BIOLOGICAL ACTIVITY OF SECONDARY METABOLITES IN OAT (*AVENA SATIVA*)

By

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Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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To my wife, Zahraalsadat

and

My daughter, Saharnaz

Abstract

Flavone-*C*-glycosides (FCGs) and saponins are important defence compounds in many species of plants. The concentration of FCGs in 72 oat accessions was determined in a pilot experiment to explore the variation of this trait. Concentration of FCG compounds ranged from 1.32 to 12.83 mg (rutin equiv.)/g dry shoot. This variation was significant in subsequent experiments evaluating 21 and 5 cultivars, respectively.

The effect of methyl jasmonate (MJ) on FCG concentration was studied on 21 accessions in more detail. No significant induction of FCGs was observed in the shoots of MJ treated and untreated plants. The activity of MJ was confirmed by showing the induction of the oat thaumatin-like gene (*PR5*) in the roots but not shoots of oat cv. Quoll.

Crude extracts from the shoots of oat cv. Quoll were tested against four species of bacteria and eight species of fungi. Bacterial growth was not inhibited by the crude methanolic extract. The mycelial growth of all *Pyrenophora* species tested, except *P. avenae* DAR 33699, was inhibited by the methanolic crude extract of oat, whereas the mycelia growth of *Fusarium graminearum, Mycosphaerella pinodes* and *Rhizoctonia solani* was not inhibited. Fractions with high concentrations of FCGs had no inhibitory effect against *Pyrenophora teres* f. *teres* or *Pyrenophora teres* f. *maculata.* The highest concentration of antifungal activity was found in fractions containing the saponins, 26-desglucoavenacoside A (26-DGA) and 26-desglucoavenacoside B (26-DGB).

A single seed descent population of oat generated from the cross between Potoroo, a cultivar resistant to cereal cyst nematode (CCN; *Heterodera avenae*), and Mortlock, a susceptible cultivar, was used to identify metabolites correlated with CCN

resistance. This population had been assessed for CCN infestation each year from 2000 to 2002 and against stem nematode (*Ditylenchus dipsaci*) in 2001 as part of an unrelated study, and showed segregation for resistance to these nematodes. In this study, 30 SSD extreme lines that showed high and low numbers of white female nematodes were selected. Three compounds were found in the methanolic extract of oat roots that had negatively significant correlation with the CCN female counts. These compounds were partially characterised by ultraviolet-visible (UV-vis) and liquid chromatography-mass spectrometry (LC-MS). No significant correlation was found between previously identified avenacins in oat roots and CCN female counts. There was also no significant correlation found between flavonoids in oat roots and CCN female counts and flavonoids in oat shoot and stem nematodes scores.

In an independent experiment the induction of methyl jasmonate on secondary metabolites in oat roots was examined. Twelve high performance liquid chromatography (HPLC)-identified peaks were induced by exogenous application of MJ. LC-MS analysis identified selected induced peaks similar to avenacoside A and/or 26-desglucoavenacoside B in oat roots. This indicates for the first time that the pathway could be active in roots as well as shoots. Two root avenacins, avenacin A-2 and avenacin B-1 were induced by MJ, one of these, avenacin A-2, negatively correlated with CCN female counts. However, the correlation was not significant based on this analysis of the extreme subset of the SSD population.

The results in this study provides several important areas for further research that in future should allow plant breeders to develop lines with better resistance to nematodes.

Abbreviations

Ara	arabinosyl
bp	base pair(s)
BYDV	barley yellow dwarf virus
CCN	cereal cyst nematode
26-DGA	26-desglucoavenacoside A
26-DGB	26-desglucoavenacoside B
cDNA	complimentary deoxyribonucleic acid
DIG	digoxygenin
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
equiv.	equivalent
ES	electrospray
FCGs	flavone-*C*-glycosides
Gga	*Gaeumannomyces graminis* var. *avenae*
Ggt	*G. graminis* var. *tritici*
glc	glucosyl
HPLC	high performance liquid chromatography
HR	hypersensitive response
HVPE	high voltage paper electrophoresis
IPTG	isopropyl-β-D-thiogalactopyranoside
JA	jasmonic acid
LB	Luria-Bertini
LC-MS	liquid chromatography-mass spectrometry
LSD	least significant difference
M	molar
MJ	methyl jasmonate
MOPS	3-(N-morpholino) propane sulfonic acid
mRNA	messenger RNA
MQ water	milliQ water

MW	molecular weight
NZ	New Zealand
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
PR	pathogenesis related
PTLC	preparative TLC
RB	randomised block
Rha	rhamnosyl
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	room temperature
R_t	retention time
SA	salicylic acid
sad	saponin-deficient
SARDI	South Australian Research and Development Institute
SBA	soft buffer agar
SDS	sodium dodecyl sulphate
SE	standard error
SN	stem nematode
SSC	sodium chloride-sodium citrate solution
SSD	single seed descent
TAE	tris acetate EDTA
TLC	thin layer chromatography
X-Gal	5 bromo-4-chloro-3-inodlyl-β-D-galactopyranoside

Chapter One

Literature Review

1.1 Introduction

Plants are subject to attack by a wide range of organisms, including fungi, bacteria, viruses, nematodes and insects. They respond to attack through different mechanisms, such as rapid production of salicylic acid (SA), methyl jasmonate (MJ), ion fluxes, strengthen physical barriers, production of pathogenesis-related (PR) proteins and secondary metabolites. Pathogens themselves employ various strategies to overcome the plant defence systems. The interaction between plant and pathogens include plant resistance, allowing plant survival, or plant susceptibility, causing yield loss or even plant death.

Use of plants with natural defence mechanisms is safe, economical and the ideal strategy for pest and pathogen control, because the risks of environmental hazards are insignificant and the need for pesticides reduced. To improve resistance in crops, a comprehensive understanding of the mechanisms of resistance is needed. The defence response of plants to pests and pathogens involves many biochemical processes. One of the well-studied mechanisms is the production of secondary metabolites.

This literature review begins with a brief review of the structure and function of two important classes of secondary metabolites, the flavonoid and saponins, with a focus on their defence functions. This review also discusses the role of PR proteins in plant defence systems and the importance of elicitors in identifying significant defence genes and metabolites in plants. The remainder of this review will focus on the important oat cultivars in South Australia and review the breeding priorities such as the

significance of nematodes including cereal cyst nematode (CCN) and stem nematode (SN) on reducing the yield of cereals will be discussed. Plant biochemical responses to the nematodes as an approach to understand resistance mechanisms will also be presented. The potential of oat compounds to control diseases that may support use of oat as a beneficial rotation crop will be considered.

1.2 Secondary metabolites

Plants produce two major groups of metabolites, 1) primary metabolites like carbohydrates, lipids, amino acids and nucleic acids that are necessary for growth and development and 2) secondary metabolites such as organic acids, phenolic compounds, saponins, flavonoids, alkaloids, volatile oils, etc. that are generally not essential for the growth and development of plant (Dixon, 2001). The high number of secondary metabolites (about 100,000) encourages scientists to use this rich diversity to improve plant and animal defence system. However, this diversity has made it difficult to employ molecular and genetic techniques for determining the function of each metabolite in plant defence systems (Dixon, 2001). Some of the secondary metabolites give plants characteristics, for example terpenoids in chilli peppers give taste and flavonoids in orange give colour and flavour (Cowan, 1999; Harborne and Williams, 2000). Some of the secondary metabolites also protect plants due to their antimicrobial properties (Cowan, 1999; Harborne and Williams, 2000). Active secondary metabolites in plant defence system can be categorised into three broad groups, 1) constitutively exposed compounds such as phytoanticipins that exist in healthy plants in their biologically active forms (Osbourn, 1996), 2) signalling molecules that activate a variety of responses, or 3) induced compounds such as phytoalexins (Dixon et al., 2002). The distinction between phytoalexins or phytoanticipins is not obvious, as some

compounds may be phytoalexins in one species and phytoanticipins in another species (Dixon, 2001).

1.2.1 Flavonoids

1.2.1.1 Chemistry and structure

Flavonoids are one of the largest group of plant secondary metabolites and are present in a wide range of plants including agricultural plants, fruits, vegetables and cereals (Schijlen et al., 2004). Over 6400 different flavonoid compounds have been described in plants (Martens et al., 2003). All flavonoids have the same basic skeleton, C6-C3-C6 (Figure 1.1) and they are classified into different groups, flavanol, flavone, flavonol, flavanone, isoflavone and anthocyanidin according to the modifications of the C-ring (Shimada et al., 2000; Heim et al., 2002; Cavaliere et al., 2005).

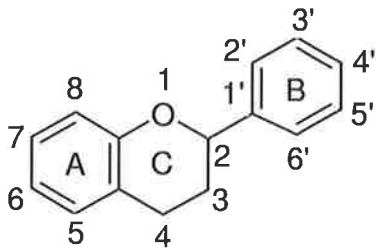


Figure 1.1 Nuclear structure of flavonoids (Heim et al., 2002).

These compounds differ in the arrangements of hydroxyl, methoxyl and glycosidic side groups. Sometimes they are found in plant as complex compounds for example, proanthocyanidin may be present in plants as high molecular weight multimers with more than 17 flavanol units (Guyot et al., 1997).

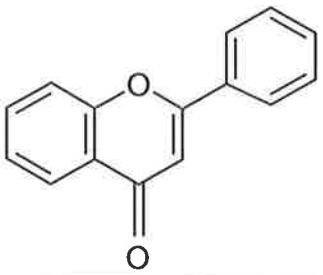
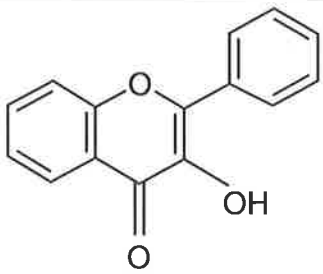
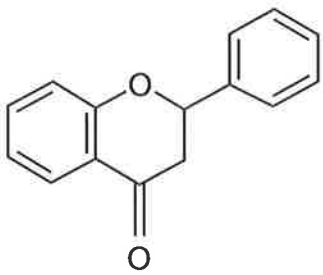
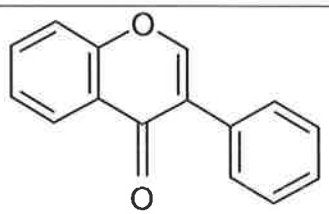
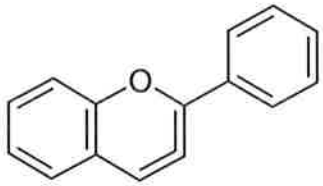
It has been shown that the different functional groups of the rings provide different functions. For example, flavones generally have higher antifungal activity than flavanone (Weidenborner and Jha, 1997). Contradictory reports of the activity of flavonoids with and without hydroxyl group have been published (Cowan, 1999). For example, it has been reported that flavonoids without hydroxyl group on their B-ring are more active against micro-organisms than those with hydroxyl group. However, other research has shown that hydroxylated flavonoids have more activity against pathogens than unhydroxylated flavonoids (Cowan, 1999). UV-B treatment results in a higher level of B-ring hydroxylation suggesting that this modification provides UV protection (Ryan et al., 2002).

1.2.1.2 Function of flavonoids in plants

Flavonoids have various functions such as antioxidants (Dixon and Steele, 1999; Harborne and Williams, 2000; Peterson, 2001), inducers of rhizobial *nod* genes in leguminous plants (Dixon and Steele, 1999; Shimada et al., 2000; Dixon et al., 2002; Shimada et al., 2003) attractors of pollinator insects by providing pigmentation and flower colour (Harborne and Williams, 2000; Moriguchi et al., 2001; Schijlen et al., 2004), stimulators of pollen germination (Dixon and Paiva, 1995), regulators of auxin transporters (Dixon and Paiva, 1995) protectors against ultraviolet light (Harborne and Williams, 2000; Ryan and Moura, 2002; Ryan et al., 2002), frost (Swiderski et al., 2004), pathogens and herbivores (Harborne and Williams, 2000; Simmonds, 2001).

One of the most important and useful effects of flavonoids is their antioxidant property. This property is attributed to their capacity to transfer electrons to free radicals like vitamin E radicals, activate key antioxidant enzymes, inhibit oxidases (Heim et al., 2002) and inhibit oxidation of proteins (Harborne and Williams, 2000). The number of flavan nuclei, the position and types of substitutions all affect the

Table1.1 Classification, structure and dietary sources of flavonoids (adapted from Heim et al., 2002)

Class	General structure	Flavonoid	Source
Flavanol		Catechin Epicatechin	Tea Tea
Flavone		Chrysin Apigenin Luteolin Rutin	Fruit skins Parsley, Celery Red pepper Citrus, Tomato skin, Buckwheat
Flavonol		Kaempferol Quercetin Myricetin	Leek, Broccoli, Endives, Grape fruits, Black tea Onion, Lettuce, Broccoli, Tomato, Tea, Berries, Olive, oil, Apple skin Cranberry Grapes
Flavanone		Naringin Naringenin Taxifolin Eriodictyol Hesperidin	Citrus, Grapefruit Citrus fruit Citrus fruit Lemon Orange
Isoflavone		Genestin Genistein Daidzin Daidzein	Soybean Soybean Soybean Soybean
Anthocyanidin		Apigenidin Cyanidin	Coloured fruit, Cherry, Raspberry, Strawberry

antioxidant properties of flavonoid (Heim et al., 2002), with the structure of the B ring in the flavan nucleus being the primary indicator of the antioxidant activity in these compounds (Pannala et al., 2001). Das and Pereira (1990) showed that a carbonyl group and a double bond between C-2 and C-3 increased the antioxidant activity.

Flavonoids have long been studied as signalling molecules for beneficial micro-organisms in the rhizosphere in leguminous plants (Dixon and Steele, 1999). Flavonoids especially 5-deoxyflavonoids and 5-deoxyisoflavonoids act as inducers of rhizobial *nod* genes in host-specific symbiotic nitrogen fixation (Shimada et al., 2003). There has been interest in producing these compounds in non-leguminous plants because of isoflavonoids protection against certain forms of cancer (Schijlen et al., 2004).

Flavonoids absorb strongly the UV region of the light spectrum, and therefore protect plants from the harmful effects of UV (Ryan et al., 2002; Ambasht and Ambasht, 2005). Markham et al. (1998) compared the response of UV-tolerant rice cv. M202 and UV-susceptible cv. Dular against high UV levels. They found that flavonoids are induced in tolerant cultivar by UV, but not in the susceptible cultivar. The effect of elevated CO_2 on flavonoid concentration was studied by Estraite et al. (1999). They discovered that high levels of CO_2 increased the concentrations of flavonoids in leaves of wheat by 14% when compared to control plants. Rising CO_2 concentrations in air increased the photosynthetic rate making carbon more available. The greater contents of flavonoids in the leaf help the plant to protect itself against UV radiation damage.

1.2.1.3 Function of flavonoids in plant defence systems

The inhibitory effects of flavonoids on feeding behaviour of pests and the growth of pathogens have been studied. Dreyer and Jones (1981) found that the more

polar phenolic fraction gave the strongest feeding avoidance for *Schizaphis graminum*. Apigenin-*C*-glycoside, which is in phloem of rice plants, can change the feeding behaviour of the brown plant hopper, *Nilaparvata lugens* (Grayer et al., 1994). Grayer et al. (1994) showed that when plant hoppers ate rice stems with high level of flavonoids, the weights of the plant hoppers decreased. Also, it has been shown that a glycosyl flavone isolated from corn silk can inhibit the growth and development of corn worm, *Heliothis zea* (Elliger et al., 1980). The flavonoid, sakuranetin, is a phenylpropanoid with inhibitory effects against *Ditylenchus angustus*, which is a damaging nematode in South Asia (Plowright et al., 1996). Five days after inoculation of a resistant cultivar with the stem nematode, the concentrations of flavanone sakuranetin and the phenylpropanoid chlorogenic had increased (Plowright et al., 1996).

The effect of flavonoids on pathogen growth has been well studied. Dillon et al. (1997) showed that there was a strong correlation between resistance to blast in rice plants and the accumulation of flavonoids. Moreover, they found that there was a genetic difference between rice cultivars in terms of phytoalexins. The sensitivity of major rice pathogens to inhibition by certain flavonoids was studied by Padmavati et al. (1997). They revealed that naringenin, a flavonoid, could inhibit the growth of *Xanthomonas* strains and prevent spore germination of *Pyricularia oryzae* in rice plants. A study on the antifungal activity of flavonoids on 34 different fungi, indicated that flavone has a higher antifungal activity than flavanone (Weidenborner and Jha, 1997). They confirmed the results of other researchers, which showed that *Fusarium* could metabolise antifungal compounds. The flavonoids that are important in plant defence of the selected crops are listed in Table 1.2.

Table 1.2 Crops known to contain flavonoids with stress protection and/or antimicrobial activity

Crop	Flavonoid	Pathogen/Stress	References
Wheat	Tricin	*Schizaphis graminum*	Dreyer and Jones, 1981
Rice	Sakuranetin	*Magnaporthe grisea*	Koda et al., 1992 Dillon et al., 1997
	Flavanone	*Ditylenchus angustus* (stem nematode)	Plowright et al., 1996 Padmavati et al., 1997
	Sakuranetin Naringenin	*Xanthomonas* sp. *P. oryzae*	
	Iso-orientin acylated glucosides	UV-B	Harborne and Williams, 2000
Maize	Anthocyanins Anthocyanins	*Helminthosporium maydis* UV-B	Padmavati and Reddy, 1999 Harborne and Williams, 2000
	Maysin	*Helicoverpa zea*	Padmavati and Reddy, 1999
	Maysin and apimaysin	*H. zea*	Lee et al., 1998
	2″-*O*-α -*L*-rhamnosyl-6-*C*-luteolin	*Heliothis zea*	Elliger et al., 1980
Oat	*O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside	*Heterodera avenae* *Pratylenchus neglectus*	Soriano et al., 2004a
Sorghum	Apigeninidin	*Colletotrichum graminicola*	Padmavati and Reddy, 1999
	Luteolinidin	*C. graminicola*	
	Flavan 3-ols	*C. graminicola*	Padmavati and Reddy, 1999
	Arabinosyl-5-*O*-apigeninidin	*Fusarium verticillioides* (synonym *F. moniliforme*)	Padmavati and Reddy, 1999
	Flavan-4-ols	*Curvularia lunata*	Grayer and Harborne, 1994
Tea	Flavone-3-ols	*Exobasidium vexans* *Vibrio cholerae*	Punyasiri et al., 2004
	Catechins	*Streptococcus mutans*	Sakanaka et al., 1989
Barley	Flavonoid 7-*O*-methyltransferase	*Blumeria graminis*	Christensen et al., 1998
Peanut	Procyanidin	*Aphis craccivora*	Grayer et al., 1992
Sugarbeet	Betavulgarin	*Rhizoctonia solani*	Elliger and Halloin, 1994
Soybean	Glyceollin	*Fusarium solani* sp. *glycines*	Lozovaya et al., 2004

1.2.1.4 Flavonoids in oat (Avena sativa)

Research on flavonoids in oat is limited to few papers that cover structure, biological activity and changes with environmental conditions. A significant variation was found among oat cultivars for the concentration of all phenolic compounds except that of ferulic and *p*-cumaric acids (Emmons and Peterson, 2001). This research also showed that the environment has a significant effect on the concentration of phenolics and total free phenolics. The abundant flavonoids in leaves of oat are apigenin-*C*-pentohexoside as major component, luteolin-*C*-pentohexoside and *O*-methyl-apigenin-*O*-deoxyhexoside-*C*-hexoside (Soriano et al., 2004). These flavone-*C*-glycosides in oat were induced by methyl jasmonate (MJ) and nematodes. Furthermore, high level of flavone-*C*-glycosides restricted the invasion of *Heterodera avenae* in oat roots (Soriano et al., 2004).

1.2.2 Saponins

Saponins are a major family of secondary metabolites that contain an aglycon hydrophobic core and sugar chains with hydrophilic property (Osbourn, 2003; Martin and Magunacelaya, 2005). These compounds are glycosylated triterpenoid, steroid or steroidal alkaloids (Hostettmann and Marston, 1995; Papadopoulou et al., 1999) with a diverse range of activities including antimicrobial, anti-insect, allelopathic, haemolytic and surfactant properties. Sapo is the Latin word for soap and reflects their surfactant properties as saponins produce stable foams when shaken with water (Hostettmann and Marston, 1995; Onning and Asp, 1996; Osbourn, 2003). Saponins are synthesised from mevalonic acid through the isoprenoid pathway (Hostettmann and Marston, 1995). Triterpenoids and steroids are produced from the cyclisation of 2,3-oxidosqualene (Qi et al., 2004; Jenner et al., 2005; Townsend et al., 2006).

Saponins are produced in a wide range of plant species particularly dicotyledonous plants (Papadopoulou et al., 1999; Osbourn et al., 2003). The presence of saponins in oat (*Avena sativa*) is noteworthy given their apparent absence from most other cereals such as rice, wheat and barley (Hostettmann and Marston, 1995).

1.2.2.1 Function of saponins in plant defence systems

Saponins have long been known to have strong biological activity. The antibacterial activity of saponins is relatively weak but their antifungal activity is strong (Okazaki et al., 2004). Extracts from oat roots have been shown to attract and then lyse zoospores of *Pythium* species and *Phytophthora cinnamomi*. Further work has shown that this property is due to the presence of avenacin (Deacon and Mitchell, 1985). α–tomatine, which is present in tomato (*Lycopersicum esculentum*), has been shown to have strong antifungal and weak antibacterial activity. The antifungal activity of this saponin was proven against *Fusarium oxysporum*, *Aspergillus* spp., *Candida albicans* and *Trichophyton* spp. (Hostettmann and Marston, 1995). *Fusarium solani* can infect ripe tomato fruits because they contain low levels of saponins, but it is not able to infect green tomato fruits as they have a high level of α–tomatine (Osbourn, 1996). Medicagenic acid glycosides are the dominant saponins in lucerne roots and were found to inhibit the growth of *Trichoderma viride, Sclerotium rolfsii, Rhizopus mucco, Aspergillus niger, Phytophthora cinnamomi* and *Fusarium oxysporum* (Hostettmann and Marston, 1995; Kohli et al., 2001).

It has been shown that the aqueous extracts of the Chilean tree, *Quillaja saponaria,* have nematicidal effects (Martin and Magunacelaya, 2005). This research also suggested that saponin and non-saponin fractions have a synergistic effect on the control of nematode populations (Martin and Magunacelaya, 2005).

The membraneolytic properties of saponins against fungi is a result of the interaction of saponins with sterols in the membrane of fungi (Osbourn, 1996; Armah et al., 1999; Kohli et al., 2001). It has been suggested that fungi with low levels of sterols in their membrane should be resistant to saponins (Kohli et al., 2001) but this has not been tested. In general, maximum antifungal activity is obtained from monodesmosidic saponins, with a single sugar chain in one side of the molecule, that contains four or five sugar residues, which can increase water solubility and thereby increase the antifungal activity (Hostettmann and Marston, 1995). However, weak haemolytic activity of bisdesmosidic saponins, with two sugar chains in both sides of the molecule, was reported (Woldemichael and Wink, 2001).

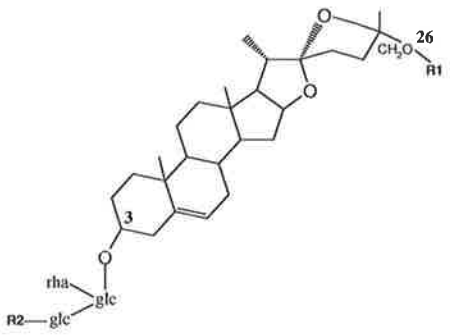
1.2.2.2 Saponins in oat (Avena sativa)

Oat is unique among cereals in producing two different families of saponins, the steroidal avenacosides in the shoots (Onning et al., 1993; Onning and Asp, 1996; Osbourn et al., 2003) and the triterpenoid avenacins in roots (Crombie et al., 1986; Crombie and Crombie, 1986).

Saponins in oat shoots: In 1953 a bitter-tasting compound with foaming and haemolytic property was isolated from the leaves of oat (Onning and Asp, 1996). Thirteen years later, Tschesche and Schmidt named this compounds avenacosides (Onning and Asp, 1996). The first two avenacosides compounds were identified as avenacoside A and avenacoside B (Figure 1.2) by Tschesche and Lauven (cited in Onning and Asp, 1996).

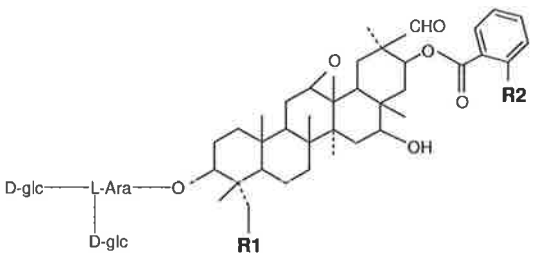
Qualitative and quantitative analyses of saponins have been performed using thin layer chromatography (TLC) (Tschesche et al., 1969; Curl et al., 1985) and high performance liquid chromatography (HPLC) (Kesselmeier and Strack, 1981). Avenacoside A and avenacoside B are bisdesmosidic saponins. They have two

A) shoots



	R1	R2
Avenacoside A	glc	H
Avenacoside B	glc	glc
26-DGA	H	H
26-DGB	H	glc

B) roots



	R1	R2
Avenacin A-1	OH	$NHCH_3$
Avenacin A-2	OH	H
Avenacin B-1	H	$NHCH_3$
Avenacin B-2	H	H

Figure 1.2 Chemical structures of saponins in oat A) shoots (Wubben et al., 1996) and B) roots (Crombie et al., 1986). 26-DGA and 26-DGB are 26-desglucoavenacoside A and 26-desglucoavenacoside B, respectively.

attachment sites for sugars, a sugar chain at C3 and a single glucose residue at C26 (Grunweller and Kesselmeier, 1985). Avenacoside A and avenacoside B are stored in the vacuoles of epidermal cells (Kesselmeier and Urban, 1983) and are converted to monodesmosidic 26-desglucoavenacoside A (Figure 1.2A, 26-DGA) and 26-desglucoavenacoside B (Figure 1.2A, 26-DGB) after removal of a glucose from the C-26 position when oat shoots are attacked or mechanically damaged (Grunweller and Kesselmeier, 1985). The deglycosylation reaction is carried out by a highly specific β-glucosidase (avenacosidase) in the plant leaves (Gus-Mayer et al., 1994).

The biological activity of 26-DGA and 26-DGB in oat was tested against a few fungi. Luning and Schlosser (1976) tested 26-DGA and 26-DGB against 15 different fungi and they measured their sensitivities using leakage of free amino acids from cells as a measure of the membranlytic activity. They found that both pathogens and non-pathogens infecting oat leaves, *Drechslera (Pyrenophora) avenacea, Septoria avenacea, Alternaria solani, Septoria cyclaminis* and *Fusarium culmorum* are insensitive to 26-DGB, whereas important pathogen such as *Gaeumannomyces graminis* var. *avenae*, *G. graminis* var. *tritici*, *Ascochyta pisi*, *F. avenaceum* are sensitive to the saponin in oat leaves. *Drechslera teres* and *D. graminea* showed low levels of leaked amino acids when exposed to 26-DGB. Luning and Schlosser (1976) were one of the researchers to suggest that the sensitivity of the fungi could be due to sterols in their membrane, based on the absence of sterols in *Pythium.*

Luning and Schlosser (1975) partially purified β-glucosidase from homogenated oat leaves by high-speed centrifuge in 10,000 *g*. In 1985, Grunweller and Kesselmeier (1985) were able to purify and characterised the specific β-glucosidase responsible for activation of avenacosides from the non-specific β-glucosidase in homogenised oat leaves. In 1994, a protein (As-P60) was isolated from oat seedlings and identified as the specific β-glucosidase responsible for activation of avenacosides (avenacosidase). The amino acid sequence of this protein showed significant sequence identity with β-glucosidases involved in defence mechanisms of dicotyledonous plants and it had 34% sequence identity with bacterial and mammalian β-glucosidase (Gus-Mayer et al., 1994).

Some fungal pathogens are able to detoxify the active saponins in oat shoots by removing the sugar chain at the C3 position (Hostettmann and Marston, 1995; Osbourn, 1996; Wubben et al., 1996). *Septoria avenae* f. sp. *avenae* (Wubben et al., 1996),

Stagonospora avenae (Morrissey et al., 2000; Bleddyn Hughes et al., 2004) and *Botrytis cinerea* (Quidde et al., 1999) are three fungi that can detoxify 26-DGA and 26-DGB. *Botrytis cinerea* can degrade and detoxify the antifungal activity of the 26-desglucoavenacosides and other saponins like α-tomatine in tomato and digitonin in *Digitalis* (Quidde et al., 1999). The gene encoding the major β-glucosidase responsible for detoxifying saponins from the foliar blast fungus, *S. avenae*, was cloned and the protein showed similarity to other saponin-detoxifying enzymes in *Septoria*, *Gaeumannomyces* and *Botrytis* (Quidde et al., 1999).

The first reports on the quantification of saponins in the shoots of oat, which was carried out using TLC (Tschesche et al., 1969), indicated that the avenacoside A and B content in oat grains was estimated to be 0.04% and more recently, the content of total saponins in rolled oat was estimated to be 0.1% (Onning and Asp, 1996). Nisius (1988) indicated that there is a natural variation among the genera of *Avena* for avenacoside. No saponins were detected in shoots of *A. clauda*, *A. pilosa* and *A. ventricosa* using TLC. Despite this research, there is still no evidence to show any correlation for sensitivity of these species to pathogens and absence of saponin in their shoots (Osbourn, 1996). Onning et al. (1993) determined the avenacoside A and avenacoside B content of 16 oat cultivars using HPLC. They found that the saponin content was significantly different among oat cultivars and it ranged from 0.02-0.05% based on dry matter.

Saponins in oat roots: An antifungal compound called avenacin was isolated from oat roots (Maizel et al., 1964). This compound is able to give resistance to oat against the wheat take-all fungus, *Gaeumannomyces graminis* var. *tritici,* previously known *Ophiobolus graminis* (Burkhardt et al., 1964; Maizel et al., 1964; Luning et al., 1978).

Four avenacins were isolated and identified as avenacin A-1, A-2, B-1, B-2 (Figure 1.2B). Both, avenacin

A-1 and B-1, show strong blue fluorescence under UV-light (366 nm) due to N-methyl anthranilate in their structures which is unique to saponins (Luning et al., 1978; Crombie and Crombie, 1986). The UV florescent property facilitated the isolation, partial quantification, screening and bioassay of these compounds. The other two root saponins, avenacin A-2 and B-2, have a benzoate group in their structures and are not florescent (Crombie and Crombie, 1986; Trojanowska et al., 2000). Avenacin A-1 and A-2 have one extra oxygen compared to avenacin B-1 and B-2, respectively as in Figure 1.2B (Begley et al., 1986). The trisaccharide chain common to all four avenacins contains two glycolyl residues and one arabinosyl residue at the C3 position (Crombie et al., 1987).

The importance of avenacins in plant protection comes from studies on the effect of wheat take-all fungus, *G. graminis* var. *tritici* (Ggt) and oat take-all fungus, *G. graminis* var. *avenae* (Gga). Oat is resistant to Ggt strains that lack avenacinase and susceptible to Gga (Crombie et al., 1986; Osbourn et al., 1991) whereas non-saponin containing cereals, wheat and barley, are susceptible to both variants of fungi (Bowyer et al., 1995). Goodwin and Pollock (1954) were able to attribute the resistance of oat to fluorescent compounds found in the root tips. The sensitivity of an oat species lacking avenacin, *A. longiglumis,* to Ggt suggests that avenacin may be a good indicator of oat resistance to fungal pathogens (Osbourn et al., 1994). The esterified avenacins with methylanthranilate group (A-1 and B-1) showed more toxicity against fungi than avenacin A-2 and B-2 (Crombie et al., 1987; Hostettmann and Marston, 1995). According to Maizel et al. (1964), avenacin at 50 μg/ml could inhibit 50% of the growth of *Candida albicans* and *Pythium irregulare*, 12.5% of *Botrytis cinerea* growth.

