Genetic and Biological Characterisation of Resistance to Root Lesion Nematode *Pratylenchus thornei* in Wheat

by

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Declaration

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This thesis is submitted in a combination of conventional and publication format. The main body of work contains three manuscripts (Chapter 2, 3 and 4) and a conventional thesis chapter (Chapter 5). A literature review establishes the field of knowledge and provides a link between publications. A general conclusion shows the overall significance of the work and future directions. The manuscripts have been prepared in the formats outlined by the journals to which they are to be submitted (excluding line numbering, Americanised spellings, table and figure lists after literature cited and font size) and thus chapters contain different formatting.

The manuscript - Characterisation of Resistance to *Pratylenchus thornei* in Wheat; Attraction, Penetration and Maturation

This manuscript will be submitted to the journal of Phytopathology. Outlined is the contributions made by each author in development of the manuscipt in terms of conceptual, experimental and documentation. The co authors give permission for this manuscript to be included in the thesis of Linsell, Katherine J. to be submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy at the University of Adelaide.

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Table of Contents

Glossary of Abbreviations

°c	Degrees Celsius
µg	Microgram
µL	Microlitre
µm	Micrometre
a.i.	After Inoculation
AUD	Australian dollars
AFLP	Amplified Fragment Length Polymorphism
BAC	Bacterial Artificial Chromosome
CCN	Cereal Cyst Nematode
cM	CentiMorgan
CR	Crushed Root
d	Days
DArT	Diversity Array Technology
df	Degrees of Freedom
DH	Doubled Haploid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
EST	Expression Sequence Tag
F1	First Filial Generation
F2	Second Filial Generation
g	Grams
h	Hours
H2	Heritability
HF	Hatching Factor
HI	Hatching Inhibitor
ITS	Internal Transcribed Spacer
J2	Juvenile Stage Two
J3	Juvenile Stage Three
J4	Juvenile Stage Four
kbp	Kilobase pair
kg	Kilogram
L	Litre
LOD	Logarithm of Odds
LRS	Likelihood Ratio Statistic
LSD	Least Significant Difference
M	Molar
MAS	Marker Assisted Select

mg	Milligram
mL	Millilitre
mm	Milimetre
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
NIL	Near Isogenic Line
PCR	Polymerase Chain Reaction
Pg	Picogram
QTL	Quantitative Trait Loci
R^2	Correlation Coefficient
RAPD	Random Amplified Length Polymorphism
RE	Root Exudate
RFLP	Restriction Fragment Length Polymorphism
RIL	Recombinant Inbred Line
RIP	Ribosome Inactivating Protein
RLN	Root Lesion Nematode
RNAi	Ribonucleic acid interference
RNase	Ribonuclease
RO	Reverse Osmosis
ROS	Reactive Oxygen Species
RT	Room Temperature
s	Second
s.e.	Standard Error
SARDI	South Australian Research & Development Institute
SCAR	Sequence Characterised Amplified Region
SDS	Sodium dodecyl sulfate
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
Taq	Thermus aquaticus DNA Polymerase
TE	Tris-EDTA
Tris HCl	Tris (hydroxymethyl) aminomethane hydrochloride
UV	Ultraviolet
w	Week
χ^2	Chi-squared

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Abstract

Root lesion nematodes of the genus *Pratylenchus* feed and reproduce in the root cortex of many plant species, including wheat. Migration through root tissue causes extensive root damage, and in turn severe reductions in growth and yield. In Australia, one of the most prevalent and widespread species affecting wheat is *Pratylenchus thornei*. Due to the wide host range of *Pratylenchus* spp. and the restrictions and inefficiency of chemical pesticides, the development of resistant cultivars has become increasingly important. Despite the identification and investigation of several resistance sources and resistance quantitative trait loci (QTL), no *P. thornei* resistance has been integrated into commercial cultivars. In addition, prior to this study, the biological resistance mechanisms of wheat against *P. thornei* were not well characterised. The identification of novel sources of genetic resistance in wheat and understanding of the biological mechanisms will allow effective combinations of genes either to be used alternatively or pyramided to generate effective and stable *Pratylenchus* resistance. The major objectives of the study were to identify genetic loci associated with *P. thornei* resistance and to investigate the associated biological mechanisms in a double haploid wheat population developed from a cross between the synthetically derived Sokoll and the Australian adapted Krichauff parental lines.

The resistance to *P. thornei* observed in the Sokoll x Krichauff wheat population is complex and under the control of several loci which suppress all nematode developmental stages. The four main components of the root invasion process by *Pratylenchus*: root attraction, penetration, endoparasitic feeding and reproduction, were investigated to determine the location, timing and role of resistance against *P. thornei*. Through analysing root invasion by each nematode life stage, it was shown that resistance in the Sokoll x Krichauff population occurs post penetration to suppress *P. thornei* motility/migration and juvenile development causing reduced reproduction (egg deposition and hatch).

Attraction and penetration assays were conducted on seedlings grown both in sand and on agar. There was no significant difference in the rate at which *P. thornei* was attracted towards resistant or susceptible roots in sand. However on agar, when both genotypes were present, there was a significantly higher attraction towards the susceptible roots indicating resistant roots may secrete repellent or toxic compounds during pre-penetration or that susceptible roots secrete more attractants. The penetration rates of *P. thornei* in resistant and susceptible roots, both on agar and in sand, did not significantly differ. No preferred root penetration zone was observed with *P. thornei*, but penetration was not random as nematodes were attracted to root regions previously invaded. In concordance with other *Pratylenchus* studies, resistance to *P. thornei* in this Sokoll x Krichauff population acts post penetration.

Analysis of *P. thornei* development in the resistant and susceptible genotypes showed that significantly fewer *P. thornei* nematodes of all stages occurred in the resistant compared to the susceptible roots. Juvenile development was suppressed as no juvenile stage two nematodes (J2) were present 35 days after inoculation in resistant genotypes. At 45 days after inoculation, forty times more *P. thornei* juvenile stage three (J3) were present in the susceptible than the resistant parent. Unlike other studies where resistance against *Pratylenchus* caused nematodes to exit roots, in this study, similar numbers of *P. thornei* J2 were still present within the resistant roots 10 days after inoculation, indicating that resistance suppresses nematode development rather than causing nematodes to leave resistant roots.

The inhibition of juvenile development resulted due to the suppression of nematode migration/motility which suppressed feeding but also due to reduced egg deposition and hatch. Simple and inexpensive assays were designed to investigate *P. thornei* motility, egg hatch and deposition in root exudates/extracts and roots grown on agar. Significantly higher numbers of *P. thornei* nematodes became non-motile when exposed to root exudates from resistant (65%) versus susceptible (30%) roots after exposure for 3 days. The effects of these compounds were found to be reversible and to specifically affect *P. thornei* but not *Pratylenchus neglectus*. In migration assays, *P. thornei* only migrated a small distance through the resistant root cortex from the point of inoculation (10 mm), but further in the susceptible roots (70 mm). *Pratylenchus thornei* reproduction was also affected by resistance. Egg deposition was up to 30% less within resistant than in the susceptible lines. About 40% less hatch occurred from eggs within and adjacent to roots of resistant versus susceptible seedlings. Similarly, hatching was decreased by 10% in resistant root exudate compared to the susceptible after 10 days of exposure. An increased hatch after dilution of root exudates and a lower hatch in resistant versus the absence of roots, indicates the presence of hatching inhibitor compounds. As these root exudates were derived from plants not exposed to *Pratylenchus*/other pathogens, this indicates resistant genotypes constitutively produce compounds that inhibit motility and reproduction.

In order to identify QTL and develop molecular markers accounting for the observed resistance, a genetic map was constructed from the Sokoll x Krichauff doubled haploid population comprising 150 lines. A total of 860 Diversity Array Technology markers and 111 microsatellite markers were used to assemble the genetic map. Two highly significant *P. thornei* resistance QTL were identified on chromosomes 2BS and 6DS, *QRlnt.sk-2B.1 and QRlnt.sk-6D*, explaining 24 and 43% of the phenotypic variation, respectively. These QTL mapped to chromosome regions previously identified to be associated with *Pratylenchus* resistance, based on common marker locations. Two significant QTL were also identified on chromosomes 4A and 5A, explaining 6 and 9% of the phenotypic variation. The population was fixed for the effects of the highly significant QTL on 2BS and 6DS and further QTL were identified on chromosomes 2B, 2D, 3A, 5B and 6B. The *QRlnt.sk-2B.1* and *QRlnt.sk-6D*

account for a large portion of the observed resistance, showing that in this population the Sokoll derived resistance to *P. thornei* is very strong and is controlled by a few loci with large effects.

There are considerable financial and labour costs associated with *Pratylenchus* phenotypic screening methods. Molecular markers employed through marker assisted selection will eliminate the need for large scale phenotyping in breeding programs and thus accelerate the development and availability of resistant cultivars. The microsatellite marker *barc183* linked to *QRlnt.sk-6D* is also associated with *P. thornei* resistance in other mapping studies in different genetic backgrounds and thus highlights the potential benefit of this marker for use in marker assisted selection. However, the highly significant QTL on 2BS and 6DS currently span large chromosomal regions, thus fine mapping is required to delimit the QTL interval to establish more closely linked markers before they can be utilised in breeding programs.

The ultimate aim of this project was to correlate a biological role with an identified *P. thornei* resistance QTL. Thus, in order to identify whether the QTL linked to *P. thornei* were associated with the observed motility and hatch inhibition, a subset of the population was phenotyped using the motility and hatching assays designed in this study. Suggestive QTL were identified on chromosomes 2B, 5B, 6B and 6D linked to hatching and motility suppression, which co-located to the *P. thornei* resistance QTL identified in this and previous studies. Although only suggestive, alignment with other QTL indicates that these resistance QTL may play a role in inhibiting *P. thornei* motility or juvenile hatching. To further define and confirm these QTL, phenotypic analysis needs to be performed on the entire population.

The biochemical characteristics of the preformed resistant root compounds causing motility and hatching suppression were investigated. Root exudates that were subjected to heat/cold treatments caused less motility suppression than compared to the untreated control, indicating these resistant root compounds are water soluble and fairly stable in nature. Flavonoids, oxidised phenols and peroxidases associated with insect resistance genes that co-located with the hatching and motility suppression QTL and the *P. thornei* resistance QTL regions have been implicated in other *Pratylenchus*-plant resistance interactions. These results indicate a potential role for these compounds in the *P. thornei* resistance observed in Sokoll x Krichauff. Further investigation is required to define the chemical nature and specific roles of resistant root compounds in the suppression of nematode development.

The results of this study show that the resistance observed in the Sokoll x Krichauff wheat population to *P. thornei* is complex and under the control of two highly significant and several minor loci, which do not affect penetration but suppress nematode feeding, development and reproduction.

Chapter 1
Literature Review

1.1 Introduction

Agricultural production is a major component of Australia's economy with 19.9 million hectares devoted to dryland wheat cropping (ABS 2011). Wheat is the most important crop in Australia with 21.8 million tonnes produced in 2009 - 2010 (ABS 2011). Nematodes are the most abundant multicellular animals on earth (Shurtleff and Averre 2000), and plant parasitic nematodes are one of the main biotic causes of plant stress and yield loss with root nematodes being the most economically important. Worldwide losses to various crops due to nematode damage annually exceed $AUD 110 billion (Bird and Kaloshian 2003) and in Australia loss due to parasitic nematodes in wheat is nearly $AUD 200 million (Murray and Brennan 2009).

Root lesion nematodes in the *Pratylenchus* genus are migratory and endoparasitic (Thompson et al. 1999). At least eight species of *Pratylenchus* are known to parasitise small grain cereals (Nicol and Ortiz-Monasterio 2004). In Australia, the two most prevalent and widespread species in wheat are *Pratylenchus thornei* and *Pratylenchus neglectus* (Thompson et al. 1981). *Pratylenchus* species feed and reproduce in the root cortex of many plants, including wheat. Migration through root tissue causes extensive root damage resulting in the formation of necrotic lesions. The resulting damage to the root system impairs the plant's ability to take up water and essential nutrients, thus causing severe reductions in growth and in turn yield (Orion et al. 1984, Farsi et al. 1993). Yield losses due to *P. thornei* and *P. neglectus* have been estimated at over $AUD 50 million and $AUD 73 million each year, respectively (Murray and Brennan 2009). Although the invasion mechanisms of some other *Pratylenchus* species have been well detailed there has been little analysis of *P. thornei* and *P. neglectus*.

As the human population grows there is in turn an increased need for food production. To meet these growing demands, it has been predicted that wheat production must increase by 60% by 2025 (IFPRI 1997). However, due to the lack of land available for agricultural use and concerns about environmental degradation, the required increases in food production must come from the land already available using fewer chemicals, water and labour (Khush 2002). Due to the wide host range of *Pratylenchus*, control through rotations with pasture crops is not a successful control mechanism. Furthermore, disease incidence in recent times has increased through practices of continuous cropping and no-till agriculture (Thompson et al. 1989). In addition, the increased costs, restrictions and damaging environmental effects of chemical pesticides have led to the increased emphasis on the development of resistant cultivars. In the future, predicted changes in global climate could make the agricultural

regions of Australia more variable and drier. Consequently, resistance to root diseases and thus better root systems will be critical for maximum access by the plant to limited soil moisture.

Tolerant cultivars maintain plant growth and yields within infested soils. However, tolerant plants allow nematode reproduction causing increased numbers within soils, which can attack subsequent less tolerant crops. Resistance to nematodes has been defined as the capacity of a cultivar to reduce nematode reproduction and thus control population levels (Thompson et al. 1999). The use of resistant and tolerant cultivars is now considered the most efficient, economical and environmentally acceptable means for *Pratylenchus* control (Castillo et al. 1998).

To date, only a limited number of sources of *Pratylenchus* resistance in wheat have been explored at the molecular level (Toktay et al. 2006). Two sources of resistance have been identified for resistance to *P. neglectus*. A single gene, *Rlnn1*, was mapped from the wheat cultivar Excalibur to chromosome 7A (Williams et al. 2002), and two Quantitative Trait Loci (QTL) on chromosomes 6D and 4D were identified in the synthetic wheat cross CPI33872 x Janz (Zwart et al. 2005). For *P. thornei*, a further five sources for resistance were identified and examined including a bread wheat line, GS50a (Thompson et al. 1999); two synthetic wheat crosses CPI33872 x Janz and W-7984 x Opata85 (Zwart et al. 2005, Zwart et al. 2006) and two Middle Eastern landraces AUS13124 and AUS4926 (Schmidt et al. 2005). QTL associated with *P. thornei* resistance were identified on four different wheat chromosomes, 2B, 3B, 4D and 6D (Thompson 2008) in these sources.

