SUITABILITY OF PHENYLALANINE AMMONIA LYASE AND CHITINASE ACTIVITIES AS BIOCHEMICAL MARKERS OF SOFT ROT RESISTANCE IN *ACTINIDIA CHINENSIS* KIWIFRUIT

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ABSTRACT

Cryptosporiopsis actinidiae causes soft rot of the kiwifruit cultivar Hort16A (*Actinidia chinensis*). This paper describes measurement of phenylalanine ammonia lyase (PAL) and chitinase, with a view to identifying possible resistance markers to characterise the host/pathogen interaction. Four *A. chinensis* genotypes were treated pre-harvest with water or *C. actinidiae* spores. Fruit was stored at 1.5°C for 18 weeks to allow for disease development. Enzyme activities were measured at harvest and after storage in the area around disease lesions and in symptomless tissue. Soft rot incidence was highest in genotype 8A and equal in the other genotypes. Pre-harvest inoculation had no effect on subsequent soft rot incidence. Chitinase activities varied with genotype, and increased significantly during storage, but there was no difference between healthy versus diseased tissue. PAL expression varied with genotype, increased during storage, and was always significantly higher in the tissue around lesions. Neither enzyme measurement was a suitable resistance marker.
Keywords: *Cryptosporiopsis*, chitinase, Hort16A, phenylalanine ammonia lyase (PAL), resistance, soft rots.

INTRODUCTION

ZESPRI International Limited, together with the breeders at HortResearch, New Zealand, are constantly looking to maintain New Zealand's competitive edge in the global market by the introduction of novel kiwifruit. When developing future commercial *Actinidia* varieties, genotypes must have a level of susceptibility to pests and diseases that does not exceed that of current commercial varieties. Ideally, new varieties should also be capable of being produced organically. Past research indicates that there is a significant amount of variation in susceptibility between *Actinidia* species to both pests and diseases (Reglinski et al. 2001; Wurms 2004; Hill et al. 2007; McKenna et al. 2006). Simple tools capable of measuring the relative or absolute levels of resistance of *Actinidia* germplasm samples to a specific pest or disease are required, to enable the requisite level of resistance to be built into future breeding and progeny screening programmes.

Cryptosporiopsis actinidiae is one of the major causal agents of soft rots, which are the main postharvest disease problem of Hort16A fruit (Manning et al. 2003). Infection occurs predominantly during flowering, with the pathogen residing in the fruit surface in a latent state until 12+ weeks of cold storage, when soft rot disease symptoms start to develop (Rheinländer et al. 2005). Soft rots are characterised by small sunken circular pits with defined margins, found anywhere on the fruit surface (Manning et al. 2003).

Two enzymes, chitinase and phenylalanine ammonia lyase (PAL), have been correlated with resistance of commercial kiwifruit varieties to *Botrytis cinerea* (Wurms 2005) and *Sclerotinia sclerotiorum* (Reglinski et al. 2001). Assays for these enzymes are reliable and affordable, so the aim of this study was to determine if they could be

used as selection tools to screen for resistance of *Actinidia chinensis* cv. Hort16A to *Cryptosporiopsis actinidiae*, since virtually nothing is known about resistance in this host/pathogen interaction.

METHODS

The four genotypes studied in this trial were Hort16A and three other mature *A. chinensis* genotypes (4C, 8A and 10B). These were selected on the basis of a similar flowering time to Hort16A, so that the stage of physiological development would be similar in each genotype at the time of inoculation. Genotypes 4C, 8A and 10B were produced by crosses of three different female *A. chinensis* parents, one of which was Hort16A, with 3 different male *A. chinensis* parents. Based on previous studies that determined the optimal inoculation time (Rheinländer et al. 2005; Fullerton et al. 2007), flowers/fruitlets of the four *A. chinensis* genotypes were spray-inoculated with a freshly prepared spore suspension (1.3–4.0 x 10^6 spores/ml of 0.01% Triton®) of *C. actinidiae,* and controls with 0.01% Triton on four occasions between blossom time (late October) and the green fruitlet stage (late January). Spore suspensions were prepared by flooding 2-week-old cultures of *C. actinidiae* on potato dextrose agar (PDA) with 0.01% Triton and adjusting final spore counts using a haemocytometer. There were 4 replicate vines, with alternate canes on the vine designated as inoculated or control treatments.