Oat mutants that lack saponin biosynthesis were generated from diploid oat, *A. strigosa*, using sodium azide and examined against Ggt (Papadopoulou et al., 1999). These saponin-deficient (*sad*) mutants were susceptible to Ggt, whereas the wild-type *A. strigosa,* showed lower sensitivity to the fungi. The involvement of avenacin in increasing the plant resistance was confirmed by assessing the F_2 progeny from the crosses between mutants and wild type oat plants (Papadopoulou et al., 1999). These mutants have provided robust tool for investigating saponin biosynthesis and should provide a greater understanding of the key enzymes and genes in the pathway in the future (Townsend et al., 2006).

As seen with fungi response to avenacoside in oat shoots, some fungi are resistant to avenacin either due to altered membrane composition or producing an avenacin-detoxifying enzyme (Bowyer et al., 1995). Fungal species like *Pythium* lack sterols in their membrane (Luning and Schlosser, 1976), therefore, saponins cannot interfere with their membranes, resulting in no toxicity (Luning and Schlosser, 1976). Among eight tested fungi, *Pythium ultimum* was the only fungus that did not show inhibition when exposed to avenacins (Turk, 2005). Bioassays of avenacin on *Fusarium avenaceum* showed that this fungi is resistant to lower levels of avenacins and sensitive to higher levels of avenacins, 50% inhibition at 200 μg/ml avenacin A-1, (Luning et al., 1978; Crombie et al., 1986).

The presence and activity of avenacinase is a determinant of pathogenicity of fungi on oat. The infection of oat by take-all fungi, Gga, is attributed to saponin-detoxifying enzyme, avenacinase (Bowyer et al., 1995; Papadopoulou et al., 1999). Osbourn et al. (1991) demonstrated that the isolates of Gga have 100 times more avenacinase activity than isolates of Ggt. They also partially characterised a single protein species of molecular weight 110 kD using SDS polyacrylamide gel

electrophoresis that was responsible for the activity. The gene encoding the avenacinase was cloned and polyclonal antisera for avenacinase has also been raised (Bowyer et al., 1995; Osbourn, 1996). It is possible that there are other mechanisms for detoxification, since Australian isolates of Ggt lacking avenacinase are able to infect oat. This property is attributed to either resistance in the level of membrane or misrecognition in clarification of the fungus (Osbourn, 1996).

The presence of avenacins in both monocots and dicots plants is limited to the genus of *Avena* and the closely related species tall oatgrass, *Arrhenatherum elatius* (Osbourn, 2003). Turk (2005) searched 189 genotypes of Poaceae for avenacins using their fluorescence property and found that none of them showed accumulation of avenacin except A*vena* spp. The distribution of avenacin in oat roots was investigated by Crombie and Crombie (1986). They studied 11 species of oat and found that the avenacin content in oat roots varied from 0.22-1.0 mg/g dry weight (DW) and the relative contents of each avenacin ranged from 47-60 % A-1; 5-7% B-1; 30-43% A-2; 3-6% B-2. They identified traces of avenacin A-1 in *Arrhenatherum elatius.* The concentration of avenacins declined with the age of *Avena sativa* plants from 5.26 mg/g DW (3-4 days old) to 0.52 mg/g (77 days old). Using the fluorescence property of avenacin A-1, Osbourn et al. (1994) found that avenacin A-1 as the major avenacin in oat roots and avenacin B-1 are localised to the epidermal cell layer of root tips. The concentration of total avenacin in the root tips (12.76 mg/g DW) is more than double that of the rest of oat roots (5.01 mg/g DW, Crombie and Crombie, 1986). Turk (2005) quantified avenacin A-1 in the root tips of 35 *Avena* genotypes using TLC. A significant difference was found among oat genotypes for the concentration of avenacin A-1 varying from 4.7 to 6.5 mg/g fresh weight.

1.3 Plant defence responses

Numerous defence mechanisms have evolved in plants to protect them against pathogens and herbivores. Some of the important mechanisms are described below.

1.3.1 Hypersensitive response

One of the best known and most powerful inducible defence responses that is activated by the invasion of pathogens is the hypersensitive response (HR) (Stuiver and Custers, 2001; Bertini et al., 2003). The HR is defined as a programmed cell death reaction restricting pathogen spread from the site of infection (Vernooij et al., 1994; Bertini et al., 2003). A HR is initiated when a gene product from the pathogen binds to a plant receptor leading to a rapid defence response (Glazebrook, 2001). The interaction between the plant and pathogens causes rapid necrosis at the site of pathogen penetration and can also induce systemic acquired resistance (Peterson et al., 2000). In the HR the first reaction after pathogen attack is an oxidative burst. The induction of HR is rapid and very important in restricting pathogen penetration.

1.3.2 Systemic acquired resistance

When pathogens and pests invade plants, the first response occurs at the site of infection. However, uninfected parts of the plant respond to the primary infection by increasing their general resistance against the same or other pathogens (Vernooij et al., 1994; Schweizer et al., 1998; Bertini et al., 2003). This non-specific resistance is known as systemic acquired resistance (SAR) and is present in many plant species and provides protection against viruses, fungi and bacteria (Ryals et al., 1994). Suprisingly, there are only a few reports on SAR in cereals (Schweizer et al., 1998; Bertini et al., 2003).

Several studies have shown that the onset of systemic acquired resistance is correlated with the accumulation of SA throughout the plant (Peterson et al., 2000). SA is thought to act as a signalling molecule (Peterson et al., 2000) that ultimately induces the expression of a set of pathogenesis related (*PR)* genes (Schweizer et al., 1998; Glazebrook, 2001). A number of *PR* genes were induced in rice by mechanical treatment (Schweizer et al., 1998). *PR4* genes were induced in wheat by SAR chemical inducers such as SA, 2, 6- dicholoroisonitric acid, benzo (*1,2,3*) thiodiazole *7*-carbothiotic acid S-methyl ester, MJ and wounding treatment (Bertini et al., 2003).

1.4 Pathogenesis related proteins

Pathogenesis-related proteins are coded by the host plant and induced by pest and pathogen attack or by other stresses (Liljeroth et al., 2001; Bravo et al., 2003). PR proteins can be grouped into 14 families based on amino acid sequences and functional properties (Hoffmann-Sommergruber, 2002). Some PR proteins are constitutively expressed in some organs of plants (Hoffmann-Sommergruber, 2002).

PR proteins can be induced in cereals and their antifungal role has been well studied in cereals. Caruso et al. (1999) found that a number of PR proteins were induced in wheat infected with *Fusarium culmorum.* They showed that two isoforms of β-1, 3-glucanase (PR2) and three isoforms of chitinase (PR3) were induced upon infection. Furthermore, activity of wheatwins (PR4) and peroxidase were increased in the early stages after inoculation. Accumulation of several PR proteins has been reported in barley in response to infection by *Bipolaris sorokiniana* including *PR1* and thaumatin-like (*PR5*) (Liljeroth et al., 2001). Agrawal et al. (2002) showed that two major PR proteins, OsPR5 and OsPR10, accumulated in rice in response to inoculation with the fungal elicitor, chitosan. Pests attack in cereal plants also induce PR proteins. Attack of barley plants by oat aphid (*Rhopalosiphum padi*) induces PR proteins

(Forslund et al., 2000). One chitinase (PR3) and four isoforms of β-1, 3-glucanase (PR2) were induced in barley by oat aphids, but PR1a and PR5a were not induced.

1.5 Elicitors

Elicitors are factors that produce a defence response by activating specific signalling pathways and genes. Elicitors can induce enzymes involved in lignification (Campbell and Sederoff, 1996) and PR protein production (Xu et al., 1994; Liljeroth et al., 2001) and they are grouped into biotic, abiotic factors and synthetic compounds.

The biotic elicitors include carbohydrates, lipids and proteins produced by pest and pathogen and stimulate a response by the plant. Abiotic elicitors include mechanical damage, drought, frost, salinity and so on. There are several key defence compounds that also induce defence responses in plants, such as SA, jasmonic acid (JA), MJ, ethylene, benzo (*1*,*2*,*3*) thiodiazole *7*-carbothiotic acid s-methyl ester and nitric oxide. This literature review will explain the important abiotic elicitors with an emphasis on MJ.

1.5.1 Salicylic acid

SA accumulates endogenously in leaves infected by pathogens and this compound induces SAR genes, *PR* genes and ultimately resistance based on observed plant protection against pathogen infection. Exogenous SA acts the same as endogenous SA in tobacco (Vernooij et al., 1994). Salicylic acid is the generator for plant defence reactions and is produced from trans-cinnamic in higher plants through the intermediate orthocumaric acid (Ryals et al., 1994). Bertini et al. (2003) showed that *PR4* genes in wheat are activated by exogenous SA application. It has been shown

that SA induces resistance in rice against blast disease, which is caused by *Magnaporthe grisea* (Song and Goodman, 2002).

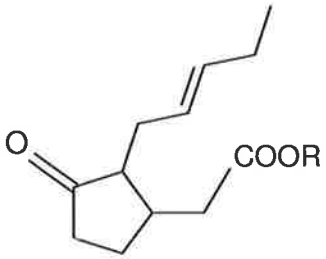
1.5.2 Ethylene

Endogenous ethylene (C_2H_4) is known as a growth and development regulator in plants (van Loon et al., 2006). This hormone has several roles in the plants including, seed germination and seedling growth, leaf abscission, organ senescence and fruit ripening (Bleecker et al., 1988; van Loon et al., 2006). It has been shown that the level of ethylene in plants is enhanced by wounding and pathogen attack (Bleecker et al., 1988; van Loon et al., 2006). Ethylene regulates plant responses to biotic stresses like pathogens and abiotic stresses such as drought (Chen et al., 2005).

1.5.3 Jasmonic acid and methyl jasmonate

Jasmonic acid (JA) and its methyl ester, MJ (Figure 1.3) play important roles in plants as growth regulators (Ananieva and Ananiev, 1997; Biondi et al., 2001; Sasaki et al., 2001), and as signal molecules in plant defence system (Thaler, 1999; Ryan and Moura, 2002). JA (Figure 1.3) is synthesised from linolenic acid in five steps (Biondi et al., 2001). It was first isolated from the fungus *Lasiodiplodia theobromae* (Creelman and Mullet, 1995). Both water deficiency and JA induce a similar response with the induction of several genes in plants, suggesting that JA could have a role in protection against drought (Creelman and Mullet, 1995).

The effect of jasmonic acid treatment on pests in the tomato was investigated by Thaler (1999). He showed that the damage on tomato leaves due to pests in plants treated with a foliar spray of JA was 60% less than control plants. Flavonoids in oat, which are shown to be involved in resistance to CCN, were induced by MJ



Jasmonic acid R=H

Methyl jasmonate $R=CH_3$

Figure 1.3 Structure of jasmonate

(Soriano et al., 2004). Saponarin, a protective compound against UV-B damage in barley, is suppressed by JA but two flavones with stronger anti-oxidative properties in young barley leaves are induced by JA (Ishihara et al., 2002).

MJ is more volatile than JA and volatility of MJ caused initial interest (Creelman and Mullet, 1995). It has been reported that MJ is able to induce the synthesis of proteinase inhibitors in both sprayed plants and nearby unsprayed plants (Farmer and Ryan, 1990). Therefore, to prevent interplant communication and any kind of drift, it is necessary to separate control and MJ-treated plants before treatment (Farmer et al., 1992).

Many experiments have been carried out to see whether *PR* genes are induced by JA and/or MJ. It has been shown that *PR4* genes in wheat can be induced by MJ (Bertini et al., 2003). *PR4* was also strongly induced in rice by JA and abscisic acid (ABA) but not SA and ethylene (Agrawal et al., 2002). In the roots of sugarcane, *PR10* was induced by MJ 24 hr after application (Bower et al., 2005). *PR5*, a thaumatin-like gene, is one of the *PR* genes that are induced by pathogens and elicitors. Over-expression of this gene in rice was found in plants resistant to the fungus *Rhizoctonia solani* when compared to control plants (Datta et al., 1999). Several *PR* genes in rice,

PR2, *PR3*, *PR5* and *PR9*, were shown to be induced and their proteins accumulated by JA and rice blast fungus, *Magnaporthe grisea* (Schweizer et al., 1997). In sunflower, the *defensin* (*PR5-1*) gene was induced by either JA and/or SA, 6 and 24 hr after application, respectively (Hu et al., 2003). Xu et al. (1994) showed that *PR1* and *PR5* in tobacco were induced by a combination of MJ and ethylene.

The increased resistance conferred by external application of MJ appears to cost plants based on the measurement of many different factors. For example, different researcher have observed reduced number of flowers (Thaler, 1999), delayed flowering and fruit ripening (Heil, 2002), reduced growth (Heijari et al., 2005) and induction of leaf senescence and reduced grain weight (Beltrano et al., 1998). Baldwin (1998) showed that if MJ-induced plants had not been attacked by herbivores, less seeds were obtained compared to control plants, therefore it is suggested that MJ should be applied when the attack is predictable and induction is necessary rather than constitutively. In a four-year experiment on the effect of JA on the yield and phenology of tomato, there was no significant difference between yield of JA-treated and control tomato when tomato were grown under natural and experimentally reduced the levels of herbivores (Thaler, 1999). There are some examples where the positive effects of MJ could compensate the negative effects of exogenous JA. For example, positive effects of JA on the reducing the plant damage by herbivores could compensate the negative effects on tomato such as reducing the number of flowers (Thaler, 1999).

1.5.4 Mechanical damage

Mechanical damage such as wounding induces many of the same defence reactions and genes as chemical compounds. In rice, wounding of one leaf, induced a number of *PR* genes (Schweizer et al., 1998). Similarly in wheat, *PR4* genes were

expressed at 1-3 days after wounding (Bertini et al., 2003). Li et al. (2001) observed that two serine protease inhibitor-encoding genes (*Inh-I* and *Inh-II*) were expressed at the site of wounding and throughout tomato plants by mechanical wounding of a single leaflet.

1.6 Plant parasitic nematodes

The crop losses due to nematodes are estimated to be about 11% in the world and cost about USD 80 billion (Agrios, 2004). More than 20 important crops suffer losses due to nematodes (Trivedi, 1998). Two out of 17 orders of nematodes, Tylenchida and Dorylaimida, are plant parasitic and members of Dorylaimida are vectors of plant viruses (Strange and Scott, 2005). Two of the devastating nematodes, *Heterodera avenae* and *Ditylenchus dipsaci,* are related to this thesis and will be described.

1.6.1 Cereal cyst nematode (*Heterodera avenae*)

This nematode was first reported on oat in Britain in 1908 by Theobald and then on wheat in 1909 and was first known as cereal root eelworm (Southey, 1956). The first recording of CCN in Australia and South Australia was made by Davidson in 1930 (Stanton and Fisher, 1985).

CCN is a destructive pathogen that can cause serious damage on wheat and oat. The grain yield losses of wheat due to CCN in south-eastern Australia have been estimated on average 8% (costing AUD 54 million in 1986/87) and in some farms it can be more than 50% (Brennan and Murray, 1988; Rathjen et al., 1998; Ogbonnaya et al., 2001). Oat yield loss due to CCN in infected paddocks could be as high as 81% in South Australia where an intolerant cultivar is sown (Zwer et al., 2005).

1.6.2 Stem nematode (*Ditylenchus dipsaci*)

This nematode can cause serious damage on more than 450 different plant species and is considered one of the most destructive nematodes (Strange and Scott, 2005). It has been reported that this nematode can reduce the oat grain yield by 80% (Grainger, 1956).

1.7 Nematode control

Different methods of control are advised to manage and restrict nematodes. A good chemical control for CCN was obtained on severely infested farms by spraying with aldicarb (Temik 10G) carbofuran at 1.5-2.5 kg/ha (Trivedi, 1998) and ethylene bromide (Stanton and Fisher, 1985). Biological control of CCN with the parasitic bacterium, *Pasteuria penetrans*, has also been advised (Trivedi, 1998). Crop rotation is a cultural method of control where non-host crops can restrict the number of CCN in the soil to minimise disease (Trivedi, 1998). Fallow management and control of host weeds during fallow can reduce the number of cysts because rain in the absence of host plants causes many cysts to hatch without forming of new generation (Amir and Sinclair, 1996). Use of resistant and tolerant cultivars is the most feasible and economical method to control nematodes due to environmental concerns and effectiveness of the method (Martin et al., 2004).

Resistance to nematodes is defined as the ability of the plant to inhibit and prevent the reproduction of nematodes whereas tolerance to nematodes is the capacity of the plant to withstand, grow and yield well in presence of nematodes (Rathjen et al., 1998). Several resistance genes for CCN have been identified and evaluated in wheat and its relatives (Ogbonnaya et al., 2001; Martin et al., 2004; Safari et al., 2005). A gene designated *Cre1* on chromosome 2B of AUS10894, *Cre3* on chromosome 2D and

Cre4 of *Aegilops tauschi, Cre2, Cre5* and *Cre6* (*CreX)* of *Aegilops ventricosa, CreR* of *secale cereal, Cre7* of *Aegilops triuncialis, Cre8* in a cultivar Festiguay and *CreF* of Frame and Yipti are some of these genes (Ogbonnaya et al., 2001; Martin et al., 2004; Safari et al., 2005). Several genes for CCN resistance, *Ha1, Ha2* from Chebec, *Ha3, Ha4* from Galeon have been reported for barley (Kretschmer et al., 1997; Barr et al., 1998; Seah et al., 2000).

1.8 Plant biochemical response to nematodes

It is well known that plants respond to nematode attack by producing nematicidal compounds either as phytoanticipins or phytoalexins. In a study on the interaction between soybean-cyst nematode (SCN), *Heterodera glycines*, and glyceollin, Huang and Barker (1991) found that glyceollin I, an isoflavone derivative compound, accumulated in a resistant cultivar after inoculation with SCN. Mixtures of saponin and non-saponin fractions of *Quillaja saponaria* are active against several nematodes including *Meloidogyne* and *Pratylenchus* (Martin and Magunacelaya, 2005) as are flavonoids in oat against *H. avenae* and *P. neglectus* (Soriano et al., 2004), sakuranetin in rice against *D. angustus* (Plowright et al., 1996), polyacetylenes in roots of safflower (*Carthamus tinctorious*) against several nematodes (Akhtar and Mahmood, 1994), 20-hydroxyecdysone in spinach (*Spinacia oleracea*) against *H. avenae, H. schachtii, P. neglectus* and *M. javanica* (Soriano et al., 2004). Sincocin is the trade name of a commercial product that combines natural products from four different plant species, prickly pear, southern red oak, mangrove and sumac and protects orange, sugar beet, cassava and sunflower against nematode attack (Chitwood, 2002). Some steroid and triterpenoid compounds in *Asparagus*, Fabaceae and *Acacia*, are reported to inhibit the growth and development of nematodes (Chitwood, 2002). JA and/or invasion of

CCN in roots of oat induce a protein, ASP45, that is detected by a wheat-germ agglutinin antibody (Oka et al., 1997). The identification of the phytochemicals that are induced by nematodes has allowed the development of a new nematicidal chemical with enhanced activity and reduced environmental concerns (Chitwood, 2002).

Incorporating the resistant genes from wild relatives is an important approach for nematode control. However, finding the most appropriate accessions in terms of resistance and tolerance genes and/or metabolites is a prerequisite. Understanding the plant's response to nematode invasion in term of producing phytochemicals should provide methods for identifying protective phytochemicals. These chemicals could be used to identify new accessions for breeding programs.

1.9 Oat (*Avena sativa*)

Oats are resistant to a number of important crop diseases, and are grown in crop rotations to limit build up of pathogens (Soriano et al., 2004) such as *Gaeumannomyces graminis* var. *tritici* (Maizel et al., 1964) and *Helminthosporium* sp. (Sebesta et al., 2001). Growing an oat crop is an effective way to reduce the population density of root lesion nematodes, *Pratylenchus thornei* (Hollaway, 2002) and alter the soil microflora to suppress pathogens such as *Verticillium* sp. (Konagai et al., 2005). Quoll, Potoroo and Mortlock are three important cultivars that are widely grown in South Australia. The characteristics of these cultivars will be described.

1.9.1 Quoll

Quoll was generated from a cross between a tall plant type designated MIOLRP-86-3 as female parent and a dwarf, hull-less seed cultivar named Bandicoot in 1987 (SARDI, 2000). Quoll is a dwarf feed cultivar that is 1) susceptible and intolerant

to CCN, 2) moderately tolerant to stem nematode, 3) moderately resistant to stem rust, 4) moderately resistant to leaf rust 5) moderately resistant to septoria, 6) moderately susceptible to barley yellow dwarf virus (BYDV) and 7) moderately susceptible to bacterial blight (Zwer et al., 2004; Zwer et al., 2005).

1.9.2 Potoroo

Potoroo was generated in 1984 from a cross between an F_1 plant obtained from the cross between NZ Cape (OX80; 226-2H) and an *Avena sterilis* line Cc 4658 (OX79; 119-20) and Echidna in South Australia. The purpose of this cross was to generate a new cultivar with resistance and tolerance to CCN (Barr et al., 1994). Potoroo is 1) resistant and tolerant to CCN, 2) moderately resistant and moderately tolerant to SN, 3) moderately resistance and tolerant to *Pratylenchus neglectus*, 4) susceptible to stem rust 5) moderately susceptible to leaf rust, 6) very susceptible to septoria and 7) moderately susceptible to BYDV (Wallwork, 2003). Potoroo is a feed grain for sheep and cattle (Zwer et al., 2004).

1.9.3 Mortlock

Mortlock is classified as a tall, milling variety of oat. This cultivar is 1) moderately susceptible and intolerant to CCN, 2) very susceptible and very intolerant to SN, 3) moderately susceptible to stem rust, 4) moderately susceptible to BYDV, 5) susceptible to leaf rust and 6) susceptible to septoria (Wallwork, 2003).

1.10 Aims of the study

Major aims of this study were:

1) Explore the extent of genetic variation of oat accessions for both constitutive and induced levels of flavonoid concentration;

2) Test the antimicrobial activity of flavonoids and other metabolites in oat;

3) Use single seed descent populations generated from Mortlock and Potoroo to identify metabolites involved in oat resistant to nematodes.

Chapter Two

Flavonoids and Saponins in Oat (*Avena sativa*); Analysis of their Biological Activity

2.1 Introduction

Oat (*Avena sativa*), like many crops, is susceptible to pests and diseases that cause substantial losses worldwide, including Australia. However, oat is resistant to a number of important crop diseases, and are grown in crop rotation to limit the built up of pathogens (Soriano et al., 2004), such as *Gaeumannomyces graminis* var. *tritici* (Maizel et al., 1964) and *Helminthosporium* sp. (Sebesta et al., 2001).

Two major groups of secondary metabolites, saponins and flavonoids are reported to confer disease resistance in oat (Osbourn et al., 2003; Soriano et al., 2004). Saponins are an important group of secondary metabolites that can protect plants from antimicrobial attack, but are lacking in many cereals (Carter et al., 1999; Haralampidis et al., 2001). Oat plants contain several saponins including avenacin in the roots, which can inhibit some pathogens (Crombie et al., 1986; Crombie and Crombie, 1986; Osbourn et al., 1994; Turk, 2005). Oat shoots contain avenacoside A and B, which are converted to active antifungal compounds, 26-desglucoavenacoside A (26-DGA) and 26-desglucoavenacoside B (26-DGB), when plants are attacked by pathogens (Luning, 1975; Laudenbach and Kesselmeier, 1982; Wubben et al., 1996).

Flavonoids, another group of secondary metabolites with important roles in plant biology, are distributed widely in the grasses (Dillon et al., 1997; Lee et al., 1998). These polyphenolics have many functions including stimulating the growth of beneficial micro-organisms (Shimada et al., 2000), protecting plants against UV stress

(Estiarte et al., 1999) and inhibiting pathogens (Dillon et al., 1997; Weidenborner and Jha, 1997; Simmonds, 2001; Punyasiri et al., 2004; Soriano et al., 2004).

One approach for finding defence genes, proteins and secondary metabolites in plants is to use elicitors particularly chemical signal molecules. Many different elicitors induce flavonoids in plants. Jasmonic acid (JA) induced two flavones with potent anti-oxidative properties in young barley leaves (Ishihara et al., 2002). Moreover, Soriano et al. (2004) showed that FCGs measured at 254 nm in the shoots and roots of the oat cv. Quoll were significantly induced by methyl jasmonate (MJ) and nematode infection. These flavonoids were identified by mass spectrometry and shown to be *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside, apigenin-*C*-hexoside-*O*-pentoside and luteolin-*C*-hexoside-*O*-pentoside. The concentration of these compounds in oat cv. Quoll increased in the roots (140%) and shoots (131%) after MJ treatment. While all three flavonoids were induced by MJ, only *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside had antinematode activity (Soriano et al., 2004).

In this chapter, we investigate the genetic variation of FCGs in the shoots of 72 oat accessions in the South Australian Research and Development Institute (SARDI) collection, which included cultivars, breeding lines and wild accessions from 13 different countries. The effect of MJ on the concentration of FCGs in the accessions will be explored. The antimicrobial activity of the oat shoot extracts containing FCGs will also be described.

2.2 Materials and Methods

2.2.1 Plant material and culture methods

One hundred and fifteen accessions of oat, supplied by the Oat Breeding Group, South Australian Research and Development Institute (SARDI) were chosen for a pilot experiment (Appendix 1). A few seeds of each entry were sown into a pot containing steam pasteurised (65°C for 45 min) University of California (UC) mix (Appendix 2). Osmocote (Scotts, Australia), a slow release fertiliser, was included at the time of sowing. Plants received Hoagland's solution (Appendix 3) every second day and tap water on alternate days.

2.2.2 Induction of secondary metabolites in oat

Seeds of oat and wheat were germinated either in a glasshouse or a growth chamber (22°C light/18°C dark, 12 hr cycle). After three weeks, half of the pots in the experiment were treated with 0.1 mM MJ solution by drenching. Control plants received ultrafiltered water (MQ, Millipore), filtered through a 0.45 µm membrane. Pots drenched with MJ were separated from control pots before treatment by transferring to a similar glasshouse or growth chamber. Plants were harvested after 3 or 5 days as indicated. The shoots and roots were separated and frozen immediately in liquid nitrogen. They were stored at -80°C until extraction.

2.2.3 Extraction and high performance liquid chromatography analyses

Samples stored at -80°C were freeze-dried and ground to a fine powder using a coffee grinder (Breville, Australia). Sixty mg of ground sample was extracted with 10 ml methanol for 48 hr by shaking in an orbital mixer at 130 rpm. Then, 3 ml MQ water was added to 7 ml methanolic extract and lipids were removed with 10 ml n-hexane

mixed at 130 rpm for 48 hr. Eight ml of the methanolic phase was evaporated in a water bath (45°C) by ventilating the tubes with compressed air. The residue was redissolved in 5 ml MQ water, 5 ml of 1-butanol (HPLC grade) was added and mixture was shaken by an orbital mixer for 24 hr. A 4 ml aliquot of the resulting butanol phase was evaporated as above. The final residue was dissolved in 0.4 ml 45% v/v methanol in water immediately prior to high performance liquid chromatography (HPLC) analysis.

Reversed-phase (RP)-HPLC was performed with a C18 column (Waters Sphersorb ODS-2, 4.6 × 150 mm, 5 μm particle column). Isocratic elution was used with methanol: water (45:55 v/v) mobile phase at 1 ml/min. Elution was monitored at UV 254 nm (Soriano et al., 2004). Quantification of the compounds was performed comparing the data for the compounds with a regression equation for a set of rutin standards (0.2, 0.4, 0.6, 0.8 and 1 mg/ml) dissolved in 45% methanol with heating.

Alternatively, separation was achieved on a C18 column (Altech Platinum EPSC18 100A, 7 × 53 mm) by gradient elution, at a flow rate 0.5 ml/min at 55°C. Starting with Solvent A (2.5% formic acid) and Solvent B (1% formic acid: 60% acetonitrile: 39% water) was increased from 0 to 40% over 18 min, subsequently from 40 to 100% over 3 min, and finally 100% Solvent B for 1 min. Compounds were monitored at both 254 and 340 nm.

2.2.4 Genetic variation of flavone-*C*-glycosides and the effect of MJ treatment

Seeds were sown in an augmented design experiment with two factors, MJ application and cultivar (Federer et al., 2001). Seeds were germinated and grown in a glasshouse in Adelaide in December 2003. The augmented design meant that only 23 of 115 oat accessions were sown in replicate (n=2) to provide an indication of

experimental error. All other accessions were sown as single pots (50 × 50 mm) of at least 6 plants initially and culled to two plants five days after sowing.

From the above pilot experiment, 21 accessions covering a wide range of constitutive and induced concentrations of flavonoids were selected. The seeds were sown in two replicates in a randomised block (RB) design in the glasshouse in July 2004. Six seeds were sown in each pot (75 × 75 mm) and after germination the number of plants was reduced to four.

In the next experiment, five out of 21 accessions in the second experiment were selected and seeds were germinated in a growth chamber to minimize changes in environmental conditions. Seeds were sown in a RB design in three replicates. Six seeds were sown in each pot (50 × 50 mm) and all plants were kept.

2.2.5 Effectiveness of MJ application on concentration of FCGs

Seeds of oat cv. Quoll were sown in four replicates (6 seeds per 125 × 125 mm). Plants were treated with MJ, using one of three methods, 21 days after sowing. Each treatment was performed in a different growth chamber, but all had similar conditions. Control plants received MQ water containing 0.1% Tween 20. Test plants were treated with MJ by: 1) drenching (0.5 mM MJ in 0.1% Tween 20); 2) vapour (6 ml 0.5 mM MJ loaded onto cotton wool until saturated and kept in a plastic bag); or 3) spray (0.5 mM MJ dissolved in dimethyl sulfoxide) (Binns et al., 2001).

2.2.6 Application of Tween 20 plus MJ on concentration of FCGs

A randomised block design was used in a growth chamber with the similar conditions described in Section 2.2.2. Seeds (n=6) of Quoll were sown in the pot (125 mm diameter) containing UC potting mix. In this experiment three treatments were

tested with 5 replicates for each; 1) Control plants (MQ water containing 0.1% Tween 20), 2) 0.1 mM MJ solution and 3) 0.1 mM MJ plus 0.1% Tween 20. Plants in each pot (75 × 75 mm) were received 50 ml of solutions by drenching, 15 days after planting. Plants were harvested 3 days after treatment and immediately frozen in liquid nitrogen. Then the samples were ground and FCGs were extracted using the method described in Section 2.2.3. Analysis was carried out using gradient elution of HPLC mentioned in Section 2.2.3.

2.2.7 Changes in concentration of FCGs after MJ application

The seeds of three oat cultivars, Possum, Quoll, Wintaroo, were sown in UC potting mix (four seeds per pot, 50 × 50 mm). Five days after sowing, two seedlings were retained. 0.1 mM MJ solution was made and 10 ml solution was drenched onto the surface of the soil around the plants 15 days after planting. Control plants received 10 ml MQ water. Plants were harvested at different days (1 to 7 days) after MJ application. Harvesting, grinding, FCGs extraction and analysis were performed as described in Section 2.2.3.