Breeding for resistance has relied on selection through screening of phenotypes which is arduous, time consuming and requires expensive glasshouse space. With advances in molecular marker techniques, through marker assisted selection (MAS), *Pratylenchus* resistance genes and QTL tightly linked to molecular markers can be efficiently and effectively introgressed into new cultivars. Molecular markers eliminate the need for large scale phenotyping and thus facilitate the rapid identification of resistance loci and allow their implementation through MAS, to accelerate the development of new resistant cultivars (Schmidt et al. 2005, Toktay et al. 2006). As *Pratylenchus* resistance in wheat is polygenic (Zwart et al. 2004), genetic analysis is required to identify the several genes controlling the expressed resistance. While several *P. neglectus* and *P. thornei* resistance QTL have been identified, to date resistance genes are not routinely selected through MAS or have been cloned. Further work is needed to identify *Pratylenchus* resistance genes to establish closely linked markers suitable for breeding.

The majority of studies analysing biological and biochemical resistance mechanisms have focussed on the sedentary endoparasitic nematodes (*Meloidogyne* and *Heterodera*), and little is known about *Pratylenchus* resistance. Elucidation of resistance at the molecular and biological level may provide clues to how and when resistance genes function. After root penetration, root knot and cyst nematodes migrate through the cortex causing little damage. Once a suitable site is reached they initiate the formation of a feeding structure, comprising large metabolically active cells from which the nematodes obtain nutrients for development. The resistance genes of sedentary nematodes such as the *Mi* gene against root knot species and the *Cre3* gene against cereal cyst nematodes, degrade or inhibit the flow of nutrients from their feeding structures preventing juvenile development and reproduction (Dropkin 1969, Seah et al. 2000). Once permanent feeding sites have been established these nematodes are generally immobile (Sijmons et al. 1994, Williamson 1999). This contrasts to migratory nematodes such as *Pratylenchus,* which migrate intracellularly through the root after penetration, feeding on cortex cells (Zunke 1990). As the mechanisms of invasion are different, the resistance mechanisms of *Meloidogyne* and *Heterodera* cannot be used to model *Pratylenchus* resistance. Therefore, investigation of specific resistance mechanisms will aid in a better overall understanding of wheat resistance to *Pratylenchus*.

Early studies investigated resistance to *Pratylenchus* with respect to the numbers and developmental stages of nematodes within the soils of resistant roots, but the mechanisms occurring within roots are unknown. These early studies showed that resistance to *Pratylenchus* did not prevent penetration and suggested that resistance mechanisms must be activated to prevent reproduction during migration, feeding or reproduction itself (Farsi et al. 1994). The identification of a resistance mechanism at a specific stage of invasion or within a particular tissue would enhance screening procedures by reducing associated costs and time. In addition, characterisation of the role of resistance mechanisms may indicate the function of identified resistance genes.

1.2 Life Cycle

All motile *Pratylenchus* life stages are parasitic as both adults and juveniles can penetrate, migrate and feed within roots (Bridge and Starr 2007). Root lesion nematodes have four juvenile stages between the egg and adult with each subsequent moult producing an increase in size and sexual development (Luc et al. 2005). The stage one juveniles moult to stage two juveniles (J2) within the egg before they hatch and emerge into the soil or root (Shurtleff and Averre 2000). The nematodes then moult through stages three (J3) and four (J4) juveniles to become fully developed adults.

Pratylenchus penetrates and feeds within the cortex and eggs are deposited singly in the cavities created by migration (Acedo and Rhode 1971, Bridge and Starr 2007). Females can

also deposit eggs in the soil (Pudasaini et al. 2008). Males are rare in *P. thornei* and *P. neglectus* populations and thus females reproduce by mitotic parthenogenesis (De Waele and Elsen 2002). Eggs have been observed as early as 5 days after inoculation (d.a.i.) (Townshend 1963). The eggs hatch or are released into the soil during root degradation (Agrios 1988).

Pratylenchus can complete its life cycle in 45 to 65 days depending on environmental factors including the amount and quality of available food sources, temperature and host species (Taylor et al. 2000). The optimum conditions for development vary with each species. However, *Pratylenchus* can complete three to six generations within roots during one crop growing season (Taylor et al. 2000). Both *P. thornei* and *P. neglectus* are able to survive drought conditions (Tobar et al. 1996). *Pratylenchus neglectus* survived in a state of reversible anhydrobiosis in dry soil for over 15 months (Meagher 1970). While in this state the nematodes are coiled which is probably a protective and a distribution mechanism (Glazer and Orion 1983, Baujard and Martiny 1994). During anhydrobiosis the nematodes are not susceptible to desiccation, extremes of temperature and chemicals (Luc et al. 2005), but they are vulnerable to mechanical damage.

1.3 Histopathology

There has been very little investigation into the invasion mechanisms of the two most common species that parasitise cereal crops, *P. neglectus* and *P. thornei*. Physical aspects of invasion such as lesion formation which are easily visualised have been reported, but nematode movement within the roots remains poorly documented. Some *Pratylenchus* species such as *Pratylenchus penetrans* which affect legume and fruit crops have been investigated in more detail (Townshend 1963, 1978, Townshend and Stobbs 1981, Townshend 1984, Townshend et al. 1989, Castillo et al. 1995, Castillo et al. 1998, Castillo et al. 1998). Where there was a lack of literature specific to *P. thornei* and *P. neglectus* the proposed invasion mechanisms (penetration and feeding) discussed in this thesis are based around histopathological models of other closely related and well documented *Pratylenchus* species such as *P. penetrans*. However, as the histopathology of different species of *Pratylenchus* is host specific, it is essential that *P. thornei* and *P. neglectus* invasion mechanisms in wheat are the focus of future investigations (Townshend 1963, 1963, Townshend et al. 1989, Thompson 1990, Zunke 1990, 1990).

The invasion process of *Pratylenchus* nematodes can be separated into six components: root recognition, probing, penetration, ectoparasitic and endoparasitic feeding and reproduction. Initially, the nematode probes the surface of the root for an acceptable penetration site and once found penetrates the root with its stylet. After a salivation period, the nematode may feed ectoparasitically on the root under certain environmental conditions.

Eventually, the nematode enters the root and migrates intracellularly while feeding endoparasitically on root cortex cells, with adults depositing eggs (Zunke 1990).

1.3.1 Probing and Root Exploration

1.3.1.1 Mechanisms of Root Exploration

Nematodes probe or search the surface of the root to find acceptable penetration sites. Root exudates are the most probable stimulus for attracting *Pratylenchus* to the root region (Baxter and Blake 1967, Wallace 1974, 1989). Prior to selection of a penetration site, contact of the lips with the root probably involves sensory perception mechanisms (Doncaster and Seymour 1973). *P. penetrans* explores the root by rubbing the lip region along the surface of epidermal cells and protracting their stylet enough to touch but not penetrate the walls (Zunke 1990). It is unknown what makes a penetration site acceptable for entry but it could be a site that physically allows easier access or it may be controlled by biochemical signals.

1.3.1.2 Zones of Penetration and Entry

There are differences in the sites and mechanisms of *Pratylenchus* root penetration. In lucerne and clover, *P. penetrans* preferred to penetrate the main roots where lateral roots ruptured the cortex and migrated through the cortex of main roots into lateral roots (Townshend et al. 1989). In other studies, *P. penetrans* aggregated and penetrated in the zone of root elongation (Troll and Rhode 1966, Zunke 1990). In lucerne, strawberry and maize roots, the preferred zone of penetration for both adults and juveniles appeared to be the dense root hair zone, with 61% of the lesions developing on the root hairs of lucerne (Townshend 1978). However, *P. penetrans* may explore and penetrate in the root hair region but may not feed on them (Ogiga and Estey 1975, Kurrpa and Vrain 1985). Using high resolution video-enhanced contrast microscopy, Zunke (1990) observed *P. penetrans* penetrating and feeding on root hairs of various hosts including rape, tobacco and potatoes. In wheat, *Pratylenchus minyus* was found in higher numbers in seminal rather than crown roots (Kimpinski et al. 1976), suggesting nematodes may enter near the root hairs of seminal roots. Preference for the root hair zone may have a biochemical basis for attraction or a physical basis allowing easier entry (Townshend 1978). Castillo et al. (1998) found that both females and juveniles of *P. thornei* penetrated the roots of chickpea without any site preference.

It is probable that *P. thornei* penetrates in the region of root hair development as suggested by Zunke (1990) for *P. penetrans*. The video-enhanced contrast microscopy used in that study provided higher resolution images than the light microscopy used by Castillo et al. (1998). In addition, *P. mediterraneus*, a closely related species, also invades primarily at the root hair region (Orion and Lapid 1993). The number of nematodes present within the root

decreased as the inoculum density increased (Baxter and Blake 1967, Townshend 1978, Griffin and Gray 1990) indicating competition for penetration sites. If *P. penetrans* prefers to enter at the root hair zone, this may limit the root surface area available for penetration, and explain reduced penetration at high nematode density (Griffin and Gray 1990).

Pratylenchus neglectus differs from both *P. thornei* and *P. penetrans* in the way that it penetrates and feeds upon root cells. *Pratylenchus neglectus* generally penetrate in a non-random pattern but is attracted principally to the root tip region (Anderson and Townshend 1976). Studies with wheat showed that *P. neglectus* first penetrates the seminal and later crown roots (Kimpinski et al. 1976). In contrast to other *Pratylenchus* species, *P. neglectus* does not primarily feed on cortical cells but tends to migrate to and feed on more mature areas of the root such as the meristemic tissue behind the root cap. This in turn causes prevention of root elongation (Anderson and Townshend 1976).

1.3.1.3 Factors Affecting Selection of Penetration Site

The plant-nematode relationships involving *Pratylenchus* species are affected by environmental conditions, which in turn affect the nematodes' pathogenic potential. Some of these factors include soil temperature, moisture content, plant age and nematode developmental stage. The moisture content of the soil plays an important role in the numbers of nematodes present and yield losses. Castillo et al. (1995) showed that *P. thornei* populations are significantly greater in soil when plants are under water stress and the soil is dry, compared to when the soil is at water holding capacity.

The amount of nematode damage to plant roots is inversely proportional to seedling age at the time of inoculation (Ogbuyi 1976). In lucerne, the number of *P. penetrans* at all migratory life stages was inversely proportional to age of root tissue. Two day old root tissue in the root hair zone was penetrated twice as much as 10 to 20 day old root tissue. As the cortex degenerates in maturing roots, penetration apparently becomes difficult. In addition, biochemical and nutrient changes within these older tissues may cause the cortex to become less favourable for habitation (Olthof 1982). The study also confirmed the earlier study by Townshend (1978) showing that females were more infective than juveniles as they penetrated the roots earlier, faster and over a wider range of soil temperatures. Townshend (1978) attributed the greater ability of females to penetrate to the size of the glands in the posterior subventral lobe. The larger size of these glands could allow them to produce greater quantities of enzymes at a faster rate than in other life stages. Klinkenberg (1963) suggested that the stylet of newly hatched juveniles was not fully developed and too weak to penetrate the strong cell walls, thus making them less invasive than the bigger and stronger adult females. Therefore, greatest penetration would be achieved in young seedling roots in soils that are dry and have higher proportions of adult *Pratylenchus* than juveniles.

1.3.2 Stylet Penetration

After cell surface exploration, the stylet is inserted slowly several times with increasingly deeper thrusts into the chosen cellular site (Kurrpa and Vrain 1985, Zunke 1990). Kurrpa and Vrain (1985) suggested that repeated thrusts were needed as the epidermis is quite elastic and difficult to penetrate. The number and duration of stylet thrusts probably differs between plant species due to differences in structure and thickness of epidermal root cell walls.

1.3.3 Salivation and Ectoparasitic Feeding

Pratylenchus penetrans salivated once the stylet was inserted to a length of about 2 µm. During salivation the median bulb of the oesophagus pulsated several times and secretions passed from the stylet into the root cell. The saliva may predigest, through enzymic action, the cytoplasmic material to be ingested (Kurrpa and Vrain 1985). During salivation the root cell contents do not appear to change but the rate of cytoplasmic streaming increases (Zunke 1990). *Pratylenchus* adults move into the root to feed endoparasitically sooner than juveniles, which remain feeding on root hairs for extended periods (Ogiga and Estey 1975, Zunke 1990). On various hosts ectoparasitic feeding can last for several minutes (Zunke 1990). It is probable that ectoparasitic feeding and its duration relies on environmental conditions at the time of invasion.

When ectoparasitic feeding is completed, the stylet is withdrawn from the root cell and the puncture hole created by nematode penetration appears to close over. A mixture of saliva and partially digested cytoplasm may harden at the insertion point and seal the hole to prevent cellular leakage (Zunke 1990). There is generally only one salivation and ingestion period before the nematode penetrates the root to feed endoparasitically (Kurrpa and Vrain 1985).

1.3.4 Root Entry and Endoparasitic Feeding

1.3.4.1 Numbers of Nematodes during Penetration

In general, *P. penetrans* enter the roots singly but in turnip (*Brassica rapa* subsp. *rapa*) occasionally it penetrated in groups. Where several nematodes entered simultaneously they migrated together and concentrated around the stelar region. Degradation of root tissue at sites of multiple invasions resulted in an open lesion into the stele (Ogiga and Estey 1975).

One of the few histopathological studies of *P. thornei* showed that in wheat, nematodes are attracted to regions of the roots previously invaded. In this species, many nematodes penetrate, feed and reproduce in groups within particular root regions (Baxter and Blake 1968). At sites of multiple invasions, large sections of the cortex degrade and collapse, resulting in exposure of the stele. This is in contrast to the mechanisms of *P. penetrans*

where nematodes usually penetrated the roots singly and fed upon distantly different areas of the cortex. Thus, different species of *Pratylenchus*, despite being closely related and similar in structure, appear to use different mechanisms to invade and cause root damage.

