BIOLOGIA PLANTARUM 39 (3): 349-353, 1997

Phenylalanine ammonia-lyase activity in alfalfa suspension cultures treated with conidia and elicitors of *Verticillium albo-atrum*

M. KOIKE and K. NANBU

Department of Agro-Environmental Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan

Abstract

Alfalfa (*Medicago sativa* L.) cell suspension cultures of *Verticillium albo-atrum* resistant and susceptible genotypes were established from leaf callus tissues. Treatment of cultures with conidia and heat-released elicitors of *V. albo-atrum* induced a large increase in the activity of phenylalanine ammonia-lyase, only in the cells of the resistant genotypes with a maximum after 12 h. In co-cultivation with the fungal conidia and resistant cell lines, the production of spores were inhibited.

Additional key words: *Medicago sativa*, resistant and susceptible genotypes.

Introduction

Many researchers have addressed the highly destructive alfalfa *Verticillium* wilt caused by *Verticillium albo-atrum* Reinke *et* Berth., but a lot of questions concerning the mechanisms of disease resistance remain to elucidate.

An apparently ubiquitous feature of plant responses to pathogen challenge is the activation of phenylpropanoid metabolism (Hahlbrock and Scheel 1989, Lamb *et al.* 1989). The comparative biochemical investigations of compatible and incompatible interaction between alfalfa (*Medicago sativa* L.) and *Verticillium albo-atrum* have been made using alfalfa callus and the fungal colony mat (Latunde-Dada and Lucas 1986, Latunde-Dada *et al.* 1987) or the fungal heat release elicitor (Koike and Shimada 1992, Koike *et al.* 1992).

The aim of the present work has been to investigate whether one of the most representative reactions of phenylpropanoid metabolism, that catalyzed by phenylalanine ammonia-lyase (PAL; E.C.4.3.1.5.), is altered in resistant and susceptible alfalfa cell lines in response to the fungus *V. albo-atrum* and its heat released elicitor.

Received 21 June 1996, *accepted* 17 October 1996.
e-mail: koike@obihiro.ac.jp

Materials and methods

Plants: Callus cultures were initiated from young leaves of alfalfa (*Medicago sativa* L.) plants. Five genotypes were used in this study; three resistant genotypes: K19-1, K45-2 and V4-3, two susceptible genotypes: K17-1 and V26-1 (K and V means their initials of cultivars Kitawakaba and Vertus, respectively). Leaves were washed in a distilled water for 2 h and them surface-sterilized in 70 % ethanol for 30 s followed by immersion in a solution of commercial bleach (0.9 cm^3 dm^{-3} sodium hypochlorite, final concentration) for 15 min and rinsed 5 - 6 times with sterilized distilled water. Leaf segments were plated on SH (Schenk and Hilderbrandt 1972) medium supplemented with 2.0 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg dm^{-3} kinetin, 20 g dm^{-3} saccharose and 8 g dm^{-3} agar. The pH of the medium was adjusted to 5.8 prior to autoclaving. Cultures were incubated in the dark at 25 ± 1 °C. Proliferating callus was routinely subcultured by transferring it every 4 weeks to the new media.

Suspension cultures were established from leaf-derived callus by transferring approximately 2 g fresh mass to 100 cm^3 Erlenmeyer flasks containing 30 cm^3 of the same media employed to initiate callus cultures without agar. Cultures were shaken on a rotary shaker at 100 rpm in darkness and subcultured every 2 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.

Pathogen culture: Cultures of *Verticillium albo-atrum* (alfalfa pathotype: Vaa-k01) were maintained on Petri dishes in previously autoclaved potato saccharose agar. Conidia were isolated from suspension cultures grown for 7 d according to publishcd procedures (Koike *et al.* 1991).

Plant-pathogen co-culture and elicitor treatment: Approximately 1 g fresh mass of 14-d-old suspension cultures was transferred to 100 cm^3 Erlenmeyer flasks containing 20 cm^3 of suspension culture medium and incubated for 7 d before the fungal spore inoculation and the elicitor treatment. Spore suspension was added to each flask to adjust its spore density 4×10 cm^{-3}. Heat-released elicitor was isolated according to Buiatti's methods (Buiatti *et al.* 1985, Koike and Shimada 1992). The elicitor concentration (10 μg cm^{-3}) was adjusted by glucose equivalent. Inoculated and treated cultures were maintained in the same conditions for the time periods denoted in Results.

