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RESEARCH PAPERS

Variability of phenylalanine ammonia-lyase and peroxidase activities in leaves of subterranean clover is determined by their susceptibility to *Kabatiella caulivora*

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Summary. *Kabatiella caulivora* is the causal organism of northern anthracnose or clover (*Trifolium* spp.) scorch disease. The activities of phenylalanine ammonia-lyase (PAL) and soluble peroxidase were determined in seedling leaves of two cultivars of subterranean clover (*T. subterraneum)* inoculated with race 1 or race 2 of *K. caulivora*. A small increase in activity of PAL was recorded in both cultivars 2–4 h post inoculation with either race. A second, large increase in PAL activity was observed only in the incompatible interaction (cv. Daliak inoculated with race 1), increasing 18-fold between 8 and 48 h post inoculation. Peroxidase activity in cv. Daliak increased rapidly within 2 h post inoculation with either race but was significantly higher in the incompatible interaction. Peroxidase activity in cv. Woogenellup increased by 4 h post inoculation with either race, but was significantly lower than that in cv. Daliak. Subsequent increases in peroxidase activity were recorded in both cultivars, however the levels remained constant in cv. Daliak infected with race 1, while activities in the other race-cultivar combinations decreased to control levels. It is hypothesised that the peak of activity of PAL at 48 h, and the rapid increase in peroxidase at 2 h are related to the race-specific resistance response of cv. Daliak to race 1 of *K. caulivora*, and that minor peaks of activity in the compatible interactions are general defence responses.

Key words: *Trifolium subterraneum*, northern anthracnose, resistance.

Introduction

Kabatiella caulivora (Kirchn.) Karak. is the fungal pathogen causing northern anthracnose or clover scorch disease of *Trifolium* spp. The disease is endemic throughout the Mediterranean basin, including Italy, France, Portugal and Morocco, in addition to the United Kingdom, North America, Japan and Australia (Anonymous, 1991). In 1990, a hitherto unknown race of *K. caulivora* was found in Western Australia (Barbetti, 1995). This new race, designated race 2 (Bayliss, 2000), can infect and cause serious disease on some varieties of subterranean clover (*Trifolium subterraneum* L.) resistant to race 1 of the pathogen (Barbetti, 1995).

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The compatible interaction between either race of *K. caulivora* and susceptible subterranean clover cultivars is characterised by large brown lesions, most commonly observed on the petiole and petiolules (section between the trifoliate leaflet and petiole) (Bayliss, 2000). Lesions of the incompatible interaction, in contrast, are restricted to small black flecks, similar to a Hypersensitive Response (HR) reaction (Bayliss, 2000). Colonisation of leaf tissue by *K. caulivora* is difficult to detect in either the compatible or incompatible interaction prior to the development of lesions, due to a period of intercellular latency which lasts for approximately 11 days (Bayliss, 2000).

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) and peroxidase (EC 1.11.1.7) activities increase in many plant species when challenged by pathogens, and also in response to stresses such as wounding. PAL is also associated with accumulation of aromatic, fungitoxic compounds (Ampomah and Friend, 1988). Both PAL and peroxidase are associated with resistance to various pathogens in a number of different hosts (e.g. Cahill and McComb, 1992; Angelini *et al*., 1993; Okey *et al*., 1997; Reuveni, 1998). Peroxidase is known to increase in activity in red clover (*T. pratense* L.) infected with *K. caulivora* with an increase in activity of 59% in resistant and 38% in susceptible tissue, compared to control plants (Sakuma, 1975). Sakuma (1975) also reported increases in the diphenol content of 31% and 19% in resistant and susceptible plants respectively, compared to healthy tissue and proposed that oxidised products of polyphenols may inhibit the stimulator for spore germination and mycelial growth of *K. caulivora*.

The role of PAL and peroxidase in protecting subterranean clover against infection with *K. caulivora* is unknown. This knowledge would improve our understanding of the host defense responses during the early stages of infection and also provide an insight into possible resistance mechanisms in the incompatible (resistant) interaction. The purpose of this study was to determine the activity of PAL and peroxidase in compatible and incompatible interactions between subterranean clover and each of the two races of *K. caulivora*. Our aim was to relate these activities to the early defence responses of each cultivar to race 1 and race 2, and to examine the possible role of each enzyme in the resistance of cv. Daliak to race 1.