2.2.8 Cloning and induction of a *PR5* gene in oat by MJ

2.2.8.1 Plant material

Seeds of oat cv. Quoll were grown in 10 plastic pots (150 mm diameter) containing five plants each as described in Section 2.2.1. After three weeks, five pots were drenched with 0.1 mM MJ. Plants were harvested 24 hr after treatment, from three pots each of control and MJ treated plants. Roots and shoots were separated and frozen in liquid nitrogen prior to extraction for flavonoid analysis at 340 nm. Plants from the

remaining two pots of each treatment were separated into shoots and roots and immediately frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

2.2.8.2 RNA extraction and quantification

Total RNA was extracted from shoots and roots of Quoll plants by Trizol, according to manufacturer's instructions (Invitrogen, Mount Waverley, Victoria, Australia). The plant material (0.2 g) was ground in liquid nitrogen in a pestle and mortar. One ml Trizol was added to the ground samples that were in 2 ml microfuge tubes and incubated in room temperature (RT) for 5 min. Then 0.2 ml chloroform was added to homogenised sample and the samples were shaken vigorously by hand for 15 sec and incubated for 3 min at RT. The tubes were centrifuged at 15,700 *g* at 4°C for 15 min. The aqueous phase was transferred to a clean 1.5 ml tube and RNA was precipitated with 0.5 ml isopropyl alcohol and incubated for 15 min at RT. Then the tubes were centrifuged at 8,600 *g* at 4°C for 10 min. The supernatant was discarded and RNA pellet was washed with 1 ml of 75% ethanol followed by vortexing and centrifuging at 6,500 *g* at 4°C for 5 min. The RNA pellet was dried in a fume hood for 15 min and redissolved in 30 µl RNAase-free water.

RNA concentration was measured by either spectrophotometer (Biospec-mini, Shimadzu, Japan) at 260 and 280 nm or NanoDrop spectrophotometer (NanoDrop® ND-1000, USA).

Quality of the RNA was assessed by running on a 1% agarose/ formaldehyde gel (0.6 g agarose melted in 44.1 ml MQ water and then 2.6 ml 10x MOPS, 0.2 M MOPS, 0.05 M NaAc, 0.01 M EDTA, pH 7.0, and 9.9 ml formaldehyde were added and mixed by swirling). RNA samples were prepared for electrophoresis by adding 32 µl of the stock solution (for 8 samples; 40 µl 10x MOPS, 70 µl formaldehyde, 200 µl formamide, 12 µl ethidium bromide, 1 mg/ml) to a tube containing 10 µg RNA mixed

in 2 µl loading buffer. The gel was run at 76 V for about 2 hr till the dye migrated to two-third of the gel length. The gel was visualised using UV light and photographed with a ruler. Finally it was put in the container containing 10x SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) and agitated slowly for 30 min.

2.2.8.3 cDNA synthesis and reverse transcription polymerase chain reaction

Genomic DNA was removed from RNA samples using the DNA free kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. 10 µg RNA, 1 µl 10x DNase I buffer, 1 µl DNase I and water (to 10 µl) were mixed well in an 0.5 ml microfuge tube and incubated at 37°C for 30 min. 5 µl DNase inactivation regent was added to the sample and mixed. The tube was spun at 11,200 *g* for 1 min to pellet the inactivate slurry. The clear aqueous phase was used for cDNA synthesis.

First strand cDNA was synthesised using Superscript III according to the manufacturer's instructions (Invitrogen, CA, USA). Two µg RNA (equal to 6 µl), 1 µl oligo (dT) primer (50 µM), 1 µl 10 mM dNTP were mixed and the volume was made up to 13 µl by adding 5 µl sterile water and incubated at 65°C for 5 min followed by putting straight onto ice. Seven µl of a master mix (4 µl 5x first strand buffer, 1 µl 0.1 M dithiothreitol (DTT), 1 µl RNase OUT and 1 µl Superscript III) was added to the tube and incubated at 55°C for 1 hr. Then 30 µl sterile water was added to the tube and kept at -20°C.

To do reverse transcription - polymerase chain reaction 19 µl of a master mix (10 µl 10x loading buffer, 4 µl 50 mM $MgCl_2$, 2 µl 10 mM dNTP, 2 µl forward primer (100 ng/µl), 2 µl reverse primer (100 ng/µl), 0.4 µl Taq DNA polymerase and to volume 95 µl with MQ water) was added to each of 3 200 µl thin walled PCR tubes and either 1 µl water as a negative control, or 1 µl of first strand cDNA, or 1 µl of 1/20 diluted first strand cDNA were added to one of the tube. PCR reaction was performed

in a PCR machine (PTC-100, MJ research, Inc., MA, USA) according to the following program: 94°C for 2 min, 35 cycles of 94°C for 20 sec, 55°C for 30 sec, 72°C for 60 sec and then held at 23°C. Primers were designated to an oat *PR5* gene, GenBank accession number L39774, (forward: 5'-GAAGCAGTCGTCCAACATCA-3' and reverse: 5'-TGTCAACTACAACGGCTGGA-3'). PCR products were run on 1% agarose gel containing ethidium bromide at about 400 µg/L and observed under UV light.

2.2.8.4 Cloning

The re-amplified PCR product was cloned to pGEM®-T easy using Promega kit (Promega, Annadale, Australia) according to the manufacturer's instructions. Ligation was carried out using the following protocol: 5 µl 2x rapid ligation buffer, T4 DNA ligase, 2 µl pGEM®-T Easy vector (25 ng/µl), 1 µl PCR product, 1 µl T4 DNA ligase and 1 µl sterile water were mixed in an 0.5 ml microfuge tube by pipetting and incubation at RT for 1 hr. Transformation was carried out using heat shock method by adding 2 µl ligated vector to 50 µl *Escherichia coli* DH5α cells (Stratagene, CA, USA) and putting on ice for 20 min. Then, the tube was placed in a water bath adjusted at 42°C for 45 sec, returned immediately to ice and left for 2 min. Finally 900 µl SOC medium (2.0 g Bacto®-tryptone, 0.5 g Bacto®-yeast extract, 1 ml 1 M NaCl, 0.25 ml 1M KCl, 1 ml 2 M Mg^{2+} stock and 1 ml glucose bring to 100 ml with sterile water) was added to the tube containing cells and incubated for 1 hr at 37°C by shaking at 200 rpm. Two M Mg^{2+} stock was made with 20.33 g $MgCl_2 \cdot 6H_2O$ and 24.65 g $MgSO_4 \cdot 7H_2O$ and MQ water to final volume of 100 ml. 100 µl SOC media containing the ligation mix of *E. coli* transformed cells (after spinning the tubes containing the cells) was spread over the LB media plates containing ampicillin + isopropyl-1-thio-β-D-galactopyranoside

(IPTG) + 5 bromo-4-chloro-3-inodlyl-β-D-galactopyranoside (X-Gal) and incubated at 37°C overnight.

Luria Bertani (LB) + agar + IPTG + X-Gal + ampicillin were provided as follows: To make 1 L LB medium, 10 g Bacto®-tryptone, 5 g Bacto®-yeast extract and 5 g NaCl were dissolved in water and pH was adjusted to 7.0 with NaOH. To make LB + agar + ampicilin, 15 g agar was added to 1 litre of LB medium, autoclaved and cooled to 50°C, prior to addition of ampicillin at final concentration 100 µg/ml. Finally medium in each Petri dish was supplemented with 40 µl 20 mM IPTG and 40 µl X-Gal.

White colonies (n=24) were touched on a patch plate and used as a template for PCR amplification. A standard PCR reaction with primer PCR 1 (5'-CAC AGG AAA CAG CTA TGA CC-3') and PCR 2 (5'-GTC ACG ACG TTG TAA AAC GAC-3') were used. The patch plate was incubated upside down at 37°C overnight. The same PCR conditions and programme were used to amplify the *PR5* gene.

Positive clones were cultured in yellow cap (10 ml) tubes containing 2.5 ml LB liquid + ampicilin medium and rotated overnight at 37°C. The grown bacteria were divided to two parts, 800 µl was mixed with 200 µl sterile 80% glycerol and kept in -80°C and the remaining was used for plasmid purification. Plasmids were purified using a QIAprep Spin Miniprep kit (QIAGEN, Doncaster, Australia) according to the manufacturer's instructions.

The concentration of each sample after purification was obtained using a NanoDrop spectrophotometer. Samples (between 500-900 ng of purified plasmid DNA) were sequenced with 6.4 pmol of one of the gene specific primers. Nucleotide and predicted amino acid sequences of two clones were analysed using a National Center for Biotechnology Information (NCBI) program and alignments were performed using ClustalW (http://www.ebi.ac.uk/clustalw/).

2.2.8.5 Digoxygenin **PR5** *probe synthesis*

A digoxygenin (DIG) labelled probe was made in two rounds of PCR using protocol described in (Finckh et al., 1991). The PCR reaction was made as follows: for 2x 100 µl PCR reactions; 20 µl 10x buffer, 8 µl 50 mM $MgCl_2$, 10 µl 5 mM dNTPs, 2 µl forward primer (200 ng/µl), 2 µl reverse primer (200 ng/µl), 0.8 µl Taq polymerase, were mixed with water (165.2 µl). Plasmid DNA obtained from miniprep was diluted 1:1000 and 1:2000. One µl diluted DNA was added to 99 µl PCR reaction. The PCR program was: 94°C for 2 min, 55 cycles of 94°C for 20 sec, 55°C for 30 sec, 72°C for 30 sec and then held at 23°C. PCR products were run on a 1% agarose gel and visualised under UV light. To do the second round, the following PCR reaction was made: for 2 PCR reactions; 10 µl 10x buffer, 4 µl 50 mM $MgCl_2$, 2 µl DIG labelling mix (Roche Diagnostics, Mannheim, Germany), 1 µl 200 ng/µl reverse primer, 0.4 µl Taq polymerase, were mixed with water (77.6 µl). Five µl first round product was added to the PCR reaction. The PCR programme was performed as in the first round.

2.2.8.6 Probe quantification

To quantify the labelled probe, a serial dilution of the labelled probe was made (1:5, 1:50, 1:500, 1:5,000, 1:50,000) in water and compared to control DNA (Roche Diagnostics, Mannheim, Germany). One µl of each dilution was blotted onto nylon membrane (#NOOH00010, Osmonics. Inc., MA, USA). DNA was fixed to the membrane by cross-linking using UV irradiation (BIO-RAD). The membrane was wetted in a small amount of Buffer 1 (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and then it was incubated in Buffer 2 which is 1% Block in Buffer 1. 1% Block was made by dissolving 10 g Block (Roche Diagnostics, Mannheim, Germany) in 100 ml DEPC treated maleic acid buffer (Buffer 1, 0.1 M maleic acid pH 7.5, 0.15 M NaCl). One µl anti-DIG antibody was added to Buffer 2 and incubated for 5 min at RT. The

membrane was washed twice in Buffer 1 for 5 min at RT by shaking. It was incubated in Buffer 3 (0.1 M Tris·Cl at pH 9.5, 0.1 M NaCl) for 2 min. The membrane, probe side up, was placed onto a cut plastic bag. The chemiluminescent substrate, CSPD (Roche Diagnostics, Mannheim, Germany), was diluted in Buffer 3 (1:100) and applied over the membrane evenly. Excess CSPD was removed prior to exposing to X-ray film and incubation at 37°C. The probe was quantified by comparing the dilutions to the DNA control.

2.2.8.7 Northern blot and hybridization of Avena sativa PR5 *(AsPR5)*

A northern blot was performed with the oat *PR5* gene. RNA from the gel was transferred to the nylon membrane using capillary action in 10x SSC, (for 1 L 10x SSC; 175.3 g NaCl, 88.2 g Na citrate at pH 7.0). RNA was fixed to the membrane by UV cross-linking (BIO-RAD) at 150 mJ. Excess salt was washed from the membrane by slow shaking in a container containing 2x SSC for 5 min. Hybridization was achieved as follows:

Pre-hybridization was carried out by adding 9 ml pre-hybridisation (Pre-hyb) solution containing 50% (v/v) formamide, 5x SSPE (3.6 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA pH 7.4), 2% Block in maleic acid buffer, 0.1% lauroyl sarcosine and 7% SDS to membrane in hybridization bottle and put into the hybridization oven (RATEK, Australia) at 42°C for 1 hr. 10 µl/10 ml DIG probe was added to pre-hyb solution in the hybridization bottle and hybridized at 42°C overnight. Post hybridization washes were twice in 2x SSC- 0.1% SDS for 5 min at RT by shaking. Then the membrane was washed twice in 0.5x SSC-0.1% SDS for 15 min at 65°C. Detection was performed by equilibrating the membrane in Buffer 1 for 2 min. The membrane was added to a bottle containing 10 ml Buffer 2. The bottle was incubated for 30 min at RT. One µl anti-DIG antibody was added to buffer 2 and left for 30 min. Anti-DIG Buffer 2 was discarded

and membrane was washed twice with Buffer 1 for 15 min prior to incubation in Buffer 3 for 2 min. The CSPD was diluted 1:100 in Buffer 3 and visualisation was as before.

2.2.9 Pathogens and culture methods

A range of fungi and bacteria (Table 2.1) were cultured for use in bioassays. A 5 mm diameter plug of a given fungus (respectively, *Fusarium graminearum, Rhizoctonia solani, Pyrenophora teres* f. *teres, P. teres* f. *maculata* and *P. avenae)* was transferred to potato dextrose agar (PDA) media (Difco^{TM}) and incubated at 25°C in the dark until mycelia were approximately 25 mm from the edge of the plates. *Mycosphaerella pinodes* was grown on PDA and incubated at 25°C, with 12 hr light/dark cycle. *Pyrenophora tritici-repentis* was grown on V8 medium, 200 ml Campbells V8 Juice, 3 g $CaCO_3$, 20 g Bacto-agar (Cheong et al., 2004).

To culture bacteria, soft buffer agar (SBA; 60% 0.2 M NaH_2PO_4, 40% 0.2 M Na_2HPO_4 and 7 g/L Bacto-agar adjusted to pH 7) held at about 60°C was inoculated with 300 µl suspension from 5-day-old broth culture of bacteria. The concentration of bacteria was adjusted to 5×10^8 cells ml^{-1} using a haemocytometer and the optical density at 600 nm (OD600) was recorded. Soft buffer agar was mixed with the bacteria by swirling. Suspensions of *Pseudomonas* were poured over Kings B medium (20 g Bacto Proteose Peptone No. 3, 1.96 g $K_2HPO_4 \cdot 3H_2O$, 1.5 g $MgSO_4 \cdot 7H_2O$, 10 g glycerol and 20 g Bacto-agar in 1 liter MQ water adjusted at pH 7.2). Suspensions of *Rathayibacter* and "Corynebacterium" were poured over M523 medium (10 g sucrose, 2 g casamino acids, 2 g yeast extracts, 2 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 15 g agar in 1 L MQ water) to give a uniform overlay.

Table 2.1 List of fungi and bacteria used in bioassays

Organism	Isolate /Strain	Original host	Source
Pyrenophora avenae	DAR 33699	*Avena sativa*	OAI
P. avenae	DAR 32137	*Avena sativa*	OAI
P. teres f. *teres*	43/96/1	*Hordeum sativum*	SARDI
P. teres f. *maculata*	19/98	*Hordeum sativum*	SARDI
P. tritici-repentis	Mar04	*Triticum aestivum*	SARDI
Fusarium graminearum		*Triticum aestivum*	SARDI
Rhizoctonia solani	317		SARDI
Mycosphaerella pinodes	12/01	*Pisum sativum*	SARDI
Pseudomonas syringae pv. *syringae*	CS201	*Pisum sativum*	SARDI
Pseudomonas syringae pv. *pisi*	CS200	*Pisum sativum*	SARDI
Rathayibacter tritici	CS21	*Triticum aestivum*	SARDI
"Corynebacterium agropyri"	CS35	*Agropyron smithii*	SARDI

OAI, Orange Agricultural Institute, NSW, Australia
SARDI, South Australian Research and Development Institute, SA, Australia

2.2.10 Bioassays of fungal pathogens

Bioassays were performed using autoclaved filter paper discs (6 mm diameter). Each paper disc was loaded with 5 × 10 µl of a 45% methanolic crude shoot extract of oat cv. Quoll that had been analysed by HPLC. The methanol was fully evaporated between applications. Methanol (45%) and/or MQ water was applied to the paper discs as a control. In some experiments, the crude extract of wheat shoots was applied for comparison. To test for fungal inhibition a loaded paper disc was placed on the PDA medium about 15 mm from the margin of the growing mycelia and 10 mm from the edge of the plate. Plates were incubated at 25°C and the radius zone of inhibition

(distance between the centre of the paper disc and margin of the inhibited mycelium) was recorded.

The gradient concentration of the shoots of oat crude extract (50 µl, 100 µl and 150 µl) was applied on *P. teres* f. *teres* in two replicates. In this experiment, 8 mm diameter paper disc was used due to high volume of some treatments. The growth of fungus was monitored every 6 hr and photographed every 12 hr.

2.2.11 Bioassays of bacterial pathogens

A loaded paper disc was prepared as described above and placed on the soft buffer agar inoculated with the respective bacteria. The plate was inverted and incubated at 25°C for about three days and the diameter of the inhibition zone was recorded.

2.2.12 Induction of active compounds in the oat shoots by MJ

In a preliminary experiment, a bioassay experiment was performed using methanolic crude extract of the material obtained in Section 2.2.6 in three replicates. Crude methanolic extract was produced from 60 mg of ground shoots of each treatment and 50 µl was loaded onto paper filter disc. *P. teres* f. *teres* was used in this experiment.

In the second experiment, the seeds of two oat cultivars, Mortlock and Potoroo were sown in the pots containing UC potting mix in 12 replicates in the growth chamber. Three weeks after sowing, replicates were divided into two groups, one group as control plants and another group moved to a growth chamber with similar condition to be treated with 0.1 mM MJ.

The plants were harvested 3 days after MJ treatment. Methanolic crude extract of the shoots was extracted as explained previously. Five mg/100 µl crude extract of

each treatment was prepared and 60 µl was loaded onto 5 mm filter paper disc. The loaded paper discs were put onto PDA medium, 10 mm away from grown mycelium of *P. teres* f. *teres*. This experiment was carried out in four replicates. The inhibition radius zone of each paper disc was recorded 15, 18 and 21 hr after inoculation.

2.2.13 High voltage paper electrophoresis

Methanolic extracts of shoots were fractionated by preparative high voltage paper electrophoresis (HVPE). Shoot extracts (300 µl) were applied along the central axis of a piece of cellulose paper (115-285 mm; 1 Chr; Whatman, UK). Several independent electrophoresis runs were performed to accumulate sufficient material. HVPE paper was placed over a glass rod to minimize surface contact between the two wells of the electrophoresis tank (Model 715;b Betheseda Research Laboratories, Gaithersburg, MD, USA) containing 0.1 M borate buffer (3.09 g H_3BO_3, 1.756 g NaOH dissolved in 1 L water and adjusted to pH 10). Electrophoresis was performed for 45 min at 300 V. Fractions were identified under UV light (UVGL-25, CA, USA) at 254, 366 nm. Relative mobility (RM_{OG}) was estimated using compounds in a standard solution (2 mg/ml orange G, 2 mg/ml xylene cyanol F.F, 2 mg/ml sodium azide, 10 mg/ml fructose and 10 mg/ml 2´-deoxyadenosine in water (Tate, 1981). Orange G (Sigma-Aldrich, St. Louis, MO, USA) was applied as an anionic standard and 2´-deoxyadenosine (Sigma-Aldrich, St. Louis, MO, USA) as a neutral standard (Tate, 1981). Bands were excised and fractions were recovered by eluting with 45% methanol. The fractions were dried under vacuum and dissolved in 4 ml deionized water. Fractions were then passed through a C18 column (SepPak Classic; Waters Corporation, Milford, MA, USA) to remove borate buffer and the compounds were eluted from the column with 5 ml of methanol. The samples were dried in the vacuum

concentrator and then redissolved in 500 µl 45% methanol. Samples were analysed by HPLC and 50 µl was assayed against different fungi for biological activity.

2.2.14 Detection, purification and bioassays of saponins

The shoots of MJ treated Quoll plants were ground and extracted using methanol, hexane, butanol and water as described in Section 2.2.3. The aqueous phase of the extraction was loaded onto 0.5 g RP-C18 preconditioned cartridge and bound material was washed with 20 ml MQ water, then sequentially eluted with 11 × 8 ml 40% methanol and finally with 4 × 8 ml 60% methanol. The compounds in the aqueous phase were analysed by thin layer chromatography (TLC) separated initially with butanol: ethanol: ammonia, 7:2:5 v/v/v, (Hostettmann and Marston, 1995), then with chloroform: methanol: water (140:70:11) and finally the plates were stained with *p*-anisaldehyde solution (Wubben et al., 1996) and baked at 130°C. The 40% methanol fractions containing single band was pooled and used for bioassay against *P. teres*. f. *teres* and liquid chromatography-mass spectrometry (LC-MS) for identification.

The butanol phase, after drying, was redissolved in water and loaded onto a 10 g RP-C18 column (SepPak; Corporation, Milford, USA) and fractions were obtained using a range of methanol concentrations (0, 40 and 60% methanol). For further purification of the saponins, the 60% methanol elution was collected, dried and dissolved in 100% methanol. This was loaded on a 160 × 20 mm C18 column (silica gel 60, Merck KGA, Darmstadt, Germany) and eluted with a solvent system of chloroform: methanol (8:2 v/v). A total of 43 fractions (8 ml) were collected. Alternatively, the 60% methanol elution of butanol phase was separated by preparative TLC using aluminium-backed plates (0.20 mm, silica 60, Merck) with chloroform: methanol: water (70:35:5.5 v/v/v) as the solvent. Collected fractions were analysed by

TLC using the same separation method as for preparative TLC. Fractions with similar compounds based on R_f (relative to front) were pooled and tested in bioassays or analysed by HPLC-mass spectrometry (LC-MS). The butanol phase was fractionated, each of the *p*-anisaldehyde positive compounds were prepared to a final concentration of 10 µg/µl. *Pyrenophora teres* f. *teres* was treated with 40 µl of each compound and the same concentration of crude extract, in three replicates. The schematic of the purification and identification of saponins and provision of samples for bioassay is described in Figure 2.1.

Bioassays with the saponins were carried out on *P. teres* f. *teres* as detailed above. The fungus was treated with 50 µl of each of four different concentrations of aqueous phase (10, 20, 40 and 80 µg/µl), crude extract (10 µg/µl) and 45% methanol as the control.

2.2.15 LC-mass spectrometry identification of saponins

Mass spectrometry (MS) was carried out with an API-300 triple quadrupole mass spectrometer equipped with an electrospray (ES) ion source (MDS-Sciex, Concord, ON, Canada). Compounds in the active HVPE fractions of crude extract were separated on reversed-phase column (Synergi Hydro-RP Phenomenex, Torrance, CA; 4 µm, 150 × 2 mm) with a flow rate of 180 µl/min for 60 min and an injection volume of 20 µl in 90% Solvent A (5% formic acid in water) and 10% Solvent B (formic acid: acetonitrile: water; 5:80:15 v/v/v). Gradient elusion was performed by increasing from 10 to 35% Solvent B over 35 min, then 35 to 60% over 15 min, held for 6 min and then increased to 100% Solvent B over 4 min. The eluent from the LC was split using a

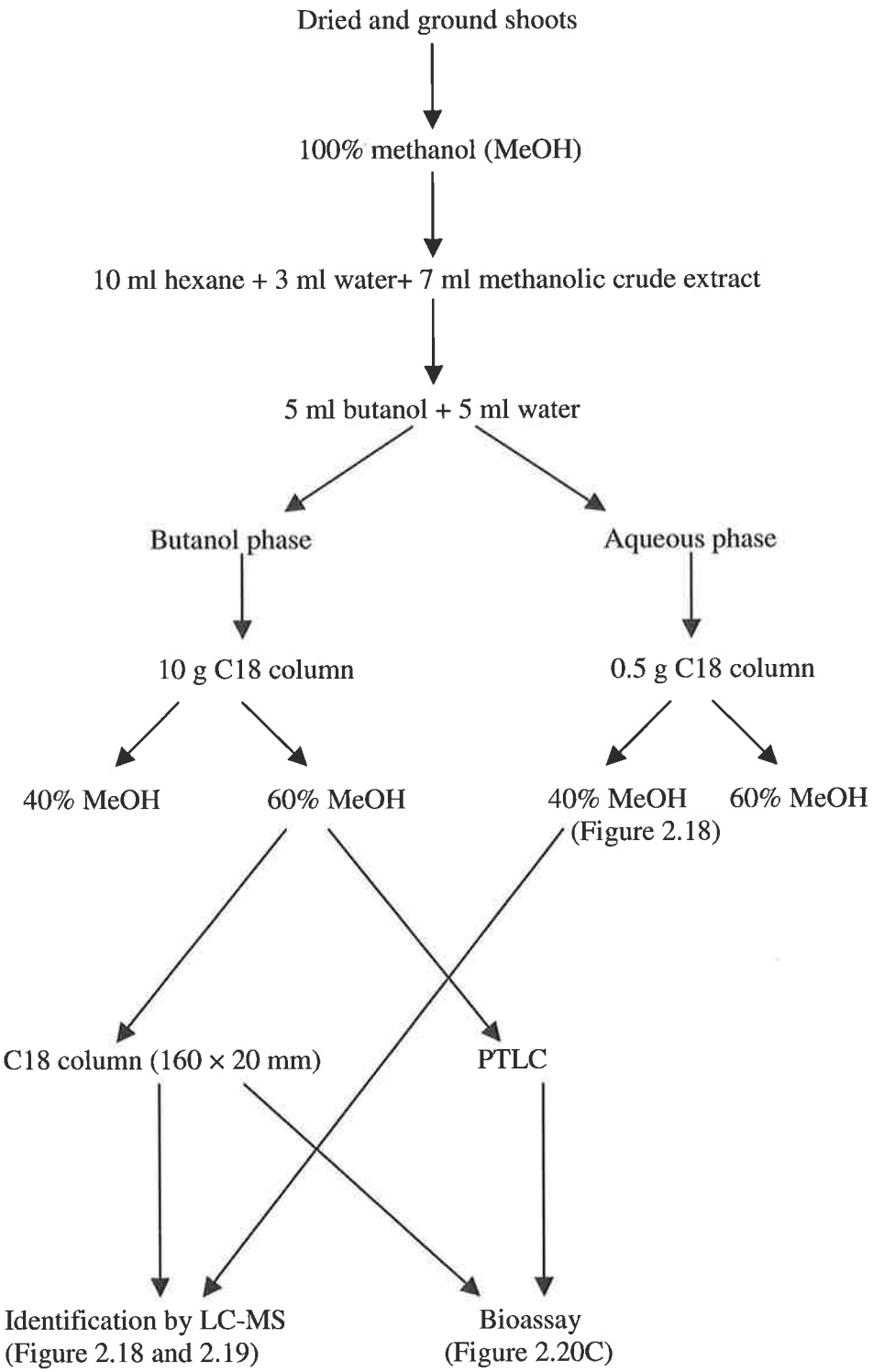


Figure 2.1 A schematic of the purification, identification and accumulation (for bioassay) of saponins in oat shoots. In all cases, TLC was performed to verify performance of purification step.

T-piece such that 22.5% of the total flow was delivered to the mass spectrometer and 77.5% to the UV detector (HP 1100, Agilent) monitoring at 254 nm and 340 nm. The mass spectrometer was operated in either positive or negative ion scan mode and scanned from *m/z* 100 to *m/z* 2000 with a step size of 0.2 Da and dwell time of 0.25 msec.

The ES needle, orifice and ring potentials were set at 5,000 V, 30 V and 250 V, respectively. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 12 units, respectively. The mass spectral data was processed using Bio-Multiview (Version 1.2β3, PE Sciex) and Analyst (Version 1.4) software.

To improve identification of the purified saponins, LC-MS was modified as follows. The elution profile started with a hold at 10% Solvent B for 5 min, increasing to 100% Solvent B from 5 to 35 min and held at 100% Solvent B for 30 min. The mass spectrometer was scanned from *m/z* 50 to *m/z* 2000. The samples were monitored at 200 nm and 210 nm.

2.2.16 Induction of active compounds in the oat shoots by homogenisation

Saponins were extracted from the shoots of 12-day-old Quoll seedlings by extracting the shoots for 1 hr with three volumes of hot methanol (60°C) as described by Wubben et al. (1996). To extract avenacoside A and B, fresh leaves that were folded once or twice in a 10 ml tube were exposed to hot methanol (60°C) directly after harvesting. To extract 26-DGA and 26-DGB, oat shoots were homogenised in one volume MQ water using a plastic homogeniser at room temperature. After 1 hr incubation at RT, they were extracted with three volumes hot methanol for 1 hr. After centrifugation at 5,500 *g* for 15 min and filtration through Whatman filter paper No 541

(55 mm), the clear supernatant containing either avenacoside A and B or 26-DGA and 26-DGB was dried under vacuum (Wubben et al., 1996). To give a concentration of 5 mg/100 µl, 15 mg of each sample was dissolved in 300 µl 45% methanol. Sixty µl of each extract was loaded onto paper disc in four applications 15 µl of each and the methanol evaporated between applications. The loaded paper discs were placed 15 mm away from the grown mycelia of *P. teres* f. *teres* (in four replicates). Forty five per cent methanol was used as a negative control. Measure of activity against the fungi was provided by calculating the radius inhibition zone.

2.2.17 Statistical analysis

Analysis of variance was performed using Genstat (Version 6.1; VSN International Ltd, Oxford, UK). Mean analysis was carried out by Duncan's multiple range test by combining control and MJ treated data and least significant differences (LSD) were also calculated. A paired *t*-test was also used to test for significant difference is in FCGs concentration between MJ treated and control plants.

2.3 Results

2.3.1 Separation and identification of flavonoids

Identification and quantification of oat FCGs has previously been monitored during HPLC using UV detection at either 254 nm or 340 nm (Soriano et al., 2004). The isocratic method (Figure 2.2A) at 254 nm has significantly shorter run times (10 min) compared to the gradient method (Figure 2.2B) at 340 nm (25 min). The isocratic method, monitored at 254 nm, produces a major peak at retention time R_t=4.3 min (Figure 2.2A). This retention time is consistent with earlier reports for flavonoids (Soriano et al., 2004). Individual flavone-*C*-glycosides can be separated with a gradient method to give three major fractions at R_t=10.3 (Fraction 1), R_t=10.8 (Fraction 2) and R_t=11.1 (Fraction 3) as shown in Figure 2.2B. The absorbance spectra of each fraction in Figure 2.2B was identical to the three different flavone glycosides (Figures 2.2C, 2.2D and 2.2E) identified by mass spectrometry (Soriano et al., 2004). Comparison of the localised absorbance maxima of each fraction and the relative order and magnitude of each eluted fraction, suggests that they are luteolin-*C*-hexoside-*O*-pentoside (267, 291 and 349 nm), apigenin-*C*-hexoside-*O*-pentoside (267 and 366 nm) and *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside (267 and 366 nm), respectively.

To ensure that the results between the two methods, isocratic at 254 nm and gradient at 340 nm, are comparable, FCGs were quantified in five accessions, each with six replicates. A highly significant correlation (r=0.952) was found between data obtained from the 254 nm and 340 nm measurements, indicating that either wavelength can be used to estimate flavone-*C*-glycosides in oat shoots (Figure 2.3). Therefore, most of the following analyses for accessions screening were performed using the isocratic method with detection at 254 nm.

