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Phenylalanine ammonia-lyase activity in suspension cultures of *Ulmus pumila* and *U. campestris* treated with spores of *Ceratocystis ulmi*

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Summary. Cell suspension cultures of a Ceratocystis ulmi-resistant (Ulmus pumila) and a -susceptible elm (U.campestris) were established from leaf callus tissue. Treatment of cultures with spores of C.ulmi induced a large increase in the activity of phenylalanine ammonia-lyase, only in the cells of the resistant species U.pumila with a maximum after 24 h. Inoculated U.pumila cells also excreted a red unidentified chemical into the culture medium. Neither responses were induced in inoculated U.campestris cultures. The results are discussed in relation to the development of the elm cell culture system as a model for studying the differential biochemical mechanisms of disease resistance in elms.

Abbreviations: BA, 6-benzylaminopurine; BSA, bovine serum albumin; 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog (1962); PAL, phenylalanine ammonia-lyase.

Introduction

For more than 60 years many workers have addressed the highly destructive Dutch elm disease, caused by the ascomycete Ceratocystis ulmi (Buisman) C. Moreau. During this period intensive efforts have been made to understand and combat this disease, but despite these efforts many questions concerning the mechanisms of disease resistance remain to be elucidated.

Jeng et al. (1983) found several phytoalexins (mansonones) in Ulmus americana after inoculation with non-aggressive isolates of C.ulmi followed by inoculation with an aggressive one, and recently Yang et al. (1989) demonstrated that fungal culture filtrates, cytoplasm or cell walls of C.ulmi contain molecules that elicit mansonone accumulation in elm callus.

However, on the callus of a susceptible elm (U.americana) the elicitors cause higher amounts of mansonone production than on the callus of a resistant elm (U.pumila). Furthermore, mansonone F, which in another study was found to constitute the bulk of the fungitoxic material produced in elm, was not very toxic to C.ulmi (Overeem and Elgersma 1970).

Since comparative biochemical investigations of compatible and incompatible interactions have made use of cell culture techniques (Apostol et al. 1989, Hahlbrock and Scheel 1989), cell cultures of a resistant elm species, U.pumila and of a susceptible one, U.campestris, were established in order to elucidate, as part of a comprehensive program, the differential mechanisms of disease resistance in elms.

An apparently ubiquitous feature of plant responses to pathogen challenge is the activation of phenylpropanoid metabolism (Hahlbrock and Scheel 1989, Lamb et al. 1989), suggesting general defensive roles for phenylpropanoid compounds, although this aspect has not yet been studied in elm trees.

Thus, the aim of the present work has been to investigate whether one of the most representative reactions of phenylpropanoid metabolism, that catalyzed by PAL, is altered in elms in response to the fungus C.ulmi.

Material and methods

Plant Cultures. Callus cultures were initiated from leaves of mature Ulmus pumila (15-20 years old) or U.campestris (30-40 years old) trees. Leaves were washed in distilled water for 2 h and then surface-sterilized in 70% (v/v) ethanol for 30 s followed by immersion in a solution of commercial bleach (0.9% (w/v) sodium hypochlorite, final concentration) for 15 min and rinsed 5-6 times with autoclaved distilled water. Leaf segments were plated on MS medium supplemented with 0.1 $mg.l^{-1}$ 2,4-D, 2 $mg.l^{-1}$ BA, 200 $mg.l^{-1}$ casein hydrolysate, 3% (w/v) sucrose and 0.7% (w/v) agar. The

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pH of the medium was adjusted to 5.7 prior to autoclaving. Cultures were incubated in the dark at 24±2°C. Proliferating callus was routinely subcultured by transferring it every 5 weeks to half strength MS media containing the same supplements.

Suspension cultures were established from leaf-derived callus by transferring approximately 2g fresh weight to 250 ml Erlenmeyer flasks containing 50 ml of the same media employed to initiate callus cultures without agar. Cultures were shaken on a rotary shaker at 110 rpm in darkness and subcultured every 3 weeks in the same medium. The cultures used in this study, unless otherwise indicated, had been maintained as suspensions for over 3 months prior to experimental work. Viability was measured according to the method described by Widholm (1972).