Materials and methods

Fungal cultures and inoculum production

Isolates of *K. caulivora* were collected, purified and stored as lyophilised cultures in glass ampoules. Isolates used in the study were WAC6019 and WAC5757 (race 1 and race 2, respectively) as determined from extensive testing on a set of host differentials (Barbetti, 1995). Cultures were rehydrated using sterile deionised water and plated onto potato dextrose agar containing 100 mg l^{-1} of aureomycin hydrochloride. Cultures were maintained at 15°C and subcultured every 5-8 d to maintain the budding, yeast-type form required for inoculation (Cole and Couch, 1958).

Inoculum was produced by inoculating sterile malt extract broth with a platinum wire loopful of conidia. Flasks were incubated on a rotary shaker at 20°C and 130 rpm for 72 h and then the concentration of conidia was estimated using a haemocytometer. Conidial suspensions were filtered and the concentration adjusted to 2.2×10^6 conidia ml^{-1} with sterile deionised water before storing at -20°C until required.

Host plant material, growth conditions, inoculation and harvest details

Seeds of the subterranean clover cultivars Daliak and Woogenellup were sown directly into steam-treated potting mix (2:1:1 composted pine bark:cocoa peat:river sand) in 100 mm free-draining, black plastic pots at a rate of 8 seeds per pot. Approximately 14 d after sowing the germinated seedlings were thinned to 4 seedlings per pot. Pots were kept in a controlled-environment room at 17/12°C (day/night, 12 h temperature and photoperiod) under a light intensity of 165 w m^{-2}. Plants were watered daily with deionised water and fertilised fortnightly with Phostrogen (Phostrogen Ltd, Deeside, Clwyd UK) (1 g l^{-1}).

Using a hand-held atomiser, four-week-old seedlings were sprayed to run-off with conidial suspensions of *K. caulivora*. Following inoculation, the plants were covered with clear plastic bags, the inner surfaces of which had been lightly sprayed with deionised water to maintain the high humidity required for disease development (Helms, 1975). There were three replicate pots of each race-cultivar combination, arranged in a randomised block design. Controls were sprayed with deionised water and covered with bags as above. The reaction

of the two cultivars to each race is indicated in Table 1.

Leaf tissue from control and infected plants was carefully harvested at the time of inoculation (0 h), and then at 2, 4, 6, 8, 24, 48, and 72 h post-inoculation (hpi). At each time point leaf tissue was immediately snap frozen in liquid N_2 and frozen leaf material stored at -80°C until analysed.

Enzyme extraction

Leaf tissue was ground in liquid N_2 using a mortar and pestle. Approximately 50 mg of tissue was transferred to a microcentrifuge tube containing 50 mg polyvinylpolypyrrolidone and 1 ml of extraction buffer [0.1 M sodium borate pH 8.8, 1 mM EDTA, 1 mM ascorbic acid, 1 mM dithiothreitol (added fresh daily), 1 mM phenylmethylsulphonyl fluoride (added fresh daily) modified from Moerschbacher *et al*. (1988)]. The suspension was vortexed to mix, centrifuged at 14,000 rpm for 10 mins and the supernatant (enzyme extract) transferred to a new tube on ice until assayed. Enzymes were extracted from three separate replicates of each race-cultivar combination at each time point.

Enzyme assays

A preliminary trial measuring both PAL and peroxidase enzyme activities was conducted to determine the optimum concentrations of substrates required. The optimum amount of enzyme extract required for the assays was determined by increasing the volume of extract in the assay while keeping the substrate concentration constant. The maximum enzyme activity of PAL and peroxidase was measured in reactions containing 10 µl of enzyme extract. The optimised conditions used in the final assay were the following.

Table 1. Types of reactions observed between two races of *Kabatiella caulivora* and two cultivars of subterranean clover (*Trifolium subterraneum*).

Subterranean clover cultivar	*Kabatiella caulivora* race	
	1	2
Daliak	-	+
Woogenellup	+	+

+, susceptible (compatible) interaction.
-, resistant (incompatible) interaction.

PAL Assay. Enzyme extract (10 µl) was added to 1490 µl of 0.1 M sodium borate (pH 8.8) containing 5 mM L-phenylalanine. The solution was incubated for 60 min at 30°C before the reaction was stopped by addition of 2 N HCl (modified from Cahill and McComb, 1992). The absorbance was read at 290 nm in a UV-VIS spectrophotometer (GBC Scientific Equipment, Arlington Heights, IL, USA) against an identical sample except that it contained L-phenylalanine instead of D-phenylalanine, and PAL activity was expressed as the concentration of cinnamic acid produced h^{-1} g^{-1} fresh weight as determined from a trans-cinnamic acid standard curve.

Peroxidase Assay. Enzyme extract (10 µl) was added to 1490 µl of 0.1 M sodium phosphate (pH 6.0) containing 7 mM guaiacol and 6 mM H_2O_2. The time taken for absorbance to increase from 0.1–0.3 at a wavelength of 470 nm was recorded and peroxidase activity expressed as the change in optical density s^{-1} g^{-1} fresh weight (modified from Moerschbacher *et al*., 1988).

