Eur J Plant Pathol (2011) 131:419–430
DOI 10.1007/s10658-011-9819-x

A quantitative PCR assay for accurate *in planta* quantification of the necrotrophic pathogen *Phytophthora cinnamomi*

Leila Eshraghi · Nader Aryamanesh · Jonathan P. Anderson · Bryan Shearer · Jen A. McComb · Giles E. St. J. Hardy · Philip A. O'Brien

Accepted: 26 May 2011 / Published online: 4 June 2011

Abstract A reliable method for measuring disease progression is important when evaluating susceptibility in host—pathogen interactions. We describe a sensitive quantitative polymerase chain reaction (QPCR) assay that enables quantitative measurement of *in planta* DNA of the necrotrophic pathogen, *Phytophthora cinnamomi,* that avoids problems caused by variation in DNA extraction efficiency and degradation of host DNA during host tissue necrosis. Normalization of pathogen DNA to sample fresh weight or host DNA in samples with varying degrees of necrosis led to overestimation of pathogen biomass. Purified plasmid DNA, containing the pScFvB1 mouse gene, was added during DNA extraction and pathogen biomass was normalized based on plasmid DNA rather than host DNA or sample fresh weight. This method is robust and improves the accuracy of pathogen measurement in both resistant (non-host *A. thaliana–P. cinnamomi*) and susceptible (host *Lupinus angustifolius–P. cinnamomi*) interactions to allow accurate measurement of pathogen biomass even in the presence of substantial host cell necrosis.

Keywords *Arabidopsis thaliana* · Internal control · *Lupinus angustifolius* · Non-host resistance

L. Eshraghi (✉) · B. Shearer · J. A. McComb · G. E. S. J. Hardy · P. A. O'Brien
Centre for Phytophthora Science and Management,
School of Biological Sciences and Biotechnology,
Murdoch University,
South street,
Murdoch, WA 6150, Australia
e-mail: L.Eshraghi@murdoch.edu.au

N. Aryamanesh
School of Plant Biology,
Faculty of Natural and Agricultural Sciences,
The University of Western Australia,
35 Stirling Highway,
Crawley, WA 6009, Australia

J. P. Anderson
CSIRO Plant Industry,
Centre for Environment and Life Sciences,
Private Bag 5,
Wembley, WA 6913, Australia

J. P. Anderson
The University of Western Australia
Institute of Agriculture,
35 Stirling Highway, Crawley,
Perth, WA 6009, Australia

B. Shearer
Department of Environment and conservation,
Science Division,
Kensington, WA 6983, Australia

Introduction

The oomycetes comprise many destructive plant pathogens including those employing a biotrophic lifestyle such as the downy mildews (e.g. *Bremia lactucae*, *Hyaloperonospora parasitica*), white rusts (*Albugo* spp.), the hemibiotrophs (e.g. *Phytophthora infestans*, *P. sojae*) and necrotrophs such as *Pythium*

spp. and *P. cinnamomi* (Hardham 2007). Due to the global impacts of plant diseases caused by *Phytophthora* species, it is the most economically important genus of plant pathogens worldwide (Erwin and Ribeiro 1996). Among these, *P. cinnamomi* with a host range of more than 3000 plant species is considered to be one of the most pathogenic and causes considerable damage to natural ecosystems, agriculture, horticulture and forestry worldwide (Shearer and Fairman 2007; Rookes et al. 2008; Brasier 2008; Hansen 2008; Hardham 2005).

Studying interactions between *Phytophthora* pathogens and their host plants is an important step in disease control strategies; however, studying interactions with native and crop plants is challenging because of the limited knowledge of their genome structure. To address this issue model plants such as *Arabidopsis thaliana* have been used extensively for studying various fungal, bacterial and oomycete pathogen–plant interactions (Glazebrook 2001; Thomma et al. 2001). However, most *Phytophthora* species, including *P. infestans* and *P. sojae,* cannot infect *Arabidopsis* suggesting that *Arabidopsis* exhibits a strong non-host resistance to these species (Kamoun 2001; Kamoun et al. 1999; Takemoto et al. 2003). Although some ecotypes of *A. thaliana* are resistant to *P. cinnamomi*, this pathogen can infect both roots and leaves of *A. thaliana*, express virulence and complete its life cycle on some *A. thaliana* ecotypes (Robinson and Cahill 2003) making *Arabidopsis* an attractive model plant to investigate mechanisms of resistance to this necrotrophic *Phytophthora* species. Although clear differences between resistant and moderately susceptible ecotypes exist, the limited extent of lesion development, even on susceptible ecotypes, makes traditional methods of visual assessment of the symptoms and spore or colony counting difficult, particularly in the early stages of infection when resistance is first being expressed.

To overcome the problems associated with visual assessments, quantitative polymerase chain reaction (QPCR) assays have been used in a number of other pathosystems (Böhm et al. 1999; McCartney et al. 2003; Schaad and Frederick 2002; Schaad et al. 2003). QPCR determination of *in planta* pathogen biomass is rapid, sensitive, specific and very efficient for detecting pathogen biomass even at early stages of infection when little pathogen biomass exists. Despite the benefits of the technique, some inherent problems, such as the requirement for a reliable normalization technique, may confound results. Many studies normalize the amount of pathogen DNA to host DNA, however, DNA degradation during plant cell collapse, such as that occurring during infection by a necrotrophic pathogen, could lead to an overestimation of pathogen biomass. To avoid this, some studies have chosen to normalize their results to sample surface area or weight (Brouwer et al. 2003; van Wees et al. 2003). However, these comparisons are still confounded by variability in DNA extraction yields between samples of the same surface area or weight (Gachon and Saindrenan 2004).

The objective of this study was to develop a sensitive QPCR assay which can be used to quantify *P. cinnamomi,* or other pathogen biomass at all stages of the necrotrophic pathogen–host interaction by avoiding problems caused by tissue necrosis including host DNA degradation and variable DNA extraction yield between samples. The method is based on the addition of plasmid DNA as an internal control during the DNA extraction stage and subsequently normalizing pathogen biomass using the internal control. Several animal, human and food studies have previously described the addition of an internal control for accurate quantification of target DNA (Damen et al. 2008; Klerks et al. 2006; Halliday et al. 2010). In this study, we tested the potential for overestimation of pathogen DNA when measuring pathogen DNA relative to sample fresh weight or host DNA without the use of an internal control, and the efficacy of the use of an internal control using two different plant–pathogen interactions, the resistant *A. thaliana–P. cinnamomi* interaction and the susceptible *Lupinus angustifolius–P. cinnamomi* interaction.

