946

BUREAU OF SUGAR EXPERIMENT STATIONS

QUEENSLAND, AUSTRALIA

FINAL REPORT - SRDC PROJECT BS79S

Identification of resistance mechanisms in sugarcane to infection by *Pachymetra*

by

T K McGhie, D Maclean, G R Smith and B J Croft

SD99002

Contact:

Mr B J Croft
BSES
Lot 207 Old Cove Road
Woodford Qld 4514
Phone: 07 5496 3357

This project was funded by the Sugar Research and Development Corporation during the 1992/93 to the 1995/96 financial years.

BSES Publication
SRDC Final Report SD99002

January 1999

TABLE OF CONTENTS

SUMMARY

In the northern sugarcane growing regions of Australia, the oomycete fungus *Pachymetra chaunorhiza*, Croft & Dick, is the causal organism of the sugarcane disease, Pachymetra root rot. At present, the only means of control is through the use of resistant sugarcane cultivars in areas where disease is present. The primary objective of this project was to provide a basis for developing improved and more cost effective management strategies for Pachymetra root rot by obtaining a basic understanding of the mechanisms that defend sugarcane against infection by *P. chaunorhiza*.

Analysis of genetic variability among 14 isolates of *P. chaunorhiza* collected from seven geographically diverse growing regions of Queensland, revealed that the population is near-monomorphic, and two closely related but distinct genetic groups (A and B) are present. Assessment of pathogenicity indicated that isolates of group A appear to be more aggressive than group B on a moderately susceptible sugarcane cultivar. A more extensive investigation to confirm these results is needed, but the existence of two strains of *P. chaunorhiza* should be considered during the assessment of Pachymetra root rot resistance ratings of commercial sugarcane cultivars.

There have been no prior studies of sugarcane defence mechanisms against Pachymetra root rot, and methods for controlled and synchronous infection of sugarcane were not available. Therefore, a technique was developed to study plant responses to challenge by using suspension-cultured sugarcane cells treated with an elicitor preparation derived from mycelia of *P. chaunorhiza*. After addition of the elicitor, differences were found in the cellular responses of a resistance cultivar and a susceptible cultivar for peroxidase, phenylalanine ammonia-lyase (PAL) activities and in the accumulation of soluble compounds.

To identify defence mechanisms consistently contributing to resistance, a selection of 10 sugarcane cultivars, ranging from highly resistant through to highly susceptible, were studied using suspension-cultured cells challenged by the *P. chaunorhiza* elicitor preparation and asynchronously-infected roots of the same 10 cultivars. Peroxidase activities were not detected in uninfected (control) roots, but induced to high levels in roots of cultivars with high to moderate resistance ratings. In contrast, PAL activities were high in control roots and showed elevated levels of activity in some susceptible cultivars. Activities of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a ubiquitous 'housekeeping' enzyme of intermediary metabolish, were used to quantify the extent of cellular damage in infected roots, and suggested that levels of PAL were elevated in the surviving cultivars. Control and infected roots of the 10 sugarcane cultivars were also assayed for UV-absorbing compounds and other methanol-soluble compounds detected by HPLC and GC, and covalently-bound phenolics in the cell wall were determined.

A number of factors, often associated with pathogen defence in other plants, showed potentially useful correlations with the Pachymetra root rot resistance ratings of the 10 test cultivars. In infected sugarcane roots these included peroxidase activity, cell wall bound phenolics, and the accumulation of the soluble phenolic compound *p*-hydroxybenzaldehyde. Other factors were also correlated with resistance ratings but

were considered to be a consequence of tissue damage in roots caused by *P. chaunorhiza*, rather than a contributor to resistance. These included G3PDH activity, and the citric acid cycle intermediates, aconitic and malic acids; which are required for normal metabolic processes in cells, and were considered to be indicators of cellular or root health.

In liquid culture, *P. chaunorhiza* growth was highest when an insoluble sugarcane cell wall preparation was added to a basal starvation medium preparation compared with adding purified carbohydrates (sucrose, pectin, xylose, carboxymethylcellulose), indicating that *P. chaunorhiza* produces cell-wall degrading enzymes in axenic culture under these conditions. Analysis of the culture media for hydrolase activities showed that appreciable levels of xylosidase activity was present when sugarcane cell walls were used as the carbon source. Analysis of the plant cell wall composition showed that xylose is the main component and that the amount of xylose, glucose and the glucose/xylose ratio in the cell walls of sugarcane cultivars were all correlated with resistance rating. Relatively low concentrations of xylose was found to be toxic to *P. chaunorhiza* in axenic culture suggesting that a high xylose content in the cell wall may contribute to resistance against *P. chaunorhiza* in sugarcane. These data are consistent with the hypothesis that resistant cultivars of sugarcane release more free xylose and less glucose at the site of challenge, which restricts fungal growth sufficiently to allow plant defence responses to be activated and reach effective levels compared with susceptible cultivars.

The main outcome of this investigation was to identify a number of potential biochemical markers for resistance. Several factors (eg G3PDH activity and organic acids) are probably related to general root health, and may be of value in developing simpler systems for assessing the extent of root damage caused by *P. chaunorhiza* when screening for resistant sugarcane clones, and in the general diagnosis of stress in sugarcane root systems. However, other factors identified by the investigation may be directly related to resistance to Pachymetra root rot (eg induced peroxidase activity, levels of soluble and cell-wall bound phenolics in infected roots, and the carbohydrate composition of sugarcane cell walls in uninfected roots), and may be useful for manipulating resistance potential in sugarcane breeding programs. Stepwise multiple regression analysis showed that the most important defence factors for predicting resistance ratings were *p*-hydroxybenzaldehyde concentrations in roots and % xylose composition of the cell wall. This project has provided background information which should assist future research on sugarcane defence against pathogens, and assist, in the development of new options for the control of Pachymetra root rot.

The project achieved all of its objectives.

Further research to confirm the relationship between the biochemical markers and resistance to *Pachymetra* will be required as well as research to develop rapid analytical techniques for the markers, before this research can be applied in the BSES breeding program. The information on genetic diversity of *Pachymetra* has already been incorporated in the routine *Pachymetra* resistance screening trials and a mix of the two genetic groups are included in each trial.

1.0 INTRODUCTION

A number of disease syndromes affecting sugarcane root systems have been recognised in Queensland. Yield Decline is the most widespread, and is defined as the progressive decline in yield of sugarcane from land under continuous production. The cause of Yield Decline is currently not known, but possible factors include pathogens and a deterioration of optimum soil structure. Poor Root Syndrome (PRS) is mainly associated with the northern areas of Queensland and is evidenced by poor root systems.

Similar to Yield Decline, all the causes of PRS are not known, however a root rot caused by the oomycete pathogen, *Pachymetra chaunorhiza*, known as Pachymetra root rot, is recognised as one component. Species of *Pythium* are also suspected components of PRs (Egan *et al.* 1984).

The causal agent of Pachymetra root rot, *Pachymetra chaunorhiza,* an oomycete fungus, was isolated (Croft and Magarey 1984; Croft 1989) and finally described by Dick *et al.* (1989). *P. chaunorhiza* produces a flaccid root rot in the primary roots of sugarcane weakening the root system, which results in decreased growth and plant death by uprooting during harvesting or lodging during storms. *P. chaunorhiza* is easily identified in roots and in soil by the presence of large oospores (about 60 μm) with a highly distinctive ornamentation. At present *P. chaunorhiza* is the only species in the genus *Pachymetra.* Other natural hosts are suspected, but have not been identified (Croft personal communication). *P. chaunorhiza* oospore numbers build up rapidly when susceptible sugarcane cultivars are grown in infested soil (Magarey and Mewing 1994) and remain viable in the soil for at least three years. Pachymetra root rot causes production losses in the order of 30% in susceptible cultivars, resistant cultivars are much less affected (Magarey 1994). Oospore numbers do not build up as quickly or to the same extent in soil under resistant cultivars as susceptible cultivars (Magarey and Mewing, 1994). In the 1970s, Pachymetra root rot caused losses estimated to be in excess of $50 million. Currently, *P. chaunorhiza* is present in 80-90% of sugarcane fields in northern Queensland (Croft personal communication).