Pathogen culture. Cultures of Ceratocystis ulmi were maintained on Petri dishes in previously autoclaved malt-extract agar. Spores were isolated from cultures grown for 8 days according to published procedures (Garcia et al. 1991).

Plant-pathogen culture. Callus.Thin callus layers of U.pumila or U.campestris cultures were prepared by spreading 2-3 g fresh weight of 30 day-old callus on the surface of solidified callus subculture medium in 35x10 mm Petri dishes with an sterile spatula. For inoculation, 15µl of fungal spore suspension ($2x10^5$ spores) was transferred to sterile moist filter paper discs (1.5 cm diameter Whatman no 1) placed on 7 day-old elm callus layers. Inoculated plates were incubated in the dark at 24°C±2.

Suspension cultures. Approximately 1 g fresh weight of 21 day-old suspension cultures was transferred to 125 ml Erlenmeyer flasks containing 25 ml of suspension culture medium and incubated for different periods at 24°C±2 (110 rpm) before inoculation. Spore suspension, (100 µl), at several concentrations, was added to each flask. Inoculated cultures were maintained in the same conditions for the time periods denoted in Results. Controls were inoculated with sterile distilled water. Observation of the germination of the spores and fungal growth in suspension cultures were carried out under a light microscope. A spore was considered to have germinated when the length of the germ tube had attained at least half the diameter of the spore (García et al. 1991).

Determination of PAL (E.C. 4.3.1.5.) activity. Elm cells were collected on Miracloth under suction and flash frozen in liquid nitrogen followed by further homogenization in borate buffer (0.05M $Na_2B_4O_7$, 0.2M H_3BO_4, 0.05M NaCl, pH 8.8) containing 5 mM mercaptoethanol and 5% (w/v) insoluble polyvinylpyrrolidone. The homogenate was centrifuged at 20000 g for 10 min and the resulting supernatant was used as enzyme preparation. All steps were performed at 2°C. PAL activity was determined spectrophotometrically according to published methods (Bolwell et al. 1985). Protein was determined by the Bradford (1976) assay with BSA as standard.

Results and Discussion

Co-culture Ceratocystis ulmi-Ulmus pumila/U.campestris

When callus layers of U.pumila and U.campestris were inoculated with fungal spores, mycelial growth was first visible on the paper disc approximately 48 h after inoculation in U.campestris callus. After one week the tissues were completely covered by fungal mycelium. By contrast, no fungal growth was observed in U.pumila cultures after 4 days of inoculation. Callus layers of this species were overgrown by the fungus only 10-15 days later. As stated by Helgeson (1983), this differential rate of fungal growth on "in vitro" cultured Ulmus sp. cells reflects a certain degree of resistance of the U.pumila tissue from which the cultures were derived.

There are a number of plant species for which the expression of disease resistance in tissue culture has been investigated, ie. tomato (Warren and Routley 1970), tobacco (Helgeson et al. 1972, Helgeson et al. 1976), soybean (Holliday and Klarman 1979), or chickpea (Keßmann and Barz 1987). The results reported above reveal that tissue cultures of elm can also be used as a model system to study host-pathogen interactions.

Although callus cultures offer several advantages for the study of disease resistance, suspension cultures are commonly employed in biochemical studies due to a more uniform contact of the pathogen with the cells.

Cell suspension cultures were established from leaf callus tissue of Ulmus pumila and U.campestris. The cell cultures grew as almost colorless, stable and fine suspensions, showing a typically sigmoidal growth curve. Figure 1 shows the changes in fresh weight of suspended cells and in both cases (species) the latent phase of growth was very prolonged. The viability of the cultures during the study period was higher than 80%.

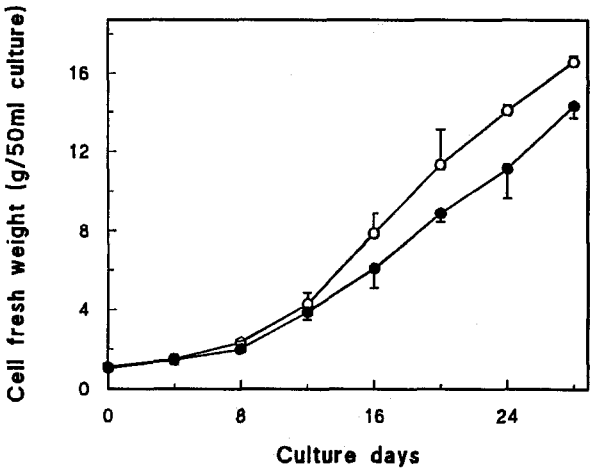

Fig. 1. Changes in cell mass of Ulmus pumila (-●-) and U.campestris (-O-) suspension cultures. Error bars = SD.