1.3.4.2 Root Entry and Cortical Migration

After penetration *P. penetrans* migrate within the root and feed endoparasitcally on cortical cells. In strawberry roots, *P. penetrans* penetrated the epidermis at various depths of the cortex between 5 and 17 d.a.i. and in lucerne within 72 hours after inoculation (h.a.i.) (Townshend 1963). *Pratylenchus penetrans* penetrates the punctured cell by tearing a hole at the pierced site and waving their bodies vigorously to force root entry (Kurrpa and Vrain 1985, Zunke 1990). Nematodes migrate intracellularly within the root in either direction from the point of entry. Generally, *P. penetrans* penetrates epidermal and the outermost cortical cells at right angles to the longitudinal axis. Once in the cortex, they migrate slowly parallel to the longitudinal axis, penetrating the transverse walls of successive cells (Ogiga and Estey 1975, Townshend et al. 1989). *Pratylenchus* may be stretched out or coiled within the cortical cells, occupying a single cell or several layers, depending on cell size (Castillo et al. 1998). During migration at each new cell the nematode punctures the cell with stylet thrusts and then pierces a row of holes over the entire end wall. Through pressing its anterior end against the weakened cell it pushes through into the adjacent cells.

1.3.4.3 Cortical Feeding

Pratylenchus can traverse several layers of cortex cells causing extensive damage while frequently feeding on cellular contents (Zunke 1990), which contrasts to the fixed feeding sites of sedentary nematodes (Taylor et al. 2000). In lucerne roots *P. penetrans* may briefly feed on cells and a small salivation zone appears around the stylet. During this brief feeding for several minutes, the cells rarely die but often have hypertrophied nuclei with a granulated appearance (Zunke 1990). Extended feeding occurring over several hours is marked by a period of salivation of 2 minutes. Salivary secretions of *Pratylenchus* contain pectic, cellulolytic and proteolytic enzymes and may be capable of breaking down cells and releasing their contents (Krusberg 1960).

Migration of *Pratylenchus* through root tissue causes collapse of invaded cells. Extended feeding causes degradation of the cellular contents and eventual cell death (Orion and Lapid 1993) which appear as necrotic lesions. Necrosis is caused by the degradation of plasma membranes leading to swelling and lysis of cellular contents (Zwart et al. 2004). In roots invaded by both *P. penetrans* and *P. thornei*, cortical cells often lack cytoplasmic content and show loss of membrane integrity, have shrunken tonoplasts and degenerated organelles, with nuclear hypertrophy and granulation (Zunke 1990, Castillo et al. 1998). Similar structural changes occur in the surrounding cortex and endodermal cells with increased tannin

deposits in the cytoplasm, tonoplast and vacuoles (Townshend et al. 1989, Castillo et al. 1998).

1.3.4.4 Endodermis and Vascular Tissue Penetration

Some studies suggest that *Pratylenchus* do not enter vascular tissues, but in certain hosts, such as turnip, *P. penetrans* invades the endodermis and stele. However, in contrast, the endodermis acted as a barrier to *P. penetrans* invasion of vascular elements in lucerne (Castillo and Essica 1965, Townshend et al. 1989), clover (Townshend and Stobbs 1981), apple (Pitcher et al. 1960), peach, maize (Ogiga and Estey 1975), strawberry (Townshend 1963, Ogiga and Estey 1975), potato, tobacco (Zunke 1990) and celery (Townshend 1963). Although *P. penetrans* was never found in the stele of celery, nematodes were observed occasionally in the endodermis (Townshend 1963). The resistance of the endodermis to *P. penetrans* in tomato (Pi 1966) and carrot (Rohde 1965) was only temporary as nematodes were found in the stele one month after inoculation. Similarly, in cabbage, *P. penetrans* was found probing endodermal tissues 2 weeks after inoculation (w.a.i.) and had penetrated the stele 4 weeks later (Acedo and Rhode 1971). In the only published study investigating the capacity of *P. thornei* to invade further tissues, *P. thornei* caused some damage to the endodermis of chickpea, but was never found in the stele or feeding on endodermal cells (Castillo et al. 1998).

1.3.4.5 Reproduction

Mature *Pratylenchus* females deposit eggs singly in the cavities created by migration (Acedo and Rhode 1971, Bridge and Starr 2007) or in the soil (Pudasaini et al. 2008) in close vicinity to the roots. There is little information on the egg laying abilities of *Pratylenchus* species. An early study found that in red clover, *P. penetrans* produced 16 to 35 eggs per female at a rate of 1 to 2 eggs per day over 55 days (Turner and Chapman 1972). Embryogenic development of *P. thornei* is completed in around 10 days (Roman and Hirschmann 1969). The first juvenile moult occurs 2 days later within the egg to produce J2 which then hatch from the egg using stylet thrusts. Egg hatching is influenced by temperature and host signals. The optimum temperature for hatching of *P. thornei* is between 18 to 22 °C (Castillo et al. 1996). Generally, not all eggs deposited will hatch. For example, a maximum of 50% of the *P. penetrans* eggs hatched even though 64% already contained J1 or J2 during *in vitro* tests under optimum conditions (Pudasaini et al. 2008). It is known that hatching in *Pratylenchus* species can occur in water without root exudates, however, some studies have shown hatching is enhanced by plant root exudates. De Waele et al. (1988) demonstrated under glasshouse conditions that hatching in *P. brachyurus* and *Pratylenchus zeae* was influenced by maize roots and Pudasaini et al. (2008) showed that maize, bean and marigold enhanced hatch of *P. penetrans*. The influence of root diffusates was not restricted to particular plant species or of a specific age, highlighting the polyphagous nature of *Pratylenchus*. As the

nematode moves and feeds within the root it undergoes development with the third and fourth moults inducing changes in the development of the reproductive system (Roman and Hirschmann 1969).

1.3.5 Symptoms

Root lesions are the main symptoms for plants invaded with *Pratylenchus* species. Generally, lesions first appear on roots as water-soaked areas as the nematodes penetrate the root epidermis. These water-soaked lesions over time form an elliptical shape 1 to 2 mm in length and change colour to olive green and finally reddish brown (De Waele and Elsen 2002). The elliptical lesions initially lie parallel to the root axis in the same orientation as the migrating nematodes but over time increase in size and merge to form large sections of discoloured tissue. Most necrosis occurs on roots near the soil surface but lateral roots may also become necrotic. In cabbage invaded with *P. thornei*, many lateral roots had a beaded appearance caused by the large number of abandoned root initials (Acedo and Rhode 1971). In strawberry roots 3 to 4 w.a.i. the crowns had not developed sufficiently to produce adventitious roots, decreasing the extent of root growth (Townshend 1963).

As *Pratylenchus* invasion causes loss of root function, above ground symptoms reflect nutrient and water deficiencies. Above ground, *Pratylenchus* symptoms are not easily determined and are affected by environmental factors (Taylor et al. 1999), but in general damaged plants show lack of vigour and stunted growth (Fulton et al. 1960). Feeding by *Pratylenchus* can facilitate the entry of other pathogens and commonly invasion is associated with fungi (Evans and Haydcock 1993) which causes increased nematode invasion and thus yield loss. For example, when the fungal pathogen *Fusarium acuminatum* is associated with *P. neglectus* greater damage to wheat plants results (Taheri 1996).

The effects of *P. thornei* and *P. neglectus* on growth are not well established. Wheat plants infested with *P. thornei* are rarely killed but show stunting, chlorotic leaves and have necrosis at leaf tips. In addition, invaded plants have reduced tillering and also fewer and smaller ears (Van Gundy et al. 1974). Premature wilting and delayed flowering have also been associated with *P. thornei* invasion of wheat (Thompson et al. 1980).

P. thornei nematodes cause degradation of large sections of the cortex, significantly reducing the absorptive capacity of the roots (Jaques and Schwass 1956). Baxter and Blake (1968) observed that *P. thornei* penetrated and fed on and destroyed both seminal and nodal roots. During early growth, loss of seminal roots can significantly reduce grain yield (Sallans 1942), as on a weight for weight basis, seminal roots absorb twice as much water as nodal roots (Goedewaagen 1942). Therefore, invaded plants can have significantly reduced yields as they are unable to efficiently uptake water.

The plant response to different species of *Pratylenchus* appears to be host specific (Nicol 1996). Differences for temporal development of symptoms of *P. penetrans* invasion were observed on different hosts. For example, 12 h.a.i. lesions on lucerne roots appeared as water-soaked areas on the upper feeder roots (Townshend 1978, Townshend and Stobbs 1981). However, lesions on strawberry roots inoculated with *P. penetrans* were observed only after 17 d.a.i. (Townshend 1963) and lesions in the proximal parts of the seminal roots of wheat appeared only after 6 w.a.i. when inoculated with *P. thornei* (Baxter and Blake 1968). Differences in pathogenicity and host tolerance could affect the timing and development of symptoms. It was initially considered that genetic diversity existed between *Pratylenchus* species (Motalaote et al. 1987) due to differences in reproductive fitness and pathogenic capabilities between populations (Tiyagi and Parveen 1992, Castillo et al. 1995). However, no significant differences in reproduction rates, measured by the final numbers of nematodes present in the root, or pathogenicity as measured by severity of necrosis, were observed among four different *P. thornei* populations on chickpea (Castillo et al. 1998). As molecular analysis has not been employed to analyse *Pratylenchus* pathogenicity, differences between species cannot be excluded.

1.4 Biological and Biochemical Resistance Mechanisms

Few studies have considered the effects of resistance genotypes on nematode biology as most focus primarily on the genetics behind plant nematode interactions. The elucidation of resistance at the molecular and biochemical level can provide clues to how and when resistance genes function. Plant responses to nematode invasion involve both biological (physical) and chemical barriers that are either preformed (constitutive) or specifically induced by the nematode, such as the highly specific resistance gene mediated defense. The preformed constitutive defense is based on phytoanticipins which are present in the plant before pathogen exposure or are produced after invasion from preformed precursors (VanEtten et al. 1994). Preformed defense compounds are synthesised during normal plant development. Phytoanticipins implicated in plant resistance to nematodes include phenols and glycosides (Zwart et al. 2004). As migratory nematodes move through the root and do not have a fixed feeding or reproductive site, the elicitation of a biochemical attack needs to be immediate to provide protection of tissues not yet invaded. Induced pathogen defense is host specific as it is initiated by pathogen derived elicitors that induce a complex signalling cascade to cue the production of defense chemicals, namely phytoalexins (VanEtten et al. 1994) either locally (at the site of invasion) or systemically (outside the invasion site) (Rojo et al. 2003). Low molecular weight antimicrobial defense compounds generally include phenylpropanoid derivatives, sesquiterpenes, polyketides and flavonoid and isoflavonoid derivatives (Hammerschmidt 1999, 1999) and their role is to interfere with pathogen

development and/or induce the production of morphological barriers that impound the pathogen (Veech 1982).

1.4.1 Resistance at Penetration

Resistance to root knot, cereal cyst and root lesion nematodes investigated in several crop species has not been reported to be associated with penetration. In a resistant wheat cultivar, significant numbers of *P. thornei* were found within the roots (Talavera and Vanstone 2001), indicating penetration had occurred. The penetration rates of *Meloidogyne incognita* in both resistant and susceptible cultivars of cotton were similar several days after inoculation (Creech et al. 1995). In addition, studies of *Heterodera* species showed the nematode could successfully penetrate and migrate to the feeding site in both susceptible and resistant cultivars of wheat, soybean and potato (Kim et al. 1987, Williams and Fisher 1993). In a resistant potato cultivar, the initial number of *Meloidogyne fallax* juveniles was less than in the susceptible cultivar, but at 60 h.a.i., similar numbers were present in the roots (Kouassi et al. 2004). This was also observed with *M. incognita* where fewer juveniles had penetrated resistant roots 24 h.a.i., but by 48 h.a.i. numbers were comparable to those in the susceptible cultivar (Cantosaenz and Brodie 1987). However, penetration of *M. incognita* in a resistant potato cultivar and in the wild grass, *Aegilops variabilis* was inhibited (Khanna and Nirula 1964) and resistance was characterised by a lower juvenile invasion rate (Yu et al. 1990). Although, as these observations were recorded only a few hours after inoculation, the effects of delayed penetration and not inhibition could have been observed in these studies. As *Pratylenchus* have been observed in resistant roots it is unlikely that penetration is inhibited, however, it may be delayed. Resistant roots may have thicker cuticles impeding or slowing root entry or have decreased secretion of chemoattractant chemicals used by nematodes to locate roots within the soil.

1.4.2 Resistance to Motility and Feeding

The ability to move is essential to invasion as a nematode must first migrate through the soil towards the root. For migratory nematodes the ability to move is critical as once it has penetrated the root it needs to migrate through the root cortex in order to feed. If feeding is suppressed the nematodes will not have enough energy to moult to the next developmental stage and thus will not mature to lay eggs. Several groups of plant derived chemicals have been associated with the suppression of nematode motility in resistant cultivars by directly acting upon the nematode including flavonoids, isoflavonoids and sesquiterpenes (Rich et al. 1977, Kaplan et al. 1980, Veech 1982, Zinov'eva and Chalova 1987). Resistance to nematode migration has also been observed by preventing nematode movement through root cells by either necrosis via phenolics and cell wall hardening via peroxidases and lignifications (Valette et al. 1998, Nithya et al. 2007, Wuyts et al. 2007).

1.4.2.1 Chemical Suppression of Motility

1.4.2.1.1 Flavonoids

Flavonoids possess a wide range of biological activities, including protecting plants against environmental stresses such as temperature extremes (drought, frost and UV exposure) (Chalker-Scott and Krahmer 1989, Rozema et al. 1997, Moore et al. 2004). Flavonoids also play a role as plant chemical defense compounds. The first flavonoid linked to nematode resistance was associated with *Pratylenchus scribneri* in lima beans (Rich et al. 1977). In resistant lima bean root tissue, the coumestans coumestrol and psoralidin were found in response to *P. scribneri* invasion. These compounds were isolated and found to inhibit *P. scribneri* motility *in vitro* at 5 µg/mL (Rich et al. 1977), which is similar to levels within root cells. The flavonoid medicarpin was correlated with resistance to *P. penetrans* in lucerne. Higher constitutive levels of medicarpin were observed in resistant roots than in susceptible. An *In vitro* analysis showed that medicarpin inhibited motility at concentrations similar to those reported for coumestrol (Baldridge et al. 1998).

