CSIRO PUBLISHING

www.publish.csiro.au/journals/app *Australasian Plant Pathology*, 2003, **32**, 53–64

Ecotypic variation in the response of *Arabidopsis thaliana* to *Phytophthora cinnamomi*

Louise H. Robinson[A,B] *and David M. Cahill*[A,C]

[A]School of Biological and Chemical Sciences, Deakin University, Waurn Ponds, Vic. 3217, Australia.

[B]Present address: Department of Plant Science, University of Adelaide, Waite Campus, Glen Osmond, SA 5064, Australia.

[C]Corresponding author; email: cahill@deakin.edu.au

Abstract. A variety of reactions to inoculation with *Phytophthora cinnamomi* ranging from high susceptibility to moderate resistance were found in 20 ecotypes of *Arabidopsis thaliana*. *P. cinnamomi* zoospores successfully colonised both root and leaf tissue of *Arabidopsis* and sporulation in the form of chlamydospores and sporangia occurred in leaves and roots of each ecotype but the number varied considerably between ecotypes. In the more susceptible ecotypes, colonisation was characterised by rapid intercellular growth and sporulation of the pathogen from 48 h post inoculation. In less susceptible ecotypes, *P. cinnamomi* was limited to a defined region within tissues. In response to *P. cinnamomi* infection, several ecotypes expressed active defence responses in both root and leaf tissue. Callose formation was closely associated with lesion restriction as was the production of the reactive oxygen species, hydrogen peroxide. The oxidative burst was not limited to the site of pathogen ingress but also occurred in distant, uninfected tissues. We have characterised an *Arabidopsis–P. cinnamomi* system that will be useful for further studies of active resistance mechanisms.

Additional keywords: non-host resistance, reactive oxygen species, callose, hydrogen peroxide.

Introduction

Plants possess several lines of defence against invaders and there are potentially thousands of resistance genes that specify for particular viral, bacterial and fungal pathogens. Resistance in many plant–pathogen interactions is accompanied by the rapid deployment of a multi-component defence response where single or multiple genes confer resistance. Resistance may also depend on the age and state of development of the host.

Phytophthora cinnamomi is a soilborne pathogen that initiates disease by killing root cells and, therefore, directly affects the ability of the host to absorb water and nutrients from the soil. Chlorosis and dieback of plant foliage are secondary symptoms that result from the reduced ability for water uptake caused by both mechanical and physiological changes to the root system (Dawson and Weste 1984; Cahill *et al.* 1986, 1989). Economically important plants that are affected by *P. cinnamomi* include *Ananas comosus* (pineapple), *Eucalyptus* spp., in particular *E. marginata* (jarrah), *Pinus* spp. (pine) and *Prunus* spp. (almond, cherry, peach and plum) (Irwin *et al.* 1995; Erwin and Ribeiro 1996) but the most dramatic impact of the pathogen is on the south-western and south-eastern coastal heath and open forest communities of Australia.

Control of *P. cinnamomi* is difficult due to its wide host range and the ability of the pathogen to survive as a saprophyte as chlamydospores in symptomless plants and in the soil. Few species are resistant to *P. cinnamomi* and when it occurs, resistance is horizontal and under polygenic control (Irwin *et al.* 1995). Although there have been great advances in recent years in our knowledge of how *P. cinnamomi* interacts with its hosts, studies have been hampered by the lack of a suitable model plant that is genetically well defined and which displays differential responses to *P. cinnamomi*.

Recently, *Arabidopsis thaliana* has been put forward as a potential model plant for use in host–pathogen interactions (Davis 1993) and specifically for species in the genus

 10.1071/AP02064 0815-3191/03/010053

Phytophthora (Kamoun *et al.* 1999). Subsequently, resistant and susceptible phenotypes have been defined in interactions with *P. porri* (Roetschi *et al.* 2001). *A. thaliana* is a member of the crucifer family (Brassicaceae or Cruciferae) and has several attributes that make it amenable to the study of plant–pathogen interactions (Price *et al.* 1994). These include its relatively small size and rapid generation time (5–6 weeks), self-fertility and its ability to produce large amounts of seed (Davis 1993). The access to approximately 240 wild isolates or ecotypes of *A. thaliana* (Alonso-Blanco and Koornneef 2000) provides a wealth of genetic material for analysis of variation in resistant phenotypes (Kunkel 1996). Additionally, *A. thaliana* has a compact and fully sequenced genome, which allows for genetic analyses to be followed up at the molecular level. For example, over 49 loci which govern pathogen specificity have been identified and a further 29 loci have been found to be involved in defence responses or post recognition events (Buell 1998).

Four distinct mechanisms of defence against various pathogens have been characterised in *A. thaliana*: the hypersensitive response (HR) (Fuchs and Sacristián 1996), resistance without HR (Adam and Somerville 1996), tolerance (Lee *et al.* 1994) and systemic acquired resistance (SAR) (Uknes *et al.* 1992). A HR to the clubroot pathogen, *Plasmodiophora brassicae*, was identified in *A. thaliana* and a single dominant gene (Fuchs and Sacristián 1996) conferred the genetic basis of resistance. Resistance without HR was found in the normally susceptible interaction between *A. thaliana* and the powdery mildew fungus, *Erysiphe cichoracearum* (Adam and Somerville 1996).

When a plant is challenged by a pathogen a number of responses have been postulated or proven to be involved in defence signal transduction (Bent 1996). For most interactions, the immediate downstream signalling events are not known in detail but involve kinases, phosphatases, G proteins and ion fluxes. Downstream signalling events rapidly activate a number of responses including the production of reactive oxygen species (ROS), defence gene transcription and ethylene biosynthesis (Dangl *et al.* 1996). There is an amplification of these responses through the generation of additional signal molecules, including ROS, lipid peroxidases and salicyclic acid (Siegrist *et al.* 2000; Venisse *et al.* 2001). In particular, ROS generation, as well as being a rapidly elicited response, is a hallmark of incompatible interactions, for example, in the interaction of *Phytophthora nicotianae* with tobacco (Able *et al.* 1998)

The oxidative burst that is associated with pathogen recognition has been well established for many plant–pathogen interactions (Mehdy 1994) and the production of ROS has been found to be a key component of plant defence (Doke 1983; Baker and Orlandi 1995; Bestwick *et al.* 1997). In the present investigation we use a sensitive and rapid technique that utilises 3,3-diaminobenzidine (DAB) which polymerises instantly and locally as soon as it comes into contact with hydrogen peroxide, in the presence of peroxidase, into a visible reddish-brown polymer (Thordal-Christensen *et al.* 1997; Tor *et al.* 2002). DAB has been used widely in studies of the subcellular localisation of hydrogen peroxide in plants. Examples are the detection of hydrogen peroxide during the HR in the barley–powdery mildew interaction (Thordal-Christensen *et al.* 1997) and the detection of ROS that mediates a systemic signal network in the *A. thaliana–Pseudomonas syringae* interaction (Alvarez *et al.* 1998).

Our objective was to investigate and characterise an *A. thaliana–P. cinnamomi* pathosystem. The approach used was to examine the interaction of *P. cinnamomi* with a range of ecotypes of *A. thaliana* and to specifically observe and measure components of the interactions such as lesion development, callose deposition and production of hydrogen peroxide, which in other systems, and in interactions of other hosts with *P. cinnamomi*, can be used to define susceptibility and resistance.

Methods

Ecotypes of A. thaliana

Wild-type *A. thaliana* seed was obtained as a set of 20 randomly chosen ecotypes from Lehle Seeds (Round Rock, TX, USA). The specific ecotypes used were (abbreviations and genetic markers in brackets): Aua/Rhön (Aa-0), Bensheim (Ben), C24, Cape Verde Islands (Cvi-0), Columbia (Col-0, no markers), Columbia (Col-3, gl1 marker, glabra), Columbia (Col-PRL, gl1 marker, glabra), Dijon-G (Dij), Estland (Est), Greenville (Gre-0), Kendalville (Kin-0), Landsberg erecta (Ler, er marker), Mühlen (Mh-0), Niederzenz (Nie), Nossen (No-0), RLD, RLD1, S96, Turk Lake (Tur) and Wassilewskija (Ws-2).