Inoculated and control fruit for biochemical analyses were sampled at harvest (early May) and after 18 weeks at 1.5°C in air. Soft rot incidence was recorded after 18 weeks of cold storage by counting the number of fruit in each tray with sunken pits and expressing this as a percentage of fruit infected per tray. There were four trays for each treatment at each sampling time.

Crude extracts for assessment of chitinase activity were prepared by homogenising fruit skin tissue in 0.1 M sodium acetate buffer (pH 5.6) at a 1:3 weight:volume ratio (Wurms et al. 1997). Each extract was produced from 3-5 pooled fruits per tray, with four trays per treatment. Only symptomless tissue was sampled to minimise the extraction of fungal enzymes (Wurms et al. 1997). At harvest there were no disease symptoms, so all extracts came from healthy tissue. After storage, tissue for the "diseased" extracts, was sampled from the zone of symptomless tissue immediately surrounding sunken pits (the most definitive symptom of infection by *C. actinidiae*), whilst "healthy" tissue was sampled either from uninfected fruits in the same tray, or in the case where all fruit within a tray were infected, from areas on the fruit that were clear of pits. Sampling healthy tissue after storage was difficult in genotype 4C since fruit tissue had softened to the point of collapse, most probably because of chilling injury, and was extensively colonised by opportunistic secondary pathogens. Chitinase activity was measured as the release of radio-labelled N-acetyl glucosamine fragments from tritiated chitin using the radioassay of Molano et al. (1977), with modifications as described by Wurms et al. (1997), except that 30-60 µl of test extract and 35 µl of tritiated chitin substrate were used. Radioactivity was measured in 3 ml of BCS scintillant (Amersham Corporation, Arlington Heights, IL, United States) on a 1218 Rackbeta scintillation counter (LKB Wallac, Wallac Oy, Finland).

Fruit skin samples for measurement of phenylalanine ammonia-lyase (PAL) were extracted for 2 h in 0.5 M potassium phosphate buffer (pH 7.5) containing 0.2 M ascorbic acid, 1.5% polyethylene glycol, 50 mM cysteine, 5 mM EDTA and 0.4 M sucrose. Immediately before measurement of enzyme activity, crude extracts were mixed with 20 mg of polyclar AT for 15 min, then cleared by centrifugation and passed through a protein desalting spin column (Pierce Biotechnology, Inc. USA). PAL activity was measured using L-[U-^{14}C] phenylalanine (Amersham) as described by Bernards & Ellis (1989).

The experiment had a nested design with "disease status" nested within "inoculation treatment" and "inoculation treatment" nested within "*A. chinensis* selection". There were four trays, each containing ca 25 fruit per treatment. Rot incidence, chitinase and PAL enzyme activities were all analysed separately, but the nested design was applied

in each case. Analysis of variance (ANOVA) and standard error of the mean (SEM) calculations were performed using SAS software, version 9.1 (SAS Institute, Cary, North Carolina, United States), with data transformation ($\log_e$ or a square root transformation) when necessary. However, ANOVA statistics applied only to the main treatment effects (cultivar type), since comparisons of the interactions between disease status, inoculum treatments and *A. chinensis* selection on rot incidence, chitinase and PAL enzymes were not possible, because of the hierarchical nature of the nested design.

RESULTS

Overall, the incidence of soft rots was significantly greater in genotype 8A (P=0.0008) than in all other *A. chinensis* selections. However, it was difficult to distinguish soft rots from other secondary rots in genotype 4C, because chilling injury, and subsequent tissue breakdown and colonisation by other microbes was so extensive. Figure 1 shows that regardless of *A. chinensis* genotype, there were no major differences in percentage rots between control and inoculated fruits.

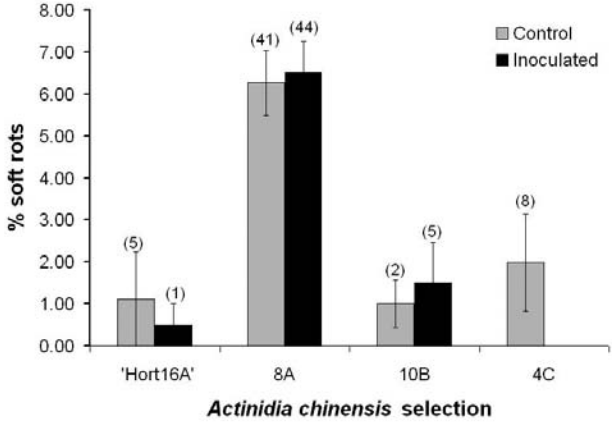

FIGURE 1: Soft rot (caused by *Crytosporiopsis actinidae*) incidence in four *Actinidia chinensis* selections, Hort16A, 8A, 10B and 4C. Disease incidence, expressed as percentage of soft rots in trays of fruit that had been inoculated pre-harvest with *Cryptosporiopsis actinidiae* or treated with water (control fruit), was assessed after 18 weeks of storage at 1.5°C. Data are presented after square root transformation, with error bars indicating SEM, and untransformed means in parentheses.