P. chaunorhiza has been the subject of an intense research effort because of the threat it poses to the sugar industry (Magarey *et al.* 1987; Croft and Magarey 1989; Magarey 1991). This research has resulted in effective disease management and reduction of production losses through the deployment of resistant cultivars. However, despite this intensive research effort, resistant sugarcane cultivars remain the only effective method of control and containment (Croft 1989). A rating procedure for resistance to Pachymetra root rot has been developed by Croft (1989). Resistance is rated by determining the percentage of rotted roots when sugarcane plants are grown in soil infested with *P. chaunorhiza.* Ratings range from 1 (resistant) to 9 (susceptible). Infected roots of a resistant sugarcane cultivar often have a bright red colour, whereas for susceptible cultivars infected roots have a flaccid, water-soaked appearance.

Although a wide variation of resistance to *P. chaunorhiza* is known in sugarcane germplasm, the mechanism of the resistance had not been studied prior to the research presented in this report. The genetics of the resistance has been examined by the analysis of progeny from selected crosses, and found to be heritable (B Croft unpublished data). Even though the resistance trait is heritable, it is not possible to attribute resistance to a single gene because of the highly aneuploid nature of sugarcane. It is believed that resistance is most likely a multigenic train (B Croft personal communication).

Although, from a disease control perspective, the use of resistant cultivars is effective, a substantial indirect cost is involved and has to be compared with the cost of implementing management strategies for direct control of diseases. The indirect costs of breeding disease resistance cultivars arise from:

- diverting breeding effort away from the primary objective of generating cultivars with higher levels of sugar production;
- restrictions placed on the selection of parents for generating crosses;
- the cost involved in rating cultivars for a large number of diseases;
- the use of less productive cultivars, albeit with higher disease resistance.

Systems or methods which lessen the impact of selecting for disease resistance will be of substantial benefit to the sugar industry.

Many disease control strategies rely on the use of resistant cultivars as the only or best option and therefore the maintenance of resistance to contain the threat of future disease outbreaks is of paramount importance. The composition of some pathogen populations can rapidly alter to overcome plant resistance with the result that the maintenance of plant resistance frequently requires extensive inputs from breeding programs (Brown 1994).

The biochemical defence factors of plants ultimately result in incompatibility (resistance) between a plant and a pathogen, and provides protection from disease. A detailed understanding of plant resistance mechanisms requires identification of the defence factor(s) employed by the plant and the degree of effectiveness. Knowledge of these defence factors has the potential to provide a suite of biochemical markers with possible application for use in screening for disease resistance in breeding programs.

The overall aim of this research program was to provide basic biochemical information to assist the Australian sugar industry in controlling Pachymetra root rot disease. Biochemical factors in sugarcane conferring resistance to Pachymetra root rot, or that correlate with resistance, were sought. Few previous studies have attempted to define the general defence mechanisms used by sugarcane to resist pathogens, and there have been no previous studies on the interaction between sugarcane and *P. chaunorhiza* at a biochemical level. Therefore, the approach adopted was to use resistant and susceptible sugarcane cultivars to survey candidate defence systems and factors, known from other

plant-pathogen systems for correlation with resistance. When appropriate, attempts were made to determine the extent of the relationship between the factor and resistance to Pachymetra root rot.

Detailed experimental methods and results can be found in the Doctorate Thesis by T K McGhie entitled 'A survey of pre-formed and induced defence mechanisms associated with resistance of sugarcane to the fungal root pathogen *Pachymetra chaunorhiza*' which was produced from the result of research conducted in this project.

2.0 PROJECT OBJECTIVES

- Develop techniques for studying the biochemical reactions of sugarcane to infection by *Pachymetra*.
- Identify biochemical mechanisms of infection by *Pachymetra*.
- Identify biochemical changes produced in sugarcane by infection with *Pachymetra*.
- Compare the effect of chemical constituents of different sugarcane varieties on *Pachymetra* oospore germination and hyphal growth.
- Determine which resistance mechanisms and responses of sugarcane are present in resistant varieties, ranked by glasshouse screening, as an aid to future breeding programs.

3.0 RESULTS AND DISCUSSIONS

3.1 Genetic diversity of *Pachymetra chaunorhiza* isolates from Queensland sugarcane fields

An analysis of genetic variability among 14 isolates of *P. chaunorhiza* collected from seven geographically diverse sugarcane growing regions of Queensland, using RAPD genetic markers has identified two background genotypes termed groups A and B. The groups were distinguished by five polymorphic RAPD bands from a total of 75 scorable bands (Figure 3.1.1). All isolates belonging to each group were apparently genetically homogeneous. Group A was found exclusively in the northern regions, whereas group B was found in both northern and southern regions (Figure 3.1.2). This near-monomorphy of *P. chaunorhiza* in Australia as assessed by RAPD markers, is consistent with a bottleneck in the introduction of this pathogen to sugarcane in Australia. Introduction could have been by either a selective movement of *P. chaunorhiza* strains from a native Australian plant (presently unknown), or selective introduction from an overseas centre of origin (presently unknown). Additionally, preliminary assessment of pathogenicity on a single test sugarcane cultivar (Q124) with medium to low levels of resistance to *P. chaunorhiza* indicated that the isolates of group A appear to be more aggressive quantitatively than group B (Table 3.1.1). Thus, the genetic difference detected by RAPD analysis may have some relationship with

pathogenicity, and further research to verify and characterise such differences are necessary to reassess current control strategies based on the deployment of resistance cultivars. A more extensive investigation of pathogenicity to determine if race differences exist among members of the *P. chaunorhiza* population using a set of sugarcane cane cultivars with a range of resistance ratings is required.

Table 3.1.1: Pathogenecity assessment for a subset of *P. chaunorhiza* isolates determined using the standard BSES method for assessing resistance in sugarcane cultivars

Isolate	Location	Pathogenicity*	Group Designation**
T123-8	Ingham	48	A
T123-3	Babinda	48	A
T123-4	Mourilyan	47	A
T123-10	Bundaberg	40	B
T123-5	Tully	36	B
T123-9	Burdekin	33	B
T123-11	Bundaberg	25	B
T123-14	Mackay	22	B

* Percent rotted roots of infected sugarcane cultivar Q124.
** Group designation determined by the presence/absence of specific PCR amplification bands (RAPDs).