Plant growth and maintenance

For leaf inoculation experiments, *A. thaliana* seed was sown on the surface of seed raising mix (Debco, Australia) in 7-cm-diameter pots pre-moistened for 30 min with distilled water (dH_2O). For use in root inoculation experiments, plants were grown in a 3:1 mix of autoclaved, sieved (1 mm^2) propagating sand (Manutec, SA) and seed raising mix that was pre-moistened to run off with Hoagland's nutrient solution (Hoagland and Arnon 1950).

Growth and maintenance of P. cinnamomi

The *P. cinnamomi* isolates used (DU002 and DU026) were from a culture collection held at Deakin University and were originally obtained from the Anglesea region, Victoria (Rose Daniel, personal communication). Isolates were grown and maintained on 20% V8-juice agar (Miller 1955) and subcultured every 5–6 days.

For zoospore inoculum production, the method of Byrt and Grant (1979) was followed. The number of zoospores/mL was determined with a bright line haemocytometer.

Inoculation procedures

For root inoculation, plants of each *A. thaliana* ecotype aged 2, 4, and 6 weeks were gently removed from their pots and adhering sand removed by rinsing gently in dH_2O. Five to ten plants were then individually placed, roots first, into the wells of 6-well cell culture plates (Costar cell culture cluster, Corning Australia, NSW). One millilitre of zoospore suspension (10^5 zoospores/mL) or 1 mL dH_2O

(controls) was dispensed into the well to cover the roots. Plants were incubated in the dark at 25°C for 12 h, removed and then placed within 90-mm-diameter sterile plastic Petri dishes that contained a filter paper disk (Whatman No. 1) pre-moistened with 2 mL dH_2O. The roots were covered with a sheet of filter paper and 1 mL Hoagland's solution applied. Plants were returned to the growth chamber and watered with nutrient solution on every second day. Roots were sampled at 24 h intervals until 96 h and then at 168 h post inoculation. A minimum of 25 plants per ecotype of 2 and 4 weeks of age and 10 plants per ecotype at 6 weeks of age were examined at each time interval. The pathogen was reisolated by plating roots onto selective medium (Kellam and Coffey 1985).

For leaf inoculation, plants of each *A. thaliana* ecotype aged 2 and 4 weeks were used. Leaves were excised and then each placed into the well of a cell culture plate, abaxial surface upwards, onto 2.5-cm-diameter filter paper disks (Whatman No. 1) pre-moistened with 0.5 mL dH_2O. Each leaf was inoculated by placing a 2.5–10 µL drop of zoospore suspension (10^5 zoospores/mL) depending on size of the leaf, along the midvein with either *P. cinnamomi* isolate DU002 or DU026 or dH_2O as the control. Following inoculation of leaves, the wells of the cell culture plates were sealed with Parafilm (American National Can, WI, USA) and incubated as previously described. A minimum of ten plants per ecotype, per age group, were examined at 6, 12, 24, 48 and 72 h post inoculation.

Microscopic examination of interactions

Interactions between *P. cinnamomi* and *A. thaliana* root and leaf tissues and cells were examined using a staining method modified from that of Vogel and Somerville (2000). Infected and control roots were stained with 100 µg/mL trypan blue in a solution of lactic acid, glycerol and water (1:1:1) for 5 min, then rinsed and mounted on a glass microscope slide in the same solution. Leaves were cleared in 96% ethanol overnight at room temperature, then rinsed in 50% ethanol followed by a rinse in dH_2O prior to staining. Roots and leaves were viewed using light microscopy (Axioskop, Zeiss Pty Ltd, Carnegie, Victoria) and interactions recorded with a digital camera (Spot Diagnostic Instruments, Inc., USA) and computer attached to the microscope. For roots, the numbers of zoospores encysted at 24 h post inoculation, the number of chlamydospores and sporangia and extent of pathogen spread were recorded. For leaves, the number of germinated cysts, and the reaction of cells to inoculation, number of chlamydospores and leaf size were recorded.

Callose staining and visualisation

Callose production within root and leaf cells of each of the 20 *A. thaliana* ecotypes was examined by staining with a 0.01% (w/v) solution of aniline blue buffered in 150 mM K_2HPO_4 (pH 9.5) (Eschrich and Currier 1964) at room temperature for 30 min. Tissues were then mounted on glass microscope slides in 50% glycerol and examined for callose production using a microscope and blue light epifluorescence. The presence of callose was shown by the production of pale blue to white fluorescence within cells and along cell walls.

Qualitative analysis of the production of hydrogen peroxide

Following inoculation or wounding, hydrogen peroxide production was measured in roots and leaves of 2-week-old plants, inoculated as described previously. Prior to inoculation of leaves, they were removed from the plants and placed, abaxial side upwards, within the wells of a cell culture plate in 3,3´-diaminobenzidine (DAB) (5 mM in dH_2O, pH 3.8, Thordal-Christensen *et al.* 1997) for 4 h. Roots were removed, and leaves examined, every 3 h up to 18 h and at 24 h following inoculation from a minimum of five plants for each time interval. Tissues were also treated with 5 mM DAB supplemented with 10 mM ascorbic acid.

Hydrogen peroxide production was detected by the reddish-brown colouration of DAB staining within cells. For each treatment, hyphal length was measured with a slide micrometer and growth rate of the pathogen calculated. To examine the effect of wounding on hydrogen peroxide production, five leaves of ecotypes Col-0, Est, Ler, Mh-0, Nie and S96 were mechanically damaged by light forceps compression and examined for DAB staining at 2, 4 and 6 h post damage.

Statistical analysis

Results are expressed as the mean and standard error of the mean for each ecotype for each variable studied. Mean values for ecotypes were compared by ANOVA using SPSS statistics software.

Results

Summary of interactions

The interactions described were based on the use of *P. cinnamomi* isolate DU026 at a density of 10^5 zoospores/mL. Isolate DU002 was also used but in preliminary experiments yielded comparable results to DU026. Therefore, only DU026 was used in further experimentation. Control plants did not show any evidence of cellular damage and were healthy.

A total of 20 *A. thaliana* ecotypes was evaluated for resistance to *P. cinnamomi*. Although all ecotypes supported colonisation of *P. cinnamomi* to some degree, disease severity varied between them and thus a continuum of interaction types was observed. Following assessment of nine interaction parameters (Table 1), the reaction to inoculation of ecotypes fell broadly into three groups. The first group consisted of the least susceptible ecotypes, for example, Est, Col-0 and Col-3, and supported low level colonisation and sporulation of the pathogen and displayed many resistance characteristics. The second, larger group of ecotypes supported a medium level of sporulation and colonisation and displayed only a few resistance characteristics. The third group comprised the most susceptible ecotypes, notably Cvi-0 and Ler, that were extensively colonised by the pathogen and supported high levels of sporulation and displayed few, if any, resistance characteristics. Table 1 shows that when examined for parameters that are associated with resistance, several ecotypes consistently placed at either end of the range of responses. For example, Cvi-0 and Ler showed susceptible characteristics in nine and seven, respectively, of the parameters assessed. Conversely, ecotype Est consistently ranked in the four least susceptible ecotypes and displayed resistance characteristics in five of the nine parameters assessed. In the accompanying figures, the ecotypes have been ranked from least susceptible to most susceptible based on their overall rankings for the parameters examined.

