibodies were raised by inoculating purified potato PAL into rabbits. The enzyme was purified as described (Havir and Hanson, 1968). The protein was inoculated in two 50-µg dosis at the following times: day 1 (subcutaneous, with complete Freund's coadjuvant) and day 11 (subcutaneous, with incomplete Freund's coadjuvant). The animal was bled at day 35. The titre of the anti-PAL serum was estimated to be 1/10,000 by immunotitration of potato PAL.

Immunotitration

Homogenates from control and infected seedlings were titrated with PAL antibodies. These were diluted in 0.1 M borate, pH 8.8, and 0.1% BSA. Aliquots of homogenates (200 µL) were treated with different dilutions of antibody, incubated at 4 °C for 30 min and centrifuged at 10,000 × g for 2 min. PAL activity was determined in the resulting supernatants.

Results and Discussion

PAL activity increased in *Citrus* seedlings treated with conidia of *T. harzianum* (Fig. 1). Heat inactivated conidia did not

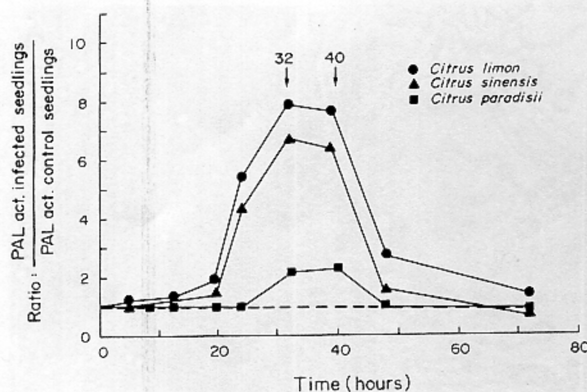

Fig. 1: PAL activation in *Citrus* spp. Kinetic response of *C. limon* (●), *C. sinensis* (▲) and *C. paradisii* (■) seedlings to infection with *Trichoderma harzianum* conidia in the absence and in the presence (dashed line) of a 1/10 dilution of the anti-conidia antibodies. PAL ratio was 1.0 for the three *Citrus* species infected in the presence of anti-conidia antibodies. The values represent the mean of at least three different experiments. Standard deviation was less than 5 %.

induce PAL activity. The maximal activation was found after 32 h of treatment for *C. limon* and *C. sinensis,* and after 40 h for *C. paradisii.* There was a difference in the degree of activation, that ranged from 2.2 for *C. paradisii* to 6.7 and 7.9 fold for *C. sinensis* and *C. limon,* respectively. After 48 h, PAL activity decreased to basal values, which fluctuated between 5 and 9 µkat kg^{-1} protein in all *Citrus* seedlings. The increase of PAL activity among the *Citrus* species tested, appears to be related to the ability of the plant system to defend itself from the fungus. It has been described that *T. harzianum* has the ability to secrete plant cell wall hydrolyzing enzymes, such as cellulases and pectinases (Pérez et al., 1991), and that these enzymes can act on intact seedlings releasing reducing sugars (Fanta et al., 1992). Therefore, these reducing sugars correspond to small oligosaccharides that might be recognized by defense systems of *Citrus* seedlings as elicitors. Other authors have shown that the degree of induction of PAL in parsley cell suspension cultures depends on the concentration of the elicitor (Hahlbrock et al., 1981). Thus, the differential results obtained with the *Citrus* species tested could be related to the concentration of the true elicitor reached during infection, or to a differential gene expression of the defense systems among these *Citrus* species.

T. harzianum has been described as a non-pathogenic fungus at the field level, and its inhibitory effect on the growth of several fungi is well known (Agrios, 1988), as well as its effect as a plant growth stimulator (Baker, 1988). PAL activation detected in *Citrus* seedlings would correspond to an hypersensitive response rather than a response to a true infection process.

The increase of PAL activity in *Citrus* seedlings, treated with fungal conidia, was completely suppressed by a 1/10 dilution of anti-conidia antibodies. No increase of PAL activity was observed up to 72 hours incubation (Fig. 1). To compare the effect of treatments, PAL activity was measured when the enzyme reached its peak activity, i.e. 32 hours for lemon and orange seedlings and at 40 hours for grapefruit specimens (Table 1). PAL activation was 50 % prevented with a 1/1,420 dilution of *T. harzianum* antiserum (Table 1). However, anti-conidia antibodies effectively suppressed PAL activation in *Citrus* seedlings only when they were added along with conidia, but not after fungal fruiting has taken place. Apparently, anti-conidia antibodies did not have a direct effect on PAL activity, but caused a 5 h delay in fungal fruiting. Direct observation of conidia reacting with the antibodies showed a significant delay in the development of the germinating tube as well as a swelling of the whole fungal structure when compared to the untreated control (Fig. 2). A similar effect on the development of the germinating tube was also observed when seedlings were treated simultaneously with fungal conidia and antibodies. Partially purified antibodies, through precipitation with ammonium sulphate (0/35 %), had the same effect (results not shown). The delay in fungal fruiting could account for the suppression of PAL activation at 32 and 40 h for lemon and orange, and grapefruit seedlings, respectively. A 5 h delay of the increase in PAL activity would have been expected if fruiting inhibition was the only effect of the antibody. On the other hand, direct treatment of lemon seedlings with commercial pectinase produced an increase of PAL activity at 18 hours, which was not affected by the presence 1/20 dilution of anti-conidia antibodies (Bravo et al., 1990). Therefore, if *T. harzianum* was secreting pectinase in the presence of antibodies, the enzyme would not be affected in its ability to induce PAL. Presumably another interaction of the antibodies with fruiting bodies of the fungus must occur in order to prevent PAL activation and infection up to 72 h, which could correspond to an inhibition of the synthesis and/or excretion of pectinase.

