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Alfalfa-*Verticillium albo-atrum* Interactions: II. *In vitro* Peroxidase and Phenylalanine Ammonia-Lyase Activities Enhanced by Treatment with Fungal Elicitors

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With the aim of ascertaining the existence of a correlation between *in vivo* resistance to *Verticillium albo-atrum* and *in vitro* responses to the fungal cell wall components, peroxidase (PO) and phenylalanine ammonia lyase (PAL) were assayed using *in vitro* cultures of three alfalfa genotypes (resistant, intermediate resistant and susceptible). Calli derived from these three genotypes treated with *V. albo-atrum* cell wall components showed varying degrees of increase in PO and PAL activities. The differential responses of these enzymes to the fungal cell wall components coincided with genetic differences among the three genotypes.

Introduction

Recently, plant tissue and cell cultures have been used for the selection of cultivars resistant to pathogens[1-4]. A protocol that has been widely used for the selection of disease resistant lines is to grow callus in the presence of a culture filtrate or toxins. A correlation between *in vivo* resistance and *in vitro* resistant reaction is required for using tissue culture techniques for the selections. Two kinds of resistant reaction, namely, resistance to toxins or fungal culture filtrates and synthesis of antimicrobial compounds (phytoalexins) can be tested *in vitro*[5].

In a previous study[6], we reported interactions between alfalfa callus and *Verticillium albo-atrum* Reinke *et* Berthold, and suggested that the antifungal activity in the callus extracts indicated by the inhibition of germ tube elongation of fungal conidia after treatment with *V. albo-atrum* cell wall components (abbreviated as Vaa elicitor) was a better criterion than the tolerance to fungal culture filtrates for the selection of resistant cultivars.

A suberization of a vascular tissue is associated with the resistance to fungal wilt diseases, and Peroxidase is known to be a key enzyme involved in suberization[7,8]. Phenylalanine ammonia-lyase is also a key enzyme in the syntheses of phenolics, phytoalexins and lignin[9].

The aim of this paper is to study the possible correlation between *in vivo* resistance of alfalfa genotypes and *in vitro* callus tissue responses (PO and PAL activities) to Vaa elicitor.

Materials and Methods

1. *Biological materials*

Three alfalfa (*Medicago sativa* L.) genotypes were used: a highly resistant genotype of cv. Vertus, an intermediate resistant genotype of cv. Kitawakaba and a susceptible genotype of cv. Europe to Verticillium wilt. The profile of each genotype is shown in **Table 1**. An isolate *V. albo-atrum* was

Table 1. Verticillium wilt resistance of three alfalfa genotypes used in this experiments

Genotype (cv.)	Wilting score[a]
Resistant (Vertus)	0. 2±0. 1
Intermediate (Kitawakaba)	2. 3±0. 2
Susceptible (Europe)	4. 7±0. 2

a: Wilting score was based on six-point severity scale where 0=lowest and 5=highest with inoculated *Verticillium albo-atrum* conidia (Koike *et al*[4]).
± indicates standard error (n=15).

obtained from Mr. R. Sato, Hokkaido National Agricultural Experimental Station, Japan.

2. *Tissue culture of alfalfa*

Calli of alfalfa were induced from young leaves as explants. They were grown on a medium of SH[10] supplemented with 2 mg/*l* 2, 4-D, 2 mg/*l* NAA, 2 mg/*l* kinetin and 0. 8% agar in an incubator at 25±1 °C. Calli were routinely transferred every 4 weeks. Callus R, IR and S represent calli derived from each genotype (R, Vertus; IR, Kitawakaba; S, Europe).

3. *Callus inoculation with colony mat of V. albo-atrum*

Callus pieces (about 1. 5 cm diameter) were transferred onto SH agar medium in 9 cm petri dishes and maintained at 20°C for 7 days prior to inoculation. Colony mats (0. 5 mm diameter) were cut from the growing edge of Potato Sucrose Agar plate cultures (three weeks) of the fungus and placed at the center of each callus. Three replicates with 5 samples in each callus line were prepared and all cultures were incubated at 20°C in the dark. The colony diameter of the fungus on each callus was measured.