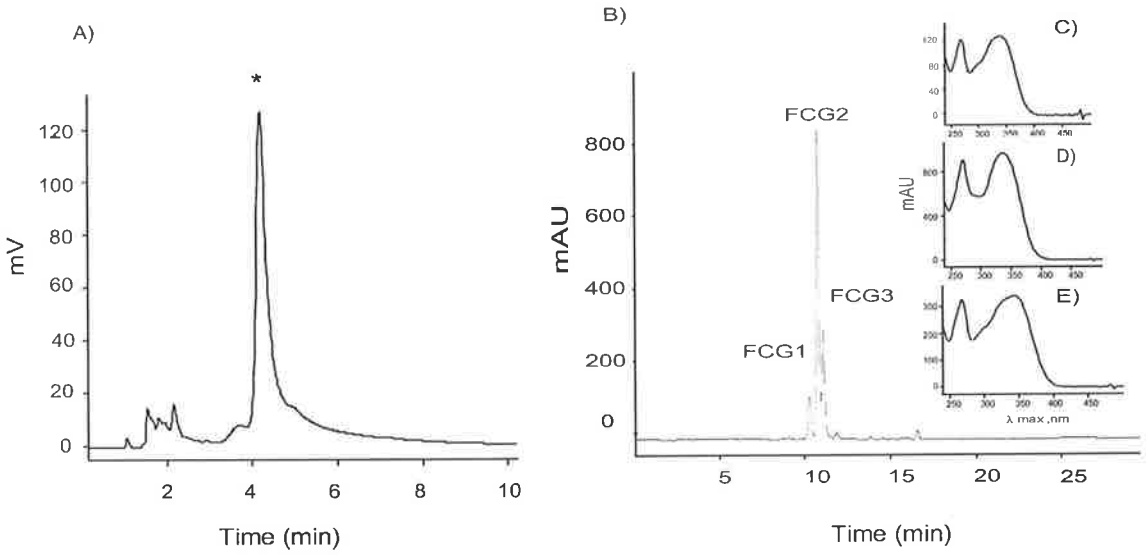


Figure 2.2 HPLC chromatogram and absorbance spectra of the flavonoids in oat (*Avena sativa*) shoot. A) Flavonoids elute as a single major peak indicated by a star, at a retention time of R_t=4.3 min, when using an isocratic elution with 45% methanol at 254 nm. B) Flavonoids can be separated into 3 major peaks using gradient elution with formic acid as Solvent A and formic acid: acetonitrile: water as Solvent B; Peaks 1, 2, 3 contain luteolin-*C*-hexoside-*O*-pentoside (FCG1), apigenin-*C*-hexoside-*O*-pentoside (FCG2) and *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside (FCG3), respectively, based on the absorbance spectra (C, D and E) that are consistent with these previously characterised FCGs (Soriano et al., 2004).

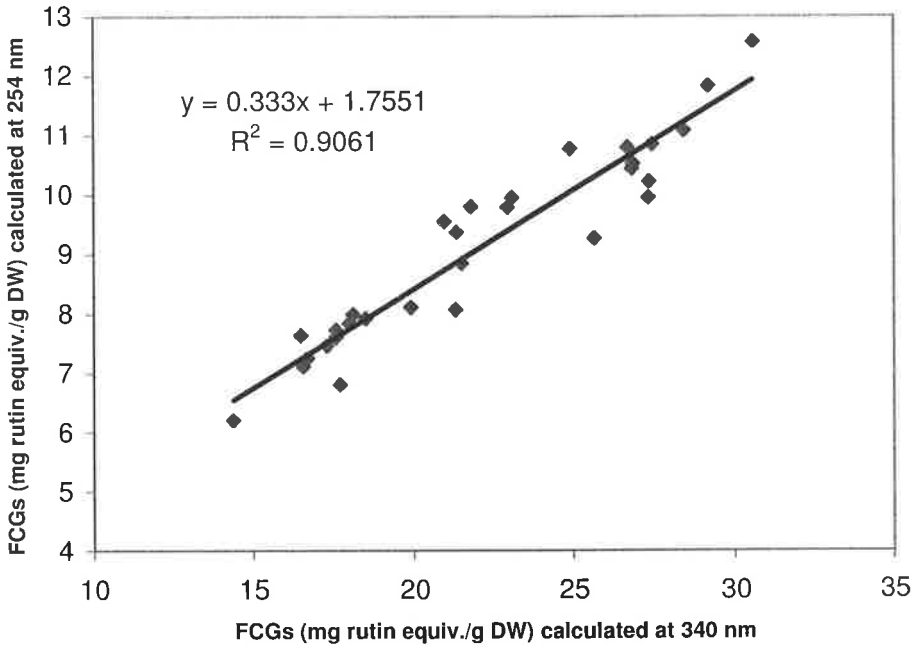


Figure 2.3 The correlation between the two methods (monitoring at 254 nm and 340 nm) for FCGs calculation in oat shoots using HPLC.

2.3.2 The effect of MJ treatment on flavone-*C*-glycosides

To determine the variation in FCG concentrations in oat, 115 oat accessions were screened in a pilot study. The accessions including important cultivars from the South Australian Oat Breeding Program such as Quoll and Potoroo and covered accessions from several countries representing a broad spectrum of environments. Of the initial 115 accessions, 43 did not germinate or insufficient material was available for extraction.

Among the 72 accessions analysed, IA91406-1 and UFRGS 988012-1, had the maximum and minimum constitutive levels of FCGs in the shoot at 12.83 and 1.32 mg (rutin equiv.)/g DW, respectively (Table 2.2). The maximum and minimum concentration of FCGs after MJ treatment was 10.45 and 2.01 mg (rutin equiv.)/g DW found in IA91406-1 and 91QK195, respectively. The average concentration of FCGs in

the shoots in control plants was 6.03 ± 0.23 and for MJ treated plants it was 6.01 ± 0.20 mg (rutin equiv.)/g DW. It was not possible to analyse the data following the planned augmented design because seeds of 43 samples did not germinate and therefore most of the replicates were lost. Although there were some accessions where FCGs were higher or lower in the MJ treated samples (Table 2.2), paired *t*-test showed treatment with MJ did not significantly induce FCGs in oat accessions.

From the 72 accessions analysed, 21 accessions (bold accessions in Table 2.2) were selected for the next experiment. The 21 accessions were chosen because they spanned a wide range of concentrations of FCGs and they had different responses to MJ (increased, decreased and unchanged). All 21 samples were grown in duplicate. Analyses of variance (ANOVA) showed significant differences among the accessions with respect to expression of FCGs in the shoot ($P<0.01$). The accessions could be classified into different groups based on FCG concentration in their shoots (Figure 2.4). Accessions IA91406-1 and Kingfisher had maximum and minimum concentrations of flavonoids, with 5.496 and 3.461 mg (rutin equiv.)/g DW, respectively. Again there was no significant difference in the mean concentration of FCGs in MJ treated and untreated plants (Figure 2.4).

To better control the environmental conditions and confirm the results, five of the 21 accessions (underlined accessions in Figure 2.4) were selected and tested in a randomised block design with three replicates in a growth chamber. Accessions IA 91406-1 and 91QK195 were selected for further analysis because of high concentration of FCGs and a slight reduction in concentration of FCGs in MJ treated plants. L'Gorskij was selected because it showed there was no change in the concentration of FCGs in response to MJ. Potoroo was selected because it showed the largest increase in FCGs in response to MJ. Finally, Quoll was chosen because Quoll is an important

cultivar in the South Australian breeding program and is also representative of accessions with low level of FCGs (level e). Analysis of the FCGs was carried out by HPLC at 254 nm. There was significant genetic variation of FCG concentration in oat accessions and mean comparison ($P<0.05$) revealed that these accessions could be classified into three groups (Figure 2.5). Accession IA91406-1 had the highest concentration of FCGs with 11.26 mg (rutin equiv.)/g DW and accession 91QK195 had the lowest concentration of FCGs with 7.57 mg (rutin equiv.)/g DW in their shoot. It should be noted that the rank of accession 91QK195 changed from the highest level of FCGs (level a, Figure 2.4) in the previous experiment to the lowest level (level c, Figure 2.5) in this experiment. The concentrations observed (Figure 2.5) are higher than the previous experiment (Figure 2.4), which possibly is due to differences in environmental conditions of growth chamber compared to the glasshouse.

There was no induction of FCGs by MJ. The lack of MJ induction was further investigated, as this was in contrast to a previous study by Soriano et al. (2004).

2.3.3 Effect of MJ application methods on the induction of FCGs

To determine whether the method of MJ application had an impact on FCGs induction, three different methods of MJ application (drench, volatile and spray) were tested on oat cv. Quoll. The concentration of FCGs was the same in control plants and in plants treated with MJ by vapour and spray treatment (Figure 2.6). MJ application by drenching significantly reduced the levels of FCGs (Figure 2.6).

Table 2.2 Flavone-*C*-glycosides concentration in the shoots of accessions sown in the pilot experiment (n=115).

Accessions	Origin	Flavone-*C*-glycoside mg (rutin equiv.)/g DW	
Sown in duplicate		Untreated	MJ treated
AC Ernie	Canada	7.37	6.21
AK-1	Japan	6.67	7.99[a]
Athabasca	Canada	8.91	5.89
Calibre	-	4.98	6.25
Carma	I.O.N.	4.75	7.23
CC6333	UK	ne	5.31
Dal	USA	ng	ng
Grise D'Hiver	France	5.90[a]	5.18[a]
IL2815	USA	5.31[a]	3.91[a]
Inta Majo	Mexico	5.10[a]	2.18[a]
L'Gorskij 1026	USSR	7.04	7.02
Matilda	Sweden	6.96[a]	6.36[a]
Wandering	Australia	6.22[a]	6.53[a]
Euro	Australia	4.43	4.49[a]
ND9308094	USA	4.64[a]	5.28[a]
Preston	USA	5.20[a]	5.28[a]
NZ2393	New Zealand	5.64	ne
Solva	UK	4.06	6.1
UFRGS987015-3	Brazil	4.08	5.10
8ZOP95	Canada	5.39[a]	4.27
A. sterilis (wild)	Australia A. N. 2	ng	ng
A. fatua (wild)	Australia A. N. 13	ng	ng
A. barbata (wild)	Australia A. N. 188	ne	ne
Sown a single pot			
AC Assiniboia	Canada	7.28	5.86
AC Baton	Canada	3.64	5.86
AC Fregeau	Canada	6.90	4.85
AC Lotta	Canada	4.70	5.67
AC Percy	Canada	6.97	5.78
Ajay	I.O.N.	ne	5.22
AK-5	Japan	4.20	5.24
Algerian	Algerian	ng	ng

[a] mean of two replicates; ng, no germination; ne, not enough material;
A. N., Accession number; I.O.N., International oat nursery.
Accessions in bold were selected for the next experiment.

Table 2.2 (continued)

Accessions	Origin	Flavone-*C*-glycoside mg (rutin equiv.)/g DW	
Sown a single pot		Untreated	MJ treated
Amagalon	USA	5.30	5.33
Appalaches	-	6.32	5.72
Arnold	-	6.91	5.57
Ariane I.N.R.A.	France	ne	4.64
Auron	-	6.04	ne
Bulgaria 84106130	Bulgaria	6.15	5.43
Avoine Nue-Nue Noise	France	6.43	6.08
Belmont AC	Canada	2.76	4.69
Blaze	I.O.N.	7.46	7.18
Border	USA	7.11	6.98
Bullion	UK	6.61	6.35
C-1/130	USA	ne	ne
C-1/370	USA	3.59	6.31
UC112	USA	7.78	4.46
UC145	USA	3.49	7.12
SAIA	Israel	ng	ng
CDC Dancer	Canada	6.95	6.96
Chapman	USA	5.26	6.33
Clintford	USA	8.17	7.08
CN 12497	UK	6.82	ne
Chinesischer Nackhthafer	-	ne	ne
Corondo	USA	6.01	5.83
CPI 115383	-	4.27	7.87
CPI 115397	-	6.31	5.81
Donald	USA	7.73	6.72
Dwarf 8	Japan	ne	8.27
Elan	USA	ne	ne
Flamings Nova	-	ne	ne
GA Mitchell	USA	ne	ne
Hendon	UK	ne	ne
IA91098-2	USA	ne	ne
IA91406-1	USA	12.83	10.45
IL92-6745	USA	ne	ne
Kingfisher	UK	9.42	8.71
Kalott	Sweden	10.51	ne
Madison	USA	8.44	8.10
Wintaroo	Australia	4.20	ne
Brusher	Australia	10.03	8.91
94046-57	Australia	8.21	ne

[a] mean of two replicates; ng, no germination; ne, not enough material;
A. N., Accession number; I.O.N., International oat nursery.
Accessions in bold were selected for the next experiment.

Table 2.2 (continued)

Accessions	Origin	Flavone-*C*-glycoside (mg rutin equiv./g DW)	
Sown a single pot		Untreated	MJ treated
Possum	Australia	9.32	7.75
Echidna	Australia	8.17	5.13
Potoroo	Australia	4.90	9.42
Mortlock	Australia	4.90	3.99
ND873364	USA	ne	3.54
ND931075	USA	4.39	3.84
Nasta	-	4.51	6.34
Neon	UK	8.20	ne
Ogle	USA	4.15	9.82
OH1022	USA	ne	7.35
Eurabbie	Australia	5.67	8.79
OT289	Canada	6.49	7.54
Ozark	USA	8.41	ne
Portage	USA	9.46	8.93
Quoll	Australia	8.88	7.51
Rossnagel 3259	Canada	7.22	4.37
NZ2101	New Zealand	7.31	6.29
NZ2742	New Zealand	1.59	4.46
Sanna	-	6.64	6.07
Trucker	USA	3.69	4.12
Tarahumara	Mexico	7.02	5.71
UFRGS-1	Brazil	4.06	4.14
UFRGS940257-1	Brazil	4.79	5.31
UFRGS940886-4	Brazil	5.12	4.03
UFRGS940548-5	Brazil	4.94	4.00
UFRGS988012-1	Brazil	1.32	5.12
1ZOP95	Canada	4.94	ne
4ZOP95	Canada	0.98	ne
13ZOP95	Canada	7.23	6.87
4671-581	Bosnia	4.58	4.30
91QK195	Australia	5.04	2.01
Yiddait	-	4.57	4.08
UPF775456	Brazil	ne	4.29
A. fatua (wild)	Australia A. N. 4	1.96	ne
A. fatua (wild)	Australia A. N. 26	4.16	ng
A. fatua (wild)	Australia A. N. 65	ng	ng
A. fatua (wild)	Australia A. N. 107	ne	ne
A. sterilis (wild)	Australia A. N. 14	ne	ng

[a] mean of two replicates; ng, no germination; ne, not enough material;
A. N., Accession number; I.O.N., International oat nursery.
Accessions in bold were selected for the next experiment.

Table 2.2 (continued)

Accessions	Origin	Flavone-*C*-glycoside (mg rutin equiv./g DW)	
Sown a single pot		Untreated	MJ treated
A. fatua (wild)	Australia A. N. 4	1.96	ne
A. fatua (wild)	Australia A. N. 26	4.16	ng
A. fatua (wild)	Australia A. N. 65	ng	ng
A. fatua (wild)	Australia A. N. 107	ne	ng
A. sterilis (wild)	Australia A. N. 14	ne	ng
A. sterilis (wild)	Australia A. N. 25	ng	ng
A. sterilis (wild)	Australia A. N. 73	ng	ng
A. sterilis (wild)	Australia A. N. 105	ng	ng
A. barbata (wild)	Australia A. N. 31	ne	ne
A. barbata (wild)	Australia A. N. 192	ne	ne
A. barbata (wild)	Australia A. N. 196	ne	ne
A. barbata (wild)	Australia A. N. 289	ne	ne

[a] mean of two replicates; ng, no germination; ne, not enough material;
A. N., Accession number; I.O.N., International oat nursery.

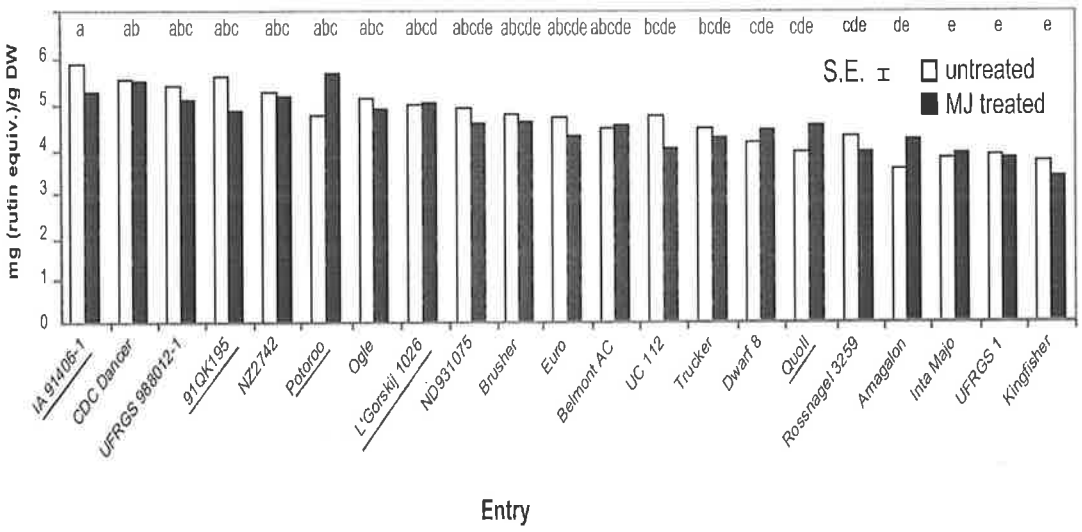


Figure 2.4 Flavone-*C*-glycoside concentrations in the shoots of 21 oat accessions. Concentration of FCGs in control and MJ treated plants grown in the glasshouse were estimated by HPLC (absorbance 254 nm). Means of accessions (n=4) were compared by Duncan's test and statistical differences among accessions ($P<0.05$) are indicated by different letters. SE represents the standard error of all samples. The underlined accessions were selected for the next experiment.

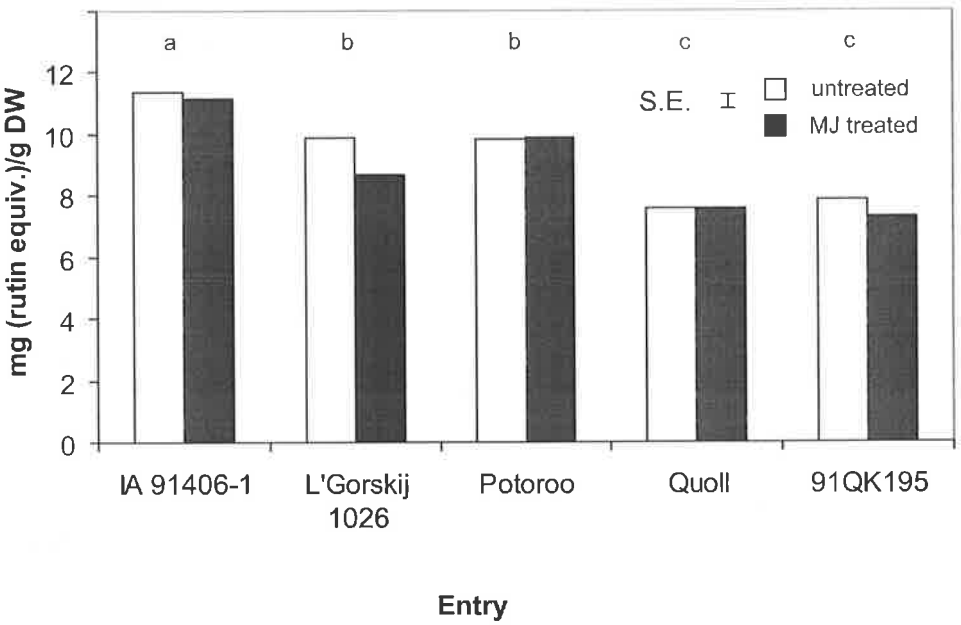


Figure 2.5 Flavone-*C*-glycoside concentrations in the shoots of five oat accessions. Concentration of FCGs in control and MJ treated plants grown in the growth chamber were estimated by HPLC (absorbance 254 nm). Means of accessions (n=6) were compared by Duncan's test, statistical differences among accessions ($P<0.01$) are indicated by different letters. SE represents the standard error of all samples.

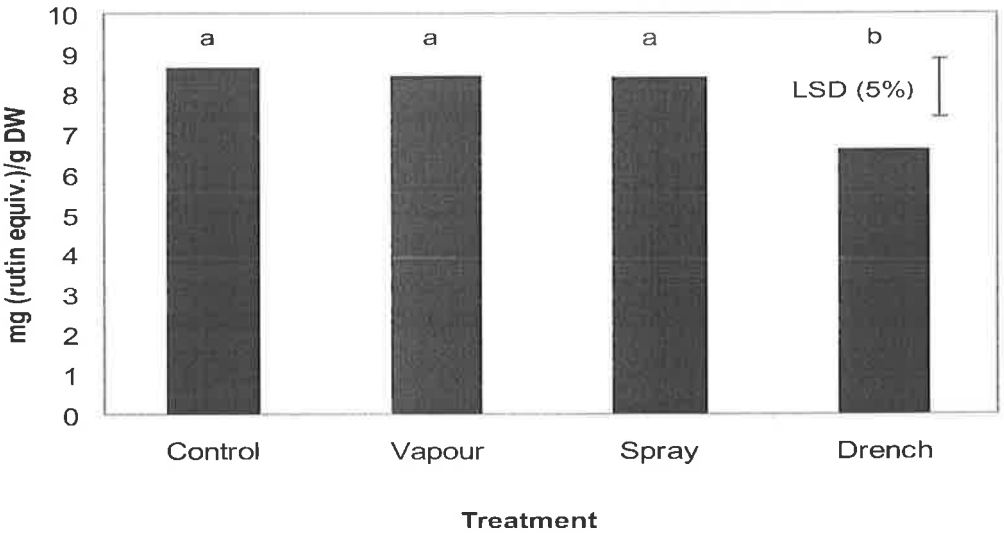


Figure 2.6 Method of MJ applications does not increase FCG concentration. Three different methods were used to apply MJ on oat cv. Quoll and the concentration of FCGs in the shoot was determined. Control plants received water. Means were calculated from four pots each containing six plants. Means were compared to the control using least significant difference (LSD). Statistical differences among treatments ($P<0.05$) are indicated by different letters.

2.3.4 Effect of Tween 20 on the induction of FCGs

Methyl jasmonate is often applied to plant roots with Tween 20 (polyoxyethylene-sorbitan monolaurate) as a non-biologically active surfactant (Vijayan et al., 1998; Shoji et al., 2000). Since Tween 20 was not used in the previous experiments and it might affect the absorption of MJ by oat roots, the addition of Tween 20 to MJ was tested on oat cv. Quoll. Results showed that there are no significant differences in the individual peaks (FCG1, FCG2 and FCG3) or total FCGs among the three MJ treatments, control (Tween 20), MJ and MJ plus Tween 20 (Figure 2.7). In other words, application of MJ with Tween 20 would not have affected the previous results.

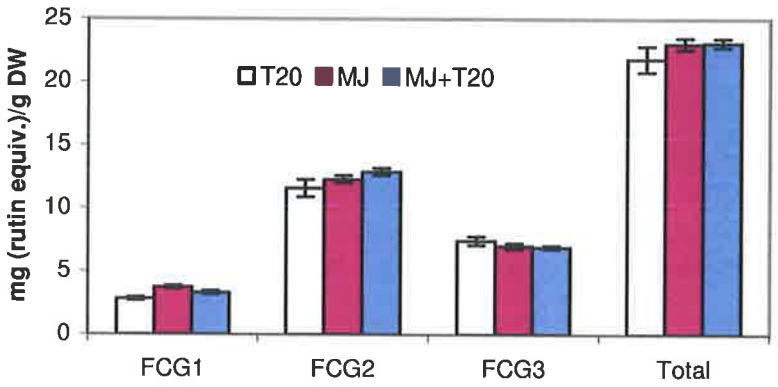


Figure 2.7 The effect of Tween 20 (T20) on the MJ induction of FCGs in oat cv. Quoll shoots. FCGs were separated by gradient elution (monitored at 340 nm) to resolve the three major FCGs in crude extracts. Mean of each peak of each treatment was calculated from five replicates. Bars represent the mean ± SE. FCG1 is luteolin-*C*-hexoside-*O*-pentoside. FCG2, is apigenin-*C*-hexoside-*O*-pentoside. FCG3 is *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside. SE represents the standard error of samples.

2.3.5 Changes in concentration of FCGs after MJ application

It is widely accepted that MJ responses are generally slow ranging from 20 hr to 5 days (Broeckling et al., 2005; Miksch and Boland, 1996; Suzuki et al., 2002). Therefore, an experiment was designed to test whether there was any spatial difference in induction of FCGs in the shoots after MJ treatment. The seeds of three oat cultivars (Possum, Quoll and Wintaroo) were sown in a RB design. Plants were harvested at 1, 2, 3, 4, 5, 6 and 7 days after MJ application. There was no significant change in the concentration of FCGs in all oat cultivars after MJ application. Peak representing FCGs showed non-significant increase (24%) in Quoll after two days and then reduced. FCGs in Wintaroo increased 2% two days after MJ application whereas, Possum did not show any increase after two days. Overall, there was no significant difference between MJ treated and control plants (day 0) in FCGs concentration and there was no overall pattern of MJ induction (Figure 2.8).

2.3.6 Cloning and induction of a *PR5* gene in oat by MJ

To provide evidence that MJ was biologically active, we tested the induction of mRNA for the pathogen-induced gene, *PR5* (thaumatin-like gene), in control and MJ treated Quoll plants. The *PR5* gene is upregulated by different elicitors in many plants (Xu et al., 1994; Lin et al., 1996). Although the *PR5* gene has not been tested for MJ induction in oat, it is expected to be induced by MJ because this response is conserved in both dicots (Xu et al., 1994; Hu et al., 2003) and monocots (Schweizer et al., 1997). Two cDNA clones of *AsPR5* were made and sequenced. Nucleotide sequences of two clones, *4AsPR5* and *8AsPR5,* were aligned to GenBank accessions, L39774, L39775, L39776 from *Avena sativa* (Figure 2.9). The predicted amino acid sequence from coding region of *AsPR5* (4 *AsPR5*) clone showed 100, 99 and 98% identity to GenBank proteins, RAST2-AVESA, RAST1-AVESA and RAST3-AVESA, respectively (Figure 2.10). The identity of *AsPR5* clone (8 *AsPR5*) was 100, 98 and 97% to RAST3-AVESA, RAST2-AVESA and RAST1-AVESA, respectively (Figure 2.10). The two clones, 4*AsPR5* and 8*AsPR5*, were similar with identity of 98% at DNA level. 8*AsPR5* clone was used as a template in PCR to make a probe. Northern blot analysis using a digoxigenin (DIG) probe indicated that PR5 gene transcripts were not induced in the shoots but were strongly induced in the roots of oat at 24 hr after MJ application (Figure 2.11). Since we had earlier only analysed shoot FCGs, we analysed the stored root tissue from the same experiment as in Figure 2.11 to investigate whether root FCGs were induced by MJ. There was no evidence of induction FCGs in the shoots of the control (26.79 ± 0.60) and MJ treated plants (26.76 ± 0.83). The level of FCGs in

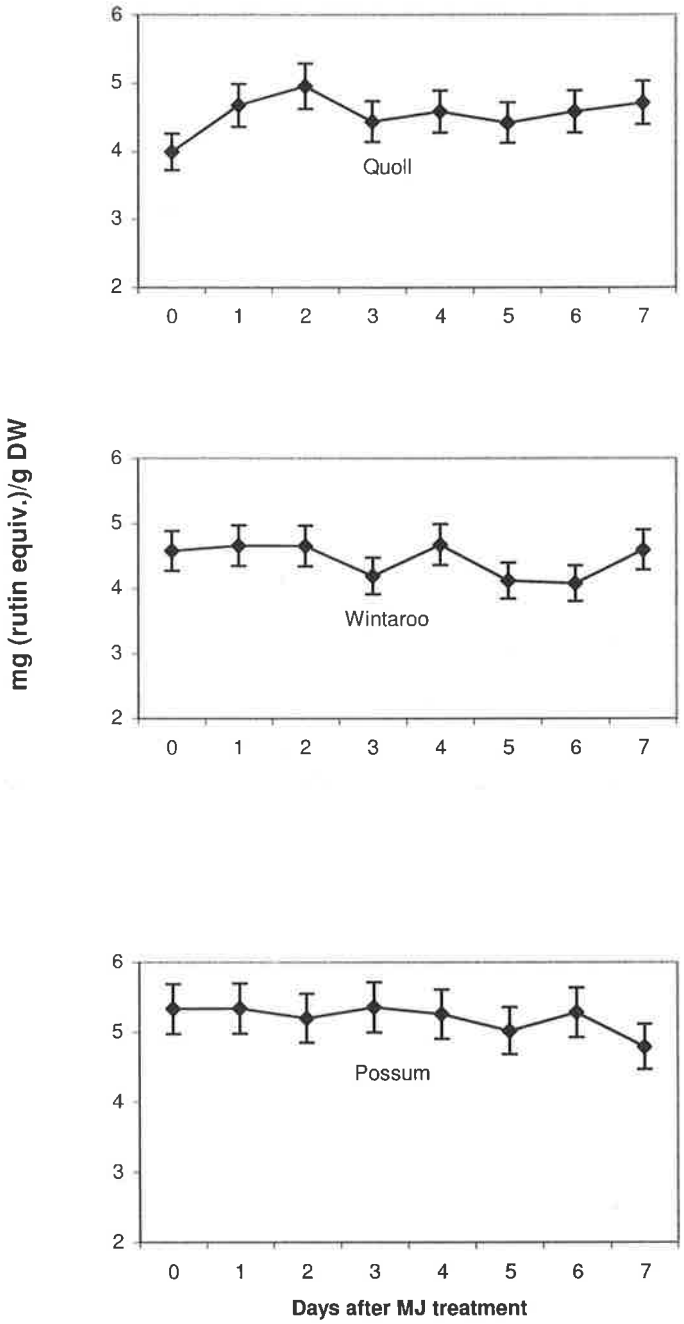


Figure 2.8 Changes in concentration of total FCGs after MJ application in three oat cultivars, Possum, Quoll and Wintaroo. Mean of each treatment calculated from three replicates. Bars represent the mean ± SE.

the roots was too low for accurate quantification (Figure 2.12). The roots contain abundant compounds at R_t>22 min that are not FCGs based on UV absorption and spectrum (data not shown).

2.3.7 Antimicrobial activity of shoot crude extracts

FCGs in oat are reported to be active against nematode (Soriano et al., 2004), but their antimicrobial activity has not been tested. Therefore, shoot crude extracts of oat were tested against bacteria and fungi. The growth of *P. teres* f. *teres* (Figure 2.13A) and *P. avenae* DAR 32137 (Figure 2.13B) was clearly inhibited by shoot crude extracts from Quoll shoots as were all other *Pyrenophora* tested (Figure 2.13D and Figure 2.13E) except *P. avenae* DAR 33699 (Figure 2.13C). Negligible inhibition was observed with Quoll crude extract in *M. pinodes* (Figure 2.13F). The growth of *P. avenae* DAR 32137, which is regarded as a compatible pathogen of oat, was inhibited with crude extract from oat shoots (Figure 2.13B). *P. avenae* DAR 33699 did not give any inhibition in this experiment (Figure 2.13C). No inhibition of *Fusarium graminearum, Rhizoctonia solani, Pseudomonas syringae* pv. *syringae, Pseudomonas syringae* pv. *pisi, Rathayibacter tritici* and "Corynebacterium agropyri" was found (data not shown).