The use of actinomycin D and cycloheximide along with fungal conidia in treatments of *Citrus* seedlings, also suppressed PAL activation, suggesting that both transcription and translation are involved in the increase of PAL activity. Also α-amanitin, an inhibitor of RNA pol II, had a strong in-

Table 1: Pal activity in *Citrus* seedlings infected with *T. harzianum* conidia.

Treatments	Relative activity			
	Control (−Conidia)	Infected (+ Conidia)		
		C. limon	*C. sinensis*	*C. paradisii*
Water	1.0	7.9	6.7	2.2
Anti *T. harzianum* conidia antibodies (1/10)	1.0	1.0	1.0	1.0
Anti *T. harzianum* conidia antibodies (1/1,420)	1.0	3.8	3.3	1.0
10 µM Ethidium bromide[a]	1.0	7.9	6.7	2.2
2 mg L^{-1} α-amanitin[b]	1.0	1.0	1.0	1.0
50 mg L^{-1} Actinomycin D[b]	1.0	0.9	0.9	0.9
2 mg L^{-1} Cycloheximide[c]	1.0	1.0	1.0	1.0

Citrus seedlings were incubated in 10 mL of Sabouraud broth previously supplemented with 1 mL of 1×10^{10} conidia L^{-1} and with the respective addenda. Controls were performed with seedlings incubated with 10 mL Sabouraud broth with the respective addenda but without fungal conidia. After incubations of 32 h for lemon and orange and 40 h for grapefruit, the seedlings were homogenated and PAL activity was measured in the protein extracts, as described in Material and Methods. The concentrations of inhibitors used did not affect fungal growth. [a] = Inhibit DNA replication. [b] = Inhibit transcription. [c] = Inhibit translation. Results are the mean of at least three different experiments whose standard deviation did not exceed 5 %.

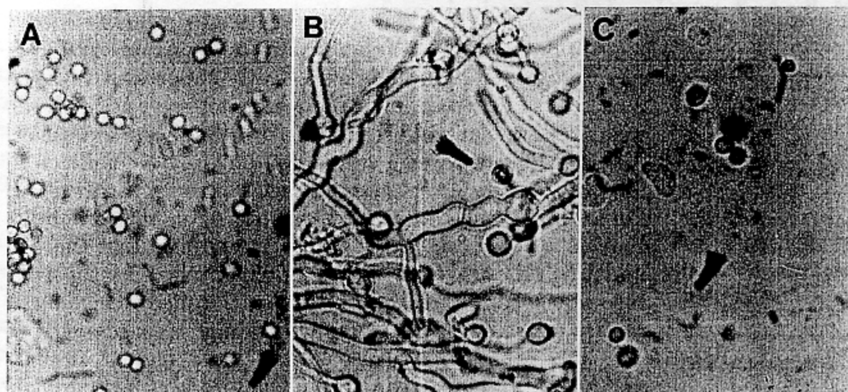

Fig. 2: *T. harzianum* incubated in Sabouraud broth at 28 °C for different times. A: Conidia at the beginning of incubation (time zero); B: Conidia incubated for 5 hours in the absence of anti-conidia antibodies; C: Conidia incubated for 5 hours in the presence of anti-conidia antibodies.

hibitory effect supporting an important involvement of transcriptional events (Table 1). Similar results have been obtained with cycloheximide and PAL activity in injured orange flavedo (Ismail and Brown, 1979), whereas actinomycin D did not have an inhibitory effect on the activation of the enzyme in orange flavedo. This difference can be easily explained considering the ultrastructure of the cells from this tissue. Flavedo cells have practically dissolved all the cytoplasmic organelles during the ripening of the fruits (Pérez and Garrido, 1985), and therefore an active transcription of genes is not expected. On the other hand, if PAL mRNA is stable enough, it would be possible to increase translation upon injury of the ripe fruits, as described (Ismail and Brown, 1979). No reports have been published for grapefruit or lemon fruits.