Response of roots and leaves to inoculation

Roots of each ecotype of *A. thaliana* that were inoculated with *P. cinnamomi* were successfully infected. Zoospores were primarily attracted to, and encysted on, cells in the zone

Table 1. Parameters measured in the interaction between *Phytophthora cinnamomi* and *Arabidopsis thaliana* and a ranking of the most and least susceptible ecotypes

Parameter	Plant organ	Highest ranked ecotypes for each parameter[A]	
		Most susceptible	Least susceptible
Chlamydospores[B]	Root	Ben, Ler, Mho, Cvi-0	S96, Aa-0, RLD, Col-0
	Leaf	Ws2, Mh-0, No-0, Cvi-0	Est, Gre-0, Aa-0, Nie
Sporangia[C]	Root	Ben, Mh-0, Ler, Cvi-0	Col-0, S96, Tur, Col-3
Hyphae[D]	Leaf	Cvi-0, Ws2, Ler, Dij	Col-3, RLD-1, Est, No-0
Cell clusters[E]	Leaf	Cvi-0, Ler, No-0, Col PRL	Col-3, RLD, RLD-1, Est
Callose papillae[F]	Root	Cvi-0, Ler, Col PRL, Col-3	RLD, No-0, Ben, C24
	Leaf	Cvi-0, Ler, Col-3, Gre-0	Mh-0, Aa-0, Nie, No-0
DAB reaction[G]	Leaf	C24, Cvi-0, Tur, Ben	No-0, Col-3, Est, Col-0
DAB positive sites away from lesion[H]	Leaf	Cvi-0, C24, Ler, Aa-0	S96, Col-0, Est, RLD

[A]The four least and most susceptible ecotypes for each parameter tested in order.
[B]The total number of chlamydospores present at 96 h after inoculation.
[C]The total number of sporangia present at 96 h after inoculation.
[D]Hyphal length in leaves 24 h after inoculation.
[E]Number of trypan-blue-stained cell clusters at a distance from the inoculation site 72 h after inoculation of 4-week-old plants.
[F]Total number of callose papillae 48 h after inoculation of 4-week-old roots and leaves.
[G]DAB-stained cell clusters at the site of inoculation 24 h after inoculation.
[H]DAB-stained cell clusters at remote sites 24 h after inoculation.

of elongation. Encystment of zoospores had occurred at 2–3 h post inoculation and there was no difference in the number of cysts on roots between ecotypes or between ages of plant (data not shown). Prior to encystment, zoospores oriented towards the root surface, shed their flagella and then encysted followed by formation of germ tubes and subsequent hyphae that successfully penetrated the cell wall of epidermal cells. Once inside the root cells, the hyphae 'swelled' to fill individual cells, before growing into adjacent cells where they again filled the cell (Fig. 1*a*). Hyphae grew rapidly and moved through the cortex to the vascular system. This pattern of colonisation and growth was consistent for each of the ecotypes and by 48 h post inoculation sporulation had occurred. Sporangia were produced on the root surface whereas chlamydospores were formed both within and outside the root.

Leaves of each ecotype supported the colonisation and growth of *P. cinnamomi*. Zoospores encysted and germ tubes successfully penetrated epidermal cells within 6 h post inoculation (Fig. 1*b*). For approximately 40% of cysts, the germ tube did not penetrate a proximate or adjacent cell. The hyphae instead grew over upper epidermal cells before penetrating cells at a distance of up to approximately 1000 µm. Following penetration, hyphae rapidly colonised cells and, within cells, developed the characteristic expanded growth described in root tissues. After removal of chlorophyll (by ethanol extraction) for trypan blue staining of pathogen structures within leaf tissues, leaves that displayed some resistance to *P. cinnamomi* had small brown macroscopic lesions (Fig. 1*c*). Within the centre of the lesions, trypan blue staining revealed numerous dead cells that had been colonised by the pathogen. Cells around the periphery of the stained cells were light brown in colour. The production of this peripheral necrosis occurred between 24 and 72 h post inoculation.

Numbers of sporangia and chlamydospores found in roots differed between individual ecotypes and with plant age. For example, at 2 and 4 weeks of age, ecotypes S96 and Aa-0 had relatively low numbers (2–5) of chlamydospores per root 96 h post inoculation compared with ecotypes Cvi-0, Ler and Mh-0 which had four to five times that number (Fig. 2*a*). There were significantly more ($P < 0.05$) chlamydospores in roots of 4 weeks of age than those 2 weeks of age. When total numbers of chlamydospores at each age were pooled and the ecotypes ranked from those that contained the least number of chlamydospores to those with the greatest number of chlamydospores, a continuum among ecotypes was apparent (data not shown). Significant differences were found between numbers of chlamydospores in ecotypes with the fewest and those with the most. Numbers of sporangia within roots of each ecotype also differed (Fig. 2*b*). There was a significant difference ($P < 0.05$) between the number of chlamydospores and sporangia in the least susceptible ecotype and the most susceptible ecotype, for both 2- and 4-week-old plants. In contrast to inoculated roots, only chlamydospores were observed within and emanating from leaf tissues. Chlamydospores were first evident at 48 h post inoculation and were present in the leaf tissue of all ecotypes (Fig. 2*c*). There was a wide range in total numbers of chlamydospores for both 2 and 4-week-old plants.

In leaves at sites up to 4 mm away from the site of inoculation, there were small and discrete regions of dead cells that were also revealed by trypan blue staining. The small clusters consisted of between 2 to 8 cells and were

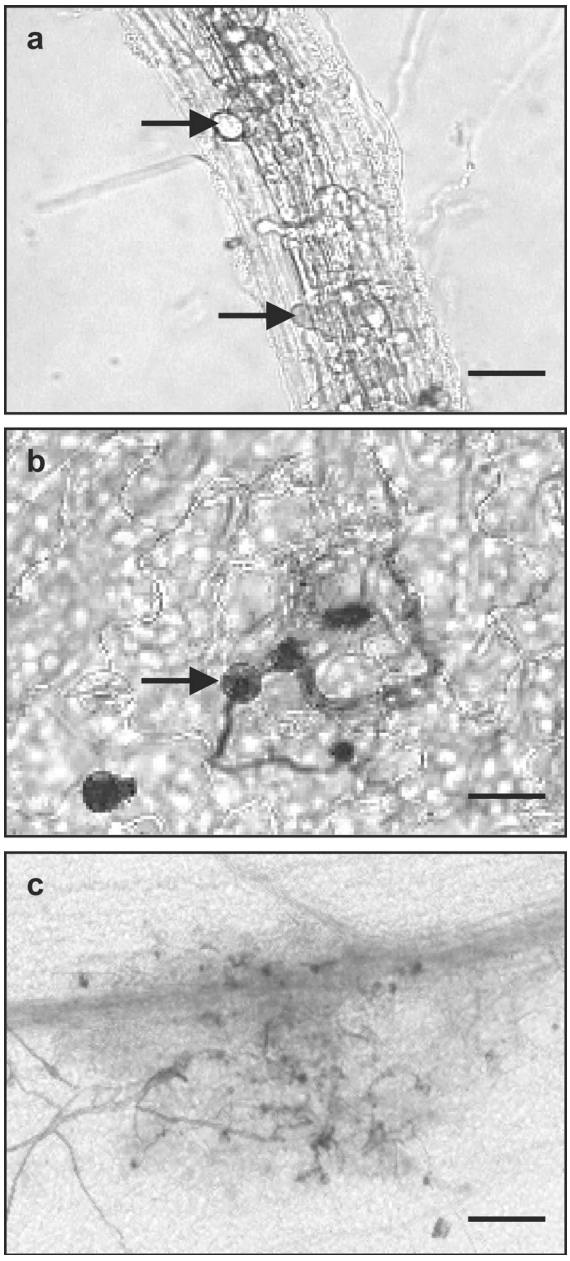

Fig. 1. Infection by *Phytophthora cinnamomi* of roots and leaves of *Arabidopsis thaliana* ecotypes. (*a*) Development of the pathogen within a root of ecotype Tur at 96 h post inoculation. Arrows show expanded hyphae and chlamydospores within and between cells. Bar represents 100 μm. (*b*) Germinated cyst (arrow) on the leaf surface and penetration into the epidermis of ecotype Est 48 h post inoculation. Bar represents 25 μm. (*c*) Localised cellular necrosis around a lesion site on a leaf of ecotype Ben 24 h post inoculation. Trypan-blue-stained hyphae are visible within the lesion site. Bar represents 100 μm.

observed in both more marginal and central regions of inoculated leaves and were often located adjacent to a vein. The cell clusters were not associated with either pathogen structures or cellular damage and were not evident in control leaves that were mock inoculated with dH_2O. There was a greater number of necrotic cells at a distance from the site of inoculation than at the inoculum site for both the least susceptible and the most susceptible ecotypes (Fig. 3*a*).