4. *Treatment of alfalfa calli with V. albo-atrum cell wall components*

A Vaa elicitor was prepared as described previously[6]. For the enzyme assay 7 day-old callus pieces (about 500 mg fresh weight) were treated with 50 μl of Vaa elicitor (0. 1 mg/m*l* as glucose equivalent) and maintained at 20°C in the dark on SH medium.

5. *Determination of enzyme activities*

Callus tissues (500 mg) treated with Vaa elicitor were homogenized in 5 m*l* 50 mM phosphate buffer (pH 6. 0). The homogenate was centrifuged at 10, 000 g for 10 min at 4°C and the activity of each enzyme in the supernatant was determined.

Peroxidase (PO): An aliquot of the callus extract (100 μl) was added to 3 m*l* of assay solution, consisting of 15 mM sodium phosphate buffer (pH6. 0), 5 mM H_2O_2 and 5 mM O-methoxyphenol (guaiacol). An increase of the optical density at 470 nm for 1 min at 25°C was recorded using a spectrophotometer. PO activity was expressed as change in absorbance min^{-1} mg^{-1} protein.

Phenylalanine ammonia-lyase (PAL): The assay buffer was 50 mM Tris-HCl (pH 8. 8). The reaction mixture (3 m*l*) consisted of 2. 5 m*l* of a 0. 2% (w/v) solution of L-phenylalanine in the assay buffer plus 0. 5 m*l* of the callus extract. The assay mixture was incubated at 40°C for 1 hour and the formation of cinnamic acid was monitored at 268 nm. PAL activity was expressed as nM cinnamic acid formed per mg protein.

The concentration of protein was determined by the method of Lowry *et al*[11].

Results

1. *Colonization on callus*

The progress of colonization is shown in **Fig. 1**. *V. albo-atrum* grew faster on callus line S than on R. The fungus grew intermediately on callus line IR. The restriction of fungal growth on callus line R was associated with a distinct brown zone surrounding the colonized tissue. No browning was seen in callus line S.

2. *Patterns of PO activity*

Fig. 2 shows PO activity in the callus extracts at different times after treatment with *V. albo-atrum* cell wall components (elicitor). PO activity up to 48 hours was higher in callus line R than in lines IR and S. PO activity reached a maximum after 48 hours in callus line R, and 72 hours in IR.

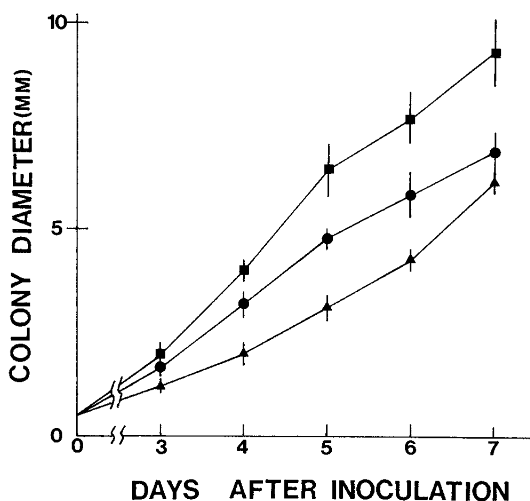

Fig. 1 *Verticillium albo-atrum* colonization on alfalfa callus.
▲—▲, Resistant genotype; ●—●, Intermediate resistant genotype; ■—■, susceptible genotype. Vertical bars indicate standard error (n = 15).

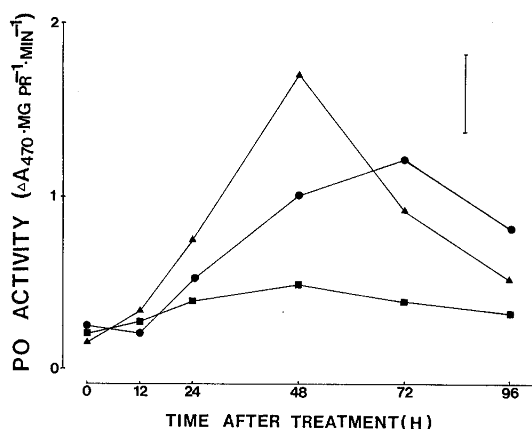

Fig. 2 Peroxidase activity in alfalfa calli after treatment with *Verticillium albo-atrum* cell wall components.
▲—▲, Resistant genotype; ●—●, Intermediate resistant genotype; ■—■, susceptible genotype. Vertical bar indicates L. S. D. (p = 0. 05).