Determination of PAL activity: Alfalfa cells were collected on Miracloth under suction and flash frozen in liquid nitrogen followed by further homogenization in Tris-HCl buffer (pH 8.8). The homogenate was centrifuged at 10 000 *g* for 10 min and the resulting supernatant was used for enzyme preparation. PAL activity was determined spectrophotometrically according to Bolwell's method (Bollwell *et al.* 1985). Protein was determined by the Lowry (1951) assay with bovine serum albumine as a standard.

Results and discussion

Effects of fungal spores on PAL activity of alfalfa cell cultures: Co-culture stimulates PAL activity in resistant cell lines (K19-1, K45-2 and V4-3) at 12 or 24 h after inoculation (Fig. 1) In the susceptible cell lines (K17-1 and V4-3), PAL activity slightly increased. There are significant differences of the PAL activity at 12 h after inoculation between resistant and susceptible cell lines derived from both cultivars.

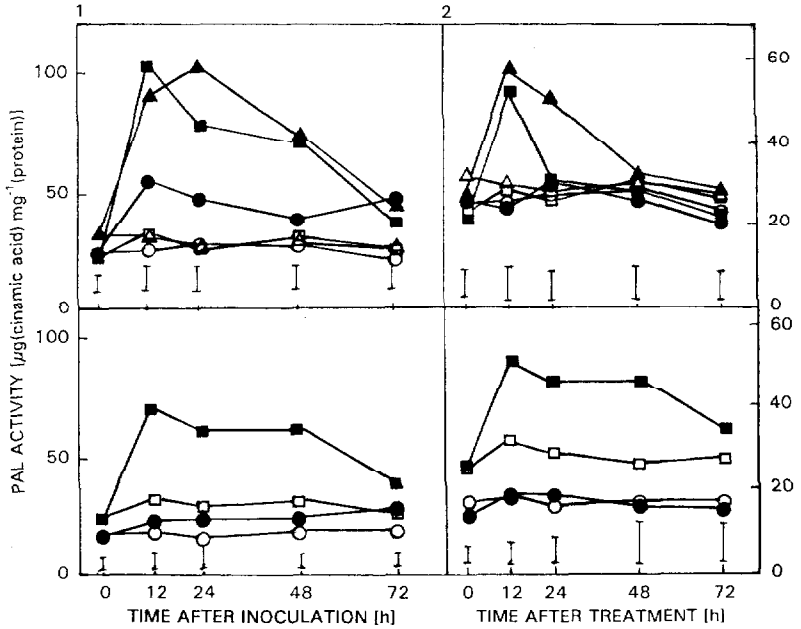

Fig. 1. Changes in the activities of phenylalanine ammonia-lyase (PAL) in alfalfa suspension cultures following treatment with *Verticillium albo-atrum* conidia. *A* - cv. Kitawakaba: susceptible genotype K17-1 (*circles*) and resistant genotypes K19-1 (*triangles*) and K45-2 (*squares*). *B* - cv. Vertus: susceptible genotype V26-1 (*circles*) and resistant genotype V4-3 (*squares*). *Closed points* - inoculated cultures, *open points* - control. *Vertical bars* indicate L.S.D. ($P = 0.05$).

Fig. 2. Changes in the activities of phenylalanine ammonia-lyase in alfalfa suspension cultures following treatment with *Verticillium albo-atrum* elicitor. *Closed points* - treated cultures, *open points* - control. For other details see Fig. 1.

In alfalfa - *V. albo-atrum* interactions, Latunde-Dada and Lucas (1986) and Koike and Shimada (1992) reported similar results using callus tissue. The results reported above reveal that suspension culture of alfalfa can also be used as a model system to study host-pathogen interactions.

Increase of the spore density was estimated after 72 h inoculation. The spore densities of susceptible cell lines were higher than those of resistant cell lines about three times (Fig. 3). This results indicated that some antifungal substances may be secreted from suspension cell, especially in resistant cell lines. Chemical identification of these compounds are currently under investigation.

Effect of heat-released elicitor on PAL activity of alfalfa cell cultures: Increase in PAL activity was found only in three resistant lines, reaching maximum at 12 h. There were no peaks in two susceptible lines (Fig. 2).