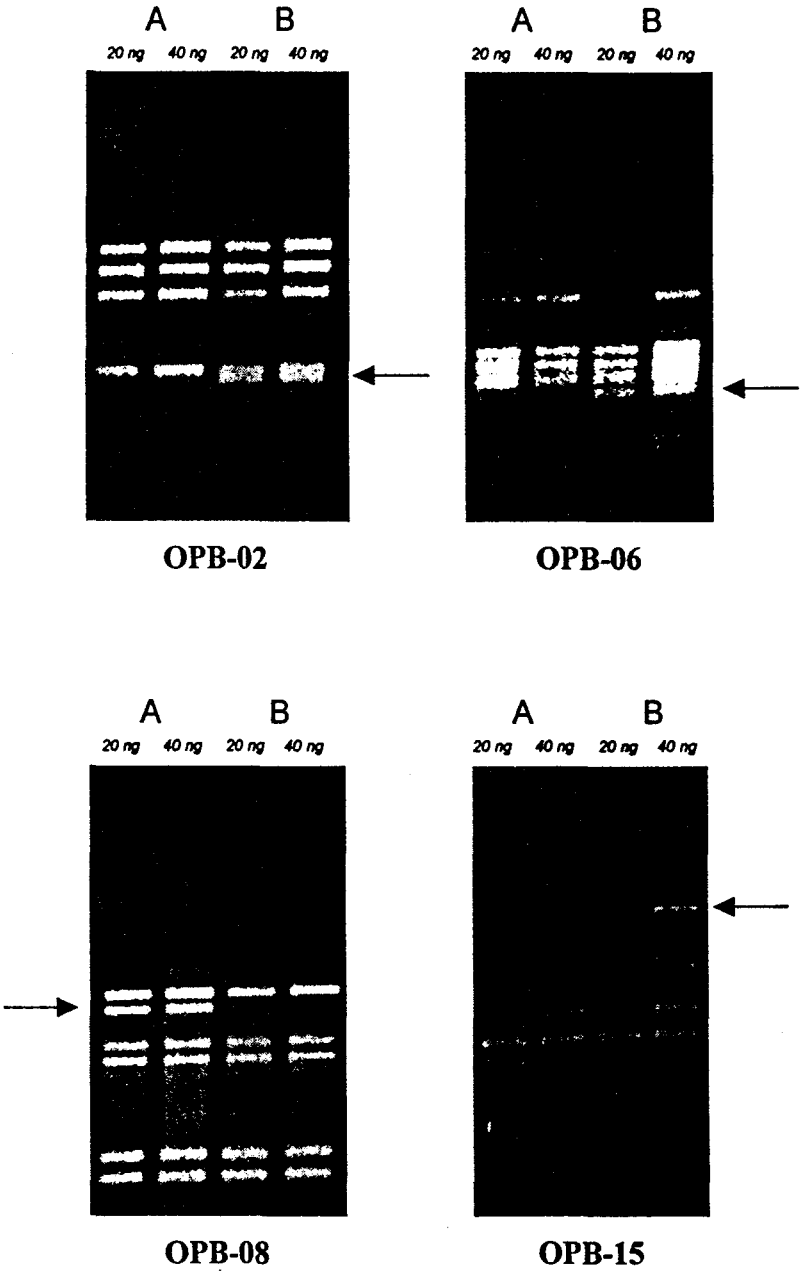

Figure 3.1.1 Agarose gels showing examples of the RAPD banding patterns produced by two isolates of *P. chaunorhiza* with four PCR primers. One isolate represents group A (T124-1A), the other represents group B (T124-2A). The arrows indicate the polymorphic PCR bands which distinguish groups.

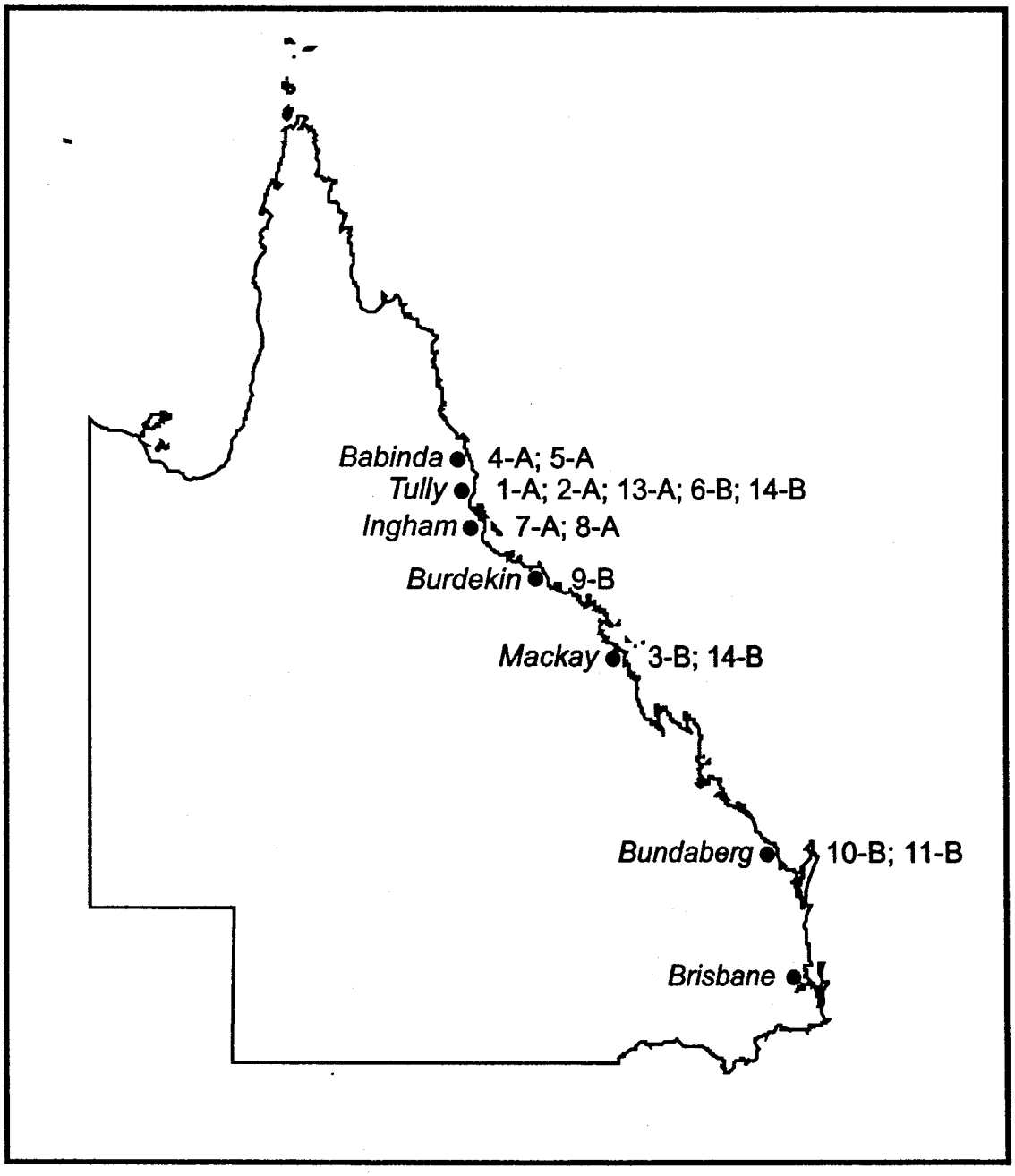

Figure 3.1.2 Map of Queensland showing the geographic origin of the *P. chaunorhiza* isolates with the isolate number and group designation (A or B) as determined by RAPD analysis.

3.2 Development of biological systems to study the interaction between sugarcane and *Pachymetra chaunorhiza*

A major goal of this research was to investigate the time-course of activation of defence systems in sugarcane roots after challenge by *P. chaunorhiza*. Although pathogen infection of plant material is the technique of choice, several attempts to develop an experimental system for the synchronous and reproducible infection of sugarcane roots by *P. chaunorhiza* were unsuccessful. As an alternative, an *in vitro* system was developed using an elicitor preparation from *P. chaunorhiza* cell walls to challenge suspension-culture sugarcane cells. An active elicitor preparation was obtained by autoclaving a cell wall fraction of mycelia of *P. chaunorhiza* to release soluble products. Fractionation of the crude elicitor preparation showed that the active component was approximately 50 kDa in size and represented only a small proportion of the total material present in the preparation. Sections 3.3 and 3.4 describe the use of the suspension-cultured sugarcane cells/*P. chaunorhiza* elicitor system for the initial characterisation of sugarcane defence responses to pathogen challenge. Similar studies have been widely and successfully used for the study of other plant-pathogen interactions.

3.3 Biochemical responses of suspension - culture sugarcane cells to addition of an elicitor preparation derived for *Pachymetra chaunorhiza*

The results presented in this section have demonstrated that the responses of cell-suspension cultures to heat-derived elicitor preparations from *P. chaunorhiza* can be used to monitor and investigate aspects of the biochemical defence reactions of sugarcane to challenge by phytopathogens. The primary objective of these experiments was to identify induced differences in the biochemical reaction of resistant and susceptible sugarcane cultivars in order to identify potential biochemical markers for resistance. Differences, some considerable, were detected in all three parameters monitored (accumulation of individual compounds, peroxidase activity, PAL activity). Increases in peroxidase activity and accumulation of phenolic compounds are frequently associated with increased resistance to fungal pathogens and were observed in the resistant sugarcane cultivar Q114 but also in the susceptible cultivar Q90 (Figure 3.3.1). However, the phenolic compounds accumulated differed between the resistant and susceptible cultivar tested. Furthermore, PAL activity increased greatly after elicitor treatment of cells of the susceptible cultivar, but not with the resistant cultivar, despite accumulation of new phenolic compounds (Figure 3.3.2). These results suggest that the expression of sugarcane defence systems are complex. The phenolic compounds luteolin, chlorogenic acid and caffeic acid differed in their toxicity to *P. chaunorhiza* (Figure 3.3.3). Luteolin actually promoted fungal growth whereas chlorogenic acid and caffeic were toxic. In subsequent sections, attempts were made to characterise the defence response in a wider selection of resistant and susceptible sugarcane cultivars, and to compare responses in both culture cells and normal root tissue.