1.4.2.1.2 Isoflavonoids

Glyceollins, products of the isoflavonoid branch of the phenylpropanoid pathway (Kaplan et al. 1980), have antimicrobial properties and have been implicated in the resistance of soybean to nematode invasion. In response to *M. incognita* invasion, glyceollin accumulated 2 to 3 d.a.i. in the resistant roots but no accumulation was observed in susceptible roots. An *in vitro* analysis found that the effect of glyceollin is nematostatic, as it inhibits *M. incognita's* respiration preventing movement and thus establishment of a feeding site (Kaplan et al. 1980). In response to *Heterodera glycines*, glyceollin accumulated in root tissues adjacent to the head of the nematode, suggesting glycoproteins on the cuticle near the head (Huang and Barker 1986) or salivary secretions act as glyceollin elicitors.

1.4.2.1.3 Sesquiterpenes

Terpenoids are a large and diverse plant chemical class derived from five-carbon isoprene units. They are involved in the plant respiratory process and electron transport but also have protective properties (Zinov'eva et al. 2001). The sesquiterpene rishitin, isolated from potato roots, had nematostatic effects on the stem nematode, *Ditylenchus destructor*, with the amount detected proportional to the resistance level observed (Zinov'eva and Chalova 1987). Sesquiterpenes were also associated with *M. incognita* resistance in cotton. After inoculation with *M. incognita*, the sesquiterpene aldehyde, gossypol, and its derivatives increased in the endoderm and cortical tissues of resistant, but not susceptible, cotton roots (Veech and McClure 1977). An *in vitro* analysis showed that after exposure to gossypol for 5 h at a concentration reflecting similar levels produced in roots (125 µg/mL), *M. incognita* larvae lost motility (Veech 1982).

1.4.2.2 Suppression of Migration and Feeding

1.4.2.2.1 Cinnamic Acids

Cinnamic acids are products of the phenylpropanoid pathway and are involved in plant resistance to nematodes (Trudgill 1991), most likely through physically blocking nematode movement within roots. A greater number of cells containing these acidic compounds were found in roots of resistant banana cultivars infested with *Radopholus similis* than in susceptible cultivars (Wuyts et al. 2007). High concentrations of hydroxycinnamic acids, including ferulic acid, caffeic esters and dopamine, were found in parenchyma and vascular cells in *R. similis* resistant roots (Valette et al. 1998). In particular, ferulic acid has been found covalently bound to cell wall polysaccharides (Wuyts et al. 2007), which may act as a physical mechanism to protect cell walls from nematode-secreted cell wall degrading enzymes (Hartley and Jones 1977) and thus prevent nematode migration and feeding.

1.4.2.2.2 Phenolics and Necrosis

Phenolic compounds have been associated with *Pratylenchus* lesion formation in a variety of hosts. Necrosis and death of cortical cells caused by *Pratylenchus* species occurs rapidly in advance of penetrated areas, preventing further nematode migration (Mountain and Patrick 1959). The release and oxidation of preformed phenolic compounds such as glycosides and glucosinolates (nitrogen-containing secondary metabolites) by plant enzymes, as a result of pathogen damage, is a common defense mechanism observed in many plants against various bacterial and fungal pathogens (Oku 1960, Hildebrand and Schroth 1964, Olah and Sherwood 1973). Preformed phenolic compounds are stored in specialised cells as inactive precursors and released into invaded tissue by plant or pathogen enzymes.

Chlorogenic acid and its oxidation products were identified as the major phenolic compound in tomato roots, both before and after invasion by *P. penetrans* and *M. incognita* (Hung and Rohde 1973). It was detected in both resistant and susceptible roots but at greater amounts in the resistant. The authors suggested that invasion results in accumulation of chlorogenic acid which is oxidised by the action of plant or nematode enzymes causing formation of the brown-coloured melanins in necrotic tissues. Necrosis, especially around the vascular tissues, may prevent nematodes from penetrating the endodermis. It was previously reported that lesion nematodes were repelled by the presence of chlorogenic acid and its oxidation products, in addition to significantly reducing their respiration (Chang and Rohde 1969). Chlorogenic acid has also been associated with resistance to the stem nematode *Ditylenchus angustus* in rice. In resistant plants, a compound is deglycosylated to form chlorogenic acid, but susceptible plants lack the enzyme required for deglycosylation (Plowright et al. 1996). Phenolic compounds were associated with the formation of necrosis in cabbage after invasion of *P. penetrans* (Acedo and Rhode 1971) and the major contributor

was identified as ferulic acid. These phenols were either synthesised in the invaded cells or released from preformed glycosidic compounds.

Mountain and Patrick (1959) suggested that root lesion formation was the result of an interaction between the nematode feeding enzyme, β-glucosidase, and plant phenolic compounds released from glycosides. Amygdalin, a host plant compound in peach roots, is hydrolysed by β-glucosidase secreted by *Pratylenchus* resulting in the release of hydrogen cyanide and benzaldehyde, which are toxic to nematodes and plant roots (Mountain and Patrick 1959), forming the yellow-red-brown products seen in the necrotic root tissue (Mace 1963). The hydrolysis of phenolic compounds by nematode secretions was later observed in *Globodera rostochiensis*, which injected β-glucosidase hydrolysing a glycoside to release phenolic aglycones that caused necrosis only in resistant roots (Giebel 1982).

Preformed compounds are often stored at sites where they may play a direct role in defense (Treutter 2005) and thus their release, oxidation and subsequent necrosis can occur at sites not yet occupied by the nematode. Endodermis and stele tissues not invaded by *Pratylenchus* are often the most discoloured (Mountain and Patrick 1959). Townshend (1963) found that the root tissue with the highest concentration of phenolic substances was the endodermis, and suggested that this may account for necrosis without nematode invasion. Necrotic tissue may serve as a physical barrier to prevent *Pratylenchus* penetration into the vascular system (Mountain and Patrick 1959, Pitcher et al. 1960, Townshend 1963, Uritani 1963). In oat roots invaded with *P. neglectus,* the concentration of three flavone-C-glycosides increased significantly compared to uninoculated controls. Induction of these compounds before inoculation with *P. neglectus* protected the plant from damage resulting in root and shoot growth similar to the uninoculated controls (Soriano 2004).

Hypersensitive reactions inducing necrosis are well characterised resistance mechanisms used by sedentary nematodes to prevent feeding. An example is the isolation of the syncytium of *Heterodera glycines*, where development was initiated in both soybean resistant and susceptible genotypes but within resistant roots a necrotic layer formed around it. This prevented further development and cell wall thickening sealed off the plasmodesmata preventing movement of nutrients and food to the developing nematodes (Kim et al. 1987). Nematodes were unable to feed or reach maturity as development was arrested at the juvenile stage. The *Mi*-gene based resistance is another example where an early hypersensitive response prevents the induction of *M. incognita* giant cell development and therefore nematode feeding (Trudgill 1991).

Tannins are polyphenolic compounds also involved in plant defense reactions. Light microscopic studies in lucerne roots identified electron dense osmiophilic deposits suspected to be tannin molecules in the cytoplasm and vacuoles in *P. penetrans* invaded cortical cells

and those adjacent to them, including the endodermis (Townshend et al. 1989). These tannin deposits may act as a physical barrier to nematode entry to the endodermis and vascular tissues (Townshend et al. 1989) preventing further migration and thus feeding.

1.4.2.2.3 Peroxidases and Lignification

In addition to the accumulation of pathogen-induced proteins and products of secondary plant metabolism, cells undergoing a hypersensitive response are often characterised by an increase in the activity of certain enzymes, which include peroxidases, polyphenol oxidase, and phenylalanine ammonia lyase (Zwart et al. 2004). Peroxidases may be involved in increased lignin biosynthesis and esterases in the primary stages of cell wall lignification (Melillo et al. 1989, Melillo et al. 1992). Lignification may act as a physical protection mechanism. It causes cell wall hardening, preventing access of nematode enzymes to cell wall polysaccharides (Ride 1978), inhibiting nutrient transport and subsequently suppressing nematode growth and development (Levinsh and Romanovskaya 1991). Vascular lignification may also act to protect nematode invasion of the xylem and prevent entry to the vascular tissues and its source of nutrients (Valette et al. 1998).

For example, the peroxidase activity in resistant banana cultivars increased in response to *Pratylenchus coffeae* significantly more than in susceptible cultivars (Nithya et al. 2007). In addition, in cabbage roots invaded by *P. penetrans,* peroxidase activity was five times greater in resistant than in susceptible roots (Acedo and Rhode 1971). Investigation of lucerne defense responses to *P. penetrans* showed increased levels of phenylpropanoid pathway transcripts of phenylalanine ammonia-lyase and caffeic acid O-methyltransferase, which are in the lignin biosynthesis branch of the pathway. A later investigation found the compound hydroxybenzaldehyde in discoloured areas of lucerne roots invaded with *P. penetrans* (Townshend and Stobbs 1981), indicating the synthesis of lignin-like substances in these regions (Pridham 1960). Differences in the levels of superoxide dismutase were evident in resistant wheat roots inoculated with *Heterodera avenae* (Andres et al. 2001) indicating it may also have a role in defense. The increased production of these peroxidases in resistant cultivars ultimately prevents nematode feeding and thus migration through the root (Sijmons et al. 1994).

1.4.3 Resistance and Effects on Reproduction

While nematodes may be able to penetrate and migrate through resistant root tissue, reproduction can be inhibited. For nematode reproduction to be successful nematodes need to develop to mature adults and deposit eggs which need to hatch at the correct time in an environment where a food source is available.

In resistant wheat roots, after 2 months, *P. thornei* densities decreased, indicating that the nematodes were unable to reproduce (Talavera and Vanstone 2001). Moulting of *P. neglectus* was significantly delayed in wheat resistant roots, compared with susceptible roots (Farsi 1996). Phytoecdysteroids have been linked to suppression of nematode moulting. Pathogens that feed upon plants with these compounds may prematurely moult or suffer metabolic damage (Dinan 2001). Mass spectrometry found that the phytoecdysteroid 20-hydroxyecdysome was induced in lucerne roots after invasion with *P. neglectus*. Exogenous application of 20-hydroxyecdysome resulted in abnormal moulting and eventual death of *P. neglectus* (Soriano 2004). Despite similar penetration by J2, several studies have found delayed or suppressed development of *M. incognita* in resistant cultivars (Reynolds et al. 1970, Moura et al. 1993). In cotton, the majority of *M. incognita* in resistant roots failed to develop to J2 or J3 (McClure et al. 1974, Jenkins et al. 1995), and thus did not mature to deposit eggs. In soybeans 10 d.a.i., sexually differentiated *M. incognita* juveniles were only observed in susceptible plants (Moura et al. 1993).

The rate of egg deposition has been associated with resistance in several plant species. For example, suppression of egg deposition for *P. neglectus* has been observed in resistant wheat cultivars. Deposition was delayed by 4 days in one resistant cultivar compared to the susceptible, and no eggs were present even 35 d.a.i. in another resistant cultivar (Farsi 1996). Similar delays in egg deposition were observed in *M. incognita* where deposition was delayed in resistant soybean cultivars by 10 days (Moura et al. 1993), and in a susceptible banana cultivar *Pratylenchus goodeyi* deposited 29.8 eggs per day compared to 18.8 in the resistant roots over the same time period (Prasad et al. 1999). In the case of *M. incognita* on soybean, those in the resistant cultivars produced 99% less eggs per egg mass than in the susceptible (Moura et al. 1993).

Nematode hatching suppression has also been observed in resistant root exudates from various plants. Root exudate from susceptible soybean cultivars stimulated more hatch and emergence of *H. glycines* than resistant cultivars (Caballero et al. 1986, Schmitt and Riggs 1991, Sikora and Noel 1996). Similarly, partially resistant clones of *solanum* hybrids generally stimulated lower hatch of *Globodera pallida* than susceptible clones after several weeks exposure to root exudates (Forrest and Phillips 1984). The isoflavonoids glyceollin and coumestrol which suppress *H. glycines* and *P. scribneri* motility have also been associated with egg hatch suppression (Trivedi et al. 1984).

1.4.4 Timing and Types of Resistance

The resistance mechanisms employed can greatly vary between different nematode and plant species. For example, characterised genes conferring resistance to sedentary nematodes vary considerably in regards to mechanism, location and timing (Williamson 1999). Sedentary endoparasitic nematodes migrate intercellularly and cause little damage until a feeding site is formed, when resistance generally occurs. Migratory endoparasitic nematodes on the other hand cause extensive cellular damage while migrating and feeding intracellularly directly after penetration. Thus, the types of resistant responses and timing of induction between migratory and sedentary endoparasitic nematodes may differ due to variations in their root invasion and the mechanisms of their associated resistance genes (Rich et al. 1977, Zinov'eva and Chalova 1986). Therefore it is essential that further studies focus on investigating migratory nematode resistance. Some similarities exist however, such as lack of resistance at penetration (Williamson and Kumar 2006). The discovery of several different *Pratylenchus* resistance quantitative trait loci (QTL) indicates that *Pratylenchus* resistance in wheat will suppress several stages of nematode invasion and involve several biochemical pathways. It is beneficial for the plant to have more than one resistance ploy against nematodes like *Pratylenchus* due to their ability to continually migrate and feed within the root system and their constant soil population pressures.

1.5 Genetic Mapping in Plants and its Applications

Plant breeding exploits genetic variation to select for traits and characteristics that are of benefit for farmers, processing industries and consumers. Numerous agronomically significant traits in cropping plants, such as disease resistance, yield and quality, are governed by many genes and thus called polygenic quantitative traits (Collard et al. 2005). For example, resistance to cereal cyst nematode, *H. avenae*, in hexaploid wheat is conferred several genes (*Cre* genes) (Slootmaker et al. 1974, Ogbonnaya et al. 2001) and resistance to *Phytophthora infestans* in potato is conferred by two closely linked resistance genes with different specificities (Huang et al. 2004). A quantitative trait is a characteristic that relies on the expression of more than one gene contributing to the final phenotype (Semagn et al. 2010). The action of these genes and their interaction with the environment varies between individuals in a population to produce a phenotype along a continuous gradient (Sham et al. 2002). This contrasts to qualitative traits, which are normally governed by a single dominant gene. The understanding of the genetics of quantitative variation is very important for plant breeding and is achieved through QTL mapping. DNA or molecular markers can be used to construct linkage maps to identify DNA regions within the plant genome that contain genes controlling a specific quantitative trait (e.g. disease resistance) and represent a QTL (Collard et al. 2005).