Chitinase activity differed significantly between the *A. chinensis* selections (P=0.005), with greatest activity in genotype 10B and Hort16A, followed by genotype 4C, and markedly less activity in genotype 8A. Figure 2 illustrates that chitinase activity increased markedly during cold storage relative to harvest in all selections, but that there were no significant or consistent differences between healthy (non-infected) and diseased (infected) tissue and no effect of the inoculation treatment.

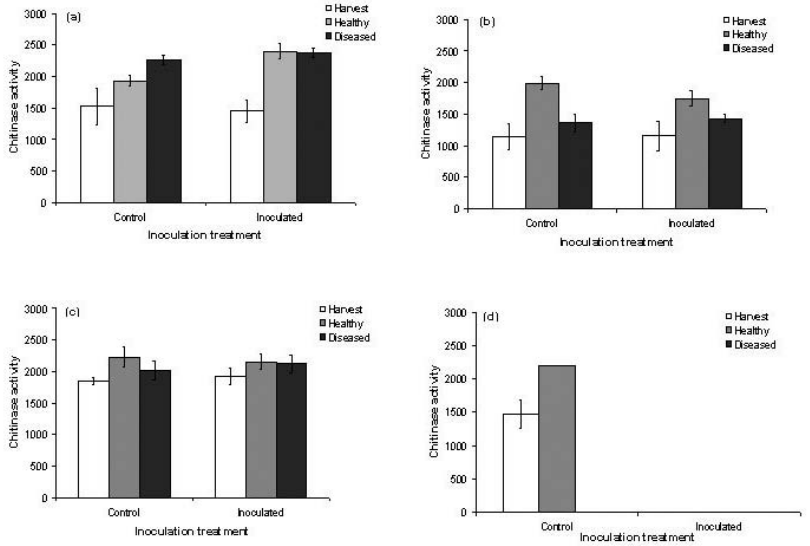

FIGURE 2: Chitinase activity in four *Actinidia chinensis* selections, (a) Hort16A, (b) genotype 8A, (c) genotype 10B and (d) genotype 4C, as measured at harvest time, and after 18 weeks of storage at 1.5°C in healthy and diseased kiwifruit fruit tissue that had been inoculated pre-harvest with *Cryptosporiopsis actinidiae* or treated with water (control fruit). Chitinase activity is expressed as ng of N-acetyl glucosamine product produced/min/g fresh weight of kiwifruit flesh, with error bars indicating SEM. Some data points are missing from genotype 4C because fruit was too degraded to measure chitinase activity.

Actinidia chinensis genotype had a significant effect (P=0.0123) on PAL activity, with activity in genotype 4C and Hort16A being greater than that in genotypes 8A and 10B. In all selections, PAL activity increased significantly with storage and in infected versus non-infected fruit (Fig. 3). Inoculation treatment did not appear to affect PAL activity (Fig. 3).

DISCUSSION

Inoculating fruit pre-harvest with *C. actinidiae* did not markedly increase subsequent disease incidence compared with that in water-sprayed controls. This indicates that natural populations of this pathogen within *A. chinensis* are reasonably well established. Given its natural prevalence and the fact that *C. actinidiae* is one of the most important pathogens of Hort16A (Manning et al. 2003), coupled with observations in the current paper that levels of resistance to this pathogen vary in different *A. chinensis* genotypes, there is a need to include tests of *C. actinidiae* resistance into future *A. chinensis* breeding experiments.

Disease assessments of the 4C genotype were extremely difficult, because the tissue had completely collapsed and had become colonised by numerous opportunistic microbes. This also affected enzyme measurements, because it was almost impossible to distinguish soft rots from other rots and to sample any healthy tissue surrounding *C. actinidiae* lesions. The inability to store genotype 4C precludes it from further commercial development.