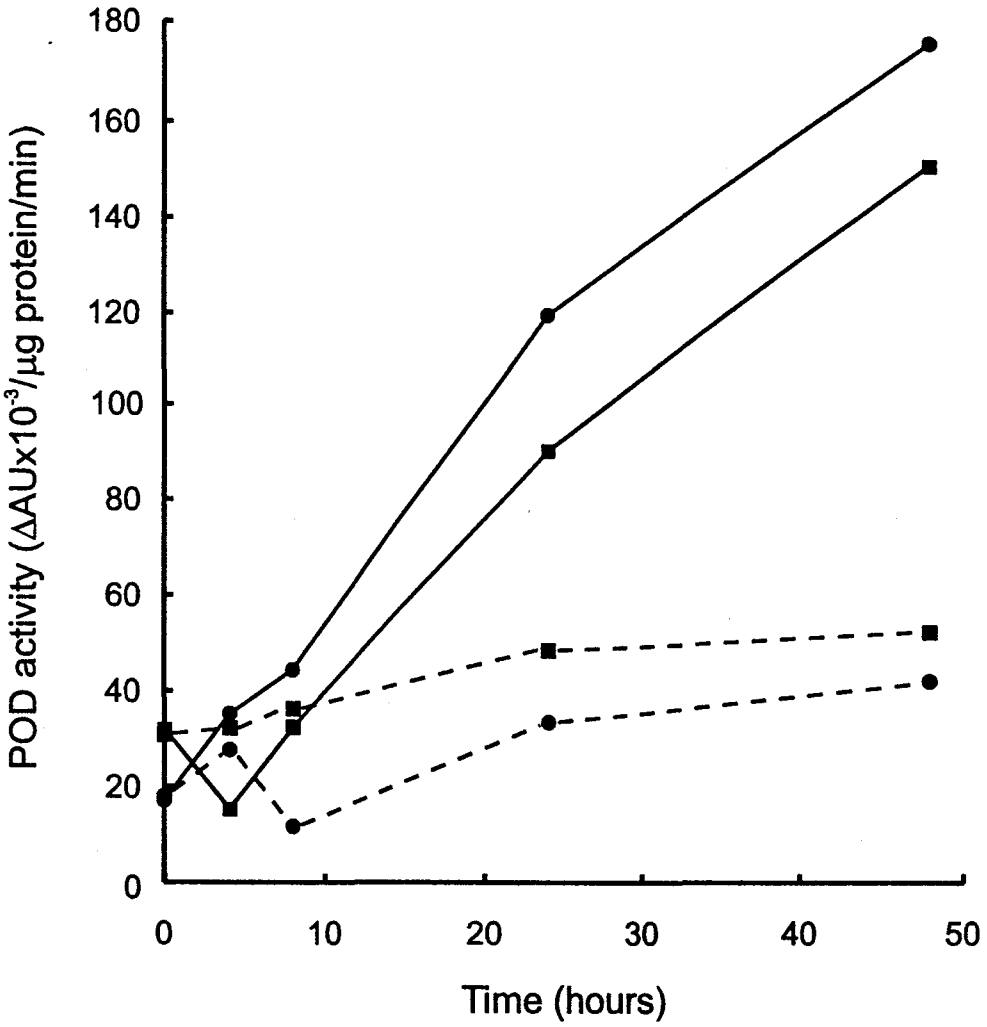

Figure 3.3.1 Peroxidase enzyme activities of Q90 (susceptible) and Q114 (resistant) suspension-cultured cells following the addition of the heat-derived elicitor from *P. chaunorhiza*. All points represent the mean activity of two independent duplicate cultures, and the mean difference between sample duplicates was 12.2 ΔAU_{430} x 10^{-3} min^{-1} (μg protein)$^{-1}$. (- - • - -) unchallenged Q114; (——•——) challenged Q114; (- - ■ - -) unchallenged Q90; (——■——) challenged Q90.

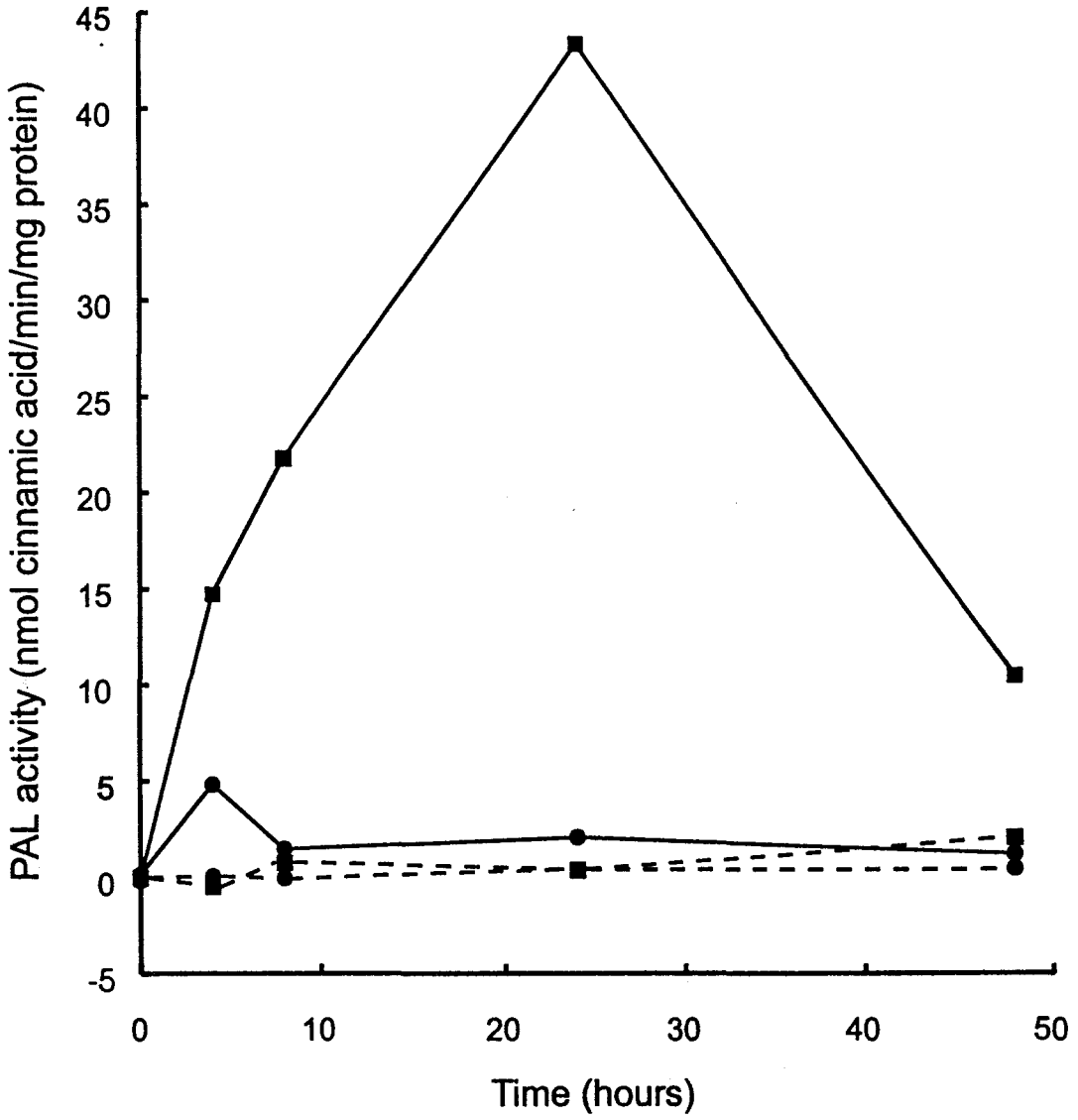

Figure 3.3.2 Phenylalanine ammonia-lyase (PAL) enzyme activities of Q90 (susceptible) and Q114 (resistant) suspension-cultured cells following the addition of the heat-derived elicitor from *P. chaunorhiza*. All points represent the mean activity of two independent duplicate cultures, and the mean difference between sample duplicates was 2.9 nmol cinnamic acid min^{-1} (mg protein)$^{-1}$. (- - • - -) unchallenged Q114; (—•—) challenged Q114; (- - ■ - -) unchallenged Q90 (—■—) challenged Q90.