The inhibitory effect of oat crude extracts on *P. teres* f. *teres* was much higher than that observed for wheat cv. Aus10348 extracts (Figure 2.13A). In contrast, both oat and wheat had similar, albeit low levels of inhibition against *M. pinodes* (Figure 2.13F).

As mentioned above, the growth of *P. teres* f. *teres* was inhibited by shoot crude extract of oat. To investigate the reaction of fungus with higher concentration of

```
L39774    GAAGCAGTCGTCCAACATCAACGTGCCCGCGGGCACCAGCGCCGGCAGGATATGGGGCCCACC
L39775    GAAGCAGTCGTCCAACATCAACGTGCCCGCGGGCACCAGCGCCGGCAGGATATGGGGCCCACC
L39776    GAAGCAGTCGTCCAACATCAACGTGCCCGCGGGCACCAGCGCCGGTAGGATATGGGGCCCACC
4AsPR5    GAAGCAGTCGTCCAACATCAACGTGCCCGCGGGCACCAGCGCCGGCAGGATATGGGGCCCACC
8AsPR5    GAAGCAGTCGTCCAACATCAACGTGCCCGCGGGCACCAGCGCCGGCAGGATATGGGGCCCACC
          ****************************************** ****************

L39774    GGCTGCTCCTTCAACAACGGGAGAGGGAGCTGCGCGACCGGCGACTGCGCCGGCGCGCTGTCC
L39775    GGCTGCTCCTTCAACAACGGGAGAGGGAGCTGCGCGACCGGAGACTGCGCCGGCGCGCTGTCC
L39776    GGGTGCTCCTTCAACAACGGGAGAGGGAGCTGCGCGACCGGCGACTGCGCCGGCGCGCTGTCC
4AsPR5    GGCTGCTCCTTCAACAACGGGAGAGGGAGCTGCGCGACCGGAGACTGCGCCGGCGCGCTGTCC
8AsPR5    GGCTGCTCCTTCAACAACGGGAGAGGGAGCTGCGCGACCGGAGACTGCGCCGGCGCGCTGTCC
          ** ********************************** ********************

L39774    TGCACCCTCTCCGGGCAGCCGGCGACGCTGGCCGAGTACACCATCGGCGGCTCCCAGGACTTC
L39775    TGCACCCTCTCCGGGCAGCCGGCGACGCTGGCCGAGTACACCATCGGCGGCTCCCAGGACTTC
L39776    TGCACCCTCTCCGGGCAGCCGGCGACGCTGGCCGAGTACACCATCGGCGGCTCCCAGGACTTC
4AsPR5    TGCACCCTCTCCGGGCAGCCGGCGACGCTGGCCGAGTACACCATCGGCGGCTCCCAGGACTTC
8AsPR5    TGCACCCTCTCCGGGCAGCCGGCGACGCTGGCCGAGTACACCATCGGCGGCTCCCAGGACTTC
          ************************************************************

L39774    ACGACATTTCGGTGATCGACGGCTTCAACCTCGCCATGGACTTCTCCTGCAGCACCGGCGTCG
L39775    ACGACATCTCGGTGATCGACGGCTACAACCTCGCCATGGACTTCTCCTGCAGCACCGGCGTCG
L39776    ACGACATCTCGGTGATCGACGGCTACAACCTCGCCATGGACTTCTCCTGCAGCACCGGCGTGG
4AsPR5    ACGACATCTCGGTGATCGACGGCTACAACCTCGCCATGGACTTCTCCTGCAGCACCGGCGTGG
8AsPR5    ACGACATCTCGGTGATCGACGGCTACAACCTCGCCATGGACTTCTCCTGCAGCACCGGCGTCG
          ******* *************** ************************************ *

L39774    CGCTCAAGTGCAGGGATGCCAACTGCCCCGATGCCTATCACCACCCCAACGATGTCGCCACGC
L39775    CGCTCAAGTGCAGGGATGCCAACTGCCCCGACGCCTATCACCACCCCAACGACGTCGCCACGC
L39776    CGCTTAAGTGCAGGGATTCCGGCTGCCCCGATGCCTATCACCACCCCAACGACGTCGCCACGC
4AsPR5    CGCTTAAGTGCAGGGATTCCGGCTGCCCCGATGCCTATCACCACCCCAACGACGTCGCCACGC
8AsPR5    CGCTCAAGTGCAGGGATGCCAACTGCCCCGACGCCTATCACCACCCCAACGACGTCGCCACGC
          **** ************* **  ********* ******************** **********

L39774    ACGCCTGCAACGGCAACAGCAACTACCAGATCACCTTCTGCCCATGAAGGCCAc--------c
L39775    ACGCTTGCAACGGCAACAGCAACTACCAGATCACCTTCTGCCCATGAAGACCCtatgccgcgc
L39776    ACGCCTGCAACGGCAACAGCAACTACCAGATCACCTTCTGCCCATGAAGACCTtacgccgcgc
4AsPR5    ACGCCTGCAACGGCAACAGCAACTACCAGATCACCTTCTGCCCATGAAGACCTtacgccgcgc
8AsPR5    ACGCTTGCAACGGCAACAGCAACTACCAGATCACCTTCTGCCCATGAAGACCCtatgccgcgc
          **** ****************************************** **          *

L39774    cgccaataaacggcgcgcgtatatacgaccgtataaatagtgtaaactgtgtaatgcttacat
L39775    cgccaataaccggcgtac--atatacgaccgtataaatagtgtaaactgtgtaatgcttacat
L39776    caccaataaacggc----------------------------------tgtgtaatgcttacat
4AsPR5    caccaataaacggc----------------------------------tgtgtaatgcttacat
8AsPR5    cgccaataaccggcgtac--atatacgaccgtataaatagtgtaaactgtgtaatgcttacat
          * ******* ****                                   ***************

L39774    cgcggtatcgtatatctgtattccagccgttgtagt---tgaca
L39775    cgcggtatcatatatctgtattccagccgttgtagtagttgaca
L39776    cgcggtatcgtatatctgtattccagccgttgtagt---tgaca
4AsPR5    cgcggtatcgtatatctgtattccagccgttgtagt---tgaca
8AsPR5    cgcggtatcatatatctgtattccagccgttgtagt---tgaca
          ********* *************************   *****
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Figure 2.9 Alignment of nucleotide sequences of two cDNA clones encoding PR5 proteins with three sequences of oat clones from GenBank. Primers were designed based on GenBank sequence L39774 and are shown in bold. Coding sequence is in upper case and the 3' untranslated region (3' UTR) is in lower case.

```
RAST1_AVESA   KQSSNINVPAGTSAGRIWGRTGCSFNNGRGSCATGDCAGALSCTLSGQPATLAEYTIGGSQDFYD
RAST2_AVESA   KQSSNINVPAGTSAGRIWGRTGCSFNNGRGSCATGDCAGALSCTLSGQPATLAEYTIGGSQDFYD
RAST3_AVESA   KQSSNINVPAGTSAGRIWGRTGCSFNNGRGSCATGDCAGALSCTLSGQPATLAEYTIGGSQDFYD
4AsPR5        KQSSNINVPAGTSAGRIWGRTGCSFNNGRGSCATGDCAGALSCTLSGQPATLAEYTIGGSQDFYD
8AsPR5        KQSSNINVPAGTSAGRIWGRTGCSFNNGRGSCATGDCAGALSCTLSGQPATLAEYTIGGSQDFYD
              ************************************************************

RAST1_AVESA   ISVIDGFNLAMDFSCSTGVALKCRDANCPDAYHHPNDVATHACNGNSNYQITFCP
RAST2_AVESA   ISVIDGYNLAMDFSCSTGVALKCRDANCPDAYHHPNDVATHACNGNSNYQITFCP
RAST3_AVESA   ISVIDGYNLAMDFSCSTGVALKCRDSGCPDAYHHPNDVATHACNGNSNYQITFCP
4AsPR5        ISVIDGYNLAMDFSCSTGVALKCRDSGCPDAYHHPNDVATHACNGNSNYQITFCP
8AsPR5        ISVIDGYNLAMDFSCSTGVALKCRDANCPDAYHHPNDVATHACNGNSNYQITFCP
              ******:******************:.*****************************
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Figure 2.10 Amino acid alignment of the two proteins predicted by the AsPR5 clones with other PR5 sequences from *Avena sativa.* Identical amino acids are shown by asterisks. Conserved amino acids are shown as colons. Semi-conserved amino acids are shown as dot. Nucleotide sequences of AsPR5 clones were translated by Sequence Manipulation Suite program (http://bioinformatics.org/sms2/trans_map.html). The amino acid sequences were aligned using "ClustalW" program. "RAST1_AVESA", "RAST2_AVESA" and "RAST3_AVESA" are the predicted proteins of clones, L39774, L39775 and L39776, respectively.

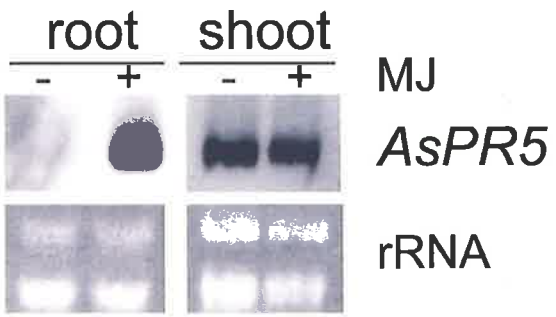


Figure 2.11 Northern blot analysis of the expression of *AsPR5* in oat cv. Quoll in response to MJ. Total RNA (10 μg) extracted from the shoots and roots of control and MJ treated plants was electrophoresed and hybridized with a DIG labeled *PR5* probe. Ribosomal RNA (rRNA) was stained with ethidium bromide to show equal loading.

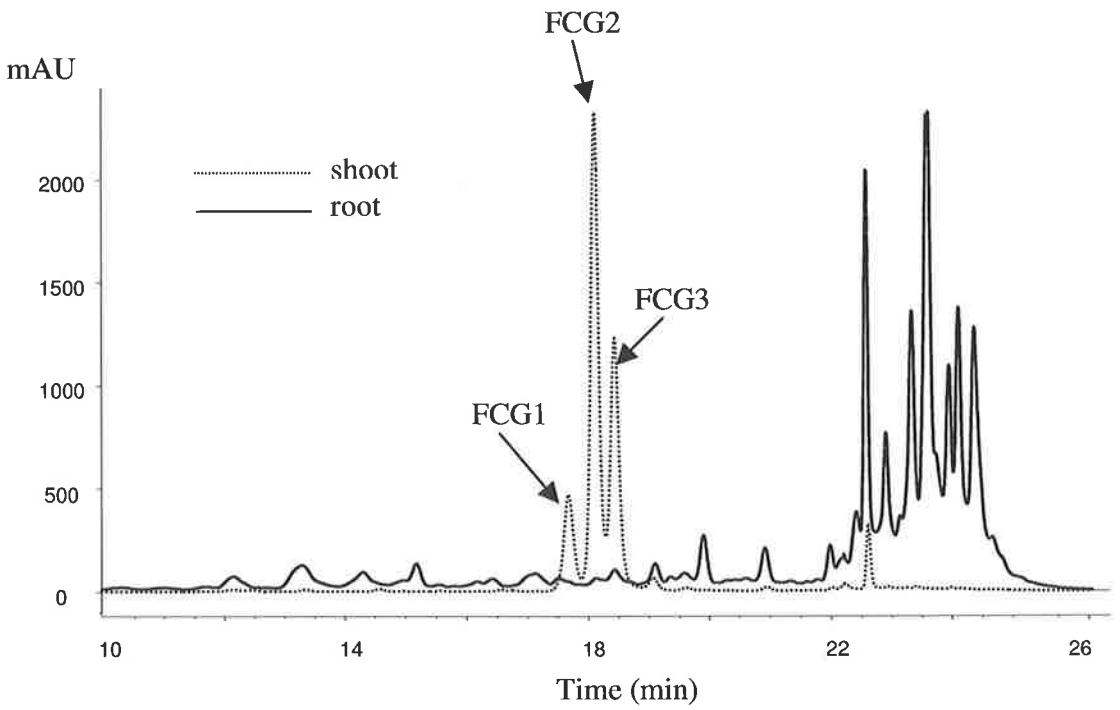


Figure 2.12 Overlay of two HPLC chromatograms obtained from HPLC analysis (monitored at 340 nm) of 20 µl shoot crude extract and 60 µl root crude extract. Retention time of FCGs here (17-19 min) is different to Figure 2.2B (10-12 min) due to minor changes in the elution gradient. The root chromatogram is indicated by a solid line and the shoot chromatogram is indicated by a dashed line.

crude extract, an experiment was performed with different concentration of methanolic crude extract. 100 µl and 150 µl crude extract showed significantly more inhibition than 50 µl (Figure 2.14). The inhibition is shown 48 hr after treatment (Figure 2.14 A). By 96 hr the mycelia had grown over the inhibition zone for the 50 µl treatment and only minor inhibition was visible for the 100 µl (Figure 2.14 B).

2.3.8 Induction of active compounds in oat shoots by MJ

To determine whether the active compounds in methanolic crude extract of oat shoots were induced by MJ, the inhibitory effect of extracted samples from different

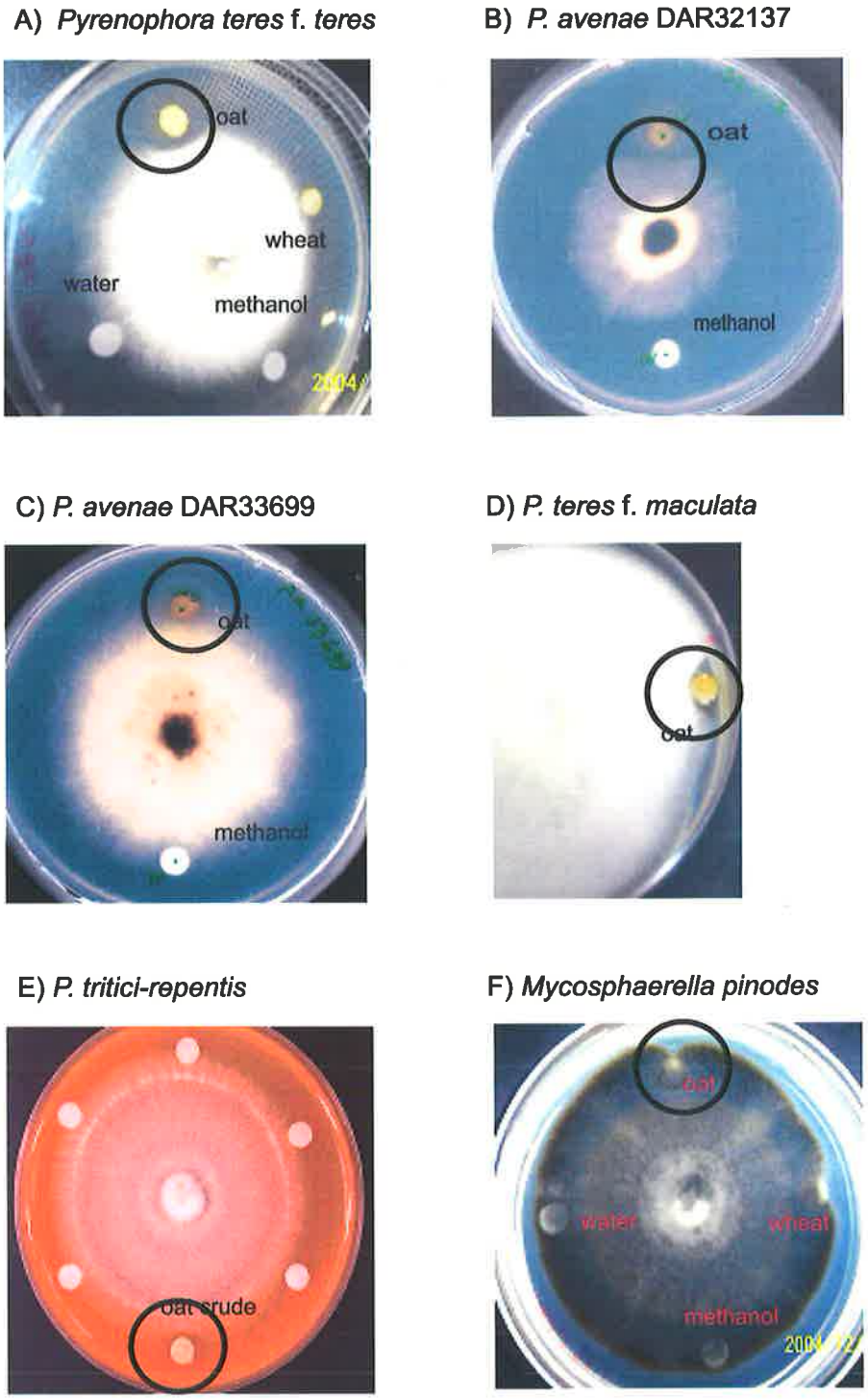


Figure 2.13 Effect of methanolic crude extract (50 µl) from oat shoots on the growth of different fungi. Methanolic extract of wheat shoot crude extract (50 µl) was also tested on *P. teres* f. *teres* and *M. pinodes*. Water and/or methanol (45%) were used as negative controls.

A) B)

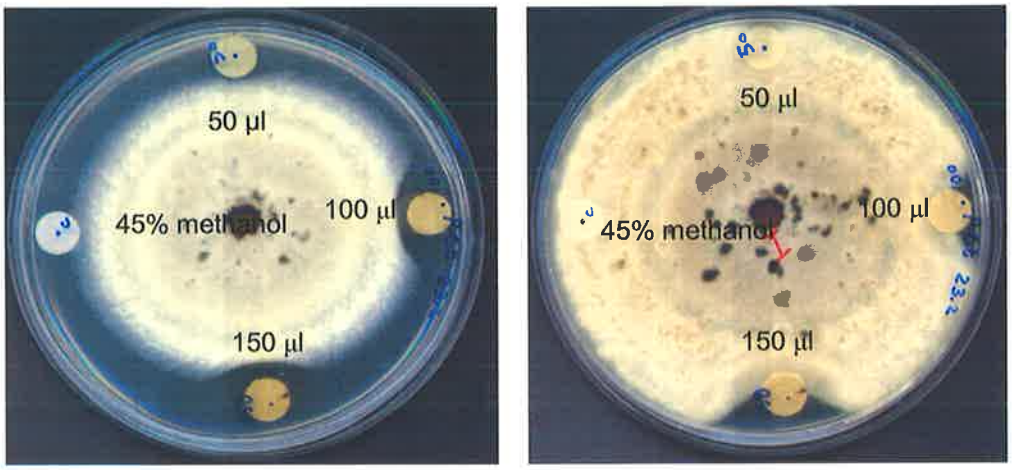


Figure 2.14 Inhibitory activity of different concentrations of shoots crude extracts from oat cv. Quoll on the mycelia growth of *P. teres*. f. *teres* A) 48 hr and B) 92 hr after treatment.

treatments on the growth of *P.teres* f. *teres* was tested.

As a preliminary experiment, the material from the experiment described in Section 2.2.6 was used. Equal volumes (50 µl) of crude extract from freeze-dried shoots (60 mg) obtained from MJ treated, MJ plus Tween 20 and control (Tween 20) plants were applied to *P. teres* f. *teres* in three replicates. The results showed that the inhibition observed with the extract from MJ treated plants were higher than extracts from control plants (Figure 2.15A).

In the second experiment, the inhibitory effect of extracts obtained from MJ treated Mortlock plants were significantly higher than the activity of extracts obtained from control Mortlock plants, but no significant difference was found between inhibitory effects of crude extracts obtained from MJ treated and untreated Potoroo plants (Figure 2.15B).

2.3.9 Fractionation of shoot extracts and testing for biological activity

In an attempt to identify the active antifungal components, the methanolic crude extract of Quoll was fractionated into 10 fractions by HVPE based on relative mobility and distinct absorbance at 254 nm (UV) and florescence at 366 nm using a UV lamp.

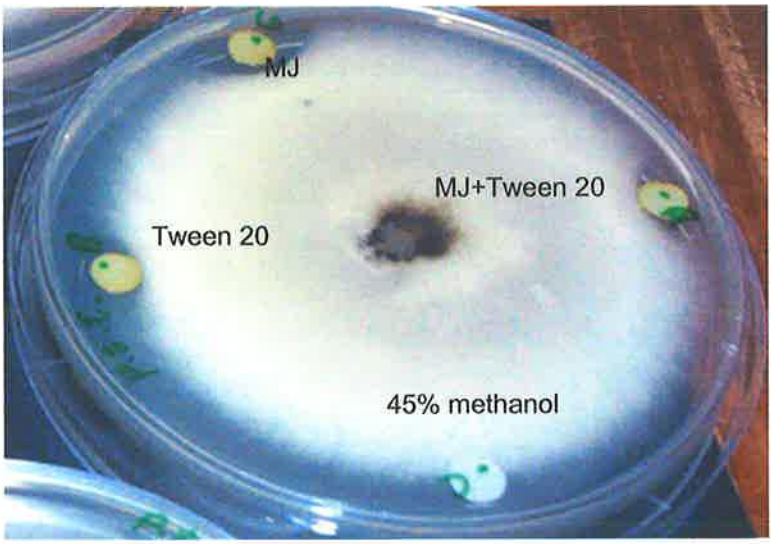
The λ_{max} of each fraction was measured as an additional criterion to distinguish different compounds (Table 2.3). All HVPE fractions were analysed by HPLC and the concentration of FCGs was calculated based on the absorbance of FCGs 1, 2, 3 (Figure 2.2B) at 340 nm. Results showed that Fraction 8 and Fraction 6, at 1.344 and 0.713 mg (rutin equiv.)/g DW, respectively, had the highest concentration of FCGs whereas other fractions contained negligible or undetectable FCGs (Table 2.3).

Each fraction was purified from the paper chromatogram and dissolved in 500 µl 45% methanol and 50 µl of each fraction was applied onto a paper disc. The ability of each fraction to inhibit the growth of *P. teres* f. *teres* and *P. teres* f. *maculata* was tested. Fractions 3, 4, 5 had inhibitory effects on the mycelia growth of *P. teres* f. *teres* (Figure 2.16A). No FCGs could be detected in Fractions 4 and 5 and only low concentrations of FCGs were found in Fraction 3 (Table 2.3). Fractions 8 and 6, with the highest concentrations of FCGs, had either no inhibitory or only a minor inhibitory effect, respectively. When *P. teres* f. *maculata* was treated with the same fractions, Fraction 3 had the highest inhibitory effect and Fractions 7 and 8 had no inhibitory effect (Figure 2.16B). These results suggest that FCGs are not responsible for the inhibition observed in the crude extract.

2.3.10 Saponins in the shoot extract of oat and antifungal activity

To determine which compounds in the active fractions are responsible to the antifungal activity, the active HVPE fractions (Fraction 3 and 4, Table 2.3) were further analysed by LC-MS. HPLC chromatograms (Figures 2.17A and 2.17B) showed that

A)



B)

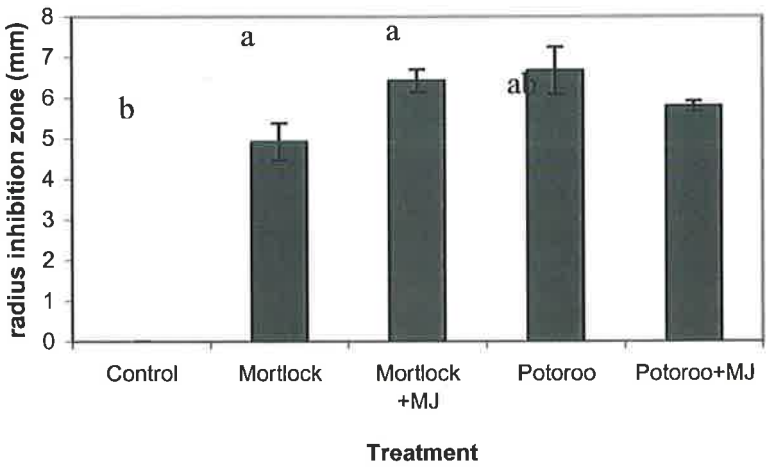


Figure 2.15 Induction of active compounds in the shoots of oat by methyl jasmonate using a bioassay on *P. teres* f. *teres*. A) Bioassay of crude extracts obtained from shoots of oat cv. Quoll under different treatments. Equal volumes (50 µl) of extracts obtained from an equivalent amount of dried tissue (60 mg). B) Bioassay of crude extracts obtained from MJ treated and untreated Mortlock and Potoroo. Equal quantity of extracts was applied (3 mg). 45% methanol was used as a control.

Table 2.3 Characterisation of fractions from oat cv. Quoll shoots fractionated by HVPE.

HVPE Fraction	Relative Mobility	λ_{max}	flavone-*C*-glycoside mg (rutin equiv.)/ml[a]
1	0.00	257.8	nd
2	0.10	204.8, 272.6	0.02
3	0.16	206.2	0.025
4	0.22	207.8	nd
5	0.26	206.1	nd
6	0.36	197.7, 256.7, 331	0.713
7	0.56	194.9, 261.6	0.013
8	0.67	204.6, 267, 350.6	1.344
9	0.81	195.2, 267.4	0.011
10	1.00	nd	nd
Crude			2.505

[a] flavone-*C*-glycosides were quantified at 340 nm

nd, not detected

most of the material eluted as a series of peaks between retention times 47 to 50 min. All major peaks in this range were investigated (Table 2.4). The peak at R_t=48.64 of Fraction 3 (Figure 2.17A) and peak at R_t= 48.58 of Fraction 4 (Figure 2.17B) contained saponins (Figure 2.17C and D) based on the ionisation patterns of saponins (Wubben et al., 1996). It was not possible to conclusively identify all of the saponins by LC-MS alone because different saponins can have the same mass and fragmentation pattern (Wubben et al., 1996). Thin layer chromatography (TLC) was used to provide a definitive identification of the alternatives identified by LC-MS. Based on LC-MS, the fraction at R_t=48.64 contains either 26-desglucoavenaside B ($C_{51}H_{82}O_{23}$, mass *m/z* 1063.2) and/or avenacoside A ($C_{51}H_{82}O_{23}$, mass *m/z* 1063.2) showing the parent ion at

m/z 1064.2 ($M+H^+$) and other ions at 902.0 ($M+H^+$-hexose), 756.0 ($M+H^+$-hexose-deoxyhexose), 431.6 ($M+H^+$-3 × hexose-deoxyhexose) (Figure 2.17C).

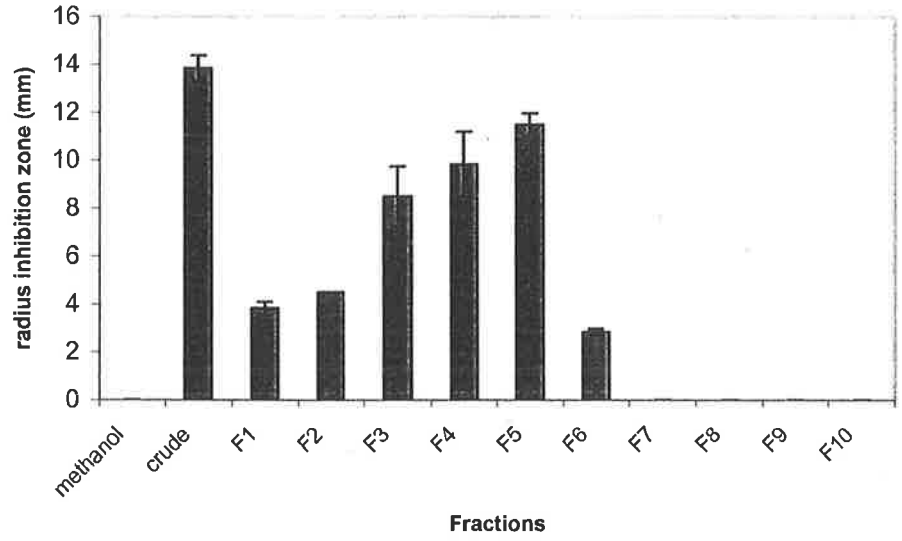
TLC confirmed (data not shown) the identification of this compounds as saponins (see Figure 2.19) for a similar TLC run. Fraction 4 contains 26-DGA ($C_{45}H_{72}O_{18}$, mass *m/z* 901.04), based on mass spectra of *m/z* 902.0 ($M+H^+$), 739.6 ($M+H^+$-hexose), 593.4 ($M+H^+$-hexose-deoxyhexose), 431.6 ($M+H^+$-2 × hexose-deoxyhexose) (Figure 2.17D). The ion of mass 431.6 in both spectra (Figures 2.17C and 2.17D) shows that the sugars are attached to an aglycone, which is either nuatigenin or isonuatigenin ($C_{27}H_{42}O_4$, mass *m/z* 430.63).

2.3.11 Purification and identification of saponins

Given 26-DGA and 26-DGB are known to be active against fungi (Wubben et al., 1996), we purified the saponins from oat to test if they were active against *P. teres* f. *teres*.

The aqueous phase of an extract from MJ treated 3-week-old Quoll plants was fractionated by C18 column using batch elution with 40 and 60% methanol to give two fractions (Figure 2.18C). Elution with 40% methanol resulted in 7 × 8 ml sequential sub-fractions. These fractions were separated by TLC and the components visualised with *p*-anisaldehyde to stain for saponins (Hostettmann and Marston, 1995). The different fractions (1 to 7) of isocratic 40% methanol elution all contained the same saponin (Figure 2.18A). The 60% methanol fraction (pooled) contained two *p*-anisaldehyde positive bands, whereas the 40% methanol fraction (pooled) contained only one band (Figure 2.18C). The 40% methanol fractions were pooled and applied to grown mycelia of *P. teres*. f. *teres* in two replicates. The results of the bioassay indicated that the 40% methanol fraction had no biological activity against the fungi

A)



B)

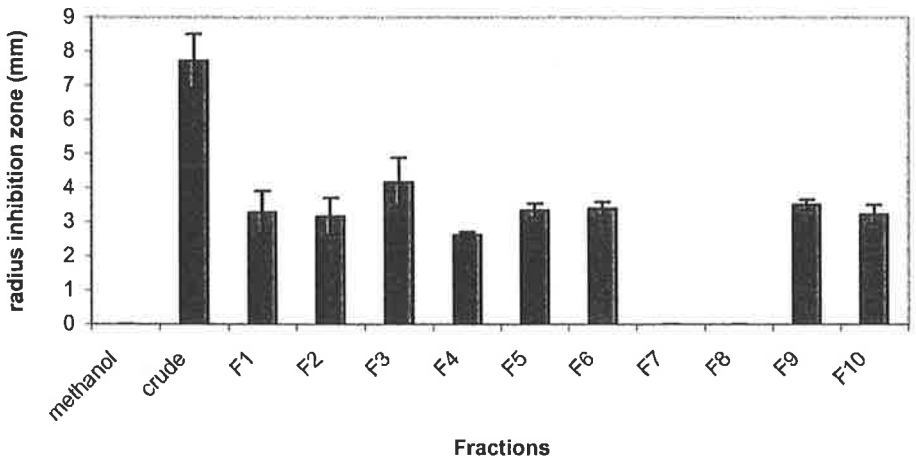


Figure 2.16 The inhibitory effect of HVPE fractions from Quoll shoots, on the growth of A) *P. teres* f. *teres* and B) *P. teres* f. *maculata.* 50 µl of each fraction was applied to each disk and 50 µl 45% percent methanol was also used as a control. Each mean was calculated from three replicates. Bars represent the mean ± SE.