QTL mapping approaches have been used to successfully identify QTL and genes associated with various quantitative traits in wheat. Examples include, resistance in wheat to cereal cyst nematodes, *Cre1-8* and *CreR* (Ogbonnaya et al. 2001), root knot nematodes, *Rkn-mn1* (Persondedryver and Jahier 1985, Yu et al. 1990, Yu et al. 1995) and root lesion nematodes (Schmidt et al. 2005, Zwart et al. 2005, Zwart et al. 2006, Zwart et al. 2010). Several QTL have been mapped controlling resistance to various fungal diseases such as *Fusarium* head blight (*Fhb1* and *Fhb2*), powdery mildew (*Pm1–Pm32*) (Liu et al. 2002, Mingeot et al. 2002, Zeller et al. 2002, Hsam et al. 2003, Xie et al. 2003) rusts including leaf, stem (Nelson et al. 1995) and stripe (Singh et al. 2000). In addition, QTL specific to wheat agronomic traits such as yield (grain number and thousand-grain-weight) (Narasimhamoorthy et al. 2006, Kumar et al. 2007), height (dwarfing *Rht)* (Gale and Marshall 1973, 1973), winter hardiness and ear emergence (vernalisation, *Vrn1* and frost resistance, *Fr1 and* photoperiod sensitivity *Ppd)*, (Galiba et al. 1995, Kato et al. 1999, Kuchel et al. 2006) and grain protein content, *Gpc-B1* (Olmos et al. 2003, Prasad et al. 2003) have also been mapped.

1.5.1 Molecular Markers

QTL mapping of resistance through the use of DNA markers (genotypic information) and resistance scores (phenotypic information) can identify the location and role of the genomic regions that contain the genes for phenotypic variation. QTL mapping is based on the screening of DNA markers for their likelihood of association with a plant trait (Young 1996). Two different alleles of a gene or molecular marker occur due to DNA mutations such as base pair substitutions, DNA rearrangements and errors in DNA replication (Paterson 1996). Markers represent the genetic differences between individuals within a population and these differences are generally visualised using gel electrophoresis or fluorescence-based techniques (Collard et al. 2005). Genetic markers are either described as codominant or dominant. Codominant markers are able to generate a product of the different alleles whereas dominant markers amplify only one allele. Being able to distinguish between both alleles enables differentiation between homozygotes and heterozygotes. Genetic differences can be detected using a range of different markers, and are grouped according to their detection methods with hybridisation or PCR amplification being the most common. Examples of hybridisation-based techniques are restriction fragment length polymorphisms (RFLPs) and of the polymerase chain reaction (PCR) based markers, random amplified length polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs). SSRs or microsatellites have been extensively used in the mapping of various plant associated traits based on their high levels of polymorphism, robustness and simple methods of detection (Semagn et al. 2010).

The development of Multiplex-Ready Technology allows the selection of numerous markers in single assays (Hayden et al. 2008) through a fluorescent-based system where each primer

pair has a unique fluorescent tag. This technology increases the throughput of the SSR marker based system. Technologies based on single nucleotide polymorphisms (SNPs) are used increasingly due to the availability of cultivar-specific DNA sequence information. The SNPs represent the most frequent type of genetic polymorphism and thus provide a high density of markers near a locus of interest (Jehan and Lakhanpaul 2006). Diversity Array Technology (DArT) is a high-throughput and highly polymorphic array based method (Jaccoud et al. 2001, Akbari et al. 2006, Semagn et al. 2006) that simultaneously genotypes several hundred to thousands of SNPs and insertion/deletion polymorphisms in a single assay. The DArT arrays score the presence and absence of DNA fragments in a genomic representation via hybridisation fluorescence. The high-throughput nature of this system allows rapid construction of genetic framework maps.

1.5.2 QTL Mapping

QTL mapping requires several steps: 1) development of a mapping population, 2) phenotyping the population, 3) genotyping the population (DNA markers) to construct linkage maps and 4) QTL analysis. QTL mapping is centred around that genes and markers segregate via chromosomal recombination during sexual reproduction (meiosis), which allows for analysis within the progeny (Paterson 1996). According to Mendel's law of independent assortment, when two genes (or DNA markers) are close together on the same chromosome they do not sort independently. They are considered linked as they are transmitted from parent to the offspring together during meiosis. If they sort independently, the segregation of an allele of one gene is independent of alleles of another gene. Such genes or markers are considered unlinked and 50% of the progeny will receive either one or the other allele (Collard et al. 2005). In terms of mapping, markers that are closely linked to a specific QTL will be inherited together from parent to progeny more frequently than those located further apart due to the lower chance of recombination events. The greater the distance between markers, the greater the chance of recombination (Collard et al. 2005). Recombination is not uniform across chromosomes as within wheat genomes recombination preferentially occurs in distal regions (Dvorak and Chen 1984, Kota et al. 1993, Lukaszewski and Curtis 1993, Hohmann et al. 1994, Delaney et al. 1995, Delaney et al. 1995, Gill et al. 1996, Dvorak et al. 1998).

1.5.2.1 Mapping Populations

QTL mapping requires a population segregating for the trait of interest so genetic differences are detectable. Relatively large population sizes (generally >150 lines) are required to ensure QTL are identified and do not produce unrealistic phenotypic effects (Beavis 1998, Melchinger et al. 1998, Utz et al. 2000). As most cereal crops are self pollinating, mapping populations are created from parents that are homozygous but heterogenous (Collard et al.

2005). Several different types of populations can be used for mapping studies. Homozygous populations such as recombinant inbred lines and doubled haploids (Semagn et al. 2006) are advantageous as they produce homozygous, fixed lines that can be multiplied without genetic change and allow analysis of replicated trials using identical genetic material (Collard et al. 2005). Backcross or F2 (second filial generation) populations, derived from crossing the F1 (first filial generation) hybrids to one of the parents or through selfing, are easy to construct. However, they are limited as they are not highly recombinant and cannot be fixed as they are heterozygous (Young 2001, Semagn et al. 2010). Markers common between linkage maps can be used to draw comparisons and to generate consensus maps, where maps constructed from different genotypes are merged (Appels 2003, Somers et al. 2004).

1.5.2.2 Phenotypic Analysis

In order to perform QTL analysis which correlates the phenotype with the genotype, the mapping population needs to be phenotyped for the trait of interest. The quality of QTL mapping relies heavily on the quality of the phenotypic data generated and thus it is important that replicated trials are conducted with reliable screening protocols to minimise variation (Young 1996). Nematode resistance screening is difficult as unlike other pathogen resistance, such as leaf rusts, where visible plant disease symptoms are evident and quantifiable, there are no characterised disease symptoms that can be visualised, measured easily or be correlated to resistance. Nematode resistance has been defined as the capacity of the plant to reduce nematode reproduction, and thus to phenotype *Pratylenchus* resistance the numbers of nematodes reproducing on wheat roots needs to be quantified.

Nematode tolerance is the ability of the plant to grow and yield whilst enduring nematode reproduction and is phenotyped by measuring yield. Therefore, phenotyping has to occur at the end of the season and generally only in the field as glasshouse space is commonly limited. In addition, many factors can influence yield and thus it is difficult to associate reduced yield with nematode damage even if there are high nematode numbers in the soil.

Traditionally, phenotypic evaluation of *Pratylenchus* resistance is measured by nematode reproduction either under controlled environmental conditions or in the field. Field evaluations have been used to phenotype selected cultivars for resistance and tolerance, but have not been used for generating phenotypic data for mapping studies. Field sites are carefully selected so that they primarily contain the target nematode and have minimal populations of other soil pathogens (nematodes and fungi). Field evaluation requires replicated trials planted on sites with quantified initial population numbers (Pi). Trials can be conducted on sites with low initial target populations which are then re-cropped with the same test cultivars for several successive wheat seasons and after each cropping season (approximately 7 months) the soil is sampled (replicated cores to 10 cm depth from each plot) and the final

number of nematodes is (Pf) quantified. The amount of reproduction is calculated by the change in nematode numbers (Pi/Pf). Alternatively field reproduction evaluations can be conducted in sites where test cultivars grown in plots with high and low *Pratylenchus* densities are compared. Susceptible cultivars are planted over several seasons to establish high soil inoculum levels. Wheat cultivars of known susceptibility and resistance are included in the experimental design to act as standard controls. These field evaluations are laborious, costly and time consuming as they require intensive labour for site management including planting, harvesting and soil collection on large scales for several years. Field studies are subject to variability due to inconsistent weather (rain, temperature, and soil moisture), inconsistent nematode populations across plots and other environmental implications (effects of other diseases and pests and soil type changes). These environmental factors can greatly influence the phenotypic assessments of traits.

A less labour intensive and faster approach is through the use of controlled environmental/ glasshouse evaluation. This method has been used extensively to phenotype large mapping populations. Plants are grown in pots arranged according to a randomised complete block design with replicates of each line. Plants are established usually in a pasteurised medium enriched with required nutrients (N, P, K, S, Ca and Zn) and inoculated with *Pratylenchus* nematodes obtained from a pure culture. The inoculum concentration varies between research groups from 2.5 to 10 nematodes per gram of soil (Thompson et al. 1999, Zwart et al. 2004). Inoculum densities need to mimic those of general field environments. Levels need to be high enough to distinguish reproductive differences between genotypes but not to cause extensive root damage preventing nematode feeding. The temperature, water, humidity and light conditions are regulated. Generally, temperatures are maintained at 22 °C, the optimum temperature for *P. thornei* and *P. neglectus* reproduction. Pots are placed on matting and watered through a self regulating bottom watering system that maintains a moisture content of approximately 55%. Plants are grown for 6 to16 weeks before either soil and/or roots are collected for nematode quantification. This method compared to field evaluation eliminates environmental variability and enables much higher throughput in considerably less time (months versus years).

With resistance screening there is always the uncertainty whether resistance identified under controlled environment/glasshouse conditions will be observed in the field. Results from a study of the initial glasshouse pot tests developed (O'Reilly and Thompson 1993) for *Pratylenchus* resistance, using sterilised soil at 5 *P. thornei*/g were able to closely rank wheat cultivars on resistance to their field rankings. Despite the ability to mimic temperature, moisture, nutrients and soil type aspects within controlled growth systems it is very difficult to recreate the same field growing systems. It has been suggested that root architecture of plants grown in pots does not reflect that of root systems in the field. The number, structure, density and depth of roots can greatly influence *Pratylenchus* penetration and thus

reproduction. An aspect that needs to be considered when comparing systems is the higher density of plant roots in pots compared to field conditions, causing higher nematode multiplication. While glasshouse screenings effectively identify resistance, it may also be useful to analyse the same material in field trials over various locations. This will evaluate the influence of soil environment and climate on the identified resistance and tolerance.

The quantification of nematodes from soil and roots has traditionally relied on extraction of live nematodes and direct counting via microscopic assessment. Extraction methods can separate and collect nematodes from soil and roots based on their size and shape through sieving, decanting, elutricating or floatation. However, the most common method for *Pratylenchus* extraction relies on their mobility and sedimentation rate. Techniques based on the Whitehead tray method and its modifications (Whitehead and Hemming 1965, Doyle et al. 1987, Thompson et al. 1995, Taylor and Evans 1998, Hollaway et al. 2000, Taylor et al. 2000) such as misting techniques (Southey 1986, Vanstone et al. 1998) have been extensively used. These methods are based on the premise that when inoculated plant material or soil is moistened with water nematodes will move out of the material and sink enabling collection. The extraction efficiency of these Whitehead methods is quite low in contrast to the newly emerged DNA quantification technology. The number of nematodes extracted from damp soils (South Australian wheat field) collected by Whitehead tray and misting chamber methods yielded 5.2 and 2.7 *Pratylenchus*/g soil respectively, compared to 21.2 *Pratylenchus*/g of soil extracted and quantified by DNA detection (Hollaway et al. 2003). DNA detection methods quantify the amount of nematode DNA present within soil or plant samples and can be designed to specifically target different genus and species. The amount of nematode DNA can be used to calculate nematode reproduction but also as a diagnostic tool to identify and quantify different nematodes within soil communities. The plant material/soil is dried and DNA from all living matter is extracted. There are a variety of different DNA kits for soil extraction (e.g. MoBio, MoBio Laboratories Inc., Carlsbad, USA, and Bio 101, Bio 101 Inc., Vista, USA) and several protocols have been published (e.g. (Steffan et al. 1988, Picard et al. 1992, Frostegård et al. 1999, Watson and Blackwell 2000 , Stults et al. 2001)). However, due to intellectual property protecting these protocols for commercial use, many are unavailable.

A targeted approach, using nematode species/genus specific primers designed from sequence data unique to the target nematode, allows the discrimination of resistance between different nematodes in the field. Real time quantitative PCR assays quantify the amount of target nematode DNA providing a sensitive and powerful method for the quantification nematodes in soil (Schena et al. 2004). Quantitative PCR is based on the detection and quantification of a fluorescent reporter signal (Bustin 2000), which increases in direct proportion to the amount of PCR product in a reaction, thus enabling absolute quantification (Gachon et al. 2004). This method has high sensitivity detecting one nematode

per gram of soil (Yan et al. 2012) and is rapid as it can process 500 g samples, with a high throughput of over 100 samples/day (Ophel-Keller et al. 2008). Standard curves are generated by adding known numbers of nematodes to soil so that the amount of nematode DNA per gram of soil can be calculated. The plants are scored resistant, moderately resistant, moderately susceptible or susceptible based on correlations to DNA amounts of check varieties in each of these categories.

Although this DNA quantification method is rapid and reliable, it is expensive, especially when large populations need to be phenotyped with several replicates. In addition, the technology is restricted in that it can only be performed by specialised technicians with specialised equipment. However, the quantification of extracted nematodes that utilises microscopic analysis relies upon tedious counting which is inefficient and introduces human error. The DNA methods have higher nematode extraction efficiencies, are more sensitive and have low extraction variations. As this technology continues to evolve, costs and accessibility will be improved making it the superior choice for nematode resistance phenotyping.

1.5.2.3 Linkage Maps

In order to genotype the mapping population and to construct a genetic linkage map, polymorphic DNA markers need to be selected as they reveal differences between the parents. Once these markers are identified the population is genotyped where DNA from each of the progeny lines is screened with all polymorphic DNA markers (Collard et al. 2005). Genetic differences between markers in individuals are observed by detection of sequence differences. Distances between markers along the chromosomes are measured in terms of recombination frequencies, which are represented as map distances in centiMorgans (cM) (Collard et al. 2005). The markers act as signs indicating the genetic distance along the chromosome and from a QTL. The progeny will inherit alleles and thus markers from either parent and in the case of a double haploid progeny, each marker will be present in a homozygous state (Semagn et al. 2006). The logarithm of odds (LOD) ratio calculates the probability that two markers are linked versus not being linked based on their recombination frequencies (Risch 1992). A linkage map is created using the recombination estimates between each pair of markers and is used to estimate positions relative to each other. Markers are then grouped into linkage groups which represent chromosomes (Collard et al. 2005). Due to unequal recombination along chromosome linkage maps (Gill et al. 1993, Gill et al. 1996) genetic distance does not correlate linearly to physical distance (Semagn et al. 2006).