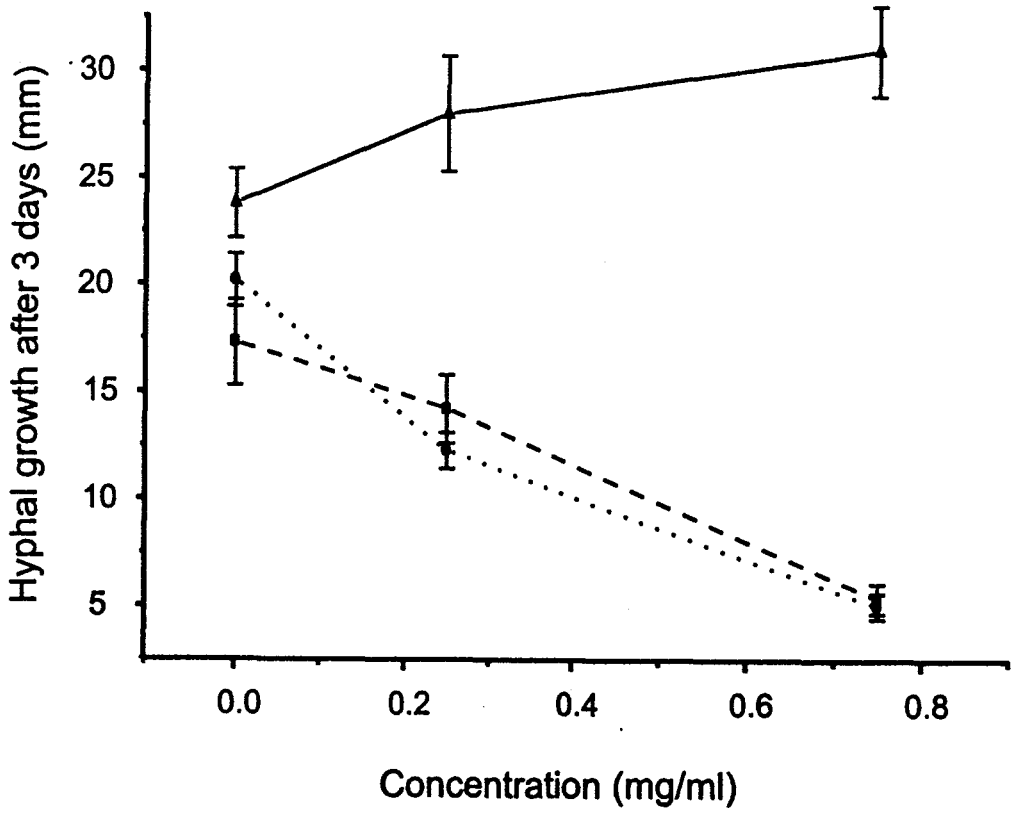

Figure 3.3.3 **The toxicity of selected phenolic compounds to *P. chaunorhiza* when incorporated into the growth medium. Each concentration was replicated 5 times and the error bars represent ± one standard deviation. Luteolin (——▲——); chlorogenic acid (- - ■ - -); caffeic acid (.....•.....).**

3.4 Investigation and characterisation of defence responses in 10 sugarcane cultivars ranging in resistance to Pachymetra root rot

The primary objective of the experiments described in this section was to extend the survey of constitutive and elicitor-induced biochemical factors detected in homogeneous suspension-cultured cells of two sugarcane cultivars by examining a larger selection of cultivars representing a range of resistance ratings. Biochemical factors consistently correlated with resistance or susceptibility were sought, both to assist sugarcane breeding programs, and if possible to identify mechanisms causally related to disease resistance. However, the heterogeneous suspension-culture sugarcane cells used for the

survey of the sugarcane cultivars described in the current experiments were either developmentally regulated or stressed compared with the homogeneous cell cultures, as indicated by elevated levels of PAL and peroxidase activity in the absence of elicitor (Tables 3.4.1 and 3.4.2). Nonetheless, the heterogeneous suspension-cultured cells still underwent pigmentation changes after elicitor treatment despite high levels of expression of these two enzymes, indicating that other metabolic changes, possibly related to resistance, were superimposed on high activities of these enzymes. Various UV-absorbing compounds (putative phenolics, flavones, and anthocyanidins) present in control and elicitor-treated cells were partly characterised by HPLC and spectral analysis (Table 3.4.3).

The survey of 10 cultivars was extended to the analysis of *P. chaunorhiza*-infected and uninfected (control) roots of pot-grown sugarcane plants. Peroxidase activity using guaiacol as substrate showed no detectable activity in uninfected roots, but was highly induced in resistant cultivars after infection (Table 3.4.1). In contrast, increases in PAL activity were correlated with susceptibility (Table 3.4.2). Furthermore, HPLC and spectral analysis of relative concentrations of methanol-soluble phenolics revealed that many compounds changed in concentration after infection. Further analyses revealed that the major alkali-labile cell-wall bound phenolics, *p*-coumaric and ferulic acid, also changed in concentration after infection of roots. Multiple linear regression analysis showed a strong relationship in infected roots linking concentrations of cell wall bound phenolics and peroxidase activity, with resistance rating to *P. chaunorhiza* (Table 3.4.4). This relationship is consistent with the hypothesis that the accumulation of cell wall bound phenolic compounds and polymerisation by peroxidase may be a contributing factor to resistance of some cultivars. This hypothesis is supported by the observation that CAD activities did not show any increase in activity with increased peroxidase activity, and therefore the additional induced peroxidase activity did not appear to be utilised for the production of lignin.

In summary, a number of factors were found to have some correlation with Pachymetra root rot resistance ratings. Some of these appear to be related, at least in part, to the damage caused by the infecting fungus, and are therefore a consequence of varying degrees of pathogen-induced damage, rather than a contributing factor to resistance. Other factors, such as peroxidase activity, soluble phenolics (Table 3.4.4) and cell-wall bound phenolics, are postulated to have a role in resistance. Both types of parameters could be potentially useful as markers for resistance in sugarcane breeding programs.

Table 3.4.1: **Phenylalanine ammonia-lyase (PAL) activities of both (a) sugarcane suspension-cultured cells 24 hours after addition of elicitor, and (b) infected sugarcane roots after growth for six weeks in soil infested with *P. chaunorhiza* oospores. Results are expressed as the rate of production of cinnamic acid (CA) from L-phenylalanine. Correlation coefficients (*r*) were calculated for the relationship between each PAL activity data set and Pachymetra root rot resistance ratings. An * indicates significance at $p>95\%$. For the suspension-cultured cells experiment, cultures were prepared in duplicate for the control and in triplicate for the elicitor-treated. The values reported are means, and the numbers in brackets represent the standard deviation (elicitor-treated), and the difference between duplicates (controls). A single composite (two independent plants) sample was analysed for each cultivar for the infected root experiment. n d = no data.**

Cultivar	Resistance Rating	PAL activity (nmol CA min^{-1} $(mg\ protein)^{-1}$)					
		a) Suspension-cultured cells			b) Sugarcane roots		
		Control	Elicitor	E/C	Control	Infected	I/C
Q114	1	25.1 (3.9)	17.5 (0.7)	0.70	261	217	0.83
Q78	1	13.0 (1.6)	17.8 (5.2)	1.37	277	212	0.77
58N829	3	11.5 (2.4)	19.9 (8.4)	1.73	231	322	1.39
Q120	3	n d	n d	n d	259	242	0.93
Q117	4	18.8(11.7)	13.8 (6.6)	0.73	149	224	1.50
Q96	5	73.6(20.1)	116.8(19.7)	1.59	217	221	1.02
Q113	5	50.7(33.4)	27.6 (4.3)	0.54	202	284	1.41
Q132	6	29.1 (0.6)	23.2 (4.5)	0.80	176	297	1.69
Q90	8	63.2 (5.3)	64.7(16.9)	1.02	278	285	1.03
Q83	9	9.4 (3.7)	23.3 (8.9)	2.47	194	546	2.81
Correlation coefficient (*r*)		0.30	0.297	0.35	-0.35	0.71*	0.70*