(Figure 2.18B). The single band of the 40% methanol fraction is avenacoside B ($C_{57}H_{92}O_{28}$, mass *m/z* 1225.3) based on LC-MS and published ionisation data for desglucoavenacoside B (Wubben et al., 1996) with the presence of one extra glucose. The MS (negative mode) of this compound indicated a strong ion at *m/z* 1224.2 ($M\text{-}H^+$) (Figure 2.18D). The ionisation pattern in positive mode of this compound was: *m/z* 1226.2 ($M+H^+$), 1063.8 ($M+H^+$-hexose), 901.8 ($M+H^+$-2×hexose), 739.6 ($M+H^+$-3×hexose), 593.8 ($M+H^+$-3×hexose-deoxyhexose), 431.0 ($M+H^+$-4×hexose-deoxyhexose) and 413.2 ($M+H^+$-4×hexose-deoxyhexose-water) as shown in Figure 2.18E. The minor band eluted by 60% methanol (Figure 2.18C) was not of sufficient purity for identification. However, this band was later isolated and fractionated from the butanol phase as follows.

The butanol phase from the shoot extract was further fractionated by C18 RP and the resulting 60% methanol fraction was further purified using C18 silica gel and resulted in more than 30 fractions. Fraction 11 to 30 contained *p*-anisaldehyde positive compounds with R_f values of 0.22, 0.31, 0.40 and 0.50 (Figure 2.19 A). The elution profiles of the different saponins overlap. The fractions containing only a single saponin (based on R_f) were pooled. For example, Compound 1 (R_f 0.50) eluted in Fractions 11-12, Compound 2 (R_f 0.40) eluted in Fractions 14-15, Compound 3 (R_f 0.31) eluted in Fractions 17, 18 and 19 (Figure 2.19A). Subsequent TLC separation of the TLC purified compounds showed each fraction contained only a single compound (data not shown).

The identity of each of the purified saponins was predicted based on published TLC profiles of saponins (Wubben et al., 1996) and confirmed by LC-MS. The MS in negative mode of Compound 1 revealed a single major ion at *m/z* 900.0 ($M\text{-}H^+$), but there were no apparent fragmentations of this ion (Figure 2.19B). In contrast, the MS in

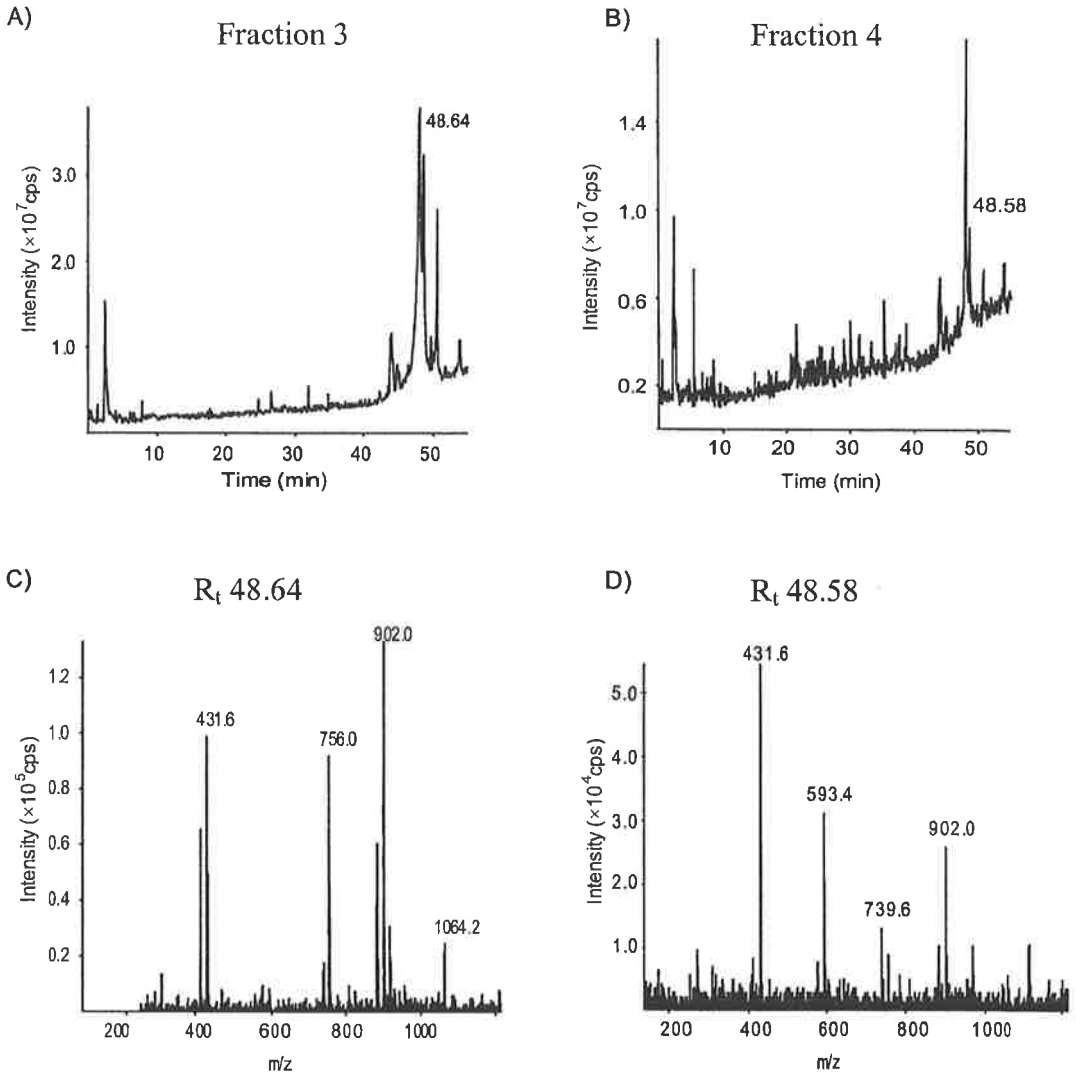


Figure 2.17 HPLC chromatogram and LC-mass spectrometry data of biologically active fractions against *Pyrenophora* species. A) and B) are HPLC chromatograms of Fractions 3 and 4 (Table 2.2), C) and D) are fragmentation patterns of the fraction R_t=48.64 and R_t=48.58, originally derived from oat cv. Quoll shoot Fractions 3 and Fraction 4, respectively. Fragmentation spectrum of the [M+H$^+$] ion, *m/z* 1064 C) indicates either the saponin 26-desglucoavenacoside B or avenacoside A; and *m/z* 902 D) indicates the saponin 26-desglucoavenacoside A (Wubben et al. 1996). R_t is retention time.

Table 2.4 Characterization by LC-mass spectrometry of biologically active fractions against *P. teres* f. *teres*

Fraction[a]	Retention time (min)	Major ions in positive mode (*m/z*)
3	43.97	1262.0, 1210.0, 1064.0, 885.8, 723.6, 577.6
	48.11	1280.0, 1064.2, 901.8,756.0, 739.6,593.6, 431.6
	48.64 (Figure 2.14C)	1064.2, 902.0, 756.0, 431.6
	50.65	1117.0, 901.8, 756.0, 739.6, 593.6, 431.6
	54.00	1118.0, 1048.0, 740.0, 593.8, 431.6
4	43.93	1210.0, 908, 577.4, 318.8
	48.01	1279.0, 901.6, 593.4, 431.6
	48.58 (Figure 2.14D)	902.0, 739.6, 593.4, 431.6
	50.82	1118.0, 902.0, 739.8,593.6,431.4, 363.8, 319.0
	53.96	1118.0, 913.6, 740.8, 593.8, 431.4, 318.8

[a] HVPE fractions (see Table 2.3 and Figure 2.17).

positive mode produced good fragmentation with the parent ion at *m/z* 901.8 ($M+H^+$),and other ions at *m/z* 739.6 ($M+H^+$-hexose), 593.8 ($M+H^+$-hexose-deoxyhexose), 431.6 ($M+H^+$-2×hexose-deoxyhexose), 413.4 ($M+H^+$-2×hexose-deoxyhexose-water) and 318.4 (Figure 2.19C), leading to identification of Compound 1 as 26-DGA ($C_{45}H_{72}O_{18}$). The MS in negative mode of Compound 2 indicated a strong ion at *m/z* 1062.2 ($M-H^+$) (Figure 2.19D). A positive MS spectrum of Compound 2 (Figure 2.19E) gave ions at *m/z* 1063.8 ($M+H^+$), 901.6 ($M+H^+$-hexose), 739.6 ($M+H^+$-2×hexose), 633.0, 593.2 ($M+H^+$-2×hexose-deoxyhexose), 431.0 ($M+H^+$-3×hexose-deoxyhexose), 412.8 ($M+H^+$-3×hexose-deoxyhexose-water), as expected for 26-DGB ($C_{51}H_{82}O_{23}$). Compound 3 had a single major ion at *m/z* 1062.0 in negative MS mode (Figure 2.19F). The fragmentation pattern for Compound 3 in positive mode was: *m/z* 1064.0 ($M+H^+$), 901.8 ($M+H^+$-hexose), 739.6 ($M+H^+$-2×hexose), 593.6 ($M+H^+$-2×hexose-deoxyhexose), 431.4 ($M+H^+$-3×hexose-deoxyhexose), 413.4 ($M+H^+$-

3×hexose-deoxyhexose-water) (Figure 2.19G). The ionisation pattern confirms that the Compound 3 is avenacoside A ($C_{51}H_{82}O_{23}$). Compound 4 (R_f 0.22) was identified in the aqueous phase as avenacoside B (Figure 2.18A, C, D, E).

2.3.12 Bioassay of saponins

When the aqueous phase extract containing only two of the four saponins (avenacoside A and avenacoside B) was used at different concentrations against *P. teres* f. *teres*, no inhibition was observed (Figure 2.20A) whereas the crude extract showed inhibition. The four purified saponins that have been visualised as single band by TLC (Figure 2.20B) were also tested in a bioassay (Figure 2.20C). Initially, 50 and 250 µg of each of the purified saponins were used in separate experiments and minor inhibition of mycelium growth was observed for 26-DGB and 26-DGA (data not shown). When the bioassay was repeated with 400 µg of each saponin it was observed that 26-DGB had a greater inhibitory activity than 26-DGA, but the crude extract had even higher activity (Figure 2.20C). The minor inhibition observed with avenacoside A and B was not complete and mycelia were present throughout the inhibition zone, whereas the zone around 26-DGA has much less mycelia.

In summary, 26-DGA and 26-DGB are active compounds against *P.teres* f. *teres* and this is consistent with other reports of activity against other fungi (Wubben et al. 1996).

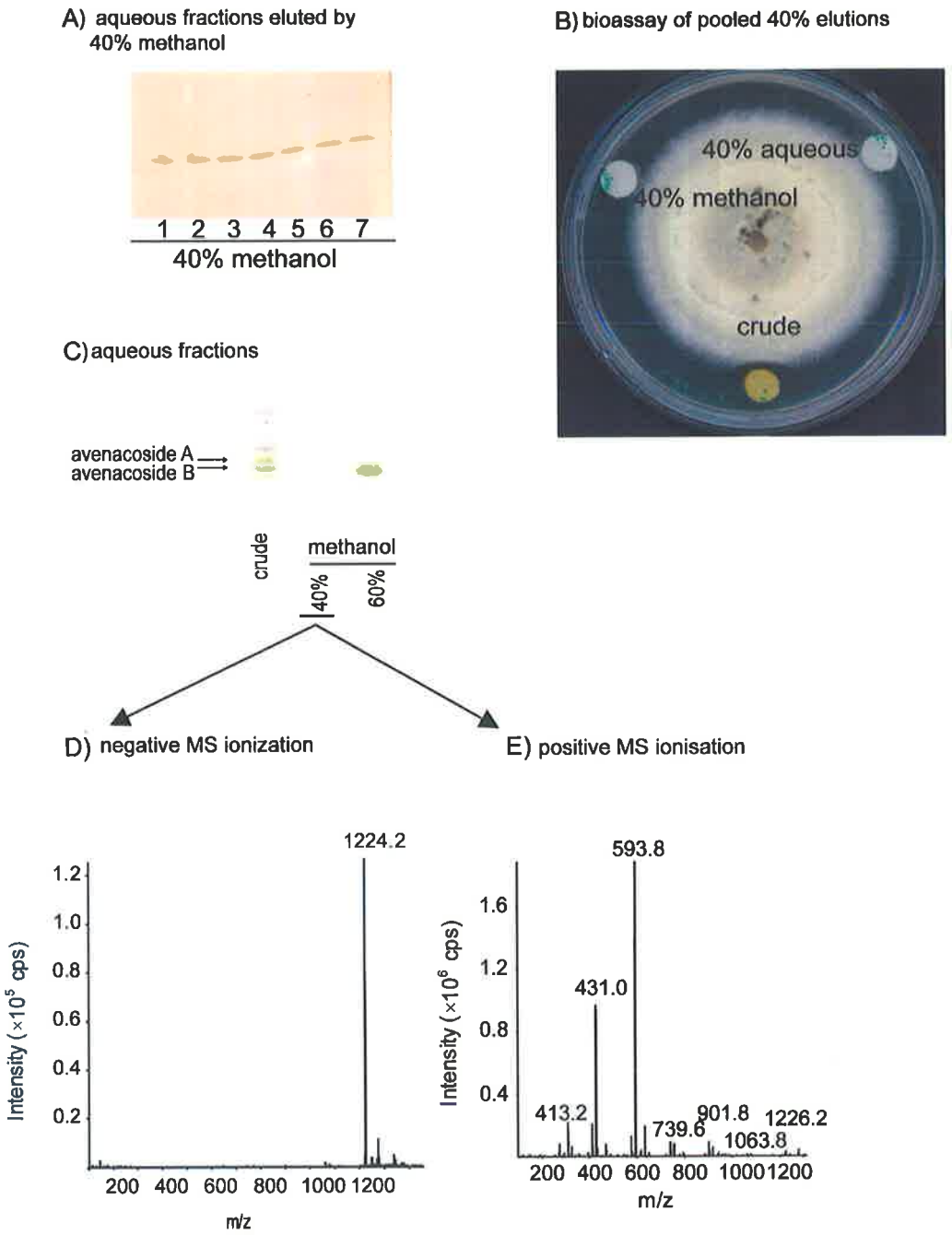
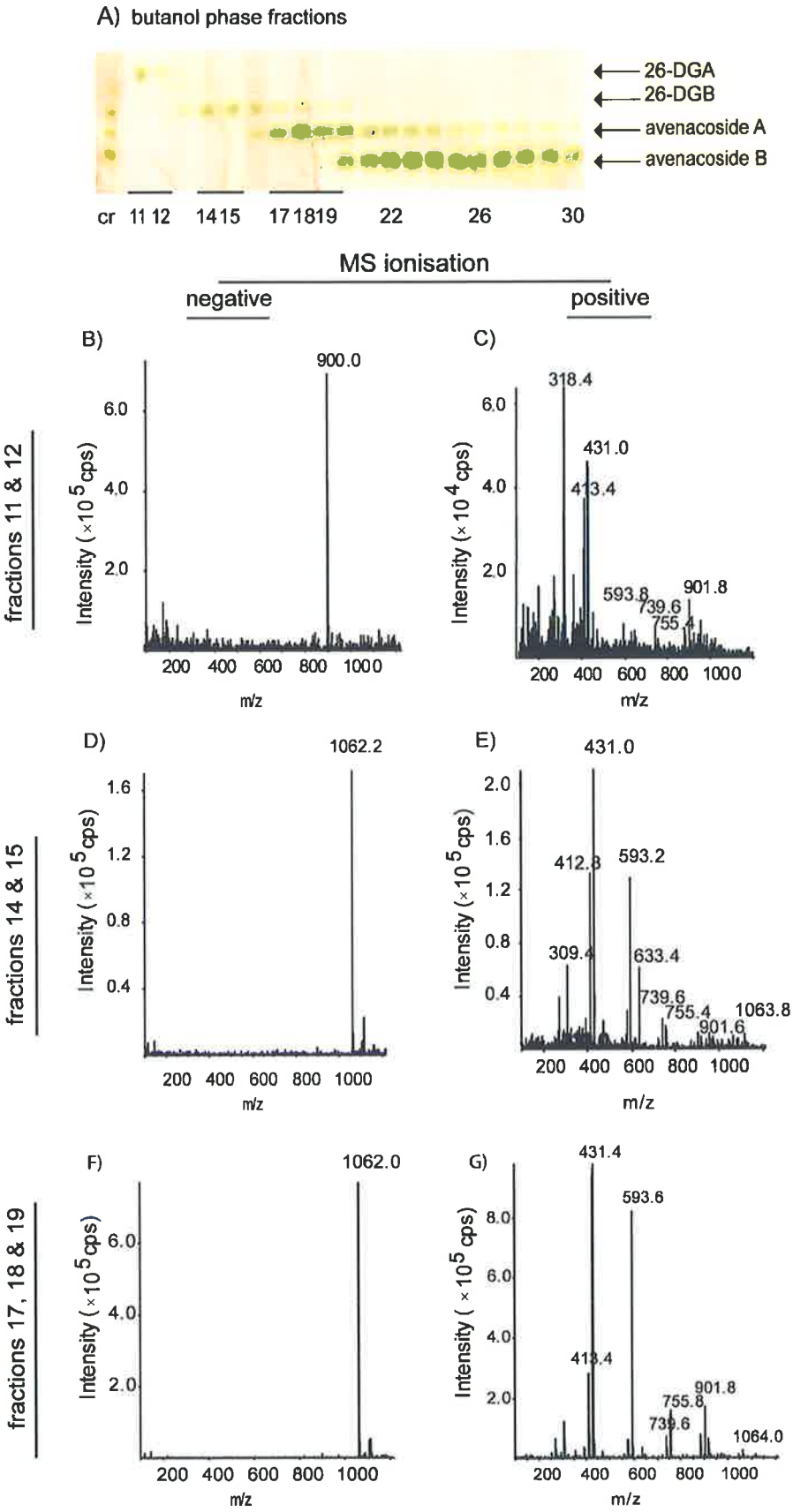


Figure 2.18 Separation and identification of the compounds in aqueous phase extract of shoots from oat cv. Quoll. A) TLC of the aqueous phase of the methanolic extract that was fractionated using C18 column and eluted with isocratic 40% methanol (7 × 8 ml) B) bioassay of pooled 40% methanol elution of aqueous phase on *P. teres* f. *teres*. C) TLC of the aqueous phase of the methanolic extract and the two fractions eluted with 40% and 60% methanol (RP-C18 column and run with butanol: ethanol: ammonia (7:2:5 v/v)). The plate was stained with *p*-anisaldehyde solution. D) MS of the $[M\text{-}H^+]$ (negative mode) ions and (E) the $[M+H^+]$ (positive mode) ions from the aqueous phase fraction eluted by 40% methanol.

Figure 2.19 Separation and identification of the compounds in butanol phase after extraction of compounds from shoots of oat cv. Quoll. A) TLC analysis of saponins from the C18 (silica gel) separation of the compounds from the butanol phase. The order of elution of the individual saponins is 26-DGA, 26-DGB, avenacoside A, avenacoside B based on previous studies (Wubben et al., 1996). The plate was stained with *p*-anisaldehyde solution and baked at 130°C. The fractions that contained each single saponin were pooled for further analysis (as underlined). cr is crude extract. MS of saponin at R_f = 0.50 (purified from TLC Fractions 11 and 12) in negative B) and positive mode C), at R_f = 0.40 of Fractions 14 and 15 in negative D) and positive mode E) and at R_f = 0.31 of Fractions 17, 18 and 19 in negative (F) and positive mode G).


A) butanol phase fractions
26-DGA
26-DGB
avenacoside A
avenacoside B
cr 11 12 14 15 17 18 19 22 26 30
MS ionisation
negative
positive
fractions 11 & 12
fractions 14 & 15
fractions 17, 18 & 19
B)
C)
D)
E)
F)
G)
Intensity (×10 5 cps)
Intensity (×10 4 cps)
m/z
900.0
318.4
431.0
413.4
593.8
739.6
755.4
901.8
1062.2
412.8
593.2
309.4
633.4
1063.8
1062.0
431.4
593.6
1064.0

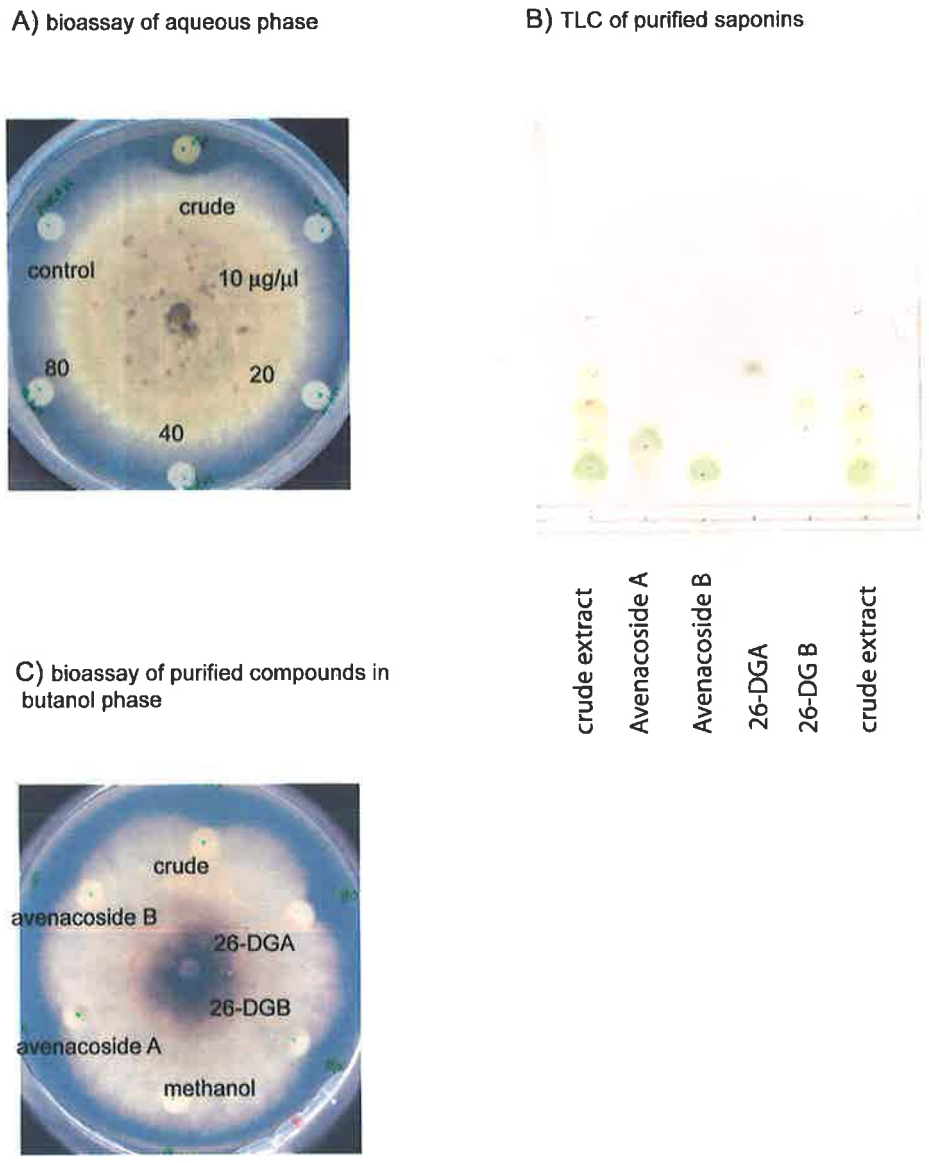


Figure 2.20 Bioassay of purified saponins from oat cv. Quoll shoot extracts against *P. teres* f. *teres*. A) Bioassay with 50 µl of 10, 20, 40 and 80 µg/µl of the aqueous phase extract from shoot, 45% methanol as negative control and crude extract (500 µg) as positive control. B) 2 µl of each purified saponins was dotted onto aluminium backed TLC plate and developed by chloroform: methanol: water (140:70:11) and stained with *p*-anisaldehyde reagent. C) Bioassay of TLC purified compounds from the butanol phase of shoot extract that were obtained from silica gel column followed by preparative TLC; 40 µl avenacoside A, avenacoside B, 26-DGA and 26-DGB (each 10 µg/µl in 45% methanol). Crude extract (400 µg) used as a positive control and 45% methanol as a negative control.

2.3.13 Induction of active compounds in the oat shoots by homogenisation

To provide independent additional support that saponins are the active compounds, the antifungal activity of extracts was compared to extracts obtained from fresh leaves with and without homogenisation. If saponins are indeed the antifungal compounds, it is expected that homogenisation of fresh leaves should give greater activity as homogenisation disrupt the vacuoles releasing the β-glucosidase which is able to convert inactive avenacosides to active form of saponins, 26-DGA and 26-DGB (Wubben et al. 1996). The biological activity of shoot extracts with and without homogenisation (as a mechanical damage) was tested. The ability of the extract from the homogenized oat shoots to inhibit the mycelia growth of fungi was significantly higher than the extract from non-homogenised shoots (Figure 2.21).

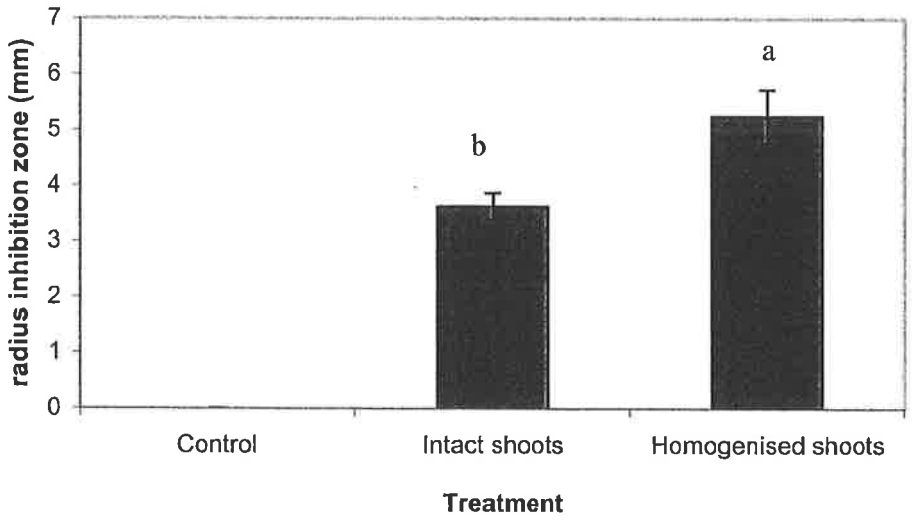


Figure 2.21 Induction of active compounds (saponins) in the shoots of oat using the bioassay on *P. teres* f. *teres* by homogenisation of the fresh shoots of Quoll before extraction. Homogenisation was performed as explained by Wubben et al. (1996). Equal quantity of extracts were applied (3 mg), 45% methanol was used as a control.

2.4 Discussion

2.4.1 Variation in FCGs in oat accessions

The study of the flavone-*C*-glycoside concentration in oat shoots revealed that there is wide genetic variation for this trait in *Avena sativa*. The variation of oat accessions for the concentrations of FCGs (*O*-methyl-apigenin-*C*-hexoside-*O*-deoxyhexoside, apigenin-*C*-hexoside-*O*-pentoside and luteolin-*C*-hexoside-*O*-pentoside) was confirmed in all three experiments both in the glasshouse (Table 2.1 and Figure 2.4) and in the growth chamber (controlled conditions, Figure 2.5). The only major inconsistency between experiments was the difference in ranking of accession 91QK195 from the first rank in the experiment with 21 accessions in the glasshouse (Figure 2.4) to the last rank in the experiment with five accessions carried out in the growth chamber (Figure 2.5). This was possibly due to the effect of environmental conditions (Emmons and Peterson, 2001) such as UV (Estiarte et al., 1999). The level of variation in the flavonoid concentration in oat shoots could be used in future breeding program. As flavonoids are generally regarded as a group of natural metabolites with antimicrobial activity (Cowan, 1999; Harborne and Williams, 2000), this variation could be used to improve cultivar resistance against pathogens including nematodes, such as stem nematode (*Ditylenchus dipsaci*) that infest the shoots (Soriano et al., 2004).

2.4.2 MJ and FCGs in oat

The pilot experiment with 72 accessions and limited replication indicated positive responses to MJ in a few accessions but overall there was no significant induction of FCGs in MJ-treated compared to control plants. Furthermore, for

accessions where MJ induction was seen, it was not reproducible in subsequent experiments. For example, the concentration of FCGs in Potoroo and Quoll increased by up to 20 and 16%, respectively, after MJ treatment in the experiment with only two replicates (Figure 2.4), but in the growth chamber experiment with three replicates, there were no significant differences in FCG concentrations between MJ treated and control plants for these two cultivars (Figure 2.5).

The absence of MJ induction is in contrast to the significant induction of FCGs by MJ previously reported (Soriano et al., 2004). One explanation is that MJ induction is unreliable and only occurs under particular circumstances. The possibility that technical issues were responsible for the differences between our results and Soriano et al. (2004) was ruled and the same cultivar (Quoll) was used in all experiments to test effectiveness of MJ application. The results presented here represent multiple independent experiments with several replications (Figures 2.4, 2.5, 2.6, 2.7 and 2.8). Three different methods of MJ application were tested and none of the methods increased FCGs concentration (Figure 2.6). One possibility was that MJ was not getting to the plants. Therefore, the effectiveness of Tween 20 as a surfactant was studied. No significant difference was found between the concentration of FCGs in the shoots of oat treated by MJ and MJ plus Tween 20 (Figure 2.7) and neither treatment had a significant affect on the induction of FCGs in oat shoots. It was confirmed that the MJ applied to oat plants was biologically active, based on the expression of *AsPR5* (Figure 2.11). In this experiment, it was shown that oat *PR5* gene is induced in roots by MJ as observed for other monocots (Schweizer et al., 1997) and dicots (Xu et al., 1994; Hu et al., 2003).

2.4.3 FCG quantification in oat roots and shoots

The concentration of FCGs in the shoot and the root of oat accessions were much lower than what was reported by Soriano et al. (2004). The concentration of the total FCGs in the shoot of oat cv. Quoll measured in this study was the range of 4-9 (Table 2.2, Figures 2.3, 2.4, 2.5 and 2.6, measured at 254 nm) and 20-27 mg (rutin equiv.)/g DW (Figure 2.3 and Section 2.3.6, measured at 340 nm) and depends on the condition of growing and harvesting. The concentration of these compounds in the roots of oat cv. Quoll was significantly lower at about 0.05-0.2 mg (rutin equiv.)/g DW (Figure 2.12). The concentration of FCGs in the root is too low to be measured accurately, so root FCGs were not measured in any of the experiments. The FCGs concentration of oat cv. Quoll has been reported as 350 and 150 mg (rutin equiv.)/g DW (measured at 254 nm) in the shoots and roots, respectively (Soriano et al., 2004). This over-estimation of FCGs could be due to a calculation error or incorrect preparation of rutin standard.

The flavonoid concentration measured at 254 nm was much lower than 340 nm due to the differences in extinction coefficient at the respective wavelengths as they were compared with rutin. The validity of using either 254 nm or 340 nm was to measure FCG in shoots was reinforced based on significant correlation (r=0.952) of FCG data obtained from both wavelengths (n=30 sample).