Materials and methods

Plant materials, growth conditions and inoculation

Arabidopsis thaliana ecotypes *Landsberg erecta* (Ler) and *Colombia* (Col-0) were purchased from Lehle Seeds (Round Rock, TX). Seeds were planted on the surface of seed raising mix (Debco, Australia) in 5 cm free-draining polyurethane pots pre-moistened with distilled water. Seeds were stratified for 3 days at 4°C in the dark before being transferred to a growth

cabinet with a 10/14 hday/night photoperiod with 100 µmol m^{-2} s^{-1} at 21±1°C. The pots were watered every second day. Leaves of four-week old *A. thaliana* seedlings were inoculated with 5 µl of 1×10^5 *P. cinnamomi* zoospores ml^{-1} on the abaxial surface and pots were transferred into a box which was sealed with aluminum foil (to maintain high humidity). After 12 h incubation in the dark at 25°C, the seedlings were transferred to a growth cabinet with a 10/14 h day/night photoperiod at 21±1°C to allow lesions to develop. Tissue samples were collected 24, 48 and 72 h after inoculation.

Blue lupin (*L. angustifolius* L., cv. Mandalup) seeds were obtained from Department of Agriculture and Food, Western Australian (DAFWA). Seeds were surface-sterilized in 70% ethanol for 2 min followed by immersion in 50% bleach solution (6.25% available chlorine) for 5 min. The sterilized seeds were germinated on filter paper pre-moistened with distilled water at 25°C in the dark for 3 days. The seedlings were transferred to filter papers pre-moistened with half strength MS medium (Murashige and Skoog 1962) in plastic Petri-dishes (14 cm in diameter) and grown for a further 4 days. The seedlings were inoculated by placing a 2 mm diameter plug of *P. cinnamomi* mycelium at the tips of roots and covering with filter paper pre-moistened with sterile distilled water. Mycelial tissue was removed from the surface of the roots and root tissue samples collected at 24, 48, 72 and 96 h after inoculation. It should be noted that different inoculation procedures were used for *Arabidopsis* and lupin and this should be taken into account if comparing the degree of susceptibility of the two species.

Inoculum preparation

Phytophthora cinnamomi (isolate MP 94.48) was obtained from the Centre for *Phytophthora* Science and Management (CPSM) culture collection at Murdoch University. The isolate was maintained on 20% V8-juice agar (Erwin and Ribeiro 1996) at 25°C in the dark and sub-cultured every 7 days. Preliminary trials found isolate MP 94.48 to show similar infection characteristics to other *P. cinnamomi* isolates tested but was more pathogenic on *A. thaliana* ecotype Ler and more consistently produced zoospores in culture (data not shown). *Phytophthora cinnamomi* zoospores were produced aseptically using the method described by Byrt and Grant (1979). The zoospore density was determined using a haemocytometer and adjusted to a concentration of 1×10^5 zoospores ml^{-1} with sterile distilled water and used for inoculation of *A. thaliana* leaves. For genomic DNA extraction, *P. cinnamomi* hyphae was harvested from patches of 5-day old hyphae grown on filter papers placed on the surface of V8 medium.

Plasmid DNA extraction

For the preparation of plasmid DNA, *E. coli* HB2151 containing the plasmid pScFvB1 (Manatunga et al. 2005) was grown in LB broth medium (Sambrook et al. 1989) with 50 µg ml^{-1} ampicillin for 24 h at 37°C with shaking. Plasmid DNA was extracted using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) following the manufacturer's instructions.

Plant DNA extraction using plasmid DNA as an internal control

Eight *A. thaliana* leaf discs (7 mm in diameter with an average total weight of 50 mg per eight discs) that included the entire area of a lesion were collected in a 2 ml tubes 72 h after inoculation. In the case of lupin, either 50 mg or 100 mg of the infected roots were weighed for each sample and transferred to a 2 ml tube. Tissue samples were homogenized using Precellys 24 lysis and homogenizer (Bertin Technologies). DNA was extracted from the tissue samples using the Nucleon™ PhytoPure™ Genomic DNA Extraction Kits (GE Healthcare) according to the manufacturer's instructions. Based on a preliminary experiment, 1 ng of ScFvB1 plasmid DNA was added to all samples just before the first centrifugation step.

Primer design

Phytophthora cinnamomi primers (forward 5′–GCT AGC AAG CAC GTA TGA GG and reverse 5′–CGC CCC AAC TAT ACG ACA AC) were designed for the PDN gene (FJ493007). Actin (AT3G18780) primers (forward 5′–CTT GCA CCA AGC AGC ATG AA; reverse 5′–CCG ATC CAG ACA CTG TAC TTC CTT) were used for amplification of both *A. thaliana* and lupin genome and the plasmid was detected using the primers forward 5′–GGA TCG GAC ATC GAG CTC AC; reverse 5′–CAC TTG AGC TGG CAC TGC AG. All primers were designed using primer 3

(v. 0.4.0) software (http://frodo.wi.mit.edu/) and tested for their specificity by (a) comparing with the genome sequences of *A. thaliana, L. angustifolius,* and *P. cinnamomi* and (b) running PCR using all other DNAs used in this study as templates. On the basis of these analyses the primers were judged to be specific for their target DNA templates. The *P. cinnamomi* primers were designed to be specific to *P. cinnamomi* and not to bind to other *Phytophthora* sequences in Genbank however when used on samples potentially containing other *Phytophthora* species, such as field soil samples, the specificity of the primers should be experimentally determined.