The germination of the spores in suspension cultures were measured 12 h after inoculation. In U.pumila cultures the germination percentage of the spores was 30% whereas in U.campestris cultures a 75% was observed. At extended periods of incubation measurement of germination percentages became difficult because C.ulmi is an hyphomycete with secondary production of conidia (Bernier and Hubbes 1990). Fungal growth, as with the callus cultures, was less pronounced in the

resistant species, U.campestris suspensions being completely overgrown by the fungus 72-84 h post-inoculation. In U.pumila this result could be only noticed 5-6 days later. Therefore, this system was used for further study.

Effect of fungal spores on PAL activity of Ulmus pumila cell cultures

The results shown in Fig. 2 reveal that the co-culture C.ulmi-U.pumila stimulates PAL activity (measured 24 h after inoculation) in elm cells regardless of the age of the culture, although maximum stimulation was noticed 4 days after subculture. This result contrasts in part to those of several reports in which the elicitation of PAL activity decreased as the cells aged (Apostol et al. 1989, Moniz de Sá et al. 1992).

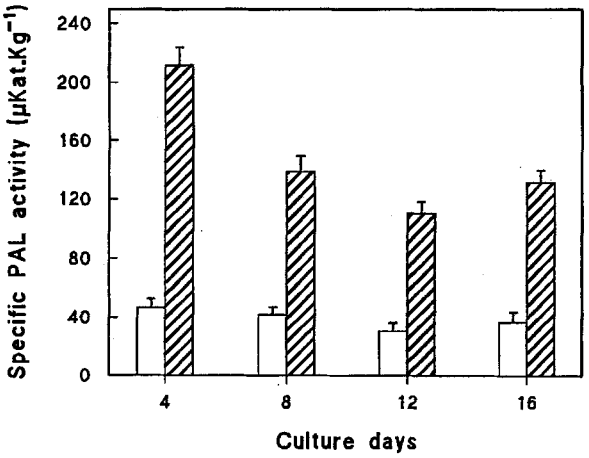

Fig. 2. PAL enzyme activity in untreated Ulmus pumila cells (open bars) and cells treated with 10^6 spores/ml culture (filled bars) at various times after subculture. Enzyme activity was measured 24 h after spore application. Error bars = SD.

As has been observed in other systems (Hahlbrock et al. 1981), the absolute values for induced enzyme activities could be directly compared only when the same batch of cells was used. To facilitate comparison of the results, in all of the ensuing experiments, PAL activity obtained with control cells was subtracted from the activity obtained after the specific treatments.

In order to determine optimal concentration of the spores for the induction of PAL activity, four day-old cultures were treated for 24 h with 10^4, 10^5, 10^6, 5.10^6 and 10^7 spores/ml culture. PAL activity (Fig. 3) increased in a dose-dependent manner before reaching a maximum. The decreased PAL activity at high spore concentration can not be only explained in terms of losses in cell viability, which was near 60% at the highest concentration tested. This result has also been observed by Hille et al. (1982) who found a reduction on the stimulatory effect of an elicitor from Phytopthora megasperma on PAL activity in cultured soybean cells. In view of these results further experiments were carried out using 10^6 spores/ml culture.