Linkage maps are unique to the population they are created from, however, comparisons can be drawn between maps if common markers are present (Collard et al. 2005). Common

markers from different maps are used to produce consensus maps where maps from different genetic sources are merged based on the alignment of common or anchor markers. Consensus maps are useful in identifying markers located within a specific chromosomal region in order to saturate a previously identified QTL region (Collard et al. 2005).

1.5.2.4 Comparative Mapping

The use of maps with common markers can allow comparative mapping where regions of synteny or conservation within and between species can be identified to reveal further details of QTL location and function. There is high conservation of the linear order of genes between different grass genomes (Hulbert et al. 1990, Ahn et al. 1993, Kurata et al. 1994, VanDeynze et al. 1995, Gale and Devos 1998, VanDeynze et al. 1998) and this can be exploited by comparison with better characterised genomes, such as rice. High-resolution, sequence-based maps between wheat and rice using mapped wheat expression sequence tags (ESTs) and rice genome sequence data are available (Sorrells et al. 2003). Comparative maps are useful to increase marker density at genetic target loci, and facilitate map-based cloning of genes (Yu et al. 2004). The International Wheat Genome Sequencing Consortium is using a chromosome-based strategy to construct physical BAC (bacterial artificial chromosome) clone maps and subsequently sequence each of the chromosomes of the hexaploid, bread wheat cultivar Chinese Spring. The first physical map of the largest wheat chromosome, 3B was recently released and low coverage sequences were made available to provide the virtual gene order for all 21 chromosomes, providing a valuable resource for comparative genomics and map-based cloning ((IWGSC) 2010).

1.5.2.5 QTL Analysis

QTL analysis is performed to identify and locate genomic regions that are associated with the trait of interest. It is based on detecting an association between the phenotype (e.g. disease resistance) and the genotype of markers. The population is separated into genotypic groups based on the genotype of each particular marker and analysed to determine if significant differences between these groups exist with regards to the trait of interest (Tanksley 1993, Young 1996). If a significant difference between the phenotypic means of the two genotypic groups exists the population is linked to the QTL at that marker locus (Collard et al. 2005). There are various methods used for detecting QTL: single marker analysis, interval mapping and composite interval mapping (Tanksley 1993, Liu 1998). Single marker analysis uses simple regression analysis to determine the percentage of phenotypic variation explained by each marker for the QTL. The major disadvantage with this method is that the further a QTL is from a marker, the less likely it will be detected due to possible recombination between the marker and the QTL. A more powerful method of QTL analysis is through interval mapping which separates linked QTL on the same chromosome (Schork et al. 1993). Presence of a QTL at a specific interval is estimated by a logarithm of odds score which represents the

likelihood of a significant QTL at that site (Collard et al. 2005). Simple interval mapping analyses intervals between adjacent pairs of linked markers along chromosomes simultaneously, rather than analysing single markers (Landere and Botstein 1989). As this method analyses one interval at a time the effects of other QTL are not considered which may bias the location and effect of QTL, to cause false identification or affect the ability to find a QTL (Haley and Knotts 1992 , Zeng 1994). Composite interval mapping identifies the most likely location of a QTL with respect to adjacent linked markers but also takes into account the effect of background markers/QTL. Analysing background markers minimises the effects of other strongly linked QTL to increase sensitivity of QTL identification (Zeng 1993, 1994). Multiple interval mapping uses multiple marker intervals simultaneously to analyse multiple QTL (Kao et al. 1999) making it the most powerful and precise QTL mapping approach.

QTL mapping provides not only the number and location of QTL but also the magnitude of their effect on the trait. Thresholds are calculated to help determine the significance of the detected QTL. Permutation tests are used to determine thresholds and are generated by randomly pairing the phenotypes and genotypes to estimate a test statistic for the detection of a QTL. The permutation analysis is repeated (usually 1000 permutations) to determine threshold values (Semagn et al. 2010). The amount of observed phenotypic variation is explained by the QTL (R^2 value). Generally, a QTL that accounts for a large proportion (>10%) of the effect of the phenotype is significant while a minor QTL accounts for much less (Lander and Kruglyak 1995, Collard et al. 2005). The heritability of a trait measures the proportion of the total phenotypic variance that is explained by the total genotypic variance. Thus, if a trait has high heritability, variation from individual to individual in a population can be explained genetically and environmental factors have little effect. Broad-sense heritability reflects all the genetic contributions to a population's phenotypic variance including additive, dominant, and epistatic interactions. Narrow sense heritability measures the proportion of the phenotypic variation due to only additive (allelic) genetic factors. Heritability is important when estimating the selection outcomes from a population. The presence of a QTL is usually confirmed by the analysis of one or more validation populations that share the donor parent of the QTL (Lander and Kruglyak 1995) which are phenotyped under various environmental conditions.

1.5.3 Marker Assisted Selection

Marker assisted selection (MAS) is the selection of individuals with the trait of interest based on their genotype rather than phenotype and is a powerful application of QTL mapping (Tanksley et al. 1989). MAS aims to accelerate the development of new cultivars through the precise transfer of genomic regions expressing the target trait (Babu et al. 2004). Molecular breeding is most useful in the selection of traits where conventional phenotypic screening

methods are laborious, time consuming, expensive and produce widely variable results between replications (Young 1996). Other applications include the identification of new and valuable wild alleles and the enhancement of their introduction into commercial cultivars through selective backcrossing. With respect to resistance breeding, MAS can be applied in the selective pyramiding of several different resistance genes against one or many pathogens (Tanksley et al. 1989). Markers that are closely linked to QTL or genes can minimise the linkage drag effect, which prevents unfavourable alleles being introgressed along with the trait of interest (Young 1996). However, QTL regions can be quite large, up to tens of centiMorgans, and can include large chromosomal regions with many genes. Therefore, before markers can be utilised by MAS, fine mapping of chromosome regions is normally required to delimit the QTL interval and to establish tightly linked markers that can be used in breeding programs (Asins 2002). The mapping of additional markers sometimes does not improve QTL resolution and therefore to enable QTL deployment flanking markers are utilised. In addition, marker reliability of linkage to QTL needs to be established by performing marker validation in independent populations from different genetic backgrounds (Semagn et al. 2010). Marker assisted selection has been successfully used to incorporate not only nematode resistance into commercial wheat cultivars as discussed later in 1.5.6, but several other high priority traits. For example, microsatellite DNA markers for *Bo1* and *Bo2*, genes for tolerance to high levels of boron (Jefferies et al. 2000) have been used extensively in Australian wheat breeding programs (Eagles et al. 2001) to combine these genes. Many markers are being utilised for leaf rust resistance in Australia and internationally (Eagles et al. 2001, William et al. 2007). For example, markers for resistance to the stem rust gene *Sr2* (Brown 1993, McIntosh et al. 1995), which is characterised by slow rust development, and markers linked with *Sr38/Lr37/Yr17*and *Sr39* have been used for pyramiding stem rust resistance genes (Ayliffe et al. 2008, Joshi and Sanghamitra 2010).

1.5.4 Map-Based Cloning

In addition to the development of markers for MAS, genetic mapping and QTL identification is the gateway towards map-based cloning. Map-based cloning or positional cloning aims to locate and sequence the target gene within the QTL region (Paterson and Wing 1993). To delimit the QTL region, fine mapping is required to firstly saturate the map near the target gene by the addition of markers and secondly to increase the size and thus the recombination frequency of the mapping population. Recombinant inbred line populations are particularly useful for fine mapping because they provide at least two-fold higher frequencies of recombination in any small chromosomal region compared to an F2 or an F1 backcross, and provide a permanent source of mapped individuals (Semagn et al. 2010). When the phenotypic variance of traits is contributed mainly by one QTL, near isogenic line populations can be constructed to remove the effect of all other linked loci contributing to the trait. Flanking markers identified on the low resolution populations are used to screen these higher

resolution populations to select for plants that are recombinant between the flanking markers. These lines are then selected for further marker saturation. As grasses show conservation of gene order and thus markers on chromosomes (Hulbert et al. 1990, Ahn et al. 1993, Kurata et al. 1994, VanDeynze et al. 1995, Gale and Devos 1998, VanDeynze et al. 1998), this synteny can be used for marker development especially in the sequenced genomes of rice and *Brachypodium* (Keller et al. 2005, Krattinger et al. 2009). Deletion lines can also be used for physical mapping of genes or molecular markers to specific intervals. Each line has a specific deletion and can be used as a physical landmark to locate a gene or marker to a specific chromosomal region (Nagy et al. 2002). If the region of interest has been sequenced markers can be developed from available information.

Once the map has been saturated the flanking markers are used to establish a physical region of the DNA within the QTL. To achieve this, sequence information needs to be identified in that region. A physical map consists of continuously overlapping contigs of genomic DNA clones representing the wheat genome (Zhang and Wing 1997). Flanking markers are used to screen these genomic libraries to identify clones containing the target gene region (Krattinger et al. 2009). Due to the large size of the wheat genome, the construction and screening of libraries can be costly in terms of time and expense (Keller et al. 2005). Additional markers are constructed based on cloned sequences to further screen libraries to identify a clone with the target region. The designing of specific markers can be difficult due to the large amount of repetitive non-coding DNA in cereals and because the uneven coverage of libraries leaves genomic gaps. Once a clone is identified within the target gene region further sequence information is required to identify the candidate gene. Generally, several clones are identified, which are partially sequenced. Sequence information is then used to search DNA databases to identify possible candidate genes. Due to the synteny discussed earlier, genes from rice or *Brachypodium* in the target region can be used to narrow the interval in wheat where the target gene is located. The International Wheat and Barley Genome Sequencing Consortia are working towards sequencing these genomes, with a 95% unassembled draft sequence of the entire wheat genome released in 2010 ((IWGSC) 2010). As more information becomes available the cloning of wheat trait defining genes will become a more realistic target within practical timeframes. Once target genes are identified and fully sequenced their role within the expression of the target trait is validated, for example, through mutant analysis (Krattinger et al. 2009), phenotype complementation in transgenic plants (Wing et al. 1994) and supportive expression data.

Once genes are cloned, gene sequences can be used to develop markers specific to that allele to identify alleles in a wide range of diverse species through allele mining (Kaur et al. 2008). Essentially allelic variations of the gene are mined in different genetic sources by identifying SNPs in coding sequences or in non-coding sequences, which alter gene expression, and thus trait expression. Allele mining can identify superior alleles, or perfect

markers, for the trait of interest and develop allele specific markers so they can be introgressed into breeding material (Kumar et al. 2010). Examples of perfect markers in wheat include the glutenin genes for gluten strength (Anderson et al. 1989), the waxy genes for starch properties (Briney et al. 1998), the puroindoline genes for hardness (Beecher et al. 2002), the *Vrn* genes for vernalisation (Yan et al. 2003, Yan et al. 2004), the *Rht* genes for dwarfing (Peng et al. 1999) and the *Lr10* and *Lr21* genes for leaf rust resistance (Feuillet et al. 2003, Huang et al. 2003).

1.5.5 Association Mapping

There are several problems associated with the fine mapping of plant genes. As plant genomes are relatively large, most DNA markers are unlinked to any given locus and most linked markers are still several cM away from the targeted gene. Secondly, as recombination is rare, mapping markers to a low resolution map requires the investigation of a large number of progeny. Thirdly, as polymorphism levels can be low, tightly linked markers might not be detected and located (Bennetzen 2000). An alternative fine mapping method has emerged in recent times through linkage disequilibrium or association mapping. Association mapping uses evolutionary recombination events in large diverse germplasm collections (of cultivars, landraces and breeders lines) (Risch and Merikangas 1996, Nordborg and Innan 2002) so that over multiple generations of recombination, correlations only to markers tightly linked to the trait of interest will remain (Vinod 2011). Association mapping determines if a particular genetic marker is more common in a particular phenotype than expected by chance. This approach provides greater fine mapping than achieved through standard biparental crosses (Oraguzie and Wilcox 2007). To screen large numbers of samples, a high throughput marker technology is required. Advances in sequencing technologies are now capable of high throughput analysis at low costs and thus allow for the rapid identification of markers, particularly SNPs. These SNP markers are useful as they provide the finest resolution of a DNA sequence, they are abundant and have low mutation rates (Syvanen 2001). Association mapping has been successfully used to map marker associations to stem, leaf and yellow rust, powdery mildew, and grain yield in five historical wheat populations to validate previously found QTL and to identify many new chromosome regions for disease resistance (Crossa et al. 2007).

1.5.6 Marker Assisted Selection for Nematode Resistance

Marker assisted selection has become an important tool for plant breeding, especially for nematode resistance. This is due to the lengthy and costly phenotypic methods required for resistance screening. As discussed in 1.5.2.2, *Pratylenchus* resistance screening methods require field/glasshouse space, maintenance of nematode cultures and extensive labour to process plants for extraction and nematode quantification. Despite the initial costs of

developing markers, in the case of *Pratylenchus* resistance where the trait is inherited by additive gene action and the great expense of phenotypic screening, the use of markers is highly desirable. The selection of resistant progeny generated by breeding programs can be simply assessed for resistance by screening DNA obtained from seeds or seedlings with genetic markers linked to resistance. This eliminates the need to select lines based on their resistant phenotypes reducing the phenotypic screening periods from several months to only several days.