2.4.4 Bioassay with crude extract fractions of oat shoots

Of the seven genera of pathogens tested only *Pyrenophora* species were inhibited by methanolic crude extract of oat containing FCGs (with the exception of one compatible *Pyrenophora* isolate that was not inhibited). Although the crude sample contained FCGs, these were not the active compounds against *P. teres* f. *teres* and *P.*

teres f. *maculata* (Figure 2.16). Further work with other fungi and bacteria is needed to understand if FCGs have antimicrobial activity in oat. Antimicrobial activity may not be the only function of flavonoids as they are reported to have many different roles in plants (Harborne and Williams, 2000).

2.4.5 Bioassay of saponins

The HVPE fractions active against *Pyrenophora* species were shown to contain saponins (Figure 2.17). The saponins were purified from the butanol phase of extracts by fractionation on silica gel and purified by TLC. Bioassays with the four purified saponins from oat shoots confirmed that, two saponins, 26-DGB and 26-DGA inhibited the growth of *Pyrenophora* species (Figure 2.20C). This is consistent with previous reports showing that 26-DGA and 26-DGB are active antifungal compounds released by deglycosylation of avenacoside A and B in infested leaves (Wubben et al., 1996). Bisdesmosidic saponins (saponins with two sugar chains), avenacoside A and B, showed weak activity against the fungi when 400 µg was used in the bioassay. It is widely believed that bisdesmosidic saponins do not show haemolytic activity and most monodesmosidic saponins, with only single sugar chain, show activity. However, weak activity of bisdesmosidic saponins has been reported (Woldemichael and Wink, 2001). The inhibition zone seen by avenacoside A and B may be due to minor contamination with other active compounds during purification. Although avenacoside A and B are not highly active, the concentration of these compounds is of interest because they are the precursors of the active metabolites.

A few days after treatment, fungi are able to spread their mycelia over inhibition zone (compare Figure 2.14A and B). This could be because of either diffusion so effectively dilutes the concentration of 26-DGA and 26-DGB in the medium or

reducing their concentration due to a specific reaction of enzymes of fungi, which hydrolysed the 26-DGA and 26-DGB to nutigenin or isonutigenin (Luning and Schlosser, 1976). If the latter was true, it seems that the ability of *Pyrenophora* species to inactivate 26-DGA and 26-DGB is different among the fungi or even isolates of one species as observed in the two isolates of *P. avenae* (Figure 2.13B and C). The difference in the inhibition of *P. avenae* isolates by methanolic crude extract of oat shoots could be due to the difference in their ability to inactivate 26-DGA and 26-DGB. This could be tested by re-extracting saponins from the medium.

The degree of inhibition observed by the purified saponins was less than in the crude methanolic extract indicating that either there are synergistic interactions between these saponins or between the saponins and other non-saponin metabolites in the crude extract. It is widely recognised that synergism between defence compounds is common and is one explanation for the large number of secondary metabolites found in plants (Berenbaum and Neal, 1985; Wang and Huang, 1999; Alonso Amelot and Calcagno, 2000; Macel et al., 2005). An example relevant to this study is the antinematode activity of *Quillaja saponaria*, which is known to involve synergism between a saponin and a non-saponin fraction (Martin and Magunacelaya, 2005). It is unlikely that saponins are the only important antifungal compounds and it is known that phytoalexins such as avenanthramides (hydroxy cinnamic acid amide) are produced in oat shoots in response to fungal attack (Mayama et al., 1981; Miyagawa et al., 1996; Okazaki et al., 2004).

2.4.6 Effect of MJ on antifungal activity of crude extracts

The signalling molecule MJ is known as a potential plant elicitor capable of inducing a wide range of plant secondary metabolites involved in plant defence

reactions (Ishihara et al., 2002; Heijari et al., 2005). While performing bioassays with *P. teres* f. *teres*, it was observed that the crude extract obtained from the shoots of MJ treated oat cv. Quoll has significantly higher activity than crude extract obtained from the control plants (Figure 2.15A). Therefore, the effect of application exogenous MJ on the antifungal inhibitory effects of crude extracts obtained from oat cv. Potoroo and Mortlock was studied. It was found that bioactive compounds, which were subsequently identified as saponins were significantly induced in Mortlock by MJ but not in Potoroo (Figure 2.15B). This highlights the complexity of plant defence responses with cultivar-dependent responses compared by pathogen defences. Further work with HPLC analysis is needed to determine whether 26-DGA and 26-DGB are induced by MJ.

Determining the contribution of saponins in plant disease resistance is a prerequisite of any manipulation of the saponin biosynthetic pathway in plants that could lead to improving the resistance of cultivars.

Chapter Three

Metabolites Correlated with Cereal Cyst Nematode Resistance in Oat (*Avena sativa*)

3.1 Introduction

Cereal cyst nematode (CCN; *Heterodera avenae*) is one of the most destructive root pathogens of cereals and is widely distributed in many cereal-growing areas. Plants react to nematode invasion in several ways including expressing defence genes, proteins and synthesis secondary metabolites all of which are widely believed to provide plants with resistance (Giebel, 1979; Huang and Barker, 1991; Oka et al., 1997; Soriano et al., 2004). Methyl jasmonate (MJ), an inducer of plant defence pathways, was found to provide protection to oat against CCN and root lesion nematode (*Pratylenchus neglectus*) by reducing the number of invading nematodes and increasing plant mass (Soriano et al., 2004). This protective effect of MJ was attributed to induced flavone-C-glycosides (Soriano et al., 2004).

Constitutive phytochemicals likely play a role in nematode resistance. Many different classes of chemicals are implicated as natural nematicidal compounds, including alkaloids (Rao et al., 1996; Zhao, 1999; Cunha et al., 2003) saponins (Deepak et al., 2002; Zinov'eva et al., 2004; Martin and Magunacelaya, 2005), phenolics (Rich et al., 1977; Oka, 2001; Soriano et al., 2004) and sulphur containing compounds (Akhtar and Mahmood, 1994; Potter et al., 1999; Zasada and Ferris, 2003, 2004).

To search for constitutive compounds associated with nematode resistance, the concentrations of flavonoids and other metabolites from individuals of a single seed

descent population (SSD) that was segregating for both CCN resistance and tolerance and stem nematode (SN) resistance was determined. To identify potential induced compounds for nematode resistance, the metabolites in MJ treated and untreated oat roots were compared.

3.2 Materials and Methods

3.2.1 Plant Material

A single-seed descent (SSD) population of 170 lines was generated by Pamela Zwer, SARDI, Australia, from a cross between Potoroo and Mortlock. This population is segregating for several different disease resistance loci including resistance to CCN and SN (unpublished data). A laboratory-based bioassay was used to obtain an indicator of CCN resistance, based on the number of white CCN females developing on the outside of the rootball (Lewis et al., 1999). The number of nematodes will be called the "CCN female count" in the rest of the thesis. For stem nematode resistance, the symptoms of the nematode were scored from 0 (no symptoms) to 5 (dead plants). A number of SSD lines were selected from the population based on either high or low CCN females count. CCN female counts (2000-2002) and SN resistance data (2001) were collected under natural conditions with supplementary irrigation in pots as part of an independent research project by John Lewis, SARDI, and were provided by Dr Kevin Williams, SARDI.

3.2.2 Plant culture and maintenance

In the first experiment (to investigate possible differences in FCG concentration), seeds of the parent lines, Mortlock and Potoroo were sown in four replicates in the growth chamber (22°C light/18°C dark, 12 hr cycle). A few seeds of

each cultivar were sown into a pot containing steam pasteurised (65°C for 45 min) University of California potting mix (Appendix 2) with Osmocote® (Scotts, Australia), a slow release fertiliser. Plants received Hoagland's solution (Beltrano et al., 1998) every other day and tap water on alternate days. After three weeks, plants were harvested. Shoots and roots were separated and frozen immediately in liquid nitrogen. They were stored at -80°C until extraction.

In the second experiment, the seeds of 58 SSD lines along with parental lines were sown in a complete RB design in three replicates in a growth chamber as mentioned above.

3.2.3 Flavonoid extraction and assessment by HPLC

Samples stored at -80°C were freeze-dried and ground to a fine powder using a coffee grinder (Breville, Australia). Sixty mg of ground sample was extracted with 10 ml methanol (HPLC grade) for 12 hr by shaking on an orbital mixer at 130 rpm. Then, 3 ml MQ water was added to 7 ml methanolic extract and lipids were removed with 10 ml n-hexane mixed at 130 rpm for 12 hr. Eight ml of the methanolic phase was evaporated in a speed vac concentrator. The residue of shoot and root samples were dissolved in 0.4 and 0.2 ml 45% v/v methanol in water, respectively, and immediately were analysed by HPLC as described in Section 2.2.3. The extraction method used here were different from Section 2.2.3 in two parts: 1) a reduced time of exposure to methanol was used from 48 hr to 12 hr that gave no significant difference in yield of FCG (Appendix A4.1) and 2) the butanol step was omitted which gave more FCGs (Appendix A4.2).

3.2.4 Search for metabolites associated with CCN female count

The availability of a single seed descent population (SSD) segregating for CCN resistace and tolerance and stem nematode resistance provides an opportunity to identify compounds that are important for resistance or tolerance. Potoroo is resistant and tolerant to CCN, whereas Mortlock is moderately susceptible and intolerant. Potoroo is moderately resistant and moderately tolerant to stem nematode, but Mortlock is very susceptible and very intolerant to stem nematode (Table 3.1, Wallwork, 2003)). Resistance to CCN was based on the number of CCN females developing on the outside of the rootball (Lewis et al., 1999). Resistance to SN was based on the symptoms of the nematodes on the plants.

Fifteen resistant and 15 susceptible SSD lines were selected from the 170 SSD lines based on the 2000 to 2002 CCN female counts (Appendix 5). Forty seeds of each SSD line were germinated in a misting cabinet in a polyvinyl chloride (PVC) pipe sieve (50 mm height and 90 mm diameter) with a nylon insect screen mesh across one end, suspended above a plastic funnel to provide good drainage and air flow. The experiment was carried out in three replicates. After five days, the root tip of main roots was excised (about 7 mm) with a sterile surgical blade and transferred into a 1.5 ml microfuge tube containing 150 µl 50% methanol (HPLC grade) in batches of 20 for each line in each replicate. Samples were frozen in liquid nitrogen and then stored at -80°C.

3.2.5 Metabolite extraction from oat roots and HPLC analysis

Each frozen batch of root tips was ground in a microfuge tube containing 200 µl 50% methanol using a plastic homogeniser (Bel-Art Products, # F19923-0001). The material was pelleted by centrifugation and re-extracted with 4 × 250 µl 50% methanol.

Each time the sample was centrifuged at 16,450 *g* for 5 min and the supernatant was collected and pooled. Samples were air-dried using a speedy vac and the final residue was dissolved in 80 µl 45% methanol and centrifuged at high speed to remove any insoluble material.

Reversed-phase (RP-HPLC) was performed with a C18 column (Waters Sphersorb ODS-2, 4.6 mm × 150 mm, 5 µm particle) using a gradient elution applied at a flow rate of 0.8 ml/min at 40°C. Samples were dissolved in 80 µL 45% methanol. Separation started with 95% Solvent A (water) and 5% Solvent B (acetonitrile) and followed with a gradient from 5 to 70% Solvent B over 17 min, and then increasing to 100% over 1 min and held at 100% for 2 min. Finally buffer B was reduced to 5% over 1 min and the column re-equilibrated for 7 min. Compounds were monitored with UV detection at 223, 255 and 340 nm. The extraction and HPLC analysis for each SSD line was performed in the same day to minimise possible degradation. The mean and standard error of the data for each line were calculated using Excel 2000. Genstat (Version 6.1; VSN International Ltd, Oxford, UK) was employed to determine the correlation between the areas under the peak for each line and the CCN female count. The significant peaks were collected, accumulated, concentrated and analysed by LC-mass spectrometry in positive and negative ionization modes.

Table 3.1 Comparison of Potoroo and Mortlock for their response to cereal cyst nematode and stem nematode (Wallwork, 2003) and levels of flavone-*C*-glycosides in the shoots

Cultivar	Response to CCN		Response to SN		FCGs mg (rutin equiv.)/g DW
Potoroo	Resistant	Tolerant	Moderately resistant	Moderately tolerant	22.49±0.41
Mortlock	Moderately susceptible	Intolerant	Very susceptible	Very intolerant	14.62±0.41

3.2.6 Induction of secondary metabolites in oat

In an independent experiment, seeds of oat cv. Quoll were germinated in six plastic pots (150 mm diameter) each containing five plants as described above. After three weeks, three pots were drenched with 0.1 mM methyl jasmonate (MJ). This experiment was initiated before I obtained the result showing that drenching with MJ produced reduced levels of FCGs compared to controls (Figure 2.6). Control plants received ultrafiltered water (MQ, Millipore), filtered through a 0.45 μm membrane. Pots drenched with MJ were separated from control pots before treatment by transferring to a different growth chamber with identical conditions (25°C light/20°C dark, 12 hr cycle). Plants were harvested and washed 24 hr after treatment. The roots and shoots were separated and frozen in liquid nitrogen prior to extraction for flavonoid analysis.

3.2.7 LC mass spectrometry identification

MS was carried out with an API-300 triple quadrupole mass spectrometer equipped with an electrospray (ES) ion source (MDS-Sciex, Concord, ON, Canada).

Compounds in the active fractions of the crude extract were separated using a reversed-phase column (Synergi Hydro-RP Phenomenex, Torrance, CA; 4 μm, 150 × 2 mm) with a flow rate of 200 μl/min for 60 min and an injection volume of 20 μl in 100% Solvent A (2.5% formic acid in water) and 0% Solvent B (formic acid: acetonitrile: water; 1:60:39 v/v/v). Gradient elution was performed by increasing from 0 to 20% Solvent B over 10 min, then 20 to 60% over 8 min, followed by 60 to 100% over 7 min, held steady for 10 min and finally decreasing to 20% Solvent B over 1 min. The mass spectrometer was operated in either positive or negative ion scan mode and scanned from *m/z* 150 to *m/z* 2000 with a step size 0.2 Da and dwell time of 0.25 msec. The electrospray needle, orifice and ring potentials were set at 5000 V, 30 V and 250 V, respectively. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 12 units, respectively. The samples were monitored using a diode array detector (DAD, HP 1100, Agilent) at 254 nm and 340 nm. The mass spectral data was processed using Bio-Multiview (Version 1.2β3, PE Sciex) and Analyst (Version 1.4) software.

3.3 Results

3.3.1 Flavone-*C*-glycosides concentration and nematode resistance

To determine if the parents of the single seed descent population were segregating for levels of FCGs, the total concentration of FCGs in the shoots of the parental lines of the population, Mortlock and Potoroo, was determined using established protocols (Sections 2.2.3 and 2.3.1). The concentration of FCGs in the shoots of Mortlock and Potoroo was determined using HPLC monitored at 340 nm. The concentration of FCGs in the parent more resistant to stem nematode, Potoroo, was significantly higher than in FCGs in susceptible parent, Mortlock (at 22.49 ± 0.41 mg (rutin equiv.)/g DW and 14.62 ± 0.41 respectively) (Table 3.1). The difference between

the parental lines suggests that it was worth screening the SSD lines to determine if these differences segregate with the CCN females count and SN scores.

Flavonoids were extracted from the roots of 20 SSD lines chosen on the basis of the resistance phenotypic extremes of CCN female count (Table 3.2) and the shoots of 12 SSD lines chosen on the basis of resistance phenotypic extremes of stem nematode symptoms on plants (Table 3.3). Flavonoids were also extracted from the parental lines (Section 3.3.1). For each sample, the concentration of the three known flavonoids, luteolin-*C*-hexoside-*O*-pentoside (FCG1 at R_t=10.3), apigenin-*C*-hexoside-*O*-pentoside (FCG2 at R_t=10.8) and, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside (FCG3 at R_t=11.1) was quantified and they were about 350 fold higher in the shoots (Table 3.3) than in roots (Table 3.2). There was neither significant correlation between the concentration of FCGs in the roots and CCN female count nor between the concentration of FCGs in the shoots and resistance to stem nematode (Table 3.4). The FCGs data was compared with the CCN female counts for each of the three years, 2000, 2001 and 2002, and the mean of counts in the three years (Table 3.4).

3.3.2 Metabolites associated with CCN resistance

To determine if other metabolites are involved in CCN resistance we extracted metabolites from the root tips of 30 SSD lines (Appendix 5). Methanolic extracts were analysed by HPLC and 16 peaks were identified (Figure 3.1A). The area under the peak was determined for each of the SSD lines in three replicates. Peaks 1, 2 and 8 were ignored because they were consistent in both resistant and susceptible lines based on visual inspection. Two out of 30 lines were removed from analysis due to high standard error and/or low recovery of all metabolites. Three of the peaks were significantly

Table 3.2. Concentration of FCGs in SSD lines based on mg (rutin equiv.)/g DW. The CCN female count of each line is provided in Appendix 5.

Line #	FCG data			
	FCG1	FCG2	FCG3	Total FCGs
High CCN count				
10	0.0062	0.0299	0.0348	0.0709
22	0.0053	0.0224	0.0300	0.0577
78	0.0077	0.0356	0.0354	0.0787
84	0.0085	0.0342	0.0346	0.0773
93	0.0045	0.0336	0.0351	0.0732
95	0.0057	0.0281	0.0362	0.0700
115	0.0018	0.0201	0.0247	0.0466
157	0.0069	0.0214	0.0331	0.0614
190	0.0063	0.0266	0.0391	0.0720
192	0.0091	0.0326	0.0419	0.0836
Low CCN count				
11	0.0052	0.0231	0.0309	0.0592
13	0.0089	0.0325	0.0429	0.0843
15	0.0075	0.0246	0.0294	0.0615
20	0.0056	0.0256	0.0349	0.0661
23	0.0092	0.0274	0.0337	0.0703
28	0.0075	0.0404	0.0391	0.0870
29	0.0031	0.0279	0.0379	0.0689
30	0.0073	0.0326	0.0346	0.0745
34	0.0041	0.0260	0.0250	0.0551
108	0.0042	0.0214	0.0334	0.0590
Parental				
Potoroo	0.0101	0.0366	0.0502	0.0969
Mortlock	0.0066	0.0247	0.0314	0.0627

FCG1, luteolin-*C*-hexoside-*O*-pentoside (Soriano et al., 2004; Figure 2.2B)

FCG2, apigenin-*C*-hexoside-*O*-pentoside (Figure 2.2B)

FCG3, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside (Figure 2.2B)

nt, not tested

Table 3.3. Concentration of FCGs in the shoots of SSD lines based on mg (rutin equiv.)/g DW and their SN scores

Selection number	Stem nematode data				
	FCG data				Nematode score[a]
	FCG1	FCG2	FCG3	Total FCGs	2001
10	2.81	11.73	9.56	24.10	1.75
23	3.39	11.19	11.51	26.09	2.25
30	3.07	11.68	10.79	25.54	0.75
35	2.75	9.73	11.43	23.91	2.25
47	2.96	9.67	11.53	24.16	1.50
73	3.23	11.67	10.30	25.20	1.50
78	2.27	11.46	8.95	22.68	4.50
93	2.18	8.86	11.89	22.93	4.67
112	3.17	10.43	12.67	26.27	4.67
128	3.50	9.79	11.73	25.02	5.00
134	2.48	11.21	11.17	24.86	4.75
175	3.66	11.37	9.50	24.53	4.75
Potoroo	3.11	11.68	9.90	24.69[b]	nt
Mortlock	2.89	9.63	11.06	23.58[b]	nt

FCG1, luteolin-*C*-hexoside-*O*-pentoside (Soriano et al., 2004; Figure 2.2B)

FCG2, apigenin-*C*-hexoside-*O*-pentoside (Figure 2.2B)

FCG3, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside (Figure 2.2B)

[a] symptoms of the nematode were scored from 0 (no symptoms) to 5 (dead plants)

[b] difference is not great as in first experiment but it is still significant ($P<0.05$)

nt, not tested

higher in the CCN resistant lines compared to the CCN susceptible lines, based on the mean of the CCN female counts. In contrast, significant correlation was not found between CCN tolerance and any of the peak area (Table 3.5).

The significant differences between CCN resistance and susceptible lines for Peak 10 (R_t=13.25 min) and Peak 13 (R_t=14.03 min) are clearly shown for one of the three replicate samples for each of the resistant line, 92, and the susceptible line, 78 (Figure 3.1B). The other significant peak (R_t=5.2 min) is not shown. The data for these

Table 3.4. The correlation of flavonoid compounds with the CCN female counts, tolerance to cereal cyst nematode and resistance to stem nematode in the extreme subset of the oat SSD population (n=12 for CCN resistance and tolerance and n=20 for SN resistance)

	Year	FCG1	FCG2	FCG3	Total FCGs
CCN female count	2000	-0.184^{ns}	-0.261^{ns}	-0.122^{ns}	-0.226^{ns}
	2001	-0.120^{ns}	0.125^{ns}	0.062^{ns}	0.069^{ns}
	2002	-0.151^{ns}	-0.042^{ns}	-0.154^{ns}	-0.119^{ns}
	Mean	-0.166^{ns}	-0.076^{ns}	-0.077^{ns}	-0.106^{ns}
Tolerance to CCN	2001	0.113^{ns}	0.192^{ns}	-0.070^{ns}	0.092^{ns}
Resistance to SN	2001	-0.132^{ns}	-0.292^{ns}	0.116^{ns}	-0.198^{ns}

FCG1, luteolin-*C*-hexoside-*O*-pentoside (Soriano et al., 2004; Figure 2.2B)
FCG2, apigenin-*C*-hexoside-*O*-pentoside (Figure 2.2B)
FCG3, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside (Figure 2.2B)
^{ns} not significant at $P<0.05$

three peaks are significant for 2000 and 2002, but not 2001, and this is presumably due to low level of female nematodes in 2001 (Appendix 5). Peak 5 (R_t=7.1 min) had significant correlation with the data 2001, but did not show significant correlation with CCN female count in other years and so remains unsupported.

Significant peaks were separated by HPLC, collected, accumulated and concentrated for further analysis and identification by LC-mass spectrometry. LC-MS of Peak 10 apparently it contains two compounds with two molecular weights, 1080 and 1043 (Table 3.6). LC-MS of Peak 13 indicated that this peak contained a single compound of molecular weight 1027 contained an aglycone of mass *m/z* 571.6 [$M+H^+$] (Table 3.6). It was not possible to determine the mass of the compound in Peak 3 or

obtain any fragmentation data despite three attempts with LC-MS and MS of direct infusion of the HPLC-purified fractions.

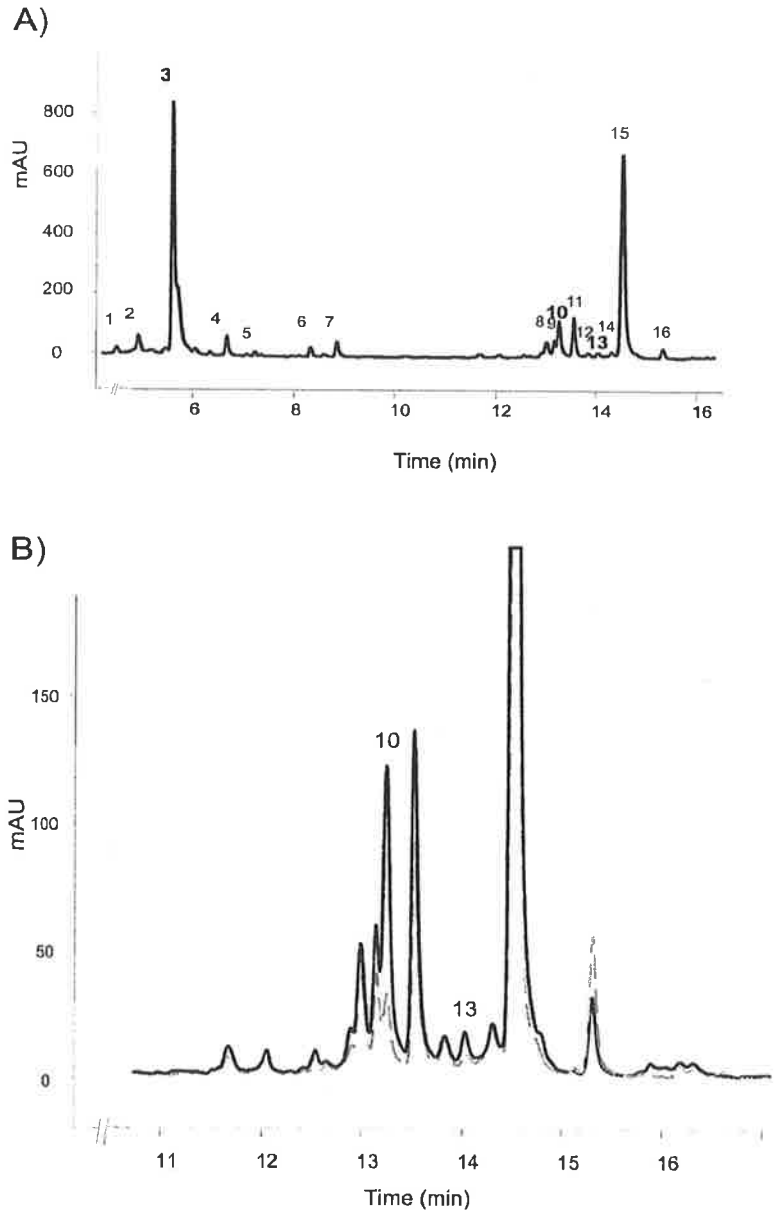


Figure 3.1 HPLC chromatogram of methanolic extracts from oat root tips. (A) Example of the separation of one replicate of Mortlock. Bold numbers (3, 10 and 13) represent the peaks that were significantly different between CCN resistant lines and susceptible lines when data for 28 SSD lines were compared. (B) A comparison of HPLC chromatograms of the CCN resistant line 92 (solid line) and CCN susceptible line 78 (dashed line). Peaks 3, 10 and 13 were significantly correlated with CCN female counts when data for all three replicates were compared (Table 3.5).

Table 3.5 Correlation of metabolites from oat roots with CCN female counts

Peak	CCN female counts				Tolerance
	mean	2000	2001	2002	2001
P3	**-0.474***	**-0.480****	-0.358^{ns}	**-0.443***	0.343^{ns}
P4	-0.106^{ns}	-0.155^{ns}	0.057^{ns}	-0.161^{ns}	-0.072^{ns}
P5	0.317^{ns}	0.268^{ns}	0.382*	0.236^{ns}	-0.207^{ns}
P6	0.141^{ns}	0.041^{ns}	0.214^{ns}	0.162^{ns}	0.073^{ns}
P7	0.046^{ns}	-0.008^{ns}	0.109^{ns}	0.046^{ns}	0.164^{ns}
P9	-0.231^{ns}	-0.154^{ns}	-0.242^{ns}	-0.254^{ns}	-0.227^{ns}
P10	**-0.456***	**-0.476***	-0.361^{ns}	**-0.393***	-0.028^{ns}
P11[a]	-0.283^{ns}	-0.179^{ns}	-0.320^{ns}	-0.303^{ns}	0.090^{ns}
P12	-0.015^{ns}	0.118^{ns}	-0.078^{ns}	-0.118^{ns}	0.094^{ns}
P13	**-0.450***	**-0.429***	-0.343^{ns}	**-0.450***	0.212^{ns}
P14	-0.034^{ns}	0.036^{ns}	0.013^{ns}	-0.151^{ns}	0.098^{ns}
P15[b]	-0.078^{ns}	-0.068^{ns}	-0.055^{ns}	-0.090^{ns}	-0.127^{ns}
P16[c]	0.003^{ns}	0.061^{ns}	-0.064^{ns}	-0.011^{ns}	0.255^{ns}

^{ns},*, **, non significant and significant at $P<0.05$, $P<0.01$ respectively.

[a] avenacin A-2; [b] avenacin A-1; [c] avenacin B-1 based on LC-MS

Bold data showed consistent significant correlation.

Table 3.6. Characterisation of two peaks correlated negatively with CCN female count in oat

Peak	Compound	λ_{max} (nm)	$(M+H)^+$ Molecular ion (*m/z*)	$(M-H)^+$ Molecular ion (*m/z*)	$(M+H-456^{a})^+$ Aglycon ion (*m/z*)	Molecular Weight
10	1	218, 247, 339	1080.8	1079	624.6	1080
	2	nd	1043.8	1041.8	587.4	1043
13	1	198, 256, 347	nd	1026.0	571.6	1027

[a] 2×hexose + 1×pentose

nd, not determined

3.3.3 Induced metabolites in oat roots

Induced rather than constitutive compounds may also be important for nematode resistance, therefore the effect of soil-drenched exogenous MJ was tested on metabolites in roots. Several peaks were induced significantly by MJ in whole roots (Figure 3.2A). The HPLC chromatogram of untreated Quoll plants highlights that many peaks are significantly lower (eg Peak 7, Peak 11 and Peak 13, Figure 3.2B). The HPLC profile obtained with Quoll (Figure 3.2) is significantly different that for SSD lines (Figure 3.1A) and it is due to using older whole root (Figure 3.2) rather than root tips (Figure 3.1A)

To identify the compounds in each peak (20 peaks) LC- mass spectrometry was employed in positive and negative modes of ionisation. Characteristics of each peak are summarised in Table 3.7. The LC-MS analysis of two of the induced peaks, Peak 13 and Peak 15 gave good fragmentation in both modes. The ionisation patterns show that they have the same mass, 1062.2, as the saponin avenacoside A or 26-desglucoavenacoside B (26-DGB) that cannot be distinguished by MS (Wubben et al., 1996). The only difference between the ionisation pattern of Peak 13 and Peak 15 compounds with the avenacoside A or 26-DGB is the presence of the fragment ion *m/z* 819.8 in negative mode. Whereas, all of the ions seen in positive mode have been reported for avenacoside A or 26-DGB. This could arise from contamination as it was found in Peak 14 (Table 3.7) or more likely Peaks 13 and 15 are not avenacosides but they are similar compounds. Induced Peaks 16 and 20 are consistent with avenacin A-2 and avenacin B-1, respectively (Table 3.7). Peak 18 was not induced by MJ but could still be identified as avenacin A-1 (Table 3.7). The ionisation data for other peaks was not enough to determine the structure of the metabolites.