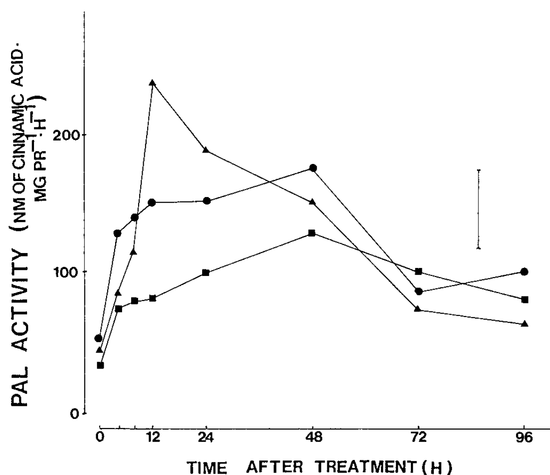

Fig. 3 Phenylalanine ammonia lyase activity in alfalfa calli after treatment with *Verticillium albo-atrum* cell wall components.
▲—▲, Resistant genotype; ●—●, Intermediate resistant genotype; ■—■, susceptible genotype. Vertical bar indicates L. S. D. (p=0. 05).

There was no peak in callus line S.

1. *Patterns of PAL activity*

PAL activities in the callus extracts measured after treatment with Vaa elicitor are shown in **Fig. 3**. Increases in the activity were found in three callus lines, while the maximum value of the activity in callus line R was higher than those of the other two.

Discussion

Significant correlations between *in vivo* resistance to pathogens and *in vitro* phytoalexin production following elicitor treatments have been found in several cases[12]. In our experiment, tissue cultures of three alfalfa genotypes (resistant, intermediate resistant and susceptible) showed genotype-specific PO and PAL activities after treatment with Vaa elicitor. These enzymes' activities which are involved in phytoalexin biosynthesis and in other defence reactions after the elicitor treatments may thus be good markers for the selection of disease resistant alfalfa cell lines to *V. albo-atrum*.

The outer heteropolymers of fungal cell wall components act as elicitors of phytoalexin synthesis[9]. There have been few reports on cultivar- or genotype-specifity of those elicitors. Washed heat-killed conidia of *V. albo-atrum* elicited strong synthesis of phytoalexin in resistant *Gossypium barbadense* cotton, but little or no synthesis in susceptible or tolerant *G. hirsutum*[13]. A good correlation has been shown between PAL activity increase in carnation callus tissues treated with *Fusarium* cell wall components and *in vivo* resistance to the fungi[5]; the correlation between PAL induction and phytoalexin production was also strict. Our restlts also indicate that fungal cell wall components may induce cultivar- or genotype-specific disease resistance reactions.

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《和文要約》

アルファルファと *Verticillium albo-atrum* の相互作用：II. 菌体エリシターに対するカルスのパーオキシダーゼとフェニルアラニンアンモニアリアーゼ活性の増加

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植物体レベルとカルスレベルにおける *Verticillium albo-atrum* に対する抵抗性の相関性を確かめる目的で，*V. albo-atrum* に対する反応の異なるアルファルファの遺伝子型（抵抗性，中度抵抗性，感受性）の個体由来カルスに *V. albo-atrum* 菌体細胞壁成分を処理し，パーオキシダーゼ（PO）とフェニルアラニンアンモニアリアーゼ（PAL）活性の変動を測定した．菌体細胞壁成分（エリシター）処理後の PO, PAL 活性の変動は，抵抗性程度が高い遺伝子型のカルスほどその活性が高く，その反応は植物体の抵抗性程度を反映していた．