Marker assisted selection for nematode resistance in wheat has been achieved for the cereal cyst (*Heterodera avenae)*, the root knot (*Meloidogyne naasi)* and the root lesion (*Pratylenchus neglectus)* nematodes. Markers for the cereal cyst nematode (CCN) resistance genes, *Cre1*, *Cre3* and *Cre6*, are completely linked and have been used in breeding programs. For example, the *CD2.2* RFLP marker, linked to *Cre1* (Ogbonnaya et al. 2001), and the *Cre3* PCR based marker (Ogbonnaya et al. 1998, Ogbonnaya et al. 2001) have been used to help select progeny with resistance to Australian and European *Heterodera* pathotypes. Markers linked to the *Cre6* gene (Ogbonnaya et al. 2001) were found in homologous sequences from the *Aegilops ventricosa Cre3* locus (Seah 1999).These markers are being used to pyramid *Cre1*, *Cre3*, and *Cre6* genes to produce lines with different combinations of resistant alleles (Eastwood et al. 1994, Williams et al. 1994, Lagudah et al. 1997, Ogbonnaya et al. 1998, Ogbonnaya et al. 2001, Ogbonnaya et al. 2001). The SCAR (sequence characterised amplified regions) marker, *Y16*, closely linked to the root knot resistance gene, *Rkn-mn1*, isolated from *Ae. variabilis,* has been used to transfer this resistance into selected bread wheat lines (Persondedryver and Jahier 1985, Yu et al. 1990, Yu and Jahier 1992, Yu et al. 1995, Barloy et al. 2000). The only marker to be currently utilised for resistance to root lesion nematodes is linked to the *P. neglectus* resistance locus *Rlnn1* identified by Williams et al. (2002), with the closest being the RFLP marker *cdo347*. This marker was used by breeding programs at the University of Adelaide throughout the early 2000's (Eagles et al. 2001). However, these RFLP markers are not suitable for marker assisted wheat breeding due to their laborious and time-consuming technique. The Australian Wheat and Barley Molecular Marker Program (AWBMMP) using an EST sequence close to this RFLP, identified through comparative mapping with rice, developed a more user friendly PCR based marker *uat0001* that is closely linked to *Rlnn1* (Mather and Chalmers 2010). This marker is now used within several Australian wheat breeding companies for *P. neglectus* resistance selection, details of which are confidential.

Resistance QTL and linked molecular markers to various nematodes have been identified and mapped in other crops (both horticultural and agricultural crops) including *Heterodera* species in beet, soybean and barley (Salentijn et al. 1995, Concibido et al. 2004, Williams et al. 2006, Dayteg et al. 2008). For example, microsatellite markers positioned only 1 to 2 cM away from the *rhg1* soybean cyst nematode resistance gene have been used extensively in

screening breeding populations (Mudje 1997, Cregan et al. 1999). Markers have been utilised for resistance selection to *Globodera* species in potato (VanderVoort et al. 1997, Bryan et al. 2002), *Meloidogyne* species in soybean, cotton, potato, cucumber and peach (Brown et al. 1996, Yamamoto and Hayashi 2002, Shen et al. 2006, Barloy et al. 2007, Ulloa et al. 2010, Devran et al. 2011), the citrus nematode *Tylenchulus* (Ling et al. 2000), the stem nematode *Ditylenchus* in sweet potato (Qina et al. 2008), the reniform nematode *Rotylenchus* in cotton (Avila et al. 2004) and finally *Pratylenchus* species in barley and oats (Townshend 1989, Schmidt et al. 2005, Zwart et al. 2005, Zwart et al. 2006, Sharma et al. 2011).

The effectiveness of molecular markers depends on the strength of their linkage to the QTL. Unless the marker itself is the gene there is a chance that recombination can occur between the marker and the resistance gene (Babu et al. 2004), which in turn may produce incorrect resistance assessment. As mentioned previously, QTL and thus marker identification can be limited by low levels of polymorphisms and complicating interactions of the environment and the QTL. However, with the recent developments in mapping technologies (SNPs and high throughput sequencing), high density genetic and physical maps will enable the development of more closely linked markers (Varshney et al. 2005, Varshney et al. 2006). These new technologies provide maximum exploitation of available polymorphisms and thus the development of closely linked markers that can be used widely. In addition, more genome sequencing efforts will lead to map-based cloning of actual resistance genes, generating perfect markers that eliminate the possibility of the breakdown of marker resistance.

1.6 Nematode Resistance Genes

Despite the identification of markers linked to resistance in many species, the only nematode resistance genes that have been sequenced and cloned are against the sedentary nematodes, *Heterodera*, *Meloidogyne* and *Globodera*. Commonly, resistance against sedentary nematodes is mediated through a gene for gene interaction, where a plant resistance gene mediates specific recognition of a nematode that expresses a matching avirulence gene (Flor 1971). Along with the induction of genes encoding hydrolytic enzymes and other chemical compounds a hypersensitive response is commonly associated with this gene for gene resistance. Through a transduction pathway, the plant signals for the rapid local cell death of invaded cells (Takken and Joosten 2000).

The first nematode resistance gene cloned was $Hs1^{pro\text{-}1}$, conferring resistance to the cyst nematode *Heterodera schachtii*, and was isolated from a wild species of beet, mapping to chromosome 1 of the sugarbeet, *Beta procumbens* (Yu 1984). The $Hs1^{pro\text{-}1}$ resistance gene confers resistance by degrading the nematode feeding structures without involving a hypersensitive response. After syncytium formation, the feeding structure degrades and the

sugarbeet cyst nematodes die as J2 due to starvation (Jung et al. 1998). The *Mi* gene cloned from tomato confers resistance against three species of root knot nematodes, *Meloidogyne incognita*, *Meloidogyne arenaria* and *Meloidogyne javanica* (Milligan et al. 1998). Molecular analysis of a 52 kbp genomic DNA region led to the isolation of two functional genes, *Mi1.1* and *Mi1.2*. Through genetic and physical mapping *Mi1.2* was localised to the short arm of chromosome 6 in tomato (Milligan et al. 1998). Extensive fine mapping in this region identified several markers closely linked to the gene (Klien-Lankhorst et al. 1991), which were used to introgress the gene into several breeding lines. The *Mi*-gene based resistance forms an early hypersensitive response (first 12 to 24 h after penetration) that prevents the induction of *M. incognita* giant cell development (Dropkin 1969, Paulson and Webster 1972). A localised necrosis around the head of the nematode occurs a few days after invasion. As resistance is only mediated after penetration and migration, secretions initiating giant cell formation from the nematode stylet may elicit the resistant response.

Through a positional cloning approach, the resistance gene, *Gpa2*, was identified giving resistance to the potato cyst nematode *Globodera pallida* (Van der Vossen et al. 2000). Using AFLP and RFLP markers, the *Gpa2* gene was mapped to chromosome 12 of potato (VanderVoort et al. 1997). Another *Globodera* resistance gene was isolated and cloned from tomato. The *Hero* gene confers broad spectrum resistance to potato cyst nematodes and provides 95% resistance to *Globodera rostochiensis* and about 80% resistance to *G. pallida* (Ernst et al. 2002). *Hero* has been mapped to chromosome 4 in a 118 kbp region containing 14 homologous genes (Ernst et al. 2002). The *Hero* gene mediated resistance is initiated after feeding site development. In potato cultivars with the *Hero* gene, a syncytium feeding site develops but a hypersensitive response follows preventing access to the xylem, inhibiting nutrient transfer, and the developing juveniles are prevented from receiving nutrients and die (Sobczak et al. 2005).

Resistance genes have also been identified and mapped for cereal cyst nematodes (CCN). Thus far, eight CCN resistance genes have been identified in wheat and its wild relatives: *Cre1* to *Cre8* (Al-Doss et al. 2010). These genes confer varying resistance to different CCN pathotypes (Barloy et al. 2007). Only two resistance genes have been directly derived from bread wheat, *Cre1* located on chromosome 2B (Slootmaker et al. 1974) and *Cre8* on chromosome 6B (Paull et al. 1998), and have been bred into many European and Australian cultivars. *Cre2*, *Cre5* and *Cre6* were transferred to wheat from *Ae. ventricosa* (Delibes et al. 1993, Jahier et al. 2001, Ogbonnaya et al. 2001) and *Cre7* from *A. truincialis* (Romero et al. 1998). *Cre3* and *Cre4* were derived from *Aegilops tauschii* (Eastwood et al. 1991, Eastwood et al. 1994). The *Cre3* gene has been mapped to the long arm of chromosome 2D and has been transferred to wheat via synthetic hexaploids (Eastwood et al. 1991, Eastwood et al. 1994). Studies have found that *Cre1* and *Cre3* resistance genes confer resistance by degrading nematode feeding structures. In cultivars with *Cre1* and *Cre3, H. avenae* was able

to induce syncytium formation, but by 33 d.a.i. the cytoplasm of the syncytium was degraded (Williams and Fisher 1993, Seah et al. 2000) preventing any further feeding.

1.6.1 Resistance Gene Clusters

Classical genetics studies indicate that (nematode) resistance genes cluster together in the genome (Michelmore and Meyers 1998). For example, the *Mi1.2* and the *HeroA* genes are found in clusters of seven and fourteen homologous copies, respectively. In addition, *Mi* maps within one cM of the fungal resistance genes *Cf2* and *Cf5* in tomato (Dickinson et al. 1993), and the potato cyst nematode resistance gene *Gpa* is tightly linked to the viral resistance gene *Rx2* and fungal resistance gene *R1* (Kreike et al. 1994). Clustering of resistance genes (and linked markers) implies that they could have evolved a common mechanism (Kanazin et al. 1996). Similarities between resistance genes may be exploited to identify sequence homologies from conserved motifs to uncover resistance loci/markers in other plants (Kanazin et al. 1996). It is likely that different *Pratylenchus* resistance genes will also map to clustered regions. Clustering of genes is also beneficial for MAS as blocks of resistance genes to various pathogens can be transferred together (Ratnaparkhe et al. 1998).

1.7 Genetic Control of *Pratylenchus* Resistance and Tolerance in Wheat

At present, *Pratylenchus* is controlled through the use of crop rotations and tolerant cultivars. However, as *Pratylenchus* species have a broad host range most rotational crops are only partially resistant. In addition, the use of tolerant hosts can lead to high densities of nematodes affecting subsequent crops. Thus, the development and use of resistant and tolerant wheat cultivars is considered the most economical and environmentally acceptable means for their control (Castillo et al. 1998).

Sedentary nematodes, particularly cereal cyst and root knot nematodes, have been the focus of research and several resistance genes have been identified and cloned. Resistance against migratory nematodes is characterised but only a few investigations revealed resistance loci against *P. thornei* and *P. neglectus.* For example, resistance to *P. neglectus* was identified and used in breeding in almonds through the use of resistant root stocks (Marull et al. 1990) and in oats (Townshend 1989). Resistance in wheat to some degree has been identified against *P. thornei* and *P. neglectus* (Thompson et al. 1989), but a limited number of *Pratylenchus* resistance sources in wheat have been explored at the molecular level.

1.7.1 Tolerance

Tolerance is defined as the capacity of a cultivar to yield in infested soils (Nelson 1973, Thompson et al. 1999). However, as tolerant cultivars allow nematode reproduction they do not reduce the numbers of *Pratylenchus* in the soil (Thompson et al. 1999). The residual *Pratylenchus* populations can attack subsequent susceptible host crops (Thompson et al. 1989, Trudgill 1991). The first source of superior tolerance to *P. thornei* in wheat lines was identified through targeted screening of cultivars. Tolerant cultivars such as Pelsart (Brennan et al. 1994), Sunvale (Ellison et al. 1995) and Baxter (Thompson et al. 1999, Thompson et al. 2008) were used to minimise the effects of *Pratylenchus*. These tolerant lines offered a 30% yield increase compared to other commercial cultivars available at the time (Thompson et al. 1995).

1.7.2 Resistance

1.7.2.1 GS50a

Resistance is defined as the capacity of a cultivar to reduce nematode reproduction (Thompson et al. 1999). Use of resistant cultivars is the best means to successfully control root lesion nematodes. The first significant source of resistance to *P. thornei* was the bread wheat line GS50a, selected from a severely infested field of the variety Gatcher (Thompson and Clewett 1986). Several lines were developed from these selections and GS50a resistance was backcrossed into commercial wheat cultivars (Thompson et al. 1994). Although the use of
GS50a resistance in adapted cultivars reduced *Pratylenchus* reproduction by more than ten-fold, it only provided partial resistance (Thompson et al. 1999). The lack of strong resistance was an incentive to locate and use more effective genetic sources.

1.7.2.2 Wild Wheat Relatives as Resistance Sources

Hexaploid bread wheat, *Triticum aestivum* (AABBDD), evolved through natural hybridisation of the diploid *Aegilops tauschii* Coss. (DD) and a tetraploid ancestor of today's *Triticum turgidum* L. subspp. *durum* (AABB) (Breiman and Graur 1995). As this was a rare event, only a limited number of genotypes from these wild progenitors were involved in the natural hybridisation. Thus, hexaploid wheat lacks the genetic diversity, including a broad range of resistance genes of its ancestors. Unique sources of resistance can be identified in wild wheat relatives such as *Ae. tauschii* and can be introgressed into durum wheat by direct crossing as *Ae. tauschii* chromosomes are homologous with those of the bread wheat D genome (Gill and Raupp 1987). Thompson and Haak (1997) first reported resistance to *P. thornei* in the wild grass *Ae. tauschii*. Several hundred *Ae. tauschii* and *T. turgidum* accessions were screened for *P. thornei* resistance, and final numbers of nematodes in the soil and in the roots were determined after 4 months of growth. In *Ae. tauschii,* 39 of 244

accessions and in *T. turgidum*, 28 accessions produced lower numbers of nematodes than GS50a.

1.7.2.3 Synthetic Wheat

Resistance can also be exploited through the construction of synthetic hexaploid wheats through hybridising tetraploid durum wheats and diploid relatives of wheat such as *Ae. tauschii* (Lagudah et al. 1993). Synthetic hexaploid wheat lines were investigated as a source of new allelic variation for advantageous resistance traits. Thompson et al. (1999) investigated *P. thornei* resistance in synthetic hexaploids and found higher resistance levels when both durum and *tauschii* parents were resistant than when just one was resistant. Thompson et al. (1999) suggested that in hexaploid wheat, *P. thornei* resistance is under the control of at least two genes, one in the D genome and the other in A or B genomes. Of the five hexaploid synthetic wheats tested, all had higher resistance to *P. thornei* than GS50a (Zwart et al. 2004). This study also established that resistance to *P. thornei* in synthetic wheats is polygenic and primarily determined by additive gene action.