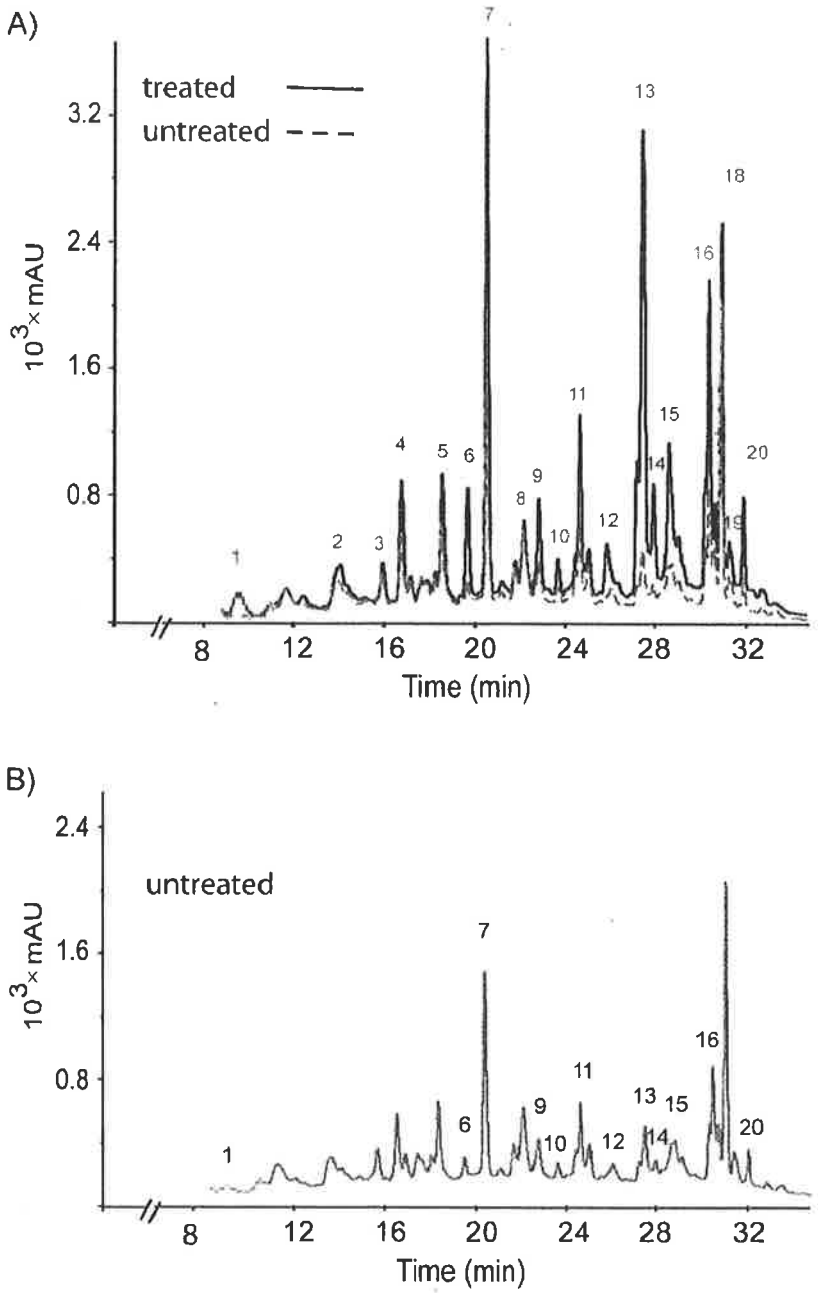


Figure 3.2 A comparison of HPLC chromatogram of methanolic extracts from the roots of MJ treated and untreated oat cv. Quoll. (A) A HPLC chromatogram overlay of MJ treated and untreated samples of Quoll plants. (B) A HPLC chromatogram of the root extracts from untreated quoll plants. Peaks induced by MJ are numbered. Separation was achieved using a C18 column with gradient elution of water as Solvent A and acetonitrile as Solvent B.

Table 3.7 LC-mass spectrometry characterisation of fractions (peaks) induced by methyl jasmonate in oat roots

Induction	Peak	R_t	Positive mode *(m/z)*	Negative mode *(m/z)*	λ_{max}>230 nm	Possible identity
Induced[a]	1	9.2	nd	nd	-	nd
	6	19.8	nd	977.8,960,944,799.8,687.6, 493.4, 449.7, 337.4	299, 323	nd
	7	20.8	nd	1375.8, 944.2, 687.8, 669.6, 643.6, 429.4, 387.6	328	nd
	9	23.1	nd	1372, 946,685.8,667.6, 315.6	275, 323	nd
	10	24.1	nd	1226.2, 735.6, 297.8, 267.4	287, 327	nd
	11	25.0	nd	1064.2, 723.4, 635.6, 486.6, 337.8, 297.6	297, 325	nd
	12	26.3	nd	1080, 902.0, 596.6, 486.6	297, 325	nd
	13	27.8	1063.8, 901. 8,755.6, 739.6 593.8, 515.6, 431.0, 413	1062.2, 819.8	300, 327	Avenacoside A or 26-DGB[b]
	14	28.2	nd	1109, 1061.8, 900.0, 819.6	300, 327	nd
	15	29.1	1063.8, 901.8, 755.6, 739.6 593.8, 515.6, 431.0, 413	1062.2, 819.8	300, 327	Avenacoside A or 26-DGB[b]
	16	30.9	1065.8, 903.8, 885.2, 741.4, 723.4, 609.6, 591.6	1064.2, 900.0	232, 280	Avenacin A-2
	20	32.5	1078.8, 916.8, 622.8, 604.6	1077.2	227, 255, 360	Avenacin B-1
Non-induced	2	14.0	nd	nd	-	nd
	3	16.2	nd	nd	-	nd
	4	17.0	nd	959.8, 799.8, 591.6, 502.4, 340.6	285, 323	nd
	5	18.8	nd	1107.8, 959.6, 813.8, 669.6	321	nd
	8	22.5	677.2,557.2, 446.8, 184.2	946, 798, 774.4, 675.6, 633.6, 591.6, 486.6, 445.6	-	nd
	17	31.1	nd	1124.8, 1064.2, 1048, 900, 861.8	-	nd
	18	31.5	1094.8, 932.6, 770.4, 638.4, 620.8	1093, 1077.0	255, 357	Avenacin A-1
	19	31.8	1094.6, 932.6, 770.6, 638.4, 620.8	1093.0, 1064.2, 1048	-	nd

[a] at least two fold induction based on comparison of three biological replicates for treated and untreated plants; [b] as described by Wubben *et al.* (1996); nd, not determined

3.4 Discussion

In searching for metabolites in methanolic crude extract of the oat root tips, three novel compounds were identified that are associated with CCN female count (Figure 3.1). These three compounds were shown to have a significant negative correlation with CCN female count by looking at 28 of 170 individual lines in the SSD population (Appendix 5). The peak area of each peak was negatively correlated to CCN female counts in 2000, 2001 and 2002. The analysis of data in multiple years is important and strengthens these findings.

The choice of roots tips for this part of the study likely contributed to the success given the limited sample size of 28 SSD lines. Root tips are the establishment and the feeding site of CCN and so they are more likely to contain important compounds (Mor et al., 1992). It was possible to obtain root tips without any damage and contamination because the plants were grown in a misting cabinet suspended in air on mesh.

Peaks 3, 10 and 13 were the peaks negatively correlated to CCN female counts (Table 3.5 and Figure 3.1). The compounds represented by these three peaks were partially characterised by UV/vis and mass spectrometry. Peak 3 (R_t=5.2 min) with maximum absorption at 198, 221 and 278 nm was the biggest peak among the significant peaks and showed 25% increase in resistant lines when compared to susceptible lines. This peak was unstable even when stored in a freezer, so that this peak became two smaller peaks in the HPLC with a similar retention time. Peaks 10 and 13 were probably saponins, based on the similarity of retention times with avenacin A-1 and avenacin B-1 standards. Peaks 10 and 13 both fragment during MS to give an ion with loss of *m/z* 456 indicating the loss of 2 hexoses and one pentose (Table 3.6).

Other peaks, not correlated with CCN resistance could be conclusively identified as the saponins, avenacin A-2 (Peak 11), avenacin A-1 (Peak 15) and avenacin B-1 (Peak 16), based on observed LC-MS data of known avenacins (Figure 3.1).

Analysis of flavonoids in the single seed descent population indicated that flavonoids are not major contributors of constitutive resistance to nematodes based on analysis of whole roots. The evidence that flavonoids are not responsible for CCN resistance is increasing, based on the findings that flavonoids are not induced by MJ (Figures 2.4, 2.5, 2.6) and the concentration of these compounds in oat roots is very low (less than 0.1 mg/g DW, Figure 2.12 and Table 3.2). However, it is possible that by analysing more SSD lines and using only root tips, a significant correlation could be observed. Alternatively, it is possible that flavonoids are not important in nematode resistance in the cultivar Mortlock and Potoroo but are important in other cultivars such as Quoll where flavonoids have been reported as providing protection against nematodes (Soriano et al., 2004).

Current interest in plant resistance to disease includes identifying inducible defence mechanisms (Osbourn, 2001), in part because these mechanisms are less likely to compromise plant yield in the absence of pathogens. MJ is an important elicitor especially in biotic stress (Staswick et al., 1998). Results of an experiment with MJ on secondary metabolites in oat roots showed significant induction of 11 fractions (Figure 3.2A and Table 3.7). This research led to the identification of two MJ inducible compounds similar to avenacoside A and/or 26-DGB, which have previously been shown to be present only in oat shoots (Wubben et al., 1996). The presence of avenacoside A derived compounds in oat have not been reported in roots, and it was thought that they were shoot specific compounds (Tschesche et al., 1969; Wubben et

al., 1996). Avenacoside A is a precursor to a potent antifungal compound, 26-DGA (Wubben et al., 1996). To our knowledge, this finding is novel and indicates the existence of saponin biosynthetic pathway in oat roots as well as oat shoots.

Avenacosides as steroidal saponins and avenacins as triterpenoid saponins were well known as antifungal compounds in oat roots (Crombie et al., 1986; Wubben et al., 1996). Previously it was suggested that avenacins are phytoanticipins, that is, constitutively produced compounds (Townsend et al., 2006). Here, it has been shown that both avenacin A-2 (Peaks 16) and avenacin B-1 (Peak 20) are induced by MJ and therefore may be more correctly classified as phytoalexins, whereas avenacin A-1 (Peak 18) was not induced by MJ. Avenacin A-2 (Peak 11 in Figure 3.1A and Peak 16 in Figure 3.2A) showed high, but not significant, negative correlation with CCN female count and it was significantly induced by MJ. Therefore, it would be worth analysing more SSD lines to determine if a significant correlation exists. In agreement with the data presented here, the induction of triterpenoid saponins in oat roots may have been expected because genes involved in saponin synthesis pathway were reported to be induced by MJ in *Glycyrrhiza glabra* and *Medicago truncatula* (Suzuki et al., 2002; Hayashi et al., 2003). This MJ induction experiment will help us to focus on inducible compounds in future experiments to determine whether they play a role in nematode resistance.

There are several benefits to identifying plant nematicidal compounds (Chitwood, 2002). For example, knowledge of the structure of protective molecules would allow them to be synthesized to provide safe, nature identical, phytoprotecants. Structural knowledge will also assist in the understanding of the biosynthetic pathway and identify targets for future genetic manipulation of genes. This study has identified more than 10 metabolites that may play a role in biotic stresses, three of which are

likely to have antinematode activity. Future studies need to focus of functional testing of the individual compounds and to determine if there are synergistic effects of the three compounds.

Chapter 4

General Discussion and Future Research

4.1 Metabolites associated with CCN resistance

4.1.1 Genetic variation for FCG concentration in oat

The original aim of this project was to determine whether there was significant genetic variation of flavonoid concentration as a potential protective factor for cereal cyst nematode resistance in oat roots and shoots. This study was initiated because flavonoids were shown to be involved in oat resistance to CCN and root lesion nematode (Soriano et al., 2004). A large number of oat accessions (72 accessions) were screened from the SARDI genetic resources including wild relatives and important breeding cultivars. In this study, it was not possible to measure the concentration of flavonoids in the roots. Roots were of primary interest to this study because a previous report suggested that flavonoids in roots gave protection against CCN (Soriano et al., 2004). In the preliminary experiments searching for genetic variation in oat accessions and the experiment described in Section 2.3.6, the FCG peaks were negligible (Figure 2.12) and were not measured. However, the flavonoids in oat roots (SSD population) were analysed and quantified using HPLC and correlated to the number of white females developing in the plants in Table 3.2. Levels of FCGs in roots varied from 0.046 to 0.096 mg (rutin equiv.)/g DW based on data in Table 3.2.

A significant variation of concentration of flavonoids was found in the shoots of a variety of oat accessions in three sequential experiments (Table 2.2 and Figures 2.4 and 2.5). IA91406-1 was the cultivar with highest level of FCGs in both glasshouse conditions (Table 2.2 and Figure 2.4) and in growth chambers (Figure 2.5). It may have

been possible to identify more consistent significant differences if the same controlled conditions were used for all of experiments. For example the difference in ranking of accession 91QK195 from the first rank in the experiment in the glasshouse with 21 accessions (Figure 2.4) to the last rank in the experiment carried out in the growth chamber with five accessions (Figure 2.5), may have been attributed to the different growth conditions.

Most of the FCG extractions performed in this thesis were performed on 5 days as described in Section 2.2.3. By reducing the time of exposure to methanol and omitting the butanol step, two days can be saved per extraction (Appendix 4). The first modification was a reduction in time of sample exposure to methanol from 48 hr to 12 hr. In fact, it may be possible to further reduce the extraction time to less than 12 hr and this should be tested before any future experiments are performed. The second modification was to omit the butanol step, which gave significantly more FCGs compared to extraction of FCGs with butanol.

4.1.2 Correlation of secondary metabolites and CCN female counts

In the study of SSD lines to find oat root metabolites correlated with CCN female count, three metabolites were found in oat root tips to have significant correlation with CCN count. In this experiment only 30 lines of 170 generated SSD lines were tested (in three replicates). The peak area of 16 peaks was tested for correlation to CCN count in 2000, 2001, 2002 and CCN tolerance data in 2001. Root tips were analysed in this experiment as root tips are the sites of establishment and feeding cells for CCN (Mor et al., 1992) and it has been reported that root tips contain antifungal compounds like avenacins (Crombie et al., 1987). Attempts to identify the compounds present in the three significant peaks using LC-MS and MS direct infusion

gave incomplete information, however the mass and absorption maxima (summarised in Table 3.6) were obtained.

Avenacin A-1, avenacin A-2 and avenacin B-1 were identified in oat roots, but none showed significant correlation with CCN count or tolerance. Further research on the effect of avenacin A-2 is suggested as it showed high but not significant correlation to CCN count and this compound showed induction by MJ in an independent experiment (Table 3.7).

The direct application of the three fractions that are correlated to low nematodes count and their synergistic effect on the development of CCN is warranted. Furthermore, it seems worthwhile to study a larger number of SSD lines.

4.2 Antimicrobial activity of flavonoids and saponins

4.2.1 Antimicrobial activity of flavonoids in oat

The antinematode activity of flavonoids was examined in this research using a single seed descent population. Results showed that none of the three FCGs had significant correlation with CCN count and resistance to stem nematode. To understand antinematode activity of flavonoids, the direct effect of FCGs on nematodes is necessary.

To extend earlier work which showed that the FCG, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside, could provide protection against CCN (Soriano et al., 2004), the possible antifungal and antibacterial activity of oat crude extract was also tested. Results showed that oat crude extract could not inhibit the growth of the tested bacteria. Therefore no more bioassay experiments were performed on bacteria. Oat crude extract containing FCGs could inhibit the mycelial growth of *Pyrenophora*

species. However, HVPE fractionation showed that fractions with high level of FCGs did not inhibit the growth of fungi. In other words, FCGs are not responsible for the inhibition of fungal growth observed in the crude extract.

4.2.2 Antifungal activity of saponins in oat

Since it was shown that flavonoids were not the active fractions, the active HVPE fractions were analysed by LC-MS. Results showed that these fractions contain saponins (Figure 2.17). The different saponins in oat shoots were purified and accumulated using PTLC and C18 column chromatography. Bioassays with purified saponins showed that 26-DGB had a greater inhibitory activity than 26-DGA, but the crude extract had even higher activity. This indicates that there is synergistic effect between either saponins or saponins and non-saponins fractions, which needs more investigation.

The reason for the recovery of mycelia growth after inhibition by saponins in the bioassays (Figures 2.14 and 2.20) deserves further study. It could be because of either diffusion leading to effectively dilution of the concentration of 26-DGA and 26-DGB in the medium or inactivation by fungal enzymes. Inactivation of saponins has been observed in oat attacking isolates of *Septoria avenae* (Wubben et al., 1996). To determine whether inactivation is involved it would be necessary to re-extract the saponins from the medium.

4.3 Methyl jasmonate and secondary metabolites in oat plants

4.3.1 MJ and FCGs in oat

It was reported that MJ could protect oat roots against major cereal nematodes by inducing flavone-C-glycosides (Soriano et al., 2004). In this study, it has been shown that MJ could induce 12 HPLC fractions in oat cv. Quoll roots (Figure 3.2 and Table 3.5) but none of these were FCGs. Furthermore, the concentration of FCGs in oat roots was too low to measure confidently. In Section 3.3.2, it was shown that there was no significant correlation between concentration of FCGs and CCN counts when analysed in a single seed descent population generated from the cross between oat cvs. Mortlock and Potoroo.

The effect of MJ on FCGs in oat shoots was also investigated. Although the concentration of FCGs in oat shoots is about 350 fold more than that in oat roots (Figure 2.12 and Tables 3.2 and 3.3), there was no induction of FCGs by MJ in any of the independent experiments. The possibility that technical issues were responsible for the differences between the findings in this thesis and those of Soriano et al (2004) was eliminated. The same cultivar (Quoll) was used in all experiments (Figures 2.6, 2.7 and 2.11) to test effectiveness of MJ application. All attempts failed to repeat the results of the earlier study, which showed that FCGs were induced by MJ.

4.3.2 MJ and the effect on other secondary metabolites in the oat roots

Exogenous application of MJ induced 12 HPLC fractions in oat roots. The induced fractions included avenacin A-2, avenacin B-1 and two compounds similar to avenacosides. Avenacins are known as antifungal compounds in oat roots (Crombie et al., 1986). LC-MS data indicated that there are two compounds in oat roots that are

very similar to avenacosides in oat shoots (Figure 3.2 and Table 3.7). This finding is novel and highlights the complexity of secondary metabolite pathways in plants.

In an independent experiment, saponins in oat cv. Quoll shoots were purified and tested against *Pyrenophora teres* f. *teres*. The results showed that 26-DGB and 26-DGA are two important antifungal compounds in oat shoots, which is consistent to other reports (Wubben et al., 1996). It was shown that antifungal compounds were also present in other oat cultivars (cvs. Potoroo, Mortlock). The induction of antifungal compounds by MJ was shown in Mortlock and Quoll but not Potoroo based on a bioassay experiment (Figure 2.15). It is not known if MJ induces the saponins in oat shoots and this needs further study. If saponins are important, screening more cultivars and wild relatives will be useful as was for flavonoids. To be more successful, it will be necessary to overcome germination issues especially for the wild accessions. The lack of germination could be due to dormancy of the seeds or loss of viability during storage. If dormancy was case, cold treatment prior to sowing is suggested (Turk, 2005).

The results in this study provides several important areas for further research that in future should allow plant breeders to develop lines with better resistance to nematodes.

Appendices

A.1 List of oat accessions obtained from SARDI, SA, Australia

No	Line (Accession number)	Source location
1	AC Ernie	Canada
2	AK-1	Japan
3	Athabasca	Alberta (Canada)
4	Calibre	-
5	Carma	International oat nursery
6	CC 6333	UK
7	Dal	Wisconsin (USA)
8	Grise D'Hiver	France
9	IL2815	Illionis (USA)
10	Inta Majo (SSR)	Mexico
11	L'Gorskij 1026	USSR
12	Matilda	Sweden
13	Wandering	WADA (Aus)
14	Euro	SARDI (Aus)
15	ND9308094	North Dakota (USA)
16	Preston	Minnesota (USA)
17	NZ2393	New Zealand
18	Solva	Wales (UK)
19	UFRGS 987015-3	Brazil
20	8ZOP95	Saskatchewan (Canada)
21	*A. sterilis* (2)	Callington (SA, Aus)
22	*A. fatua* (13)	Naracoorte (SA, Aus)
23	*A. barbata* (188)	Pinery (SA, Aus)
24	AC Assiniboia	Canada
25	AC Baton	Canada
26	AC Fregeau	Canada
27	AC Lotta	Canada
28	AC Percy	Canada
29	Ajay	International oat nursery
30	AK-5	Japan
31	Algerian	Algerian
32	Amagalon	Minnesota (USA)
33	Appalaches	-
34	Arnold	-
35	Ariane I.N.R.A.	France
36	Auron	-
37	Bulgaria 84106130	Bulgaria
38	Avoine Nue-Nue Noise	France
39	Belmont AC	Canada
40	Blaze	International oat nursery
41	Border	Washington (USA)
42	Bullion	UK
43	C-1/130	Minnesota (USA)

No	Line (Accession number)	Source location
44	C-1/370	Minnesota (USA)
45	UC 112	UC Davis CA (USA)
46	UC 145	UC Davis CA (USA
47	SAIA	Israel
48	CDC Dancer	Canada
49	Chapman	Florida (USA)
50	Clintford	Indiana (USA)
51	CN 12497	UK
52	Chinesischer Nackhthafer	-
53	Corondo	Texas (USA)
54	CPI 115383	-
55	CPI 115397	-
56	Donald	Wisconsin (USA)
57	Dwarf 8	Japan
58	Elan	Georgia (USA)
59	Flamings Nova	-
60	GA Mitchell	Georgia (USA)
61	Hendon	UK
62	IA 91098-2 (High oil – β glucan)	Iowa (USA)
63	IA 91406-1 (High oil –β glucan)	Iowa (USA)
64	IL92-6745	Illionis (USA)
65	Kingfisher	UK
66	Kalott	Sweden
67	Madison	Wisconsin (USA)
68	Wintaroo	SARDI (SA, aAus)
69	Brusher	SARDI (SA, Aus)
70	94046-57	SARDI (SA, Aus)
71	Possum	SARDI (SA, Aus)
72	Echidna	SARDI (SA, Aus)
73	Potoroo	SARDI (SA, Aus)
74	Mortlock	WADA (Aus)
75	ND873364	North Dakota (USA)
76	ND931075	North Dakota (USA)
77	Nasta	-
78	Neon	UK
79	Ogle	Illinois (USA)
80	OH1022	Ohio (USA)
81	Eurabbie	NSW (Aus)
82	OT 289	Ontario (Canada)
83	Ozark	Arkansas (USA)
84	Portage	Wisconsin (USA)
85	Quall	SA (Aus)
86	Rossnagel 3259	Saskatchewan (Canada)
87	NZ2101	New Zealand
88	NZ2742	New Zealand
89	Sanna	-
90	Trucker	South Dakota (USA)

No	Line (Accession number)	Source location
91	Tarahumara	Mexico
92	UFRGS 1	Brazil
93	UFRGS 940257-1	Brazil
94	UFRGS 940886-4	Brazil
95	UFRGS 940548-5	Brazil
96	UFRGS 988012-1	Brazil
97	1ZOP95	Saskatchewan (Canada)
98	4ZOP95	Saskatchewan (Canada)
99	13 ZOP95	Saskatchewan (Canada)
100	4671-581 (Hi β-glucan)	Bosnia
101	91QK195 (Slow rusting)	QLD (Aus)
102	Yiddait	-
103	UPF 775456	Brazil
104	*A. fatua* (4)	Callington (SA, Aus)
105	*A. sterilis* (14)	Naracoorte (SA, Aus)
106	*A. sterilis* (25)	Cummins (SA, Aus)
107	*A. fatua* (26)	Cummins (SA, Aus)
108	*A. barbata* (31)	Port Lincoln (SA, Aus)
109	*A. fatua* (65)	Ardrossan (SA, Aus)
110	*A. sterilis* (73)	Ardrossan (SA, Aus)
111	*A. sterilis* (105)	Balaklava (SA, Aus)
112	*A. fatua* (107)	Balaklava (SA, Aus)
113	*A. barbata* (192)	Tro wells (SA, Aus)
114	*A. barbata* (196)	Brra (SA, Aus)
115	*A. barbata* (289)	Laweros (SA, Aus)

A.2 Formulation of University of California (UC) mix

The ingredients of UC soil was mixed by Glasshouse Services, SARDI, Plant Research centre, Waite Campus.

400 L coarse washed sand sterilized at 100°C for at least 30 min.

300 L Euroturf peatmoss added and mixed at 80°C

Calcium hydroxide	700 g
Calcium carbonates	480 g
Nitrophoska (Germany) (15:4:12)	600 g

Total nitrogen 15% (5% NH_4, 4% NO_3, 1% NH_2, 5% IBDU)
Total phosphorus 3.9%
Potassium sulphate 12.4%
Magnesium carbonate 1.25%
Dicalcium phosphate 3.4%
Sulphate 5.3%
Iron oxide 0.3%
Copper oxide 0.0002%
Zinc oxide 0.007%
Calcium borate 0.01%
Molybdenum oxide 0.0003%

A.3 Hoagland's solution (Hydroponic Sales and Services, SA, Australia)

1.2 g Part A and 0.8 g Part B per litre water was applied.

Elements	Part A (% w/w)	Part B (% w/w)
Nitrogen as nitrate	6.9	14.5
Nitrogen as ammonia	1.7	1.0
Total nitrogen	8.6	15.5
Phosphorus water soluble	3.8	-
Potassium as nitrate	20.3	-
Calcium	-	20
Magnesium	3.7	-
Zinc	0.001	-
Copper	0.005	-
Manganese	0.1	-
Iron	0.4	-
Molybdenum	0.005	-
Boron	0.02	-
Sulphur	4.9	-

A.4 Optimising the method of FCGs extraction

A4.1 Materials and methods

A4.1.1 Effect of exposure time to methanol on the concentration of FCGs

FCGs were extracted from Quoll shoots (60 mg) using different lengths of time in methanol, 12, 24, 36 and 48 hr. Four replicates were performed at each time point. After incubation, 7 ml of methanolic solution was taken and added to 3 ml MQ water then exposed to 10 ml n-hexane for 48 hr to remove the lipids. Eight ml of the methanolic phase was transferred to a 10 ml culture tube and evaporated using a speed vac concentrator (Savant Instruments Inc. Hicksville, NY, USA). The residue was redissolved in 5 ml MQ water with a vortex mixer and 5 ml of 1-butanol (HPLC grade) was added, and shaken for 24 hr. Four ml of butanol phase was evaporated as above. The final residue was dissolved in 0.4 ml 45% methanol for assay by HPLC (Section 2.2.3, gradient elution).

A4.1.2 Effect of butanol on the concentration of FCGs

Six samples of Quoll shoots (60 mg) were exposed to 10 ml methanol for 48 hr and then 7 ml methanolic extract was added to 3 ml MQ water and exposed to 10 ml n-hexane for 48 hr. Eight ml of methanolic phase of each sample was taken and divided into 2 × 4 ml in 10 ml glass culture tubes. Both samples were dried using a speed vac concentrator. The residue of one tube was dissolved in 2.5 ml MQ water and then 2.5 ml butanol was added and incubated overnight on an orbital mixer incubator (130 rpm). Finally 2.4 ml butanol phase was taken, dried, and then residue was dissolved in 400 μl 45% methanol. The residue of the other sample was dissolved in 400 μl 45% methanol. Samples were analysed using HPLC method described in Section 2.2.3.

A4.2 Results

A4.2.1 Changes in the method of FCG extraction

The extraction method of Soriano et al. (2004) used 48 hr methanol to extract FCGs. To test whether it was possible to reduce the time of sample exposure to methanol, shoot samples were treated for four different times (12, 24, 36 and 48 hr). Results indicated that there are no significant differences for peaks representing FCGs and total FCGs among the treatments (Figure A4.1).

To see if all of the FCGs were effectively extracted into the butanol phase, a comparison of the butanol and aqueous phase of flavonoid extraction (Section 2.2.3) showed that FCGs were still present in the aqueous phase, which was previously discarded (about 20% of total FCGs). The FCGs concentration in each fraction was quantified. The aqueous fraction (without butanol) contained 28.14±1.84 mg rutin equiv./g DW significantly ($P<0.01$) higher than FCGs concentration in extracted samples with butanol (23.36±1.56 mg rutin equiv./g DW). The samples extracted by butanol contained fewer additional peaks than samples extracted without butanol when analysed by HPLC (data not shown). Despite the presence of other peaks, they were at a different retention time and they did not interfere with FCG quantification. These findings helped to save time and should provide a more accurate estimation of FCGs than the initial experiments.

By reducing time of exposure to methanol and omitting the butanol step, two days were saved for each extraction.

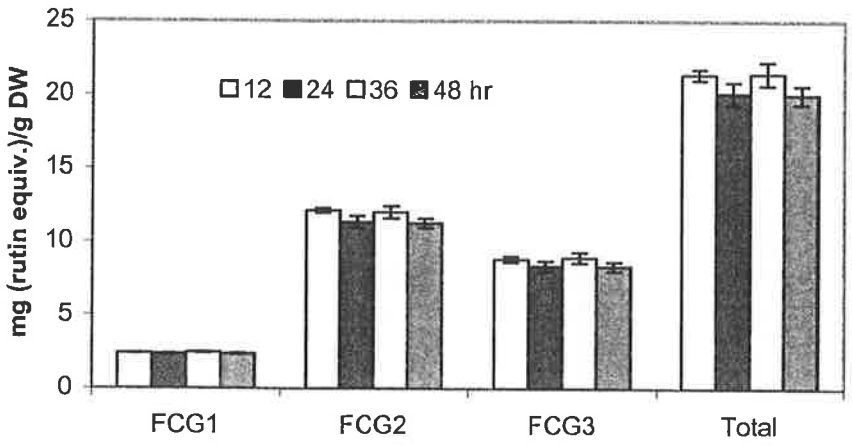


Figure A4.1 Effect of extraction time with methanol on FCG extraction. Mean of each treatment for each peak calculated from four replicates. Bars represent the mean ± SE. FCG1, is luteolin-*C*-hexoside-*O*-pentoside. FCG2, is apigenin-*C*-hexoside-*O*-pentoside. FCG3, is *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside.

A.5 CCN count data used to identify extreme individuals in SSD population.

The most susceptible lines had mean CCN counts of between 7 to 13, whereas the most resistant individuals had CCN counts less than 1. In many of the susceptible lines, for example lines 22, 74, 151 and 154, the CNN count was lower in 2001 than in 2000 and 2002.

	CCN Count					
Line #	2000	2001	2002	Mean	Expt 1[1]	Expt 2[2]
High CCN count						
10	6.80	7.00	9.20	7.67	+	+
22	13.80	4.40	10.00	9.40	+	+
35	8.20	3.60	10.40	7.40	-	+
74	13.40	3.60	11.40	9.47	-	+
78	7.70	7.60	9.40	8.23	+	+
84	6.30	7.80	7.00	7.03	+	+
93	5.70	15.00	13.20	11.30	+	+
95	12.80	14.00	5.00	10.60	+	+
111	14.00	13.25	8.20	11.82	-	+
115	15.70	10.80	11.60	12.70	+	+
157	12.90	5.00	8.80	8.90	+	+
190	16.80	8.60	8.40	11.27	+	+
192	7.90	9.80	6.80	8.17	+	+
152	11.60	6.40	6.00	8.00	-	+
174	17.90	5.40	18.00	13.77	-	+
Low CCN count						
11	0.00	0.00	0.60	0.20	+	-
13	0.40	0.00	0.00	0.137	+	+
15	0.10	0.00	0.20	0.10	+	-[3]
20	0.00	0.00	0.50	0.17	+	+
23	0.00	0.00	2.80	0.93	+	-
28	0.00	0.00	0.25	0.08	+	+
29	0.10	0.00	0.00	0.03	+	+
30	0.30	0.00	0.20	0.17	+	+
34	1.50	0.00	1.25	0.92	+	-
47	0.00	0.00	0.00	0.00	-	+
80	0.10	0.00	0.20	0.10	-	+
83	0.10	0.00	0.00	0.03	-	+
92	0.00	0.00	0.00	0.00	-	+
108	0.00	0.40	1.00	0.47	+	-
114	0.00	0.00	0.00	0.00	-	-[3]
116	0.30	0.00	0.00	0.10	-	+
125	0.00	0.00	0.00	0.00	-	+
126	0.00	0.00	0.40	0.13	-	+
189	0.10	0.00	0.40	0.17	-	+

[1] Experiment 1 examined FCG concentration in whole roots (Table 3.2)
[2] Experiment 2 examined metabolites from root tips by RP-HPLC (Table 3.5)
[3] Grown for experiment 2, but not analysed due to high standard error and/or low recovery of all metabolites

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