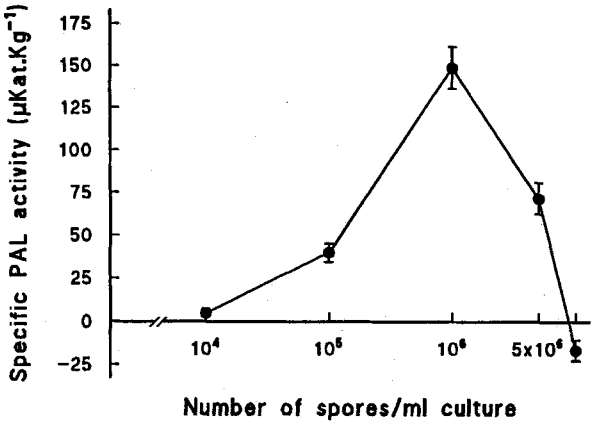

Fig. 3. Levels of PAL enzyme activity in four-day old Ulmus pumila cells treated with different spore concentrations. Results are from two independent experiments, each in duplicate (±SD), and all values were corrected for levels of enzyme activity in untreated cells (specific activity 40.5 ±5.4 µkat.kg^{-1} protein) . Enzyme activity was measured 24 h after elicitation.

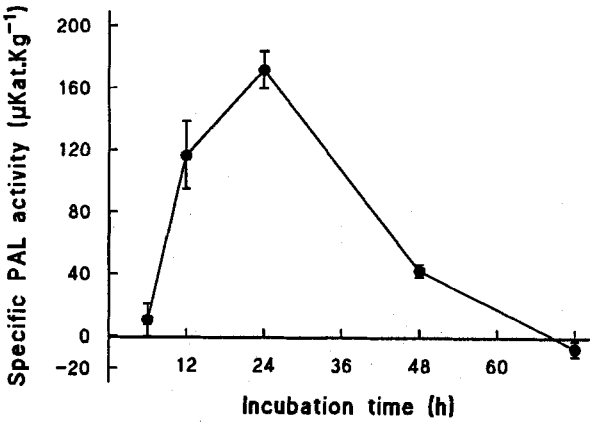

Fig. 4. Changes in PAL activity following treatment of Ulmus pumila cells with 10^6 spores/ml culture. Results are from two independent experiments, each in duplicate (±SD), and all values were corrected for levels of enzyme activity in untreated cells (specific activity 40.5±5.4 µkat.kg^{-1} protein).

The time course of activation of PAL activity after exposure of 4 day-old cells to the spores from 0 to 72 h, is shown in Fig. 4. Enzyme activity above basal levels was only detectable after 6-12 h, reaching a maximum at 24 h. After three days of treatment, activity levels were equal or less than those of the controls. By days 4-6, cell death occurred (data not shown).

It should be noted that at 12 h post-inoculation cultures turned red. This phenomenon was even noticed in cultures inoculated with 10^4 or 10^5 spores, although the reddish coloration was observed later (data not shown). Chloroform extracts of the medium or of the cells

extracted with aqueous methanol were applied to silica gel plates containing fluorescent indicator. Following development with toluene: ethyl acetate: acetic acid (50:30:4) , a red spot (Rf= 0.25), visible to the naked eye, appeared only in the medium of inoculated cultures. Chemical identification of this compound is currently under investigation.

The induction of enzymes of phenylpropanoid metabolism after a fungal attack has been frequently demonstrated in herbaceous species (Zahringer et al. 1978, Ebel 1979, Hille et al. 1982), but relatively little is known about this in woody plants (Moniz de Sá et al. 1992). Our results demonstrate, we believe for the first time, that an enzyme of this metabolic pathway is induced in elm cells after inoculation with spores of the pathogen Ceratocystis ulmi.

Effect of fungal spores on PAL activity of Ulmus campestris cell cultures

When U.campestris cells at different stages of growth, were inoculated with different concentrations of fungal spores cell death occurred 72h post-infection at the highest concentration tested (10^7 spores/ml culture). However, with 10^4, 10^5 or 10^6 spores /ml culture, cells remained viable for at least two-three days, cell viability at 48 h being near 65% at 10^6 spores/ml culture. In cultures inoculated with these spore concentrations, PAL activity, measured 3, 6, 12, 24 and 48 h after inoculation, remained at the same levels as those detected in untreated cells.

The results presented in this work show that inoculation of U.pumila cell cultures with spores of C.ulmi induces an increase in PAL activity and the excretion of a red compound into the medium. However, these responses were not observed in U.campestris inoculated cultures. The question of the significance of these differential responses in the mechanism of resistance to Dutch elm disease is raised. These preliminary data open a way to studying this aspect in depth.

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