QPCR amplification

The QPCR assays were conducted using a Light-Cycler 480 (Roche Applied Science, Mannheim, Germany). All DNA samples were analyzed by QPCR assays using the specific primers and SYBR Green 1 (Roche Applied Science, Mannheim, Germany). QPCR was performed in a volume of 10 µl containing 5 µM forward primer, 5 µM reverse primer, 5 µl of 2× SYBR Green 1 Master Mix and 20 ng of DNA template. Thermal cycling conditions were as follows; 2 min at 50°C, 2 min at 95°C followed by 40 cycles each consisting of 5 s at 95°C, and then 10 s at 55°C and 10 s at 72°C. Melting curve analysis was carried out using the following program: 95°C for 10 s, followed by a constant decrease in temperature from 95°C to 55°C at a rate of 3.3°C per 30 s. The amplification efficiency for each target gene was checked via the slope of a standard curve constructed from amplification of 10-fold dilution series of DNA (10,000 pg, 1,000 pg, 100 pg, 10 pg and 1 pg). The QPCR assay was tested with DNA from pure tissues of *A. thaliana*, *P. cinnamomi*, plasmid, and *L. angustifolius.*

Results

Assay specificity and sensitivity

To assess the specificity of primers for the plasmid pScFvB1, the primers were tested for amplification in the presence of DNA from *P. cinnamomi*, *A. thaliana*, or lupin, and in a no-template control. No amplification occurred in any of these assays (data not shown). Similarly no amplification products were obtained when the *P. cinnamomi* specific primer pair (FJ493007) was used with *A. thaliana*, pScFvB1, or *L. angustifolius* DNA templates. Amplification with the plant actin primer pair occurred only in the presence of *A. thaliana* DNA or lupin DNA (data not shown).

To test the sensitivity of amplification with the *P. cinnamomi* primer pairs and the effect of plant and plasmid DNA on amplification by these primers, a series of 10-fold dilutions of *P. cinnamomi* (ranging from 10 ng to 1 pg) were prepared. To each PCR reaction, 200 pg of plasmid DNA and 20 ng of *A. thaliana* DNA were added and QPCR was performed. In additional, *P. cinnamomi* (100 pg) was assayed in the absence of non-target DNA (*A. thaliana* and plasmid) as a control. The concentrations of *A. thaliana* and plasmid DNA were chosen to give Ct values ranging from 18 to 20 based on a preliminary experiment (data not shown). The results showed that the presence of the plant and plasmid DNA in the QPCR reaction did not affect amplification of *P. cinnamomi* DNA over this range of concentrations as low as 1 pg for *P. cinnamomi*. Furthermore, amplification of 10-fold dilution series of plasmid DNA (ranging from 1 ng to 0.1 pg) was not affected by the presence of 20 ng of *A. thaliana* DNA and 1 ng of *P. cinnamomi* DNA compared to controls without non-target DNA (*A. thaliana* and *P. cinnamomi*).The efficiency of amplifications for *P. cinnamomi* and plasmid primers were 98% (slope = −3.399; R^2= 0.999) and 96.5% (slope = −3.434; R^2=0.999), respectively.

Testing the inhibitory effect of DNA extracts on plasmid amplification

To determine the presence of PCR inhibitors in the DNA extracts from the pathogen culture, and plant, amplification of plasmid DNA (10 pg) was carried out in the presence of a 10-fold dilution series of *P. cinnamomi* and 20 ng of *A. thaliana* DNA. Furthermore, the amplification of plasmid DNA (10 pg) was also measured in the presence of DNA extracts from infected *A. thaliana* leaf samples (eight leaf discs; around 50 mg) or lupin roots (100 mg) in four biological samples. The Ct value for amplification of the plasmid in the absence of *P. cinnamomi* and *A. thaliana* DNA (control) was 19.28, and for amplifi-

cation of the plasmid in the presence of five dilution series of *P. cinnamomi* and 20 ng of infected/non-inoculated *A. thaliana* DNA was 19.31 to 19.46, with the plasmid ΔCt ranging from 0.03 to 0.18 (Table 1). The Ct value for amplification of the plasmid in the presence of infected/non-inoculated lupin DNA ranged from 19.18 to 19.42 (Table 1). The similarity in the Ct values shows that the plasmid amplification was not affected by the presence of *A. thaliana,* lupin or *P. cinnamomi* DNA, nor DNA extracted from necrotic tissue.

Table 1 Testing the efficiency of plasmid DNA amplification in the presence of DNA extracts from *Phytophthora cinnamomi* culture, inoculated/non-inoculated *Arabidopsis* or *Lupinus angustifolius* tissues with *P. cinnamomi* using QPCR

Infection[a]	*Arabidopsis* DNA	*P. cinnamomi* DNA (pg)	Ct[b] value for plasmid	ΔCt[c]
−	20 ng	10,000	19.32	0.04
−	20 ng	1,000	19.31	0.03
−	20 ng	100	19.31	0.03
−	20 ng	10	19.32	0.04
−	20 ng	1	19. 34	0.06
+	20 ng	Unknown	19.36	0.08
+	20 ng	Unknown	19.46	0.18
+	20 ng	Unknown	19.34	0.06
+	20 ng	Unknown	19.36	0.08
	Lupin DNA			
−	20 ng	Unknown	19.18	−0.1
−	20 ng	Unknown	19.39	0.11
−	20 ng	Unknown	19.25	−0.03
−	20 ng	Unknown	19.42	0.14
+	20 ng	Unknown	19.33	0.05
+	20 ng	Unknown	19.22	0.06
+	20 ng	Unknown	19.30	0.02
+	20 ng	Unknown	19.37	0.09
–[d]	–	–	19.28	–

[a] Infected leaves of *A. thaliana* or roots of lupin were collected 72 h after inoculation with *P. cinnamomi* (+) or distilled water (−)

[b] Ct (threshold cycle) is the number of PCR cycles at which a fluorescence intensity crosses a threshold set as a statistically significant increase in the SYBR fluorescence over all reactions within a plate

[c] ΔCt = (Ct of plasmid in samples tested) – (Ct of plasmid DNA in reactions without *Arabidopsis,* lupin or *P. cinnamomi* DNA)

[d] Control is the pure plasmid DNA in the absence of both plant and pathogen DNA

Using plasmid DNA as an internal control for measuring pathogen biomass

We demonstrated the efficiency of using plasmid DNA as the internal control in two different plant—pathogen pathosystems including the resistant *A. thaliana–P. cinnamomi* interaction and the susceptible *L. angustifolius–P. cinnamomi* interaction.