Callose production during the interactions

Fluorescence induced by aniline blue was evident in roots from 12 h post inoculation and was present to some degree in all ecotypes. The formation of callose 'appositions' were often associated with the failure of the pathogen to continue growing along the same plane, resulting in the pathogen turning 90° and growing into an adjacent cell. For some ecotypes, only one or a few papilla were observed but in others many epidermal cells and cells of the zones of division and elongation displayed whole cell fluorescence or fluorescence along entire walls. Following inoculation, the number of papillae in the roots of each ecotype increased over the subsequent 48 h. Ecotypes Ler and Cvi-0 had low papilla numbers and C24 and Ben the highest at 48 h after inoculation (Fig. 3*b*).

Callose was found in leaves from 6 h after inoculation and, as in roots, the number of callose papillae varied with ecotype (Fig. 3*c*). In a comparison of the number of papilla formed in leaves of 2- and 4-week-old plants at 72 h post inoculation, there was no significant difference found between ages. Ecotypes Cvi-0 and Ler had the least, and ecotypes No-0 and Nie the greatest, number of papillae formed. Callose induction was not observed in control leaves. All ecotypes had brightly staining papillae within cells beneath the inoculation site and callose was primarily located in epidermal cells (Fig. 4). In leaves of several ecotypes, papilla formation was restricted to a single cell within which the pathogen became confined (Fig. 4*a*, *b*) but in more susceptible ecotypes, papillae were located in many cells in association with hyphal spread (Fig. 4*c*, *d*).

Controls for the DAB assay for detection of hydrogen peroxide

Experiments were conducted to confirm that the histochemical reagent for hydrogen peroxide, 3,3'-diaminobenzidine (DAB) could be used as a specific marker for hydrogen peroxide production in *A. thaliana*. In an *in vivo* leaf assay, a strong hydrogen peroxide dependent DAB reaction was produced in excised, non-inoculated leaves that were pre-incubated in DAB before being placed into 0.1% Triton-X100 followed by incubation in hydrogen peroxide at concentrations of 0.01, 0.1, 1 and 10 mM. Within 2 min, visible DAB polymerisation was evident in the 10 mM treatment and by 10 min in the 1 mM treatment. Within 30 min, multiple DAB stained sites were evident in leaves treated at 1 mM and 10 mM concentrations, but no visible DAB polymerisation could be observed in leaves at concentrations of 0.01 or 0.1 mM hydrogen peroxide. At 2 h post treatment with 0.1% Triton-X100, all leaves at all concentrations of hydrogen peroxide showed visible DAB polymerisation.

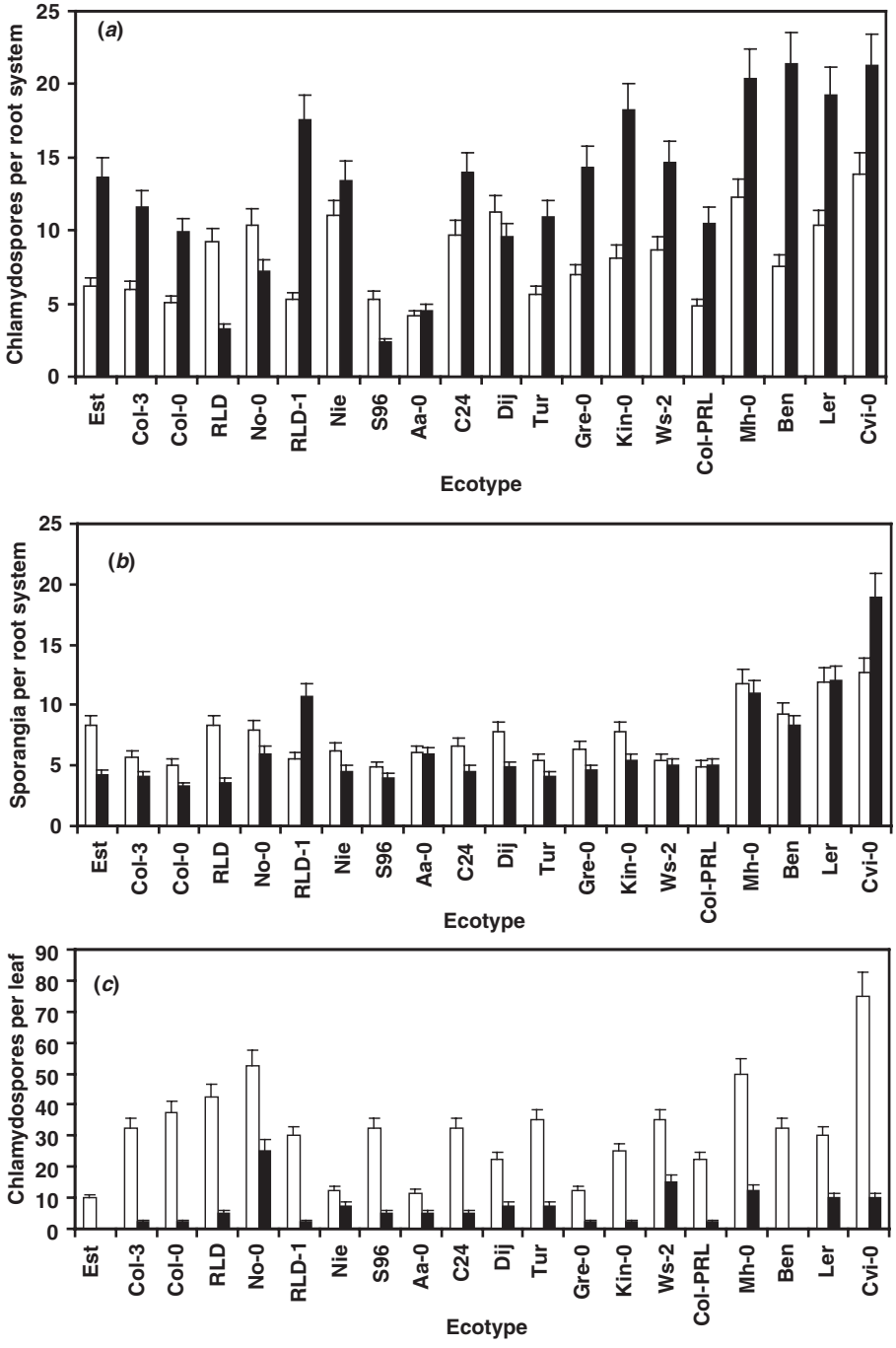

Fig. 2. Number of chlamydospores and sporangia in roots and the number of chlamydospores in leaves of 20 ecotypes of *Arabidopsis thaliana* 96 h after inoculation with *Phytophthora cinnamomi*. Data are based on a minimum of 25 plants per ecotype at 2 and 4 weeks (white and black bars, respectively) after inoculation; (*a*) number of chlamydospores in roots, (*b*) number of sporangia in roots, (*c*) number of chlamydospores in leaves. The standard error of the mean is shown for each ecotype.