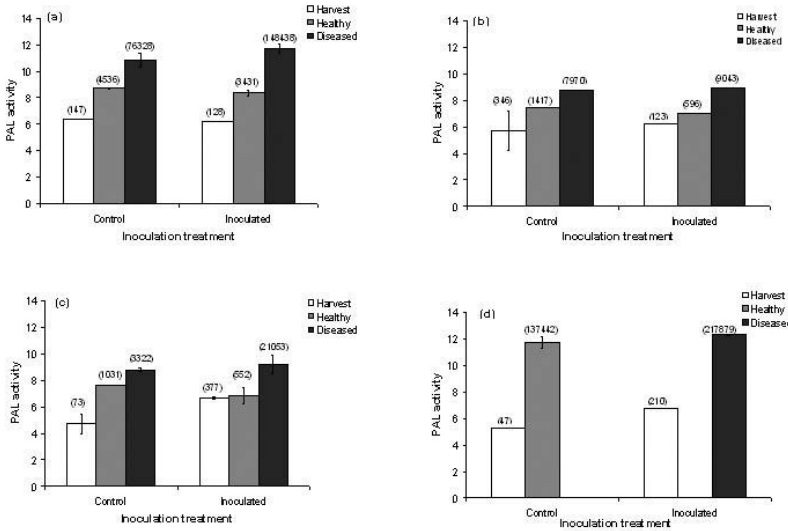

FIGURE 3: Phenylalanine ammonia lyase (PAL) activity in four *Actinidia chinensis* selections, (a) Hort16A, (b) 8A, (c) 10B and (d) 4C, as measured at harvest time, and after 18 weeks of storage at 1.5°C in healthy and diseased kiwifruit fruit tissue that had been inoculated pre-harvest with *Cryptosporiopsis actinidiae* or treated with water (control fruit). PAL activity is expressed as counts per minute (CPM) of ^{14}C-labelled cinnamic acid product produced/g dry weight of kiwifruit flesh, with error bars indicating SEM. Some data points are missing from genotype 4C because fruit was too degraded to measure PAL activity. Data are presented after log$_e$ transformation, with error bars indicating SEM, and untransformed means in parentheses.

Although results are very preliminary, with only 4 *A. chinensis* genotypes being examined, this study suggests a continuum of levels of soft rot resistance, which is a characteristic of quantitative resistance (Young 1996), rather than a discrete absence/presence of rots. Quantitative resistance in kiwifruit has also been observed for other important pests (McKenna et al. 2006; Hill et al. 2007) and diseases (Reglinski et al. 2001; Wurms 2004). Use of general defence genes as predictors of host resistance is a valuable and often-used tool in developing breeding lines with durable quantitative resistance, where inheritance of traits cannot be linked to simple Mendelian ratios (Young 1996). Chitinase and PAL have been successfully used as markers of host resistance in crops as diverse as apple, rice, potato, bean and tobacco (Geffroy et al. 2000; Wulff et al. 2003; Liu et al. 2004; Abo-Elyousr et al. 2006; Ott et al. 2006). Although expression of chitinase activity has been correlated with *A. deliciosa* cv. Hayward kiwifruit resistance to *Botrytis cinerea* (Wurms 2005), chitinase activity does not appear to be a marker for resistance of *A. chinensis* against *C. actinidiae* infection. This conclusion is based on the observations that elevated chitinase activity during storage correlated with decreased resistance, and that the presence of infection did not stimulate chitinase activity. Genotype 8A had the least chitinase activity and the most soft rots, and there was no link between the level of chitinase expression and susceptibility to *C. actinidiae* in the other *A. chinensis* selections.

PAL activity is also unsuitable as a resistance marker for this host/pathogen interaction. PAL activity increased significantly with cold storage whilst fruit became more susceptible, and there was no cause/effect relationship between PAL activity of the various selections and incidence of rots caused by *C. actinidiae*. There was no difference between inoculated and control treatments in terms of PAL activity, but this is not surprising as disease incidences were also similar in both treatments. However, *C. actinidiae* infection did stimulate significant PAL activity. One explanation for this might be that the PAL assay is measuring pathogen as well as kiwifruit PAL activity in infected tissue. It is known that the chitinase assay can distinguish plant and fungal chitinases provided that sampling is restricted to apparently healthy tissue surrounding disease lesions (Wurms et al. 1997), but it is not known whether this is the case for the PAL assay. Consequently, PAL induction by the pathogen does not constitute strong proof of its role in resistance, and would require verification by molecular assays.

In conclusion, a simple bioassay of resistance has not yet been found, but given the prevalence of this pathogen and the problem it poses the industry (Manning et al. 2003), further studies are merited.

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