For both enzymes, pathogen-induced activity was calculated as the measured activity minus the activity in control plants (control activities are indicated in Table 2), at each time point. Differences in induced activity between cultivars and races were compared by analysis of variance using a general linear model (Minitab®, Minitab Inc, State College, PA, USA). Data were transformed, where required, to satisfy assumptions of normal distribution and common variance.

Results

PAL activity in infected plants

Inoculation of seedlings with race 1 induced PAL activity in both cultivars by 2 h post-inoculation (hpi). Race 2 also induced an increase in PAL activity in cv. Daliak by 2 hpi but an increase in cv. Woogenellup inoculated with this race was not observed until 4 hpi, when maximum activity was recorded (Fig. 1a). Induced activity of PAL in cv. Woogenellup inoculated with race 1 remained relatively constant from 2 to 6 hpi (no significant difference in activity at $P<0.05$), reached a maximum at 6 hpi, and decreased to control levels by 72 hpi. Activity of PAL in cv. Woogenellup induced by race 2 decreased nine-fold between 8 and 24 hpi before increasing again at 48 hpi and remained constant

Table 2. Activities of phenylalanine ammonia-lyase (PAL) (nmol cinnamic acid produced h^{-1} g^{-1} f wt) and peroxidase (ΔOD s^{-1} g^{-1} f wt) in leaves of control plants of subterranean clover cultivars Daliak and Woogenellup (mean ± standard error, n = 3).

Time (h)	cv. Daliak		cv. Woogenellup	
	PAL	Peroxidase	PAL	Peroxidase
0	13,086 ± 1931	8.1 ± 1.0	12,846 ± 997	3.6 ± 0.4
2	13,466 ± 1749	1.5 ± 0.2	13,512 ± 1216	6.1 ± 0.5
4	7,246 ± 1279	5.5 ± 0.04	8,013 ± 812	3.3 ± 0.6
6	9,126 ± 312	7.4 ± 0.4	8,826 ± 895	2.3 ± 0.1
8	10,326 ± 137	4.7 ± 0.1	7,373 ± 1027	2.8 ± 0.1
24	6,413 ± 832	6.7 ± 0.5	8,733 ± 1623	3.2 ± 0.2
48	5,746 ± 1297	3.1 ± 0.1	4,713 ± 662	2.4 ± 0.2
72	7,499 ± 535	6.3 ± 0.6	8,159 ± 1105	3.1 ± 0.2

until the final measurement at 72 hpi (no significant difference between 48 and 72 hpi at $P<0.05$) (Fig. 1b).

Following the initial rapid increase in activity in cv. Daliak, PAL activity in seedlings inoculated with race 1 remained constant from 4 to 6 hpi (no significant difference at $P<0.05$), decreased almost to control levels at 8 hpi, before an approximately 18-fold increase to maximum recorded activity at 48 hpi, but subsequently decreased to control levels at 72 hpi. Race 2 in cv. Daliak had a peak of PAL activity at 6 hpi, decreased at 8 hpi, then had a three-fold increase between 8 and 48 h before starting to decrease again.

Peroxidase activity in infected plants

A rapid increase in activity of peroxidase was observed in cv. Daliak within 2 hpi with either race, particularly after inoculation with race 1. In contrast, induced peroxidase activity in cv. Woogenellup was significantly lower at 2 hpi, with no increase in activity in seedlings inoculated with race 1 until 4 hpi when maximum peroxidase activity was recorded, and a small increase at 2 hpi with race 2 (Fig. 2a). Seedlings of cv. Woogenellup then had a decrease in peroxidase activity, before a second increase in activity at 24 hpi, with this activity being the maximum recorded peroxidase activity in this cultivar inoculated with race 2. Activity of peroxidase in seedlings of cv. Woogenellup inoculated with either race decreased to control levels by 72 hpi (Fig. 2b).

From the maximum recorded peroxidase activities at 2 hpi in cv. Daliak, activities decreased to levels similar to those in cv. Woogenellup. In cv. Daliak seedlings inoculated with race 1, a second increase in activity occurred at 24 hpi with no significant subsequent variation in the level of activity up to the final measurement at 72 hpi. In seedlings inoculated with race 2, a second increase in activity occurred at 6 hpi before declining to control levels at 24 hpi, with a third increase in activity at 48 hpi, then again decreasing to control levels (Fig. 2b).