Generation of hydrogen peroxide in roots and leaves after inoculation or wounding

In infected and control roots that were stained with DAB, no polymerisation indicative of hydrogen peroxide production was found in any of the 20 ecotypes. DAB was readily taken up by the roots and dispersed in the vascular tissue but only light staining of tissue with DAB could be observed (result not shown). In leaves, DAB was taken up via

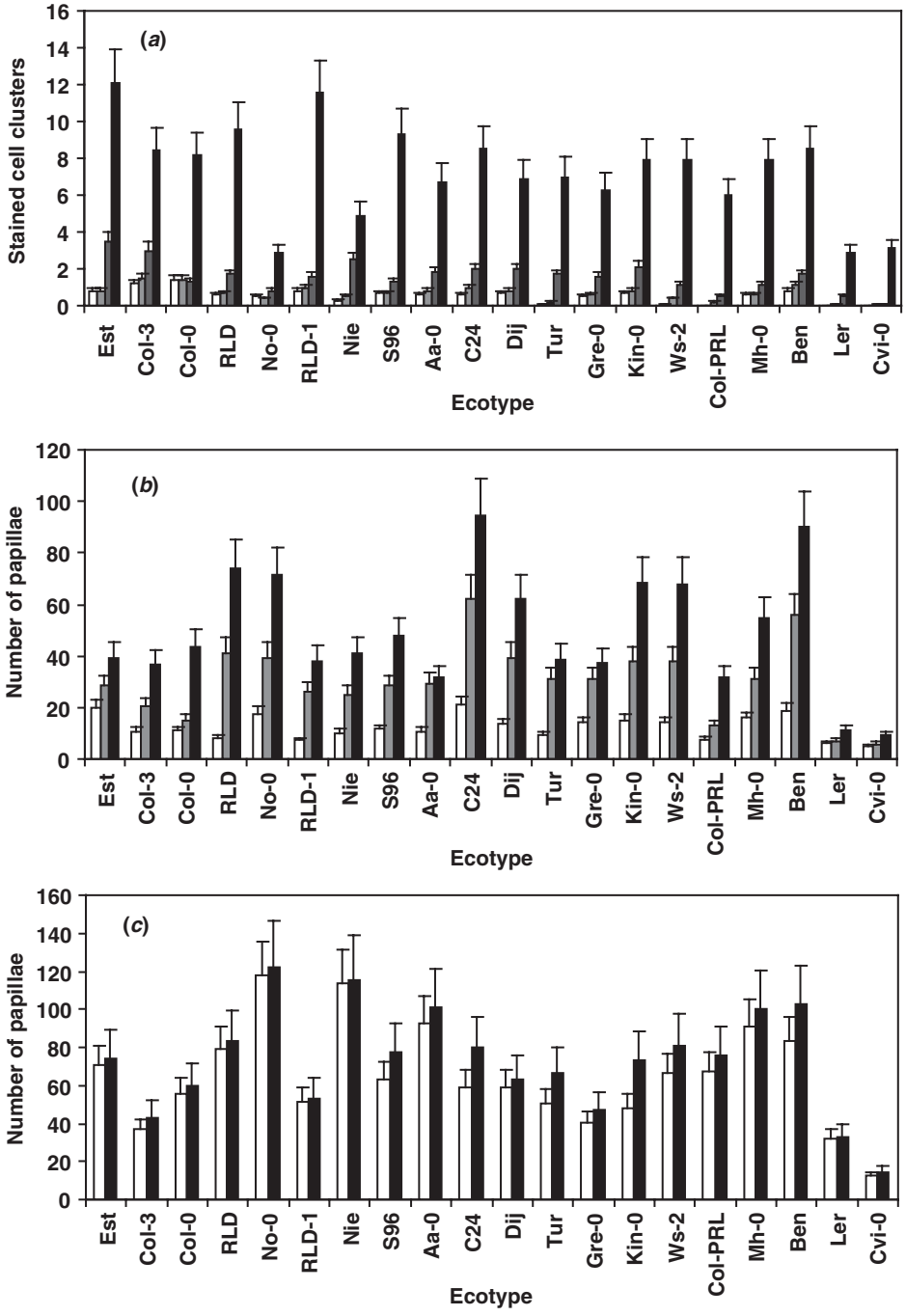

Fig. 3. Number of trypan-blue-stained cell clusters in leaves and callose papillae in roots and leaves of *Arabidopsis thaliana* following inoculation with *Phytophthora cinnamomi*. (*a*) Stained cell clusters at the inoculation site and at a distance from the inoculation site in leaves of 2 and 4 week old ecotypes of *A. thaliana*. Data for 2-week-old (white bars) and 4-week-old (lightly shaded bars) leaves at the inoculation site and 2-week-old (heavily shaded bars) and 4-week-old (black bars) leaves away from the inoculation site 72 h post inoculation. (*b*) Number of callose papillae in roots of *A. thaliana* ecotypes following inoculation with *P. cinnamomi* in 4-week-old roots at 12 h (white bars), 24 h (shaded bars) and 48 h (black bars) h after inoculation. (*c*) Comparison of the production of callose in 2-week-old (white bars) and 4-week-old (black bars) leaves at 72 h after inoculation. Error bars are standard error of the mean.

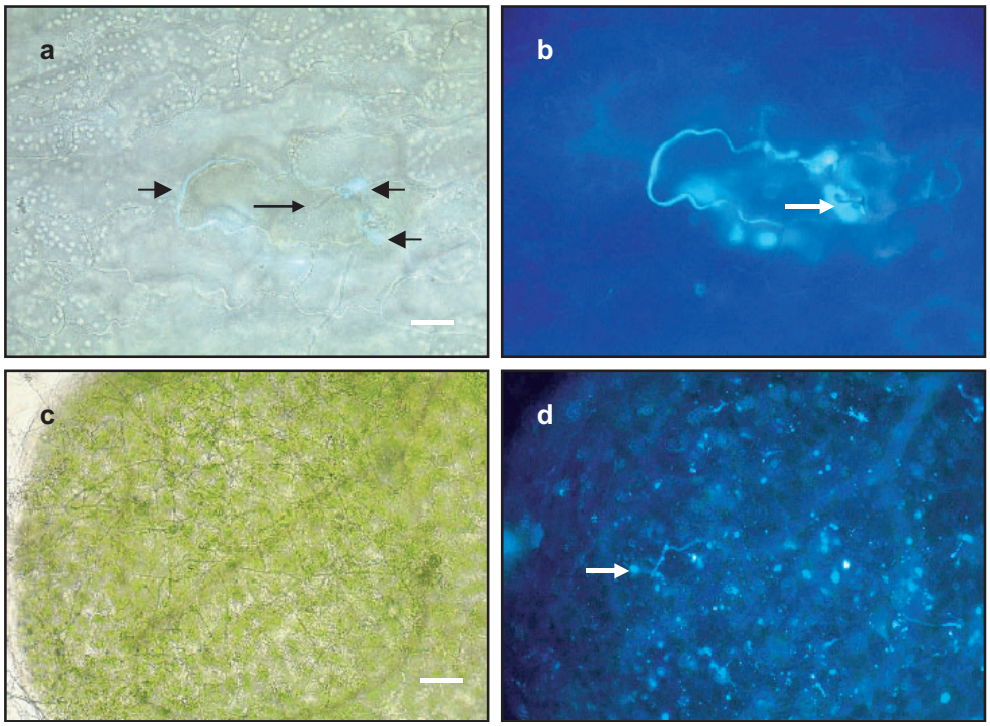

Fig. 4. Callose deposition in leaves of *Arabidopsis thaliana* ecotypes following inoculation with *Phytophthora cinnamomi*. (*a*) Aniline-blue-induced fluorescence in a combined bright field and blue-light-induced fluorescence image of a 4-week-old leaf of ecotype Col-PRL 48 h post inoculation. Light blue areas are callose (large arrows), small arrow shows single hypha within a cell. Bar represents 25 µm. (*b*) Same preparation as in (*a*) but showing blue-light-induced fluorescence of callose in a single cell, along cell walls and sites of hyphal penetration (arrow). (*c*) Bright field image of a 2-week-old leaf of ecotype Nie 72 h post inoculation showing *P. cinnamomi* cysts and hyphae on and within the leaf. Bar represents 50 µm. (*d*) Same image as in (*c*) except showing blue-light-induced fluorescence of callose. Arrow points to a hypha. Scale as in (*c*).