The concentrations of actinomycin D and of cycloheximide used in the *Citrus* system are lower than those reported for pea seedlings. In the latter system, 300 mg L^{-1} cycloheximide or 450 mg L^{-1} actinomycin D completely inhibited the synthesis of pisatin if added with the inducer, thus suggesting a similar behavior for PAL activity. In addition, the authors report that low concentrations of actinomycin D, such as 10 mg L^{-1}, stimulates pisatin synthesis and PAL activity (Schwochau and Hadwiger, 1969; Hadwiger and Schwochau, 1971). This effect was not observed in the *Citrus* system.

Ethidium bromide, an inhibitor of nuclear, mitochondrial, and chloroplast DNA polymerases, did not affect the induction of PAL. This result suggests that DNA replication is not related to PAL induction in infected *Citrus* seedlings, and that probably no extra copies of the gene are required for the synthesis of the enzyme. On the other hand, ethidium bromide and other compounds interacting with DNA intercalating compounds induce PAL activity and accumulation of pisatin in pea seedlings (Hadwiger and Schwochau, 1971). Thus the differences observed for the effect of replication, transcription and translation inhibitors in different plant species could be explained by specific genetic characteristics.

In order to discard an inhibitory effect on PAL activity, of the different compounds used in seedling treatments, these were added at the same concentrations to a standard assay medium, using homogenates from control seedlings or the commercial enzyme. Antibodies against fungal conidia (1:2000 to 1:10 dilutions), 10 µM ethidium bromide, or

Table 2: Immunotitration of PAL activity in lemon seedlings infected with *T. harzianum* conidia.

Treatment	($I_{0.5}$)* (Antisera dilution) Control (−Conidia)	Infected (+Conidia)	Ratio (Control/Infected)
Water	1:3,980	1: 501	7.9
Anti *T. harzianum* conidia antibodies (1/10)	1:3,980	1:3,180	1.2
10 µM Ethidium bromide[a]	1:3,980	1: 505	7.9
2 mg L^{-1} α-amanitin[b]	1:3,980	1:3,980	1.0
50 mg L^{-1} Actinomycin D[b]	1:3,980	1:4,975	0.8
2 mg L^{-1} Cycloheximide[c]	1:3,980	1:3,965	1.0

* $I_{0.5}$ corresponded to the antisera dilution necessary to decrease PAL activity to 50 %.

Citrus seedlings were incubated as described in Table 1 but after the homogenization of the seedlings, the protein extracts were immunotitrated as described in Material and Methods. The concentrations of inhibitors used did not affect fungal growth. [a] = Inhibit DNA replication. [b] = Inhibit transcription. [c] = Inhibit translation. Results are the mean of at least three different experiments run in quadruplicates. Standard deviation did not exceed 5 %.

2 mg L^{-1} α-amanitin, or 50 mg L^{-1} actinomycin D or 2 mg L^{-1} cycloheximide, did not affect PAL activity.

Homogenates from seedlings with different treatments were immunotitrated with anti-PAL antibodies, in order to confirm the effect of antifungal antibodies and transcription and translation inhibitors (Table 2). Results show that the treatment with fungal conidia produces an increase in the mass of PAL. The inhibitory effect of α-amanitin, actinomycin D and cycloheximide on PAL activity correlated with the suppression of the mass increase of the enzyme. These results suggest that antibiotics can block the «*de novo*» synthesis of the enzyme. On the other hand, anti-conidia antibodies also prevent any «*de novo*» expression of PAL gene, probably by eliminating the signal that triggers plant defense mechanisms.

The screening of PAL activity in a visually healthy *Citrus* seedling population showed that it ranged from 5 to 30 µkat kg^{-1}. Further incubation of homogenates from these seedlings in Sabouraud broth demonstrated microbial development in samples which contained high PAL activities, whereas those from samples which contained low PAL activ-

ity remained uncontaminated. Therefore, the level of PAL activity might be used to screen a population of plant material to ascertain stress conditions, either from microbial contamination or otherwise, thus behaving as a molecular sensor. As described by other authors, PAL activation occurs under several stress conditions (Loschke and Hadwiger, 1981; Hahlbrock and Scheel, 1989).

The results presented show that *T. harzianum* induce PAL activity in *Citrus* seedlings, probably as a response in the hypersensitive reaction of the seedlings towards the fungus. The increase of PAL activity can be prevented by using α-amanitin, actinomycin D and cycloheximide, suggesting that the observed activation was due to an increase in transcription and/or translation of the PAL gene. PAL immunotitrations confirm that these antibiotics can block the «*de novo*» synthesis of the enzyme. The use of *T. harzianum* anti-conidia antibodies suppresses PAL activation, probably through elimination of the interaction between plant surface and microorganism, altering the signal that triggers the plant defense mechanisms. It is noteworthy that the use of antibodies can delay fungal germination. The production of transgenic plants that could synthesize antibodies as has been described (Hiatt, 1990), or the development of new antibody-like molecules that prevent fungal fruiting might be very interesting and could possibly be used for the control of fungal diseases in the future.

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