Discussion

For a plant to resist infection by a pathogen it needs to be able to recognise the invader and initiate a rapid defence response. Resistance of subterranean clover to *K. caulivora* race 1 appears to be associated with a peak of PAL activity 48 h after inoculation. An increase was also observed in both cultivars inoculated with race 2 at this same time point, however at significantly lower activities, indicating that the magnitude of the response may be the important factor in determining the level of host resistance. Production of PAL has been shown to be correlated with resistance of plants to fungal pathogens including resistance of *Eucalyptus calophylla* to *Phytophthora cinnamomi* (Cahill and McComb, 1992), of cacao to *Phytophthora palmivora* (Okey *et al.*, 1997) and of *Brassica napus* to *Leptosphaeria maculans* (Chakraborty *et al.*, 1993).

The rapid, significant increase in activity of soluble peroxidase in the incompatible interaction at

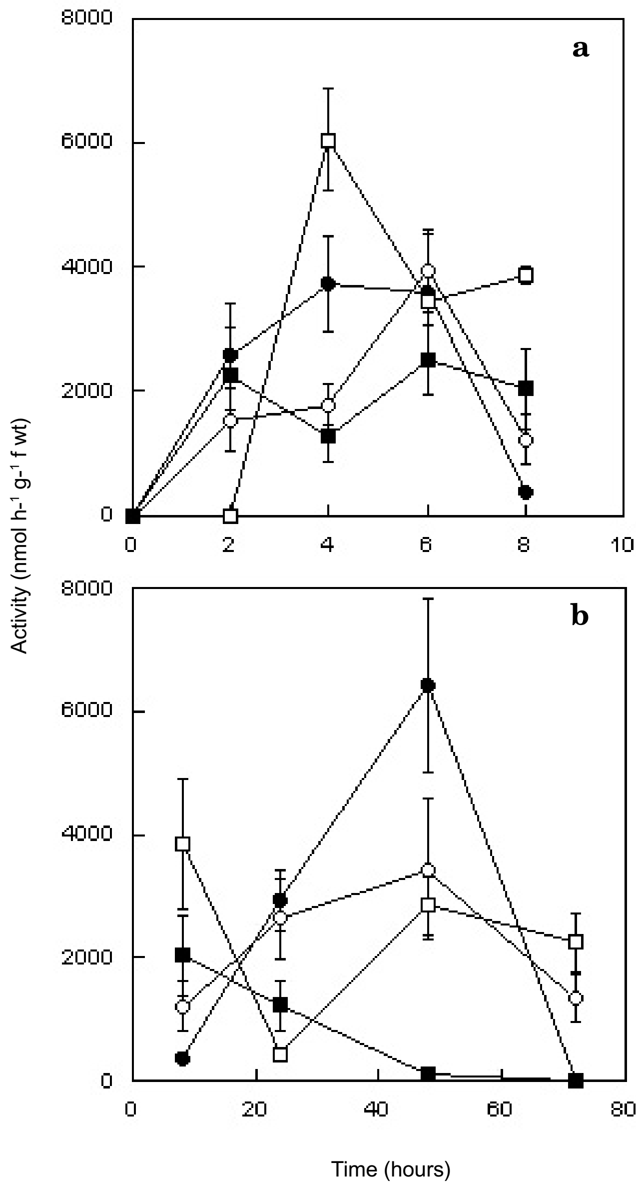

Fig. 1. Pathogen-induced phenylalanine ammonia-lyase (PAL) activity (nmol cinnamic acid produced h^{-1} g^{-1} f wt) in leaves of subterranean clover (*Trifolium subterraneum*) cultivars Daliak and Woogenellup (a) 0–8 and (b) 8–72 h post inoculation (hpi) with *Kabatiella caulivora* race 1 and race 2. Bars indicate standard errors (n=3). Activities of control plants are indicated in Table 2. Woogenellup/race 1, filled square; Woogenellup/race 2, open square; Daliak/race 1, filled circle; Daliak/race 2, open circle.