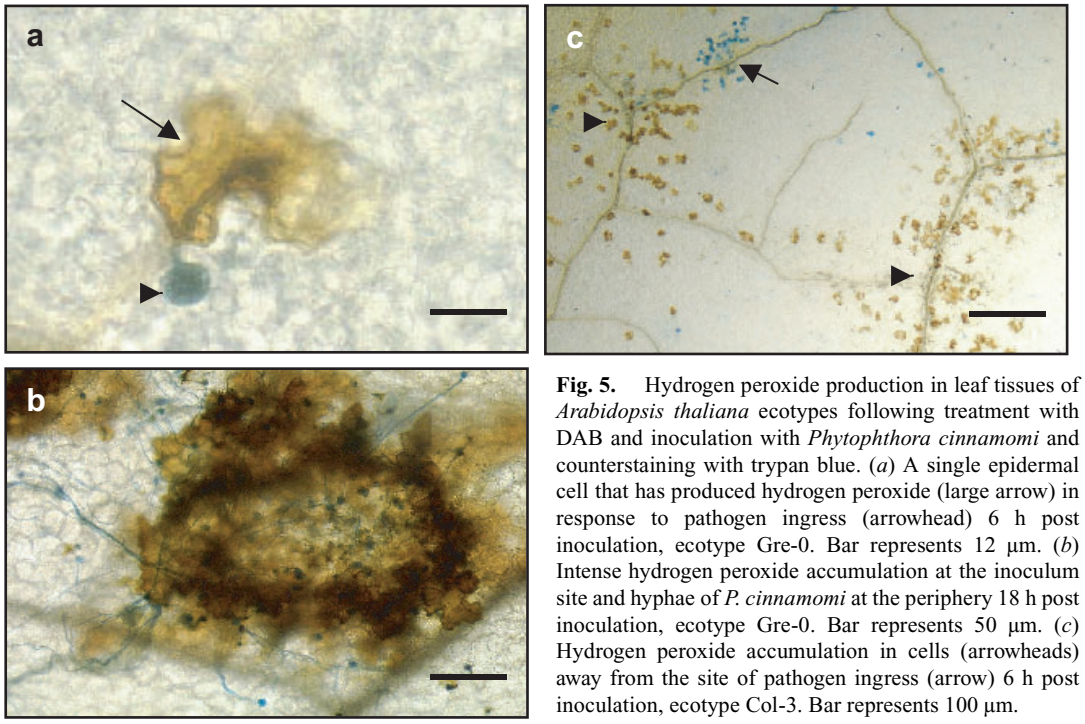

Fig. 5. Hydrogen peroxide production in leaf tissues of *Arabidopsis thaliana* ecotypes following treatment with DAB and inoculation with *Phytophthora cinnamomi* and counterstaining with trypan blue. (*a*) A single epidermal cell that has produced hydrogen peroxide (large arrow) in response to pathogen ingress (arrowhead) 6 h post inoculation, ecotype Gre-0. Bar represents 12 µm. (*b*) Intense hydrogen peroxide accumulation at the inoculum site and hyphae of *P. cinnamomi* at the periphery 18 h post inoculation, ecotype Gre-0. Bar represents 50 µm. (*c*) Hydrogen peroxide accumulation in cells (arrowheads) away from the site of pathogen ingress (arrow) 6 h post inoculation, ecotype Col-3. Bar represents 100 µm.

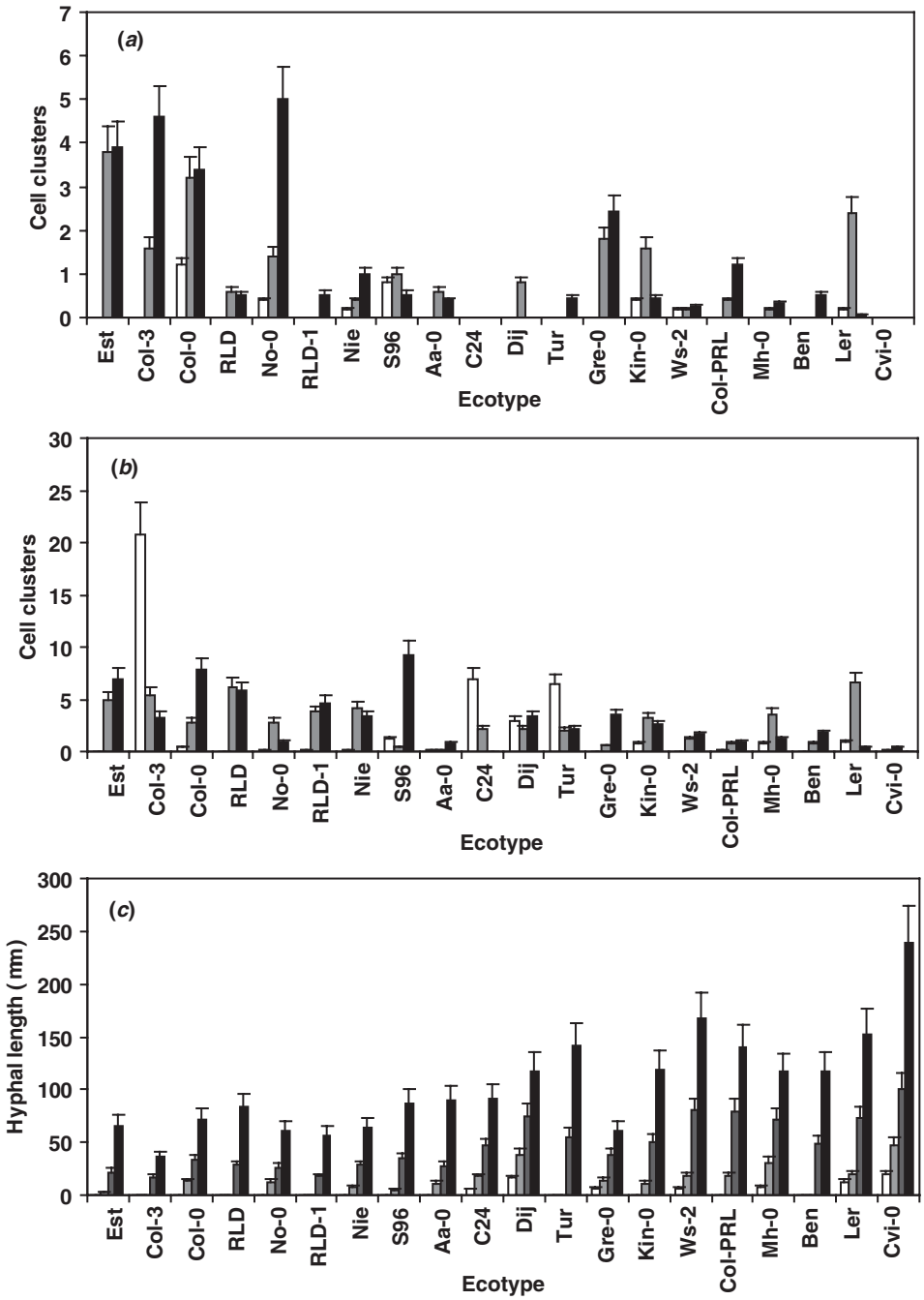

Fig. 6. Hyphal length and hydrogen peroxide production in leaves of *Arabidopsis thaliana* ecotypes following inoculation with *Phytophthora cinnamomi*. The number of DAB positive cell clusters (*a*) at the site of inoculation and (*b*) at remote sites 6 h (white bars), 12 h (shaded bars) and 24 h (black bars) after inoculation. (*c*) Length of hyphae at 3 h (white bars), 6 h (lightly shaded bars), 9 h (heavily shaded bars), and 12 h (black bars) after inoculation. Error bars are standard error of the mean.

the excision site at the petiole and distributed throughout the leaf. Hydrogen peroxide production was detected in leaf tissue as a vivid reddish-brown colouration, visible to the naked eye within 3 h of pathogen challenge. At early times (up to 9 h) after inoculation of the more resistant ecotypes, hydrogen peroxide was found in epidermal cells that had been penetrated by the pathogen (Fig. 5*a*). In ecotypes where a defined lesion was present, staining was not restricted to single cells but occurred in many cells and cell clusters beneath the inoculum site (Fig. 5*b*). In ecotypes that had restricted lesions, cells surrounding the lesion site were darkly stained with DAB. DAB polymerisation was also

found from 3 h post inoculation at discrete sites away from pathogen ingress and in distribution similar to the periveinal cell death described for trypan blue treated leaves (Fig. 5*c*). Leaves incubated with the hydrogen peroxide scavenger, ascorbic acid, in conjunction with DAB prior to inoculation showed no DAB staining at any time after inoculation. No DAB staining was found in control leaves.