Table 3.4.2: **Peroxidase activities of both (a) sugarcane suspension-cultured cells 24 hours after addition of elicitor, and (b) infected sugarcane roots after growth for six weeks in soil infested with *P. chaunorhiza* oospores. Correlation coefficients (r) were calculated from the relationship between each peroxidase activity data set and Pachymetra root rot resistance ratings, an * indicates significance $p > 95\%$. For the suspension-cultured cells experiment, cultures were prepared in duplicate for the control and in triplicate for the elicitor-treated. The values reported are means, and the numbers in brackets represent the standard deviation (elicitor-treated), and the difference between duplicates (controls). A single composite (two independent plants) sample was analysed for each cultivar for the infected root experiment. n d = no data.**

Cultivar	Resistance Rating	Peroxidase activity ($\Delta Aux10^{-3}$/min)					
		a) Suspension cultured cells			b) Sugarcane roots		
		Control	Elicitor	E/C	Control	Infected	Infected
		(μg protein)$^{-1}$			(μg protein)$^{-1}$		(mg fresh weight)$^{-1}$
Q114	1	106 (16)	176 (17)	1.66	0	273	1.9
Q78	1	48 (2)	57 (3)	1.19	9	359	4.6
58N829	3	181 (36)	182 (34)	1.01	0	351	2.3
Q120	3	n d	n d	n d	0	243	2.0
Q117	4	158 (54)	168 (76)	1.06	0	437	3.4
Q96	5	90 (10)	99 (11)	1.10	0	574	4.1
Q113	5	174 (8)	237 (21)	1.37	0	0	0
Q132	6	115 (10)	151 (7)	1.31	0	317	2.1
Q90	8	121 (12)	123 (12)	1.02	0	0.6	0
Q83	9	105 (77)	116 (16)	1.10	0	2.4	0.06
Correlation coefficient (r)		0.14	-0.44	-0.44		-0.534	-0.645*

Table 3.4.3: Summary of components detected in elicitor treated suspension-cultured sugarcane cells. Correlation coefficients (r) were only calculated between the Pachymetra root rot resistance ratings and the concentration of a component when the component was detected in three or more cultivars, an * indicates significance at p >95%. sh = shoulder; + = maxima > specified wavelength.

Peak ID	Retention Time	Spectral max (nm)	Correlation Coefficients (Control)	Correlation Coefficients (Elicitor)
SC1	15.6	(295sh), 310	0.303	0.225
SC2	18.1	(295sh), 325 (chlorogenic acids?)	0.805*	0.267
SC3	18.5	300		
SC4	20.7	(300), 330	0.488	
SC5	25.8	268		
SC6	26.4	(295sh), 310		
SC7	29.4	268		-0.498
SC8	31.1	(290), 320		
SC9	33.0	376 (flavone?)		
SC10	33.2	280		
SC11	33.6	290, 315		
SC12	38.7	290, 320		0.689*
SC13	40.6	376 (flavone?)		0.589
SC14	46.9	(295sh), 316 (oxidation product?)		
SC15	47.9	(295sh), 331		
SC16	53.0	277, 325, 400+ (anthocyanidin?)		0.694*
SC17	55.3	(295sh), 316 (oxidation product?)		

Table 3.4.4: Summary of components detected in sugarcane roots uninfected and infected with *P. chaunorhiza*. Correlation coefficients (4) were calculated between the established Pachymetra root rot resistance rating and the concentration of components only when the component was detected in three or more cultivars, an * indicated significance at $p > 95\%$. Possible compound identification (i) phenolic eg benzaldehyde; (ii) cinnamic acid eg chlorogenic acid; (iii) flavone eg luteolin.

Peak ID	Retention Time	Spectral max (nm)	Correlation (Control)	Correlation (Infected)
R1	11.5	(295), 324 (ii)		
R2	12.1	272 (i)	0.591	-0.769*
R3	14.4	(290), 312 (ii)		
R4	16.5	284 (i)	0.547	-0.675*
R5	17.0	(295), 324 (ii)	0.798*	
R6	17.7	(295), 324 (ii)	0.623	
R7	21.6	314 (i)		
R8	22.0	312 (i)		
R9	27.9	(310), 318 (ii)		-0.209
R10	28.9	270, 348 (iii)		
R11	30.1	268, 352 (iii)		
R12	30.3	268, 352 (iii)		
R13	32.1	278 (i)	-0.348	0.035
R14	32.7	288, 376 (iii)		0.534
R15	34.0	(300), 322 (ii)		-0.185
R16	40.2	376 (iii)		-0.758*
R17	43.5	346 (iii)		-0.510
R18	46.0	288 (i)		-0.560
R19	46.5	(300), 316 (ii)		-0.282

3.5 Production of cell wall degrading enzymes by *Pachymetra chaunorhiza* and characterisation of the carbohydrate composition of sugarcane cell walls

The experiments described in this section have shown that a partly purified preparation of sugarcane cell walls was a better carbon source for mycelial growth of *P. chaunorhiza* than sucrose and various purified polysaccharides (Table 3.5.1). When sugarcane cell walls were present in the growth medium, *P. chaunorhiza* released cell wall degrading enzymes capable of disrupting the structure of sugarcane cell walls, and releasing carbohydrates for sustained fungal growth.

Carbohydrate analysis of sugarcane cell walls confirmed that the composition is similar to other monocotyledonous plants, and that the monosaccharide components should represent suitable substrates for the cell wall degrading enzyme activities produced by *P. chaunorhiza*. Interestingly, the content of both xylose and glucose in the cell wall was found to correlate with resistance to Pachymetra root rot, with resistance being favoured by a low glucose/xylose ratio (Table 3.5.2). Furthermore, growth of *P. chaunorhiza* in axenic culture was found to be inhibited by free xylose (Figure 3.5.1). These results are consistent with the hypothesis that the rate of dissolution of the cell walls, and the balance of nutrients so released, may be critical to the outcome of the interaction between sugarcane and *P. chaunorhiza* during challenge, and suggest that the presence of greater amounts of xylose in the cell wall of sugarcane contributes to resistance.

Table 3.5.1: Glycosidase activities detected in the *P. chaunorhiza* culture media with different carbon sources. *P. chaunorhiza* was grown in each medium for seven days. The media were collected for enzyme analysis and dialysed to remove monosaccharides. Each value represents the mean of duplicate assays.

Carbon sources	Carbon source %	glucosidase ΔA/min/L	xylosidase ΔA/min/L	Galactosidase ΔA/min/L	Arabinosidase ΔA/min/L
basal medium		3	1	3	0
cell wall (Q90)	0.5	20	613	7	1
sucrose	1.0	4	65	2	0
pectin	1.0	0	9	2	0
xylan	1.0	1	1	6	1
carboxymethyl cellulose	1.0	1	31	3	0

Table 3.5.2: Composition of neutral cell wall sugars in roots of 10 sugarcane cultivars, representing a range of Pachymetra root rot resistance ratings. Correlation coefficients (r) indicate the relationship between the established resistance rating and each monosaccharide, an * indicates significance at p > 95%. A single composite (2 independent plants) sample was analysed for each cultivar.