The *A. thaliana–P. cinnamomi* combination

To test the efficiency of this method, we took two different approaches. In the first approach, either 100 ng or 1,000 ng of *P. cinnamomi* DNA were added into tubes containing 0.1, 0.05 or 0.025 g of non-inoculated *A. thaliana* leaves and DNA extracted with the addition of 1 ng of plasmid DNA just before the first centrifugation step. The concentrations of the plasmid DNA (1 ng) added to samples were calculated in such a way that after re-suspending DNA samples in 100 μl of distilled water, the QPCR fluorescence passed the threshold line (Ct value) close to cycle 20 when using 20 ng of sample in the PCR reaction.

In the second approach, 0.1, 0.05 or 0.025 g of non-inoculated *A. thaliana* leaves were weighed and collected in 2 ml tubes. Then, either 0.01 g or 0.005 g *P. cinnamomi* hyphae (2-fold differences) was added to the tubes containing the *A. thaliana* leaves. One nanogram of plasmid DNA was then added to each sample and DNA extraction was carried out as previously described.

Phytophthora cinnamomi biomass was calculated based on either plasmid or *Arabidopsis* DNA using Ct values according to Gao et al. (2004) with some modifications as follows:

$$\Delta Ct_{P.\ cinnamomi} = Ct_{P.\ cinnamomi} - Ct_{Plasmid/Arabidopsis}$$

$$\Delta\Delta Ct = \Delta Ct_{P.\ cinnamomi(S1)} - \Delta Ct_{P.\ cinnamomi(S2)}$$

S1 and S2 refer to different amounts of *P. cinnamomi* mixed with *Arabidopsis* leaf samples.

Fold Pc amount $= E^{-\Delta\Delta Ct}$ where E is the efficiency of primer amplification for *P. cinnamomi*

The results of these approaches are presented in Table 2. In both approaches, there was no significant difference between the obtained and the expected ΔΔCt values when the *P. cinnamomi* DNA amount

Table 2 Accuracy of QPCR using plasmid as internal control in *Arabidopsis thaliana–Phytophthora cinnamomi* combination

Plasmid DNA (ng)	*Arabidopsis* leaf (g)	*P. cinnamomi*	Mean ΔCt	SE ΔCt	ΔΔCt	$E^{-\Delta\Delta Ct}$ (Fold Pc Amount)	EXP. Fold	χ^2 Value	*P* Value
Values based on plasmid as an internal standard									
1	0.1, 0.05, 0.025	1,000 ng DNA	2.85	0.24	3.39	9.9	10	0.830	0.362
1	0.1, 0.05, 0.025	100 ng DNA	6.25	0.22					
1	0.05	0.01 g hyphae	3.56	0.02	0.77	1.7	2		
1	0.025	0.005 g hyphae	2.79	0.16					
Values based on *Arabidopsis* DNA as an internal standard									
1	0.1, 0.05, 0.025	1,000 ng DNA	4.97	0.11	3.45	9.94	10	0.771	0.380
1	0.1, 0.05, 0.025	100 ng DNA	8.42	0.22					
1	0.05	0.01 g hyphae	2.06	0.18	1.3	2.41	2		
1	0.025	0.005 g hyphae	0.76	0.09					

ΔCt = (Ct of *P. cinnamomi*) – (Ct of plasmid or *A. thaliana*); the values are the mean of five biological replicates

SE ΔCt is the standard errors of ΔCt

ΔΔCt is the variation between ΔCts of *P. cinnamomi* pairs (either *P. cinnamomi* DNA or *P. cinnamomi* hyphae)

E is the efficiency of *P. cinnamomi* primer amplification (1.95)

was normalized to *Arabidopsis* DNA (χ^2=0.771, *P*=0.380) or to plasmid DNA (χ^2=0.830, *P*=0.362).

The lupin–*P. cinnamomi* combination

To demonstrate the validity of this method in the susceptible lupin–*P. cinnamomi* pathosystem, the above two approaches were repeated using lupin tissue.

In the first approach, *P. cinnamomi* DNA (100 ng or 1,000 ng) was added to tubes containing 0.1 g or 0.05 g of non-inoculated lupin root tissue and 1 ng plasmid DNA added prior to the first centrifugation step of DNA extraction. In the second approach, *P. cinnamomi* hyphae (0.01 g or 0.002 g) were added into tubes containing 0.1 g or 0.05 g of non-inoculated lupin root tissue. The results for analysis of the lupin-*P. cinnamomi* combination are presented in Table 3.

In both approaches, there was no significant difference between the obtained and expected ΔΔCt values when the *P. cinnamomi* DNA amount was normalized to lupin (χ^2=0.845, *P*=0.358) or plasmid DNA (χ^2=0.841, *P*=0.359) showing that in samples with no pathogen infection, normalization to plasmid DNA is a valid method of pathogen biomass quantification.

Measurement of pathogen biomass in infected plants

The results showed that the relative amount of pathogen can be measured according to either plasmid or plant DNA as internal controls in the absence of an infection when pathogen DNA or hyphae is added to the plant DNA extraction. To answer the question of whether degradation of host DNA in infected tissue would lead to an overestimation of pathogen biomass when normalized to plant DNA, we conducted the following experiment.