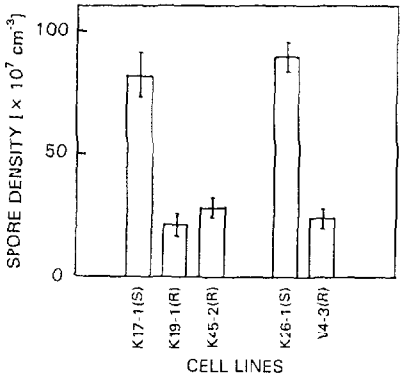

Fig. 3. Spore (conidia) density ($\times 10^7$ cm^{-3}) after 72 h inoculation of *Verticillium albo-atrum* spore in resistant (R) and susceptible (S) cell lines. *Vertical bars* indicate standard errors.

Significant correlation between *in vivo* resistance to pathogen and *in vitro* increase of phenylpropanoid metabolism following elicitor treatments have been found in several cases (Buiatti *et al.* 1985, Koike and Shimada 1992). But these reports were concerned with the callus culture with elicitor. Our results demonstrate, we believe for the first time, that an enzyme of this metabolic pathway is induced in alfalfa suspension cells after treatment with heat-released elicitors of *V. albo-atrum*.

The results presented in this work show that resistant alfalfa cell lines inoculated and treated with spores and elicitor of *V. albo-atrum* induce an increase in PAL activity. However, these responses were not observed in susceptible cell lines. The question of the significance of these differential responses in the mechanism of resistance to alfalfa *Verticillium* wilt disease is raised. These pathogen or elicitor - suspension culture systems provide valuable tools for research in *Verticillium* wilt disease resistance.

References

Bolwell, G.P., Bell, J.N., Craamer, C.L., Schuch, W., Lamb, C.J., Dixon, R.A.: L-phenylalanine ammonia-lyase from *Phaseolus vulgaris*. Characterization and differential induction of multiple forms from elicitor-treated cell suspension cultures. - Eur. J. Biochem. **149**: 411-419, 1985.

Buiatti, M., Scala, A., Bettini, P., Nascaari, G., Morpurgo, R., Bogoni, P., Pellegrini, G., Gemelli, F., Venturo, R.: Correlation between *in vivo* resistance to *Fusarium* and *in vitro* response to fungal elicitor and toxic substances in carnation. - Theor. appl. Genet. **70**: 42-47, 1985.

Hahlbrock, K., Schell, D.: Physiology and molecular biology of phenylpropanoid metabolism. - Annu. Rev. Plant Physiol. Plant mol. Biol. **40**: 347-369, 1989.

Koike, M., Nanbu, K., Shimada, T.: Alfalfa-*Verticillium albo-atrum* interaction: *In vitro* responses of alfalfa callus to fungal culture filtrates and cell wall components. - J. Jap. Grassland Sci. **37**: 412-419, 1992.

Koike, M., Shimada, T.: Alfalfa-*Verticillium albo-atrum* interaction. II. *In vitro* peroxidase and phenylalanine ammonia-lyase activities enhanced by treatment with fungal elicitor. - Plant Tissue Cult. Lett. **9**: 81-85, 1992.

Koike, M., Yoshida, Y., Kagaya, Y., Shimada, T.: *In vitro* selection and somaclonal variation in alfalfa *Verticillium* wilt. - Plant Tissue Cult. Lett. **8**: 152-157, 1991.

Lamb, C.J., Lawton, M.A., Dron, M., Dixon, R.A.: Signal and transduction mechanisms for activation of plant defense against microbial attack. - Cell **56**: 215-224, 1989.

Latunde-Dada, A.O., Lucas, J.A.: Influence of temperature on host resistance and fungal sensitivity to medicarpin in lucerne callus lines infected with *Verticillium albo-atrum*. - Physiol. mol. Plant Pathol. **28**: 89-97, 1986.

Latunde-Dada, A.O., Dixon, R.A., Lucas, J.A.: Induction of phytoalexin biosynthetic enzymes in resistant and susceptible lucerne callus lines infected with *Verticillium albo-atrum*. - Physiol. mol. Plant Pathol. **31**: 15-23, 1987.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Shenk, R.U., Hilderbrandt, A.C.: Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell culture. - Can. J. Bot. **50**: 199-204, 1972.