Cultivar	Resistance Rating	Monosaccharide composition of purified sugarcane cell wall root material				
		Ara %	Xyl %	Glu %	Gal %	Glu/Xyl
Q114	1	20.53	50.02	20.91	8.54	0.41
Q78	1	13.93	62.10	17.86	6.11	0.29
58N829	3	16.80	52.20	24.23	6.78	0.46
Q120	3	14.79	54.75	23.00	7.47	0.42
Q117	4	21.26	49.17	21.54	8.03	0.44
Q96	5	16.86	51.56	24.99	6.59	0.48
Q113	5	17.95	51.57	23.79	6.69	0.46
Q132	6	12.57	50.94	30.17	6.33	0.59
Q90	8	16.75	51.63	23.21	8.41	0.45
Q83	9	19.00	44.14	30.21	6.66	0.68
Correlation coefficient (r)		0.026	-0.643*	0.751*	0.053	0.783*

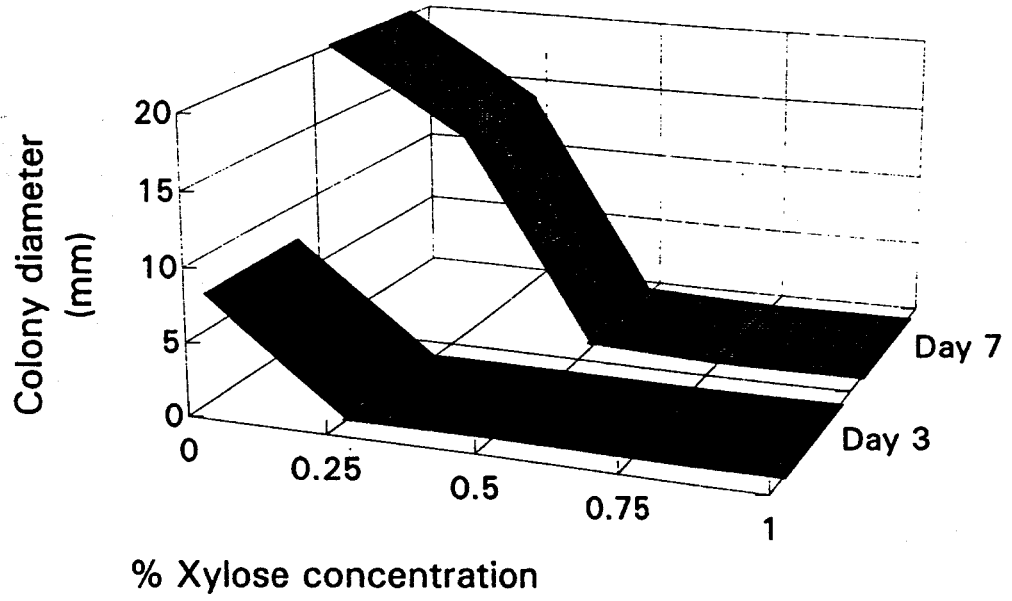

Figure 3.5.1: Growth of *P. chaunorhiza* with increasing concentrations of free xylose added to solid CMA medium. The xylose was added to CMA prior to autoclaving. The diameter of the *P. chaunorhiza* colony was recorded after three and seven days. Each concentration was tested in triplicate, the points plotted represent mean values. The experiment was repeated a second time with similar results to those shown in this figure.

3.6 Identification of phenolic compounds produced by sugarcane as a response to infection by *Pachymetra chaunorhiza*

Several constitutive and induced components present in infected roots of 10 sugarcane cultivars have been identified. The major compounds extracted from sugarcane tissue with aqueous methanol were the organic acids, aconitic acid and malic acid which represent key compounds involved in intermediary metabolism (citric acid cycle), together with a number of unidentified compounds. The citric acid cycle intermediates appear to be useful markers of root health, and their concentration in infected roots showed strong inverse correlation with the resistance rating of sugarcane cultivars. In addition *p*-hydroxybenzaldehyde was also identified and shown to represent peak R4 (Section 3.5). The concentration of *p*-hydroxybenzaldehyde present in infected roots also correlated with Pachymetra root rot resistance ratings.

In general, attempts to isolate, purify, and identify phenolic components that accumulated in suspension-cultured sugarcane cells following addition of elicitor were unsuccessful. Attempts were made to purify a number of putative phenolic compounds that eluted early during HPLC. Difficulties with purification procedures resulted in small amounts of material being isolated and purified, and only FAB-MS analysis was performed to identify purified peaks. Mass fragments corresponding to sugars or aglycones of glycosides were observed. Additional hydrolysis experiments were performed on an early eluting fraction that had been partially purified by preparative HPLC. Hydrolysis experiments and FAB-MS analysis suggested that at least one of the phenolic compounds was a glycoside, and that the attached carbohydrate group appeared to sucrose. The aglycone of this putative glycoside was observed on HPLC chromatogram traces but not in GC chromatogram traces, and was not identified.

3.7 Multiple regression analysis of defence factors produced by sugarcane

To explore the possibility that a number of defence factors contribute to resistance in sugarcane cultivars a backward stepwise multiple regression analysis (Statistix for Windows) was performed, with seven defence factors as the independent variables and resistance ratings as the dependent variable. The seven independent variables were: cell wall % xylose, % glucose, and % glucose/% xylose ratio all of roots; peroxidase activity in infected roots; *p*-hydroxybenzaldehyde concentration in infected roots, and the concentrations of cell wall bound ferulic and *p*-coumaric acids in infected roots. In a backward stepwise regression all the independent variables are added into the regression analysis and them the least significant variable discarded and the regression analysis repeated. After a number of such steps the most important variables for predicting resistance ratings were obtained and an optimum relationship between independent and dependent variables was produced.

The function generated by multiple regression analysis contained only *p*-hydroxybenzaldehyde concentration *(p=0.0058)* and % xylose in the cell wall *(0.0117)*. The function was as follows:

Resistance rating = (p-hydroxybenzaldehyde x -0.00141) + (% xylose x -0.32746) + 24.4824

The resistance ratings calculated from this function correlated with the actual resistance ratings, and the coefficient of determination (R^2) was 0.816, indicating that 82% of the variation in resistance ratings can be attributed to these two putative defence factors. Thus, by combining several putative defence factors such as composition of cell wall carbohydrates and accumulation of phenolic compounds in infected roots, Pachymetra root rot resistance ratings might be predicted with acceptable accuracy.

3.8 Conclusion

The research described in this thesis has identified a number of biochemical factors that may contribute to the expression of resistance in sugarcane to Pachymetra root rot. Cell culture techniques have been developed and assessed for investigating general biochemical responses of sugarcane to pathogens. An initial assessment of the genetic diversity among *P. chaunorhiza* populations found that there are two genetically distinct groups of *P. chaunorhiza* in Australian sugarcane fields and this aspect should be more thoroughly investigated to determine the impact on the strategy of controlling Pachymetra root rot by deploying resistant cultivars.

The most significant outcome of this research has been to identify a number of biochemical markers which are potential alternatives to the current systems used to rate sugarcane cultivars for resistance to Pachymetra root rot. These markers could potentially improve the management of Pachymetra root rot in sugarcane by leading to the development of simplified tests for resistance in breeding programs. Importantly, although this research has significantly increased the basic understanding of how sugarcane responds generally to challenge by pathogens and specifically to *P. chaunorhiza,* this understanding is still very limited particularly when compared with other commercial field crops. The research presented in this thesis is one of the first investigations of biochemical defence mechanisms to pathogens in sugarcane, and provides fundamental information as a basis for potentially fruitful areas of future research.

4.0 POSSIBLE FUTURE RESEARCH

The research described in this thesis has investigated the response of sugarcane to challenge by *P. chaunorhiza.* The induced response of sugarcane can be viewed as general (non-host) resistance, implying that the sugarcane responses are part of a general response to stress. Thus, further investigations using the experimental systems designed for this research should provide a greater understanding of how sugarcane interacts with

its environment and responds to general stresses imposed by pathogens, pests and other environmental factors.

Presented below is a series of possible future research approaches which may lead to improvements in the management of Pachymetra root rot, or may lead to a greater understanding of how sugarcane responds to environmental stress.

4.1 Further characterisation of resistance mechanisms to Pachymetra root rot

The research presented in this thesis is the first investigation of defence mechanisms to Pachymetra root rot in sugarcane and has identified some unusual features associated with resistance, as well as some mechanisms typically associated with pathogen defence in other host-pathogen systems. There are many areas in which the current understanding of the sugarcane/*P. chaunorhiza* interaction, as described in this project can be extended.

Firstly, because the difference in host cell was carbohydrate composition (glucose/xylose ratio) and represented a novel and previously unreported hypothesis for defence mechanisms, the processes by which xylose appears to have such a profound effect on *P. chaunorhiza,* and whether xylose toxicity is causally related to cell wall xylans, needs to be elucidated. A detailed understanding of these processes may lead to the identification of novel, hitherto unknown approaches for the control of disease.