Arabidopsis thaliana and lupin were grown and inoculated as described. 50 mg of *A. thaliana* leaf (eight discs; 7 mm in diameter) were collected as a biological sample from infected areas of inoculated leaves. Five independent biological samples were assessed for pathogen biomass quantification. For lupin, either 50 mg or 100 mg of infected root tissue was collected in 2 ml tubes and eight independent biological samples collected. DNA extraction was conducted as described previously. Relative pathogen biomass was measured based on both plant and plasmid DNA. The results showed that there was considerable overestimation when pathogen biomass was calculated based on plant DNA for both the *A. thaliana–P. cinnamomi* and lupin–*P. cinnamomi* pathosystems. In all infected samples tested, there were differences between ΔCt values when normalization was based on host DNA compared to that of based on plasmid DNA. The calculated overestimation ($E^{-\Delta\Delta Ct}$) of pathogen biomass for *Arabidopsis* samples ranged from 1.56 to 3.19 fold (Table 4). Overestimation of pathogen biomass for the lupin samples was much greater than that of *Arabidopsis*

Table 3 Accuracy of QPCR using plasmid as internal control in *Lupinus angustifolius–Phytophthora cinnamomi* combination

Plasmid DNA (ng)	Lupin root (g)	*P. cinnamomi*	Mean ΔCt	SE ΔCt	ΔΔCt	$E^{-\Delta\Delta Ct}$ (Fold Pc Amount)	EXP. Fold	χ^2 Value	*P* Value
			Values based on plasmid DNA as an internal control						
1	0.1, 0.05	1,000 ng DNA	1.87	0.11	3.33	9.42	10	0.841	0.359
1	0.1, 0.05	100 ng DNA	5.20	0.07					
1	0.1, 0.05	0.01 g hyphae	−1.89	0.09	2.34	4.82	5		
1	0.1, 0.05	0.002 g hyphae	−4.23	0.16					
			Values based on lupin DNA as an internal control						
1	0.1, 0.05	1,000 ng DNA	0.33	0.16	3.51	10.58	10	0.845	0.358
1	0.1, 0.05	100 ng DNA	3.83	0.19					
1	0.1, 0.05	0.01 g hyphae	−3.41	0.34	2.35	4.85	5		
1	0.1, 0.05	0.002 g hyphae	−5.75	0.26					

ΔCt = (Ct of *P. cinnamomi*) − (Ct of plasmid or lupin); the values are the mean of five biological replicates

SE ΔCt is the standard errors of ΔCt

ΔΔCt is the variation between ΔCts of *P. cinnamomi* pairs (either *P. cinnamomi* DNA or *P. cinnamomi* hyphae)

E is the efficiency of *P. cinnamomi* primer amplification (1.95)

Table 4 Measurement of *Phytophthora cinnamomi* DNA in infected samples collected from the resistant *Arabidopsis thaliana–P. cinnamomi* pathosystem and susceptible lupin– *P. cinnamomi* pathosystem

Sample amount	Total DNA yield (μg)	Values based on plant DNA $\Delta Ct = Ct_{Pc}–Ct_{At/Lupin}$	Values based on plasmid DNA $\Delta Ct = Ct_{Pc}–Ct_{Plasmid}$	ΔΔCt	$E^{-\Delta\Delta Ct}$
	Arabidopsis–P. cinnamomi				
50 mg (8 leaf discs)	17.1	7.12	8.16	−1.05	2.03
50 mg (8 leaf discs)	21.1	7.20	7.87	−0.67	1.56
50 mg (8 leaf discs)	19.6	7.78	8.71	−0.93	1.88
50 mg (8 leaf discs)	10.3	6.96	8.54	−1.58	2.90
50 mg (8 leaf discs)	9.7	6.84	8.55	−1.72	3.19
	lupin–*P. cinnamomi*				
50 mg	6.1	10.21	5.95	−4.26	17.72
50 mg	3.3	10.17	6.34	−3.83	13.32
50 mg	8.0	11.29	7.44	−3.86	13.55
50 mg	8.2	11.59	7.98	−3.61	11.42
100 mg	37.0	12.30	8.95	−3.35	9.59
100 mg	24.7	10.63	7.97	−2.66	6.03
100 mg	20.1	9.27	6.54	−2.73	6.32
100 mg	25.4	6.31	3.35	−2.96	7.40

Ct (threshold cycle) is the number of PCR cycles at which a statistically significant increase in the SYBR fluorescence (greater than background) can be detected

ΔCt is the variation between the mean of five Ct values obtained after amplification of *P. cinnamomi* DNA and the mean of Ct values obtained after amplification of *A. thaliana* or plasmid DNA

ΔΔCt is the difference between ΔCt of *P. cinnamomi* based on *Arabidopsis* DNA and ΔCt of *P. cinnamomi* based on plasmid DNA

E is the efficiency of *P. cinnamomi* primer amplification (1.965)

$E^{-\Delta\Delta Ct}$, is the fold pathogen biomass difference between ΔCt values obtained for *P. cinnamomi* using either *Arabidopsis* or plasmid DNA as internal control

ranging from 6.03 to 17.72 fold (Table 4). This high overestimation range in lupin root occurs since *P. cinnamomi* extensively infested lupin root tissues within 72 h causing considerable tissue necrosis. These results suggest that the calculation of pathogen biomass based on plant DNA can be misleading (usually overestimated) in situations where extensive plant cell death has occurred, such as during infection by necrotrophic pathogens.

Differences between DNA yield during DNA extraction

There were some variations between DNA yields during DNA extraction in both lupin and *Arabidopsis* samples (Table 4). DNA yields for *Arabidopsis* leaves ranged from 9.7 to 21.1 μg when eight discs per sample were used for DNA extraction. In the case of lupin, the DNA yield was from 3.3 to 8.2 μg and 20.1 to 37.0 μg from 0.05 g to 0.1 g of infected root tissue, respectively. These results showed that normalization of pathogen biomass only based on tissue weight or tissue surface area can generate biased results. Adding plasmid as an internal control during DNA extraction makes it possible to normalize not only DNA extraction yield but also relative pathogen biomass.

Relative and total measurement of pathogen biomass over time in susceptible/resistant hosts

Leaves of four-week-old *A. thaliana* ecotypes; Ler and Col-0 were inoculated with *P. cinnamomi* zoospores as described. For each ecotype, five biological samples were collected and snap frozen at 24, 48 and 72 h after inoculation. Each biological sample was a bulk of four randomly chosen infected leaf discs (7 mm in diameter). QPCR was conducted and relative and total pathogen biomass calculated as follows:

Relative pathogen biomass

$$= [\text{pathogen DNA amount in sample (pg)}]/[\text{plasmid DNA amount in sample (pg)}].$$

Total pathogen biomass in initial sample

$$= \text{pathogen DNA in reaction (pg)} \times \text{plasmid dilution factor}$$

Plasmid dilution factor

$$= \text{plasmid amount used in DNA extraction (pg)}/\text{plasmid amount in QPCR reaction (pg)}.$$

There were considerable differences in relative pathogen biomass between ecotype Ler and Col-0 when pathogen DNA was normalized to host DNA (Fig. 1a). Ecotype Col-0 was highly resistant to *P. cinnamomi* and showed no significant differences among relative pathogen DNA over the different time points; whereas ecotype Ler was moderately susceptible to *P. cinnamomi*, showing a significant (P= 0.001) increase in pathogen DNA between 24 and 72 h (Fig. 1a).