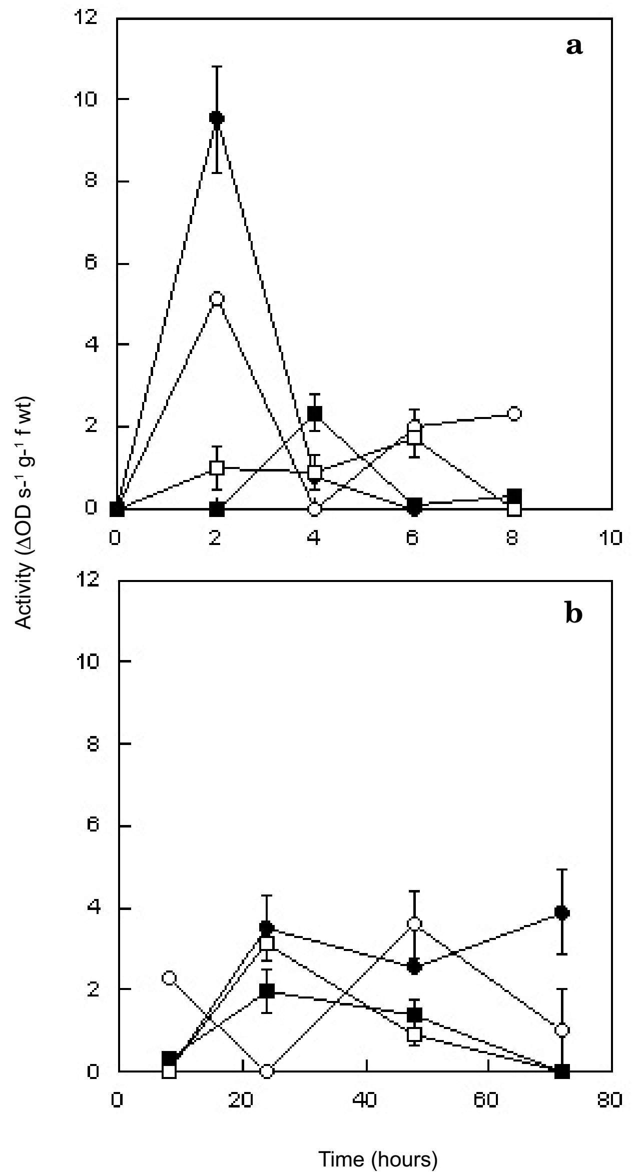

Fig. 2. Pathogen-induced peroxidase activity (ΔOD s^{-1} g^{-1} f wt) in leaves of subterranean clover (*Trifolium subterraneum*) cultivars Daliak and Woogenellup (a) 0–8 and (b) 8–72 h post inoculation (hpi) with *Kabatiella caulivora* race 1 and race 2. Bars indicate standard errors (n=3). Activities of control plants are indicated in Table 2. Woogenellup/race 1, filled square; Woogenellup/race 2, open square; Daliak/race 1, filled circle; Daliak/race 2, open circle.

approximately 2 hpi also appears to be related to host resistance. While the induced peroxidase activity in the incompatible interaction remained relatively constant, the activity in the other race-cultivar combinations declined to control levels. This suggests that a sustained level of activity contributes to the resistance of cv. Daliak to race 1. An early increase in peroxidase activity, similar to that in our study, was recorded in the tropical legume *Stylosanthes humilis* inoculated with *Colletotrichum gloeosporioides* by Harrison *et al*. (1995), who suggested that the early induction might have a possible role in limiting the number of successful infections. In our study however, it is unlikely that production of peroxidase so early after inoculation is associated with the inhibition of penetration of the host by the pathogen. In the *S. humilis*-*C. gloeosporiodes* interaction, spores germinate within 3–6 h after inoculation, whereas in the subterranean clover-*K. caulivora* interaction, spore germination is not observed until 24 hpi (Bayliss, 2000).

The initial increase in production of PAL in all cultivar-race combinations 2-4 hpi may be a general defense response. A similar pattern of PAL activity was recorded in two cultivars of barley infected with *Bipolaris sorokiniana*, with an initial increase in enzyme activity at 16 h after inoculation (Peltonen and Karjalainen, 1995). However, in the resistant cultivar there were two extra phases of PAL activity, at 24–32 and at 40 hpi.

The multiple increases and decreases in both PAL and peroxidase enzyme activities over the course of our study were similar to other reports (e.g. Harrison *et al.,* 1995; Zhang *et al*., 1997). A biphasic pattern of production of peroxidase in *S. humilis* infected with *C. gloeosporioides* was suggested to be related to the growth of the pathogen, with early production of the enzyme during the biotrophic growth phase and a second peak of production during the necrotrophic phase (Harrison *et al*., 1995). *K. caulivora* has a similar progression of pathogenesis in subterranean clover, with an intercellular latent period before the onset of massive cell collapse (Bayliss, 2000). Thus, the patterns of enzyme production we observed may correlate with the growth of the pathogen and substances it produces during invasion. Alternatively, there may be more than one gene encoding an enzyme, resulting in differential expression, as suggested for peroxidase in *Stylosanthes* (Harrison *et al*., 1995). Isozymes of each enzyme may also be expressed at different times. In future studies electrophoresis may be one way to detect the expression of defence enzymes, particularly peroxidase, in subterranean clover.

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