The amount of hydrogen peroxide produced in leaves as measured by the number of DAB positive cells varied between ecotypes (Fig. 6). For example, ecotypes Cvi-0 and C24 had produced no hydrogen peroxide at 24 h after inoculation while ecotypes Est, Col-0, Col-3, and No-0 had many cell clusters that produced hydrogen peroxide (Fig. 6*a*). There was a clear difference in the number of DAB cell clusters between the ecotypes that had the greatest number of clusters compared with those that had the least at, and away from, the inoculum site (Fig. 6*b*). Hyphal lengths were reduced in those ecotypes that showed the most DAB staining compared with those which showed the least (compare Fig. 6*a* with Fig. 6*c*). There was a large difference in hyphal length, for example from 36 µm in Col-3 to 239 µm for Cvi-0, at 12 h after inoculation that showed an inverse correlation with the number of DAB stained cell clusters.

Measurement of hyphal length within leaves after treatment with DAB in combination with ascorbic acid showed that the growth of *P. cinnamomi* was greater between 3 and 12 h post inoculation compared with leaves treated with DAB alone (data not shown).

Ecotype versus interaction parameter correlations

When all ecotypes were compared with the parameters measured, three parameters stood out as being consistently correlated with the degree of susceptibility; hyphal length in leaves, the number of trypan-blue-stained cell clusters at and away from the inoculation site in leaves, and number of DAB-stained cell clusters (signifying hydrogen peroxide production) in leaves. There also appeared to be a correlation between ecotype susceptibility and the number of sporangia and chlamydospores in roots. Ecotypes Ler and Cvi-0 consistently displayed the most susceptible characteristics for each parameter examined. There was no apparent close correlation of susceptibility with papilla formation.

Discussion

This study investigated the interaction between what would normally be regarded as a non-host plant, *A. thaliana*, and the soilborne oomycete *P. cinnamomi*. Roots and leaves of each *A. thaliana* ecotype tested were penetrated by *P. cinnamomi* and the reaction to penetration and progress of the pathogen differed between ecotypes and organ infected. *P. cinnamomi* is not usually thought of as a leaf pathogen but we have clearly shown that it has the ability to penetrate and colonise leaves of certain *A. thaliana* ecotypes. There was a range of responses for each tested parameter that was used to describe the interactions in both roots and leaves. There was always a distinct separation and consistent grouping of those ecotypes that displayed resistance from those that displayed susceptible characteristics. The interactions were characterised by differences in levels of sporulation, lesion type, callose deposition and production of the reactive oxygen species, hydrogen peroxide. Interactions within roots and leaves differed and an age-related resistance response was evident. Previously a range of responses to *P. cinnamomi*, from resistance to susceptibility, has been demonstrated for a wide variety of Australian native species (Cahill *et al.* 1989). Variation in resistance has also been shown for horticultural species such as *Persea americana* (avocado) (Phillips *et al.* 1984) and for the forest species *Eucalyptus marginata* (Cahill *et al.* 1992). This is the first study to show variation in resistance within well-defined and characterised ecotypes of a single species.

A. thaliana is a widely distributed species and, as a result, shows a diversity of phenotypes and thus genetic variation that is important for adaptation to specific conditions (Alonso-Blanco and Koornneef 2000). Considerable variation has been found for potentially adaptive traits such as resistance to biotic stresses including insects, fungi, bacteria and viruses (Kunkel 1996). It has been used extensively as a model organism and has numerous advantages as a system to analyse the interactions of plants with invading organisms (Davis 1993).

In the only other published study on the interaction of *A. thaliana* with a member of the genus *Phytophthora*, *P. porri*, a species that is normally considered to have a narrow host range, it was found that *A. thaliana* is a true host (Roetschi *et al.* 2001). Clear compatible and incompatible states were described within a set of eight ecotypes. We have now shown that like *P. porri*, *P. cinnamomi* can infect both roots and leaves of *A. thaliana* and can complete its life cycle within susceptible ecotypes. However, in contrast to the *P. porri–A. thaliana* pathosystem where distinct incompatible (with a typical HR or trailing HR) and compatible states were established with the various ecotype-isolate combinations, our results show that for *P. cinnamomi* the interactions are characterised by different degrees of susceptibility. All ecotypes were found to support sporulation of *P. cinnamomi* to some degree but there was wide variation in the numbers of sporangia and chlamydospores produced during the interactions. There was also a difference between roots and leaves with both chlamydospores and sporangia being produced in the former but sporangia being formed only rarely in the latter.

It is well known that plant species can only be successfully infected by a rather limited number of pathogens (Honée 1999). Failure of a pathogen to successfully infect a plant species is based on non-host resistance. This type of resistance is pathogen non-specific and is often based on preformed structural barriers or toxic

compounds (Osbourn 1996). *A. thaliana* has not been shown to be a natural host of *P. cinnamomi* in the wild. Though the non-host interactions that were observed in this study between *A. thaliana* and *P. cinnamomi* show that *P. cinnamomi* is able to express virulence in *A. thaliana* and complete its life cycle, *A. thaliana* can, in turn, mount active defence responses.

By exploiting the genetic diversity found in *A. thaliana*, we have found that there was a significant difference between the number of *P. cinnamomi* sporulation structures, in the production of hydrogen peroxide and callose and in overall susceptibility between the least susceptible ecotypes (Col-0, Col-3, Est, S96) and the most susceptible ecotypes (Cvi-0 and Ler). In the least susceptible ecotypes cell death, restricted lesion formation and the production of hydrogen peroxide were hallmarks of their ability to display lower sporulation levels in both roots and leaves and in compromising pathogen growth and colonisation. In the least susceptible ecotypes, the virulence of *P. cinnamomi* was reduced in older plants (4 week old) and shown by a significant decrease in pathogen sporulation in both root and leaf tissue. In contrast there was no significant difference between 2- and 4-week-old plants in the most susceptible ecotypes. Mature plant resistance is often suggested to be general (horizontal) or polygenic, with a medium level of resistance displayed to all races of a pathogen by a plant species (Manners 1993). If all of the *A. thaliana* ecotypes tested had displayed increased resistance to *P. cinnamomi*, then age related general resistance could have been the attributing factor. However, differences between the ecotypes in resistance with age suggest that there is a more complex mechanism involved in the *A. thaliana–P. cinnamomi* interaction.

Callose deposition was observed from 6 h post inoculation in all *A. thaliana* ecotypes, a finding similar to that in incompatible interactions with *P. porri* (Roetschi *et al.* 2001). In the present study, the extent of production varied between ecotypes with no clear correlation with susceptibility. Similarly, in the *A. thaliana–Erysiphe cichoracearum* interaction, callose deposition was observed in both compatible and incompatible interactions and to a similar extent at penetration sites suggesting that their formation was not a limiting factor to the powdery-mildew invasion of *A. thaliana* (Adam and Somerville 1996).

Hydrogen peroxide production was detected in leaf tissue as a rapid response to infection. DAB polymerisation (hydrogen peroxide accumulation) was observed at 3 h post inoculation and in the most susceptible ecotypes (Cvi-0 and Ler) was significantly reduced ($P < 0.05$) compared with the more resistant ecotypes (Col-0, Col-3, Est and S96). Interaction between *A. thaliana* leaves and *P. cinnamomi* also revealed a pattern of cell 'death' distant from site of pathogen ingress in all ecotypes. There was accumulation of both trypan blue and hydrogen peroxide in the same small clusters of cells at sites away from the inoculation site. The inoculation of *A. thaliana* leaves with an avirulent strain of *Pseudomonas syringae* has been shown to induce secondary oxidative bursts in cells distant from pathogen ingress (Alvarez *et al.* 1998). The oxidative burst at the inoculum site induced systemic cellular changes and in addition to bursts in distant tissue was required for the establishment of systemic acquired resistance.