There was a significant (P<0.001) difference between the two ecotypes (Ler and Col-0) of *A. thaliana* with regard to total pathogen biomass. In ecotype Ler, the total pathogen biomass reached 3.7 ng pathogen DNA in 72 h compared to 1.1 ng in ecotype Col-0 (Fig. 1b).

Root tips of 4-day-old blue lupin (*L. angustifolius*) seedlings were inoculated using *P. cinnamomi* mycelial plugs. Root samples (0.1 g) were collected at 24, 48, 72 and 92 h after inoculation. QPCR results revealed that lupin was highly susceptible to *P. cinnamomi* showing a large increase in pathogen biomass in the course of 24 h. Relative pathogen biomass reached more than 7 ng within 96 h (Fig. 1c).

In lupin, pathogen growth was much faster than that in *A. thaliana* and total pathogen biomass reached 335 ng by 72 h (Fig. 1d). The results of relative pathogen biomass (normalized based on plasmid) and total pathogen biomass were highly (r=0.98 P< 0.001) correlated. The visual assessments of lesion size (Fig. 1e and f) supported the QPCR results (Fig. 1a and c).

Discussion

Early detection and quantification of pathogen biomass is one of the essential steps for determination of disease resistance. To date, several QPCR assays have been developed to detect different oomycete pathogens *in planta* such as *Phytophthora infestans* and *Phytophthora citricola* (Böhm et al. 1999), *Phytophthora capsici* (Silvar et al. 2005), *P. cinnamomi* (Kong et al. 2003), and other pathogens (Lievens et al. 2006;

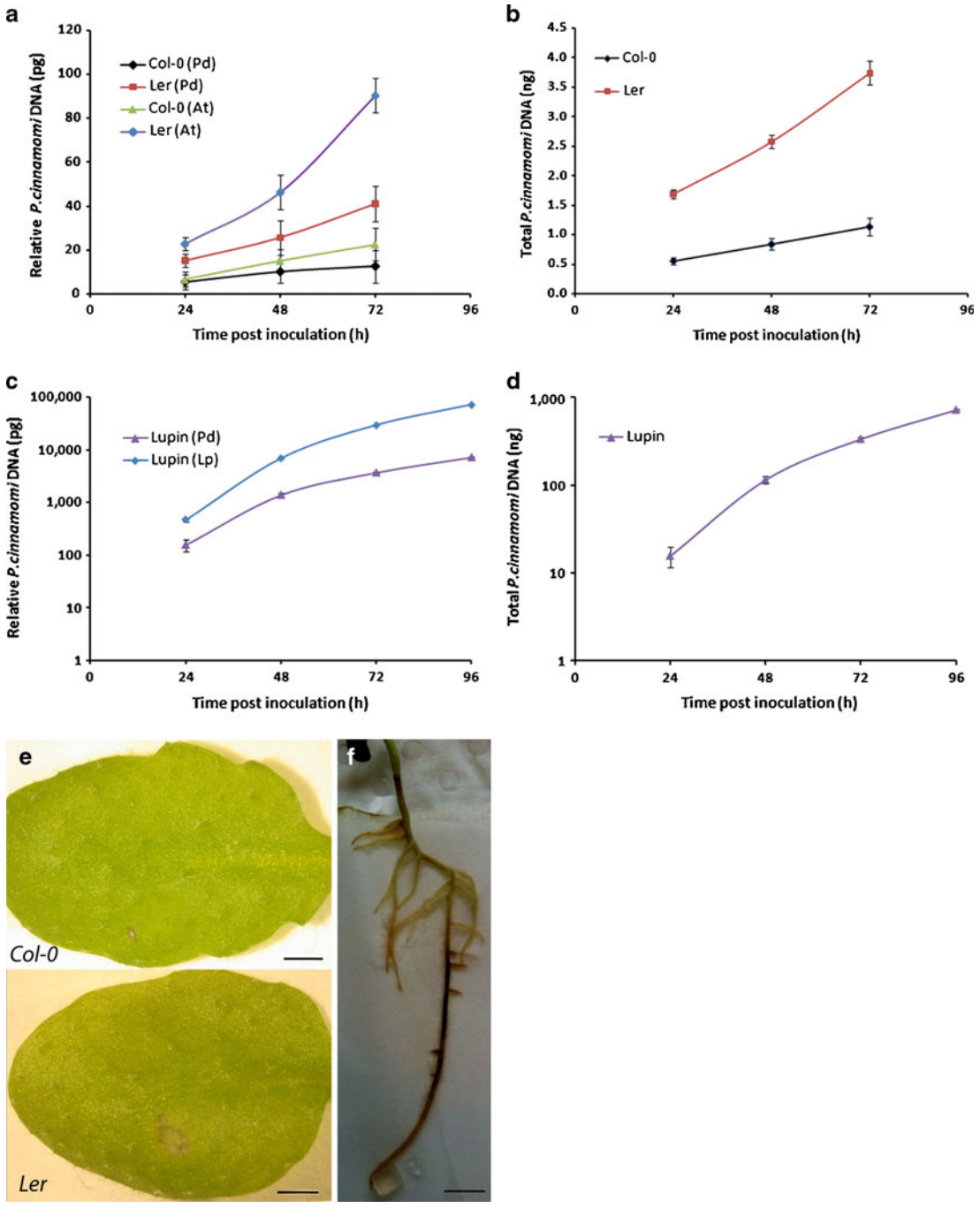

Fig. 1 Relative and total QPCR quantification of *Phytophthora cinnamomi* biomass over time in *Arabidopsis thaliana* and lupin normalized based on plasmid DNA or host plant DNA. (**a**) *P. cinnamomi* biomass (pg DNA) relative to the plasmid DNA (Pd) or *Arabidopsis* DNA (At) in *A. thaliana* ecotypes Ler (moderately susceptible) and Col-0 (resistant) after inoculation with *P. cinnamomi* zoospores. (**b**) The total amount of *P. cinnamomi* biomass (DNA) in the initial infected tissue samples from *A. thaliana* leaves. (**c**) *P. cinnamomi* biomass (pg DNA) in lupin (susceptible) relative to plasmid DNA (Pd) or lupin DNA (Lp). (**d**) The total amount of *P. cinnamomi* biomass (DNA) in the initial infected tissue samples from lupin roots. Data represent the mean of four biological replicates and bars represent the standard error of the mean. Lesion development in *P. cinnamomi* infected tissues in *A. thaliana* ecotypes Ler and Col-0 (**e**) and lupin (**f**). The scale bars for (**e**) and (**f**) are 2 mm and 1 cm, respectively