Secondly, a number of biochemicals are produced and accumulated by sugarcane in response to challenge by *P. chaunorhiza,* but only a few have been identified. Positive identification of more of these compounds, and assessment of their toxicity to *P. chaunorhiza* will provide a better understanding of how different sugarcane cultivars respond to challenge by pathogens. The responses detected are likely to be general responses to physiological stress, and probably represent factors relevant to resistance of sugarcane to a variety of other pests and diseases. For example, resistance to canegrub could be associated with the induction of generalised stress reactions in sugarcane roots.

Thirdly, peroxidase was identified as a defence factor and has been widely implicated in defence against pathogens. The exact role of peroxidase in many host/pathogen systems is not clear although there is growing evidence that peroxidase is associated with the generation of antimicrobial active oxygen species. As foreign genes can now be relatively easily introduced into sugarcane a selection of peroxidase genes could be introduced into sugarcane under the control of various promoter and targeting sequences, to provide a system for probing and investigating the role of peroxidase in defence responses of plants and could identify future disease control options.

4.2 Characterisation of the *P. chaunorhiza* elicitor and induction of resistance

A great many biotic and abiotic compounds have been shown to elicit defence reactions in plants (Kuc 1995) and have potential for use as control agents for diseases (Benhamou *et al.* 1994; Benhamou 1996). The application of elicitor preparations can induce effective defence responses prior to challenge by a pathogen, and can lead to both local and systemic acquired resistance (SAR). In some plant species, the application of elicitor can result in a hypersensitive response in which plant cells die by a process known as programmed cell death. The reaction of sugarcane cells to the *P. chaunorhiza* elicitor resulted in the accumulation of a series of potentially antimicrobial compounds, and did not result in hypersensitive cell death. Consequently, the application of an elicitor or other SAR-inducing agent to sugarcane plants could present a viable option of disease control particularly in irrigated sugarcane fields. Alternatively, characterisation of the elicitor used in this study and identification and characterisation of additional elicitors, could indicate a potential for the introduction of a gene into sugarcane (by conventional breeding or transgensis) capable of inducing a continuous low level of defence response activation and enhance resistance to Pachymetra root rot.

4.3 Virulence and biochemical differences among groups *P. chaunorhiza*

Two genetically different groups of *P. chaunorhiza* have been detected within the Australian population and initial experiments indicate they differ in pathogenicity. This result needs to be confirmed urgently because the existence of groups of isolates of different virulence phenotypes would have substantial implications regarding the method used to assess cultivars to obtain resistance ratings, and on the deployment of cultivars in the field.

If the two groups of *P. chaunorhiza* are shown to differ in virulence, it would be useful to explore biochemical differences between the groups, which might indicate how the different groups of *P. chaunorhiza* are affected by the various sugarcane defence mechanisms identified by this research. In particular, more knowledge of the cell wall degrading enzymes produced by the two groups of *P. chaunorhiza* genotypes, and their response to xylose, would be useful.

An increased understanding of the cell wall composition of sugarcane would help define the interaction between *P. chaunorhiza* and sugarcane, and provide potential for enhancing sugarcane resistance to Pachymetra root rot. Little is known about the structure of sugarcane cell walls particularly polysaccharide components and how they vary between cultivars and wild sugarcane parents. A better understanding of the composition and physiology of cell walls would almost certainly be of benefit to other aspects of the sugar industry; for example in the utilisation of bagasse (a fibre by-product of sugar production) for the production of novel products.

4.4 Assessing resistance rating from correlated resistance factors

This research has identified a number of putative defence related factors and indicators of general root health that correlate with resistance ratings of sugarcane to Pachymetra root rot. A number of these factors have potential for replacing, or supplementing the current procedure for determining resistance rating by assessing the proportion of rotted roots. Of the putative defence factors identified, the xylose composition of the cell wall offers the greatest potential advantage in sugarcane breeding programs. Not only did the current resistance rating correlate well with xylose composition in cell walls, but importantly the xylose composition of cell walls is a pre-formed defence factor. Therefore, determination of xylose in the cell wall could potentially provide a means of mass screening of sugarcane cultivars for resistance to Pachymetra root rot without the need for infection in carefully controlled experimental pot trials. To implement such a system however, the xylose-toxicity hypothesis presented in this thesis, needs to be confirmed in further investigations which consider the relationship between cell wall carbohydrate composition and resistance to Pachymetra root rot of cultivars under different environmental conditions, at different stages of growth and at different nutritional states.

5.0 PUBLICATIONS

McGhie, T K, Masel, N P, Maclean, D J, Croft, B J and Smith, G R 1996. 'Progress towards understanding sugarcane resistance to the root pathogen *Pachymetra chaunorhiza*'. Proceedings of the 1996 Conferences of the Australian Society for Biochemistry and Molecular Biology and the Australian Society of Plant Physiologists, POS-149-02.

McGhie, T K, Masel, N P, Maclean, D J, Croft, B J and Smith, G R 1997. 'Biochemical responses of suspension-cultured sugarcane cells to an elicitor derived from the root pathogen *Pachymetra chaunorhiza*'. *Australian Journal of Plant Physiology* 24:143-149.

McGhie, T K 1998. A survey of pre-formed and induced defence mechanisms associated with resistance of sugarcane to the fungal root pathogen *Pachymetra chaunorhiza*. PhD Thesis, University of Queensland.

6.0 REFERENCES

Benhamou, N, Lafontaine, P J, and Nicole, M (1994). Induction of systemic resistance to Fusarium Crown and Root Rot in Tomato plants by seed treatment with chitosan. *Phytopathology* 84, 1432-1444.

Benhamou, N (1996). Elicitor-induced plant defence pathways. *Trends in Plant Sciences* 1, 233-240.

Brown, J K M (1994). Molecular and population genetics of barley powdery mildew. In 'Advances in Molecular Genetics of Plant-Microbe Interactions, Volume 3', (Eds Daniels, M J, Downie, J A, and Osborne, A E) pp. 191-198. (Kluwer Academic Publishers: Dordrecht).

Croft, B J, and Magarey, R C (1984). Pathogenic fungi associated with Northern Poor Root Syndrome of sugarcane. *Proceedings of the Australian Society of Sugar Cane Technologists 1984 Conf.* pp. 55-61.

Croft, B J (1989). A technique for screening sugarcane cultivars for resistance to *Pachymetra* root rot. *Plant Disease* 73, 651-654.

Croft, B J, and Magarey, R C (1989). A review of research into *Pachymetra* root rot, an important new fungal disease of sugarcane. *Proceedings International Society of Sugar Cane Technologists* 20, 686-694.

Dick, M W, Croft, B J, Magarey, R C, de Cock, A W A M, and Clarke, G (1989). A new genus of the Verrucalvaceae (Oomycetes). *Botanical Journal of the Linnean Society* 99, 97-113.

Egan, B T, Hurney, A P, Ryan, C C, and Matthews, A A (1984). A review of the northern poor root syndrome of sugarcane in north Queensland. *Proceedings of Australian Society of Sugar Cane Technologists 1984 Conference* pp 1-9.

Kuc, J (1995). Phytoalexins, stress, metabolism, and disease resistance in plants. *Annual Review of Phytopathology* 33, 275-297.

Magarey, R C, Taylor, P W J, and Ryan, C C (1987). Distribution of the root rot fungus involved in poor root syndrome in canefields from Ingham to Rocky Point. *Proceedings of the Australian Society of Sugar Cane Technologists* pp 105-107.

Magarey, R C (1991). The effect of varietal resistance on the epidemology of Pachymetra Root Rot. *Proceedings of the Australian Society of Sugar Cane Technologists* pp 95-102.

Magarey, R C (1994). Effect of Pachymetra root rot on sugarcane yield. *Plant Disease* 78, 475-477.

Magarey, R C, and Mewing, C M (1994). Effect of sugarcane cultivars and location on inoculum density of *Pachymetra chaunorhiza* in Queensland. *Plant Disease* 78, 1193-1196.