The source of hydrogen peroxide production is unclear, but the initial production detected in and around callose papillae in epidermal cells of some of the more resistant ecotypes suggests that the principal site is on the extracellular surface of the plasma membrane or within the apoplasm, indicative of a cell wall peroxidase-driven oxidative burst. If the source of hydrogen peroxide is from the cell wall, it is possible that it had a direct toxic effect on the pathogen. The addition of exogenous hydrogen peroxide to *P. cinnamomi* zoospores resulted in reduced germination and growth of the pathogen (data not shown). Hydrogen peroxide production at the inoculation site may have contributed to the inhibition of *P. cinnamomi* growth and colonisation because when leaves were treated with ascorbic acid (an antioxidant), the pathogen grew more extensively and colonised greater amounts of leaf tissue.

In conclusion, we have defined a *P. cinnamomi–A. thaliana* pathosystem that displays a number of features that will be useful in elucidating further resistance in plants to a pathogen that has an extremely wide host range and few species that are resistant. The susceptible and resistant responses found in both roots and leaves of *A. thaliana* will provide an opportunity for genetic and molecular analysis, such as in the use of recombinant inbred lines, that has to date been impossible using current approaches.

Acknowledgements

This research was supported by grants to D. Cahill from the Australian Research Council and Deakin University.

References

Able AJ, Guest DI, Sutherland MW (1998) Use of a new tetrazolium-based assay to study the production of superoxide radicals by tobacco cell cultures challenged with avirulent zoospores of *Phytophthora parasitica* var. *nicotianae*. *Plant Physiology* **117**, 491–499.

Adam I, Somerville SC (1996) Genetic characterisation of five powdery mildew resistance loci in *Arabidopsis thaliana*. *The Plant Journal* **9**, 341–356.

Alexander D, Goodman RM, Gut-Rella M, Glascock C, Weymann K, Friedrich L, Maddox D, Ahl-Goy P, Luntz T, Ward E, Ryals J (1993) Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proceedings of the National Academy of Sciences USA* **90**, 7327–7331.

Alonso-Blanco C, Koornneef M (2000) Naturally occurring variation in *Arabidopsis*: an underexploited resource for plant genetics. *Trends in Plant Science* **5**, 22–29.

Alvarez ME, Pennell RI, Meijer P-L, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* **92**, 773–784.

Apostol I, Heinstein PF, Low PS (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. *Plant Physiology* **90**, 109–116.

Baker CJ, Orlandi EW (1995) Active oxygen in plant pathogenesis. *Annual Review of Phytopathology* **33**, 299–321.

Bent AF (1996) Plant disease resistance genes: function meets structure. *The Plant Cell* **8**, 1757–1771.

Bowling SA, Guo A, Cao H, Gordon AS, Klessig DF, Dong X (1994) A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. *The Plant Cell* **6**, 1845–1857.

Buell RC (1998) *Arabidopsis*: a weed leading the field of plant–pathogen interactions. *Plant Physiology and Biochemistry* **36**, 177–186.

Byrt PY, Grant BR (1979) Some conditions governing zoospore production in axenic cultures of *Phytophthora cinnamomi* Rands. *Australian Journal of Botany* **27**, 103–115.

Cahill DM, Bennett IJ, McComb JA (1992) Resistance of micropropagated *Eucalyptus marginata* to *Phytophthora cinnamomi***.** *Plant Disease* **76**, 630–632.

Cahill DM, Legge N, Grant BR, Weste GM (1989) Cellular and histological changes induced by *Phytophthora cinnamomi* in a group of plant species ranging from fully susceptible to fully resistant. *Phytopathology* **79**, 417–424

Cahill DM, Weste G, Grant BR (1986) Changes in cytokinin concentrations in xylem extrudate following infection of *Eucalyptus marginata* with *Phytophthora cinnamomi* Rands. *Plant Physiology* **81**, 1103–1109.

Dangl JL, Dietrich RA, Richberg MH (1996) Death don't have no mercy: cell death programs in plant–microbe interactions. *The Plant Cell* **8**, 1793–1807.

Davis KR (1993). *Arabidopsis* as a model plant system. In '*Arabidopsis thaliana* as a model for plant–pathogen interactions'. (Eds KR Davis, R Hammerschmidt) pp. 1–3. (The American Phytopathological Society Press: St Paul)

Dawson PD, Weste GM (1984) Impact of root infection by *Phytophthora cinnamomi* on the water relations of two *Eucalyptus* species that differ in susceptibility. *Phytopathology* **74**, 486–490

Dietrich RA, Delany TP, Uknes SJ, Ward ER, Ryals JA, Dangl JL (1994) *Arabidopsis* mutants simulating disease resistance response. *Cell* **77**, 565–577.

Doke N (1983) Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissue to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiological Plant Pathology* **23**, 345–357.

Eschrich W, Currier HB (1964) Identification of callose by its diachrome and fluorochrome reactions. *Stain Technology* **39**, 303–307.

Fuchs H, Sacristián MD (1996) Identification of a gene in *Arabidopsis thaliana* controlling resistance to clubroot (*Plasmodiophora brassicae*) and characterisation of the resistance response. *Molecular Plant–Microbe Interactions* **9**, 91–97.

Hoagland DR, Arnon DI (1950) 'The water culture method for growing plants without soil.' California Agriculture Experimental Station Circular No. 347.

Irwin JAG, Cahill DM, Drenth A (1995) *Phytophthora* in Australia. *Australian Journal of Agricultural Research* **46**, 1311–1337.

Kamoun S, Huitema E, Vleeshouwers VGAA (1999) Resistance to oomycetes: a general role for the hypersensitive response? *Trends in Plant Science* **4**, 196–200.

Kellam MK, Coffey MD (1985) Quantitative comparison of the resistance to Phytophthora root rot in three avocado rootstocks. *Phytopathology* **75**, 230–234

Lee S, Stenger DC, Bisaro DM, Davis KR (1994) Identification of loci in *Arabidopsis* that confer resistance to geminivirus infection. The *Plant Journal* **6**, 525–535.

Manners JG (1993) 'Principles of plant pathology (2nd edn).' pp. 143–152. (Cambridge University Press: Cambridge)

Mehdy MC (1994) Active oxygen species in plant defence against pathogens. *Plant Physiology* **105**, 467–472.

Miller PM (1955) V-8 juice agar as a general purpose medium for fungi and bacteria. *Phytopathology* **45**, 461–462.

Osbourn AE (1996) Preformed antimicrobial compounds and plant defence against fungal attack. *The Plant Cell* **8**, 1821–1831.

Phillips D, Grant BR, Weste GM (1984) Histological changes in roots of an avocado cultivar, Duke 7, infected with *Phytophthora cinnamomi. Phytopathology* **77**, 691–698

Price RA, Palmer JD, Al-Shehbaz IA (1994) Systematic relationships of *Arabidopsis*: a molecular and morphological perspective. In 'Arabidopsis'. pp. 7–19. (Cold Spring Harbor Laboratory Press: USA)

Roetschi A, Si-Ammour A, Belbahri L, Mauch F, Mauch-Mani B (2001) Characterisation of an Arabidopsis–Phytophthora pathosystem: resistance requires a functional PAD2 gene and is independent of salicylic acid, ethylene and jasmonic acid signalling. *The Plant Journal* **28**, 293–305.

Siegrist J, Orober M, Buchenauer H (2000) Beta-aminobutyric acid-mediated enhancement of resistance in tobacco to tobacco mosaic virus depends on the accumulation of salicylic acid. *Physiological and Molecular Plant Pathology* **56**, 95–106

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localisation of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. *The Plant Journal* **11**, 1187–1194.

Tor M, Gordon P, Cuzick A, Eulgem T, Sinapidou E, Mert-Turk F, Can C, Dangl JL, Holub EB (2002) Arabidopsis SGT1b is required for defense signaling conferred by several downy mildew resistance genes. *The Plant Cell* **14**, 993–1003.

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J (1992) Acquired resistance in Arabidopsis. *The Plant Cell* **4**, 645–656.

Venisse JS, Gullner G, Brisset MN (2001) Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora. Plant Physiology* **125**, 2164–2172.

Vogel J, Somerville S (2000) Isolation and characterisation of powdery mildew-resistant *Arabidopsis* mutants. *Proceedings of the National Academy of Sciences USA* **97**, 1897–1902.

Received 18 March 2002, accepted 25 September 2002