Brouwer et al. 2003). In these QPCR assays, the pathogen biomass was measured and normalized either to host plant DNA or the sample surface or weight of collected sample. However, measurement of the pathogen biomass relative to the amount of plant DNA may lead to an overestimation of pathogen biomass especially at late stages of infection, where host DNA degradation is often associated with host cell death (Brouwer et al. 2003; van Wees et al. 2003). Furthermore, the validity of pathogen biomass measurements based on sample surface or weight may also be significantly affected by variable DNA extraction yield between the samples (Gachon and Saindrenan 2004).

The use of host plant DNA for normalisation of pathogen biomass was shown to lead to overestimation of growth of *P. cinnamomi*, in both resistant (*A. thaliana)* and susceptible (lupin) interactions. Moreover, the degree of overestimation was shown to be related to the degree of tissue necrosis, as susceptible lupin, with extensive tissue necrosis, showed a higher impact on the accuracy of pathogen DNA quantification than resistant *Arabidopsis* (Fig. 1). The occurrence of overestimation during the quantification of *in planta* levels of necrotrophic pathogens relative to plant DNA has been suggested previously; however, solutions including normalizing pathogen DNA according to tissue weight or surface area were implemented in some pathosystems (Brouwer et al. 2003; van Wees et al. 2003). In our study, DNA yield was shown to vary considerably in samples for which the equivalent amount of starting tissue was used, even when the degree of disease did not differ significantly according to lesion size suggesting the potential for inaccuracy in relative pathogen quantification.

To address these issues as they relate to the *Arabidopsis–P. cinnamomi* pathosystem, a QPCR based assay that enables the measurement of both relative and total amount of *P. cinnamomi* was developed. The method described in this study where 1 ng of plasmid DNA was added during DNA extraction, accounted for the variation in DNA yields and enabled the calculation of the total amount of pathogen DNA present in the initial leaf or root sample. Furthermore, the broad dynamic range of the QPCR technique allowed useful information to be gained from both resistant/moderately susceptible interactions (e.g. *A. thaliana–P. cinnamomi*) where pathogen biomass may remain low throughout the experiment and susceptible interactions (e.g. *L. angustifolius–P. cinnamomi*) where *in planta* pathogen biomass may reach high levels.

The results demonstrating the utility of adding an internal control DNA during sample DNA extraction are in accordance with previous results from several studies investigating human and animal systems (Damen et al. 2008; Beld et al. 2004; Erdner et al. 2010). In a recent study, Diguta et al. (2010) also noted the potential for inaccuracy of pathogen quantification using normalization to host DNA or sample fresh weight and they added intact yeast, *Yarowia lipolitica,* as an internal control prior to DNA extraction for detection of the fungal pathogen *Botrytis cinerea* on grapes. However, variation in the efficiency and reproducibility of a given DNA extraction method for extracting the yeast DNA may still influence the accuracy of pathogen quantification. The use of previously purified and quantified plasmid DNA in the methods described in this study avoids this possible additional variation. Plasmid DNA was chosen as the internal control because a) the small size of the plasmid requires a little amount of plasmid (1 ng) to be added to each sample, and b) a QPCR target sequence within the plasmid can be selected that has no homology to either host or pathogen to ensure the specificity of the primers. The plasmid used in this study contains the mouse ScFvB1 sequence and thus should be useful for a wide range of plant—pathogen systems, having no significant homology in known plant and fungal genomes. We showed that plant and plasmid DNA in the QPCR reaction did not affect amplification of pathogen DNA over a range of different concentration as low as 1 pg and similarly that plasmid DNA amplification was not affected by extracts from *P. cinnamomi*, plant host or necrotic tissue in the QPCR. The use of internal controls in the form of intact bacteria containing plasmid vector or plasmid DNA have been successfully demonstrated in several studies quantifying human or animal diseases/pathogens (Damen et al. 2008; Klerks et al. 2006; Halliday et al. 2010).

In conclusion, we demonstrate the potential for overestimation of pathogen biomass both in resistant interactions, with limited pathogen growth and necrosis, and susceptible interactions, with a high degree of necrosis, when pathogen DNA is normalized to either sample weight or host DNA. The addition of plasmid

DNA into the biological samples during DNA extraction was an efficient way to overcome issues of variation in DNA extraction efficiency and degradation of host DNA. This method described herein should be applicable to a wide range of plant—pathogen systems.

Acknowledgements We thank Murdoch University and Australian Commonwealth Government for providing International Postgraduate Research Scholarship (IPRS) and Murdoch University Research Scholarship (MURS). We thank Invitromics Pty Ltd for contributing to some of the cost associated with this project.

References

Beld, M., Minnaar, R., Weel, J., Sol, C., Damen, M., van der Avoort, H., et al. (2004). Highly sensitive assay for detection of enterovirus in clinical specimens by reverse transcription-PCR with an armored RNA internal control. *Journal of Clinical Microbiology, 42*, 3059–3064.

Böhm, J., Hahn, A., Schubert, R., Bahnweg, G., Adler, N., Nechwatal, J., et al. (1999). Real-time quantitative PCR: DNA determination in isolated spores of the mycorrhizal fungus *Glomus mossae* and monitoring of *Phytophthora infestans* and *Phytophthora citricola* in their respective host plants. *Journal of Phytopathology, 147*, 409–416.

Brasier, C. M. (2008). The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology, 57*, 792–808.

Brouwer, M., Lievens, B., Van Hemelrick, W., Van den Ackerveken, G., Cammue, B. P. A., & Thomma, B. P. H. J. (2003). Quantification of disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR. *FEMS Microbiology Letters, 228*, 241–248.

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

Damen, M., Minnaar, R., Glasius, P., van der Ham, A., Koen, G., Wertheim, P., et al. (2008). Real-time PCR with an internal control for detection of all known human adenovirus serotypes. *Journal of Clinical Microbiology, 46*, 3997–4003.

Diguta, C. F., Rousseaux, S., Weidmann, S., Bretin, N., Vincent, B., Guilloux-Benatier, M., et al. (2010). Development of a qPCR assay for specific quantification of Botrytis cinerea on grapes. *FEMS Microbiology Letters, 131*, 81–87.

Erdner, D. L., Percy, L., Keafer, B., Lewis, J., & Anderson, D. M. (2010). A quantitative real-time PCR assay for the identification and enumeration of Alexandrium cysts in marine sediments. *Deep-Sea Research Part II-Topical Studies in Oceanography, 57*, 279–287.

Erwin, D., & Ribeiro, O. K. (1996). *Phytophthora diseases worldwide*. Minnesota: APS.

Gachon, C., & Saindrenan, P. (2004). Real-time PCR monitoring of fungal development in *Arabidopsis thaliana* infected by *Alternaria brassicicola* and *Botrytis cinerea*. *Plant Physiology and Biochemistry, 42*, 367–371.

Gao, X., Jackson, T. A., Lambert, K. N., Li, S., Hartman, G. L., & Niblack, T. L. (2004). Detection and quantification of *Fusarium solani* f. sp. *glycines* in soybean roots with real-time quantitative polymerase chain reaction. *Plant Disease, 88*, 1372–1380.

Glazebrook, J. (2001). Genes controlling expression of defense responses in *Arabidopsis*. *Current Opinion in Plant Biology, 4*(4), 301–308.

Halliday, E., Griffith, J. F., & Gast, R. J. (2010). Use of an exogenous plasmid standard and quantitative PCR to monitor spatial and temporal distribution of *Enterococcus* spp. in beach sands. *Limnology and Oceanography: Methods, 8*, 146–154.

Hansen, E. M. (2008). Alien forest pathogens: *Phytophthora* species are changing world forests. *Boreal Environment Research, 13*, 33–41.

Hardham, A. R. (2005). Pathogen profile: *Phytophthora cinnamomi*. *Molecular Plant Pathology, 6*, 589–604.

Hardham, A. R. (2007). Cell biology of plant-oomycete interactions. *Cellular Microbiology, 9*, 31–39.

Kamoun, S. (2001). Nonhost resistance to *Phytophthora*: Novel prospects for a classical problem. *Current Opinion in Plant Biology, 4*, 295–300.

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

Klerks, A., van Bruggen, A. H. C., Zijlstra, C., & Donnikov, M. (2006). Comparison of methods of extracting *Salmonella enterica* serovar enteritidis DNA from environmental substrates and quantification of organisms by using a general internal procedural control. *Applied and Environmental Microbiology, 72*, 3879–3886.

Kong, P., Hong, C. X., & Richardson, P. A. (2003). Rapid detection of *Phytophthora cinnamomi* using PCR with primers derived from the Lpv putative storage protein genes. *Plant Pathology, 52*, 681–693.

Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Cammue, B. P. A., & Thomma, B. P. H. J. (2006). Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. *Plant Science, 171*, 155–165.

Manatunga, V., Sanati, H., Tan, P., & O'Brien, P. A. (2005). Maceration of plant tissue by fungi is inhibited by recombinant antipectinase antibodies. *European Journal of Plant Pathology, 112*, 211–220.

McCartney, H. A., Foster, S. J., Fraaije, B., & Ward, E. (2003). Molecular diagnostics for fungal plant pathogens. *Pest Management Science, 59*, 129–142.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum, 15*, 473–497.

Robinson, L. H., & Cahill, D. M. (2003). Ecotypic variation in the response of *Arabidopsis thaliana* to *Phytophthora cinnamomi*. *Australasian Plant Pathology, 32*, 53–64.

Rookes, J. E., Wright, M. L., & Cahill, D. M. (2008). Elucidation of defence responses and signaling pathways induced in *Arabidopsis thaliana* following challenge with

Phytophthora cinnamomi. *Physiological and Molecular Plant Pathology, 72*, 151–161.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual*. New York: Cold Spring Harbor.

Schaad, N. W., & Frederick, R. D. (2002). Real-time PCR and its application for rapid plant disease diagnostics. *Canadian Journal of Plant Pathology, 24*, 250–258.

Schaad, N. W., Frederick, R. D., Shaw, J., Schneider, W. L., Hickson, R., Petrillo, M. D., et al. (2003). Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. *Annual Review of Phytopathology, 41*, 305–324.

Shearer, B. L., & Fairman, R. G. (2007). A stem injection of phosphite protects *Banksia* species and *Eucalyptus marginata* from *Phytophthora cinnamomi* for at least 4 years. *Australasian Plant Pathology, 36*, 78–86.

Silvar, C., Díaz, J., & Merino, F. (2005). Real-time polymerase chain reaction quantification of *Phytophthora capsici* in different pepper genotypes. *Phytopathology, 95*, 1423–1429.

Takemoto, D., Jones, D. A., & Hardham, A. R. (2003). GFP-tagging of cell components reveals the dynamics of subcellular re-organization in response to infection of *Arabidopsis* by oomycete pathogens. *The Plant Journal, 33*, 775–792.

Thomma, B. P. H. J., Penninckx, I. A. M. A., Broekaert, W. F., & Cammue, B. P. A. (2001). The complexity of disease signaling in *Arabidopsis*. *Current Opinion in Immunology, 13*, 63–68.

van Wees, S. C. M., Chang, H. S., Zhu, T., & Glazebrook, J. (2003). Characterization of the early response of *Arabidopsis* to *Alternaria brassicicola* infection using expression profiling. *Plant Physiology, 132*, 606–617.