A glasshouse phenotypic trial showed that wheat lines that were tolerant/resistant to *P. thornei* were susceptible to *P. neglectus*, indicating that the mechanisms conferring resistance to *P. thornei* do not confer resistance to *P. neglectus* (Farsi et al. 1995, Taylor et al. 1999). As both *Pratylenchus* species are often found in the same field in mixed populations (Thompson et al. 2010), the development of wheat cultivars with resistance to both species is desirable. A genetic map constructed with microsatellite markers was used to identify QTL associated with resistance to both *P. thornei* and *P. neglectus* in a doubled haploid population derived from a resistant synthetic hexaploid wheat line (CPI133872) crossed with susceptible Janz bread wheat (Zwart et al. 2005). Two resistance QTL on the distal end of chromosome 6DS, *QRlnt.lrc-6D.1* and *QRlnn.lrc-6D.1*, were associated with resistance to *P. thornei* and *P. neglectus*, respectively, (Zwart et al. 2005) and linked to the codominant microsatellite marker *barc183* (Table 1). Zwart et al. (2005) also identified a novel QTL on chromosome 2B associated with both *Pratylenchus* species which can be used to provide dual resistance (Table 1). This region may contain one gene controlling resistance for both species (Rossi et al. 1998), or two closely linked genes for each species (Takken and Joosten 2000).

A second major QTL was identified for *P. thornei* on chromosome 6DL (*QRlnt.lrc-6D.2*). Two other minor QTL for *P. thornei* resistance were found on chromosomes 6A and 2B (Q*Rlnt.lrc-6A.1* and *QRlnt.lrc-2B.1*). In addition to the *P. neglectus* resistance QTL on chromosome 6D, two other QTL were observed on chromosomes 4D and 4B (*QRlnn.lrc-4D.1* and *QRlnn.lrc-24B.1*). The presence of these QTL was not observed in material collected from both years analysed and each QTL explained less than 15% of the phenotypic variation (Zwart et al.

2005) (Table 1). Toktay et al. (2006) used microsatellite markers previously identified as linked to *Pratylenchus* resistance and mapped the synthetic wheat population of a cross between CROC and Pastor. Two resistance loci on chromosomes 1B and 3B were found with the partially resistant parent CROC lacking the resistance locus on chromosomes 2B and 6D (Toktay et al. 2006).

The CPI133872 x Janz population analysed by Zwart et al. (2005) used a framework map with a limited number of molecular markers (1 marker per 17 cM) to detect resistant associations. With subsequent development of marker and mapping technologies, higher density maps were constructed. Over 200 DArT markers were integrated with the SSR and AFLP genetic map of CPI133872 x Janz to increase overall marker density and coverage. This allowed for more accurate QTL location estimation by reducing the QTL interval size significantly (up to 10 cM) and identifying further flanking markers that might be useful for selection (Zwart et al. 2010).

QTL analysis was performed on a more densely mapped synthetic derived wheat population than previously analysed (Zwart et al. 2006), comprising RFLP and microsatellite markers. An ITMI recombinant inbred line population was derived from a cross between the resistant synthetic hexaploid wheat (W-7984) and a susceptible bread wheat Opata 85. Two major QTL for *P. thornei* resistance were found on chromosomes 6DS and 2BS, with both QTL explaining 20 to 25% of the phenotypic variation (Table 1). Both the D and B genome contribute to this *P. thornei* resistance as in both regions the QTL resistance alleles were inherited from the synthetic hexaploid parent (Zwart et al. 2006). The microsatellite marker *barc183* on chromosome 6DS was previously associated with *P. thornei* resistance in the CPI33872 x Janz population (Zwart et al. 2005, Zwart et al. 2006) indicating it is useful for MAS of resistance in genetically different backgrounds (Zwart et al. 2006). The 6D locus was also identified in the GS50a wheat line (Vicars et al. 1999). The QTL on chromosome 2B for *P. thornei* resistance identified by Zwart et al. (2006) were also detected in other mapping populations (Schmidt et al. 2005, Zwart et al. 2005), but lack of common markers meant no comparisons could be made between the QTL location and other resistance genes (Zwart et al. 2006).

1.7.2.4 Middle-Eastern Landraces

As all wheat cultivars investigated had only partial resistance to *P. thornei*, better sources of resistance were sought from bread wheat relatives. As *P. thornei* occurs in Middle-Eastern countries (Fortuner 1977, Nicol et al. 2003), the origin of cultivated wheat , it is likely *P. thornei* resistance was selected for and evolved in Middle-Eastern wheat landraces.

Populations derived from the landraces AUS13124 and AUS4926 were known to have resistance to *Pratylenchus* and were crossed with the susceptible Janz cultivar, and mapped

with AFLP and microsatellite markers (Schmidt et al. 2005). A resistance locus on chromosome 2B was only detected in the AUS13124 x Janz population and accounted for about 8% of the phenotypic variation (Table 1). In addition, a novel *P. thornei* resistance QTL on chromosome 3B was identified in both AUS13124 and AUS4926 (Table 1). A synthetic AUS4930 x Pastor population was investigated with microsatellite markers and three QTL were identified on chromosomes 1B, 2B and 6D (Toktay et al. 2006). This provides further evidence that the most commonly identified genomic regions associated with *P. thornei* resistance are located on chromosomes 6D and 2B.

Sheedy and Thompson (2009) investigated 274 accessions of Iranian wheat landraces and identified 25 accessions more resistant than GS50a. Thompson et al. (2009) found additional sources of *P. thornei* resistance from screening two wheat accession collections from the West Asian and North African regions. An additional 13 bread wheat and 10 durum accessions had resistance better or equal to GS50a. These landraces are part of the primary gene pool of wheat and can be integrated with modern wheat cultivars to use resistance genes. The 23 accessions were analysed for mode of inheritance and two to six resistance genes were used in five different parental crosses, further confirming the polygenic nature of *P. thornei* resistance (Thompson and Seymour 2011).

1.7.2.5 Pratylenchus neglectus Resistance

Resistance to *P. neglectus* has been investigated to a lesser degree than that of *P. thornei*. Most *Pratylenchus* wheat resistance studies conducted in Australia focus on *P. thornei*, as it is the more damaging species in northern Australian wheat systems in terms of monetary yield loss (Thompson 2008). However, *Rlnn1*, the first root lesion nematode resistance gene of any *Pratylenchus* species to be mapped, was identified on chromosome 7A (Williams et al. 2002) (Table 1). In addition, *P. neglectus* resistance QTL on chromosome 6D, (*QRlnn.lrc-6D.1*) 4D and 4B (*QRlnn.lrc-4D.1* and *QRlnn.lrc-4B.1*) (Zwart et al. 2005) were identified as discussed in 1.7.2.5 (Table 1). The major resistance gene, *Rlnn1,* was first identified in the wheat cultivar Excalibur closely linked to an AFLP marker. Then, using comparative mapping, an associated RFLP marker, *cdo347*, was identified at the 7AL chromosomal location of the *Rlnn1* gene (Williams et al. 2002). The RFLP marker, *cdo347*, was previously mapped to 7A and associated with *P. neglectus* resistance in other wheat populations and also with the leaf rust resistance gene *Lr20* on 7A (Nelson et al. 1995, Parker et al. 1998). However, Schmidt et al. (2005) found that in two Middle Eastern landrace populations the association between this gene and resistance was only detected through single marker regression and not composite interval mapping. In addition, no marker-trait associations were found with markers to 7AL in the CPI133872 synthetic line crossed with Janz population (Zwart et al. 2005). This indicates that this *Rlnn1* resistance gene does not segregate in these populations. More recently, using an expression sequence tag (EST) sequence close to the RFLP *cdo347*, the AWBMMP identified and developed, through comparative mapping

with rice, a PCR based marker, *uat0001,* that is closely linked to *Rlnn1* (Mather and Chalmers 2010).

1.7.3 Future Approaches To Identify Resistance To *Pratylenchus*

Although these identified resistance QTL provide useful sources of resistance, further work is required before they can be implemented. As discussed in Section 1.7.2, most of the *Pratylenchus* resistance QTL identified are minor and explain little of the phenotypic variation and in addition, are not associated with tightly linked markers, preventing incorporation of resistance to commonly used wheat cultivars using MAS. Therefore, their validity needs to be tested in other populations which have been generated with the resistant parental line. Further fine mapping is required to delimit the QTL size in order to identify more closely linked markers. As most of these resistance sources have been isolated in wild wheat relatives, several rounds of backcrossing may be required to remove detrimental traits associated with the resistance QTL or to introduce high priority agronomic traits that may be lacking.

The limiting factor in identifying new sources of *Pratylenchus* resistance and the development of resistant cultivars has been the phenotyping of resistance. The biological assays used to screen for root lesion nematode resistance as discussed in 1.5.2.2 are laborious and costly (Barloy et al. 2000). However, with the development of PCR diagnostic approaches phenotypic resistance data can more easily and reliably detect and quantify *Pratylenchus* within soil and roots (Ophel-Keller et al. 2008). Together with the increasing availability of high quality phenotypic data, genomic wheat sequences and large mapping populations, this will enhance the construction of high quality genetic maps to improve the ability to identify resistance QTL and develop closely linked resistance markers. This in turn will allow gene pyramiding to provide effective resistance combinations. When incorporating resistance into new cultivars through backcrossing, resistant markers through MAS will eliminate the need to select progeny lines based on their resistance phenotypes. Molecular markers remove the need for large scale phenotyping thus facilitating the rapid identification of resistance loci, accelerating the development of new resistant cultivars (Schmidt et al. 2005, Toktay et al. 2006). In addition, advances in map-based cloning will help characterise *Pratylenchus* resistance genes and their functions.

Table 1: Previously mapped *Pratylenchus* resistance QTL in wheat. The table shows the donor parent (Inherited Origin), the chromosomal location of the resistance QTL (Chromosome/QTL), the *Pratylenchus* species (*Pt* - *Pratylenchus thornei* or *Pn* -*Pratylenchus neglectus*), the flanking markers, the percentage of phenotypic variation (% Var) explained by the QTL and the likelihood ratio statistic (LRS).

Reference	Inherited Origin	Chromosome/QTL	Species	Flanking Markers	% Var	LRS
Thompson et al. 1999 & Vicars et al. 1999	GS50a	6D	*Pt*	np	np	np
Williams et al. 2002	Excalibur	7A	*Pn*	*AGC/CCT179* *cdo347* *psr121* *psr680* *schfc3*	8	20.8
Zwart et al. 2005	CPI133872	*QRlnt.lrc-6D.1*	*Pt*	*barc183* *barc173*	22-24	23.9-42.5
	Janz	*QRlnn.lrc-6D.1*	*Pn*	*barc183* *barc173*	11-14	17.3-17.8
	CPI133872	*QRlnt.lrc-2B.1**	*Pt*	*wmc25* *wmc154*	7	15.2
	CPI133872	*QRlnn.lrc-2B.1**	*Pn*	*wmc25* *wmc154*	7-11	9.4-12.4
	CPI133872	*QRlnt.lrc-6D.2**	*Pt*	*gdm98* *gpw95010* *barc21*	8-13	16.5-24.6
	CPI133872	*QRlnt.lrc-6A.1**	*Pt*	*psp3029* *gwm459*	9	11.3-11.7
	CPI133872	*QRlnt.lrc-3D.1**	*Pn*	*gwm161* *gwm183* *gwm664*	11	12.6
	CPI133872	*QRlnt.lrc-4B.1**	*Pn*	*gwm66* *wmc47*	10	17.2
	Janz	*QRlnt.lrc-4D.1*	*Pn*	*wmc52* *wmc331* *barc98*	10-15	11.6-15.8
Schmidt et al. 2005	AUS13124	2B	*Pt*	*gwm319* *gwm494.2* *ACT/CTC.1* *gwm191.2*	6-13	9.4-13.7
	AUS13124	6D*	*Pt*	*gwm469* *gdm36* *gwm518* *gdm132*	4-6	4.9-9.3
	AUS13124	3B*	*Pt*	*gwm133* *gwm340.2*	7-24	5.2-20.1
	AUS13124	1B*	*Pt*	*gwm153.1* *gwm153.2*	3-6	3.3-3.6
	AUS4926	3B	*Pt*	*gwm112.2* *gwm66.1* *gwm213.2* *gwm133.1* *AGC/CAT.1*	36	10.3
	AUS4926	2B*	*Pt*	*wmc25.5* *wmc25.4* *gwm428*	2-3	2.7-3.4
	AUS4926	6D*	*Pt*	*gdm98.1* *gdm98.2* *gdm132*	2-2	2.6-2.7

Table 1: Continued.

Reference	Inherited Origin	Chromosome/QTL	Species	Flanking Markers	% Var	LRS
Zwart et al. 2006	W-7984	6DS	*Pt*	*psr964* *psr889* *barc183*	11-23	4.4-8.5
	W-7984	2BS	*Pt*	*cdo447* *bcd348* *gwm210*	19-5	2.0-7.4
Zwart et al. 2010	CPI133872	6DS	*Pt*	*barc183* *cfd49* *cfd135*	18-27	28.1-46.5
	Janz	6DS	*Pn*	*barc183* *cfd49* *cfd135*	9	10.6-17.9
	CPI133872	6DL	*Pt*	*gdm98* *gpw95010* *barc21*	6-15	11.0-23.5
	CPI133872	2B	*Pt*	*wPt-2410* *wPt-6706* *wPt-6311* *wPt-8737*	13-22	19.8-36.3
	CPI133872	2B	*Pn*	*wPt-2410* *wPt-6706* *wPt-6311* *wPt-8737*	11-16	12.4-27.6
	CPI133872	3D	*Pn*	*gwm2* *gwm664* *gwm314*	8	15.6
	CPI133872	4B	*Pn*	*gwm368* *gwm66* *wmc47*	13	25.3
	CPI133872	4D	*Pn*	*wPt-5809* *wPt-431*	13	24.4

np - not provided

* - below the significance threshold

1.8 Conclusion

This review demonstrates that the histopathology, invasion and resistance mechanisms of *P. thornei* have not been extensively analysed. The investigations of other *Pratylenchus* species in other plant species suggest that in wheat *P. thornei* resistance may suppress several stages of nematode invasion post penetration and involve several biochemical pathways. While numerous *P. thornei* and *P. neglectus* resistance QTL have been identified in wheat, there are currently no commercially available resistant cultivars. Thus, there is a need to identify further sources of *P. thornei* resistance to identify major QTL and most importantly to develop markers that can be utilised by plant breeders in order to incorporate this resistance into commercial cultivars. The elucidation of resistance at the molecular and biochemical level can provide clues about the function of *P. thornei* resistance.

1.9 Aims

The principal aims of this study are to identify genetic loci associated with *P. thornei* resistance and to investigate the associated biological resistance in a synthetic wheat population. In order to characterise biological resistance utilised by the wheat plant to *P. thornei*, the mechanisms and how they affect nematode root invasion will be investigated at each invasive stage. To investigate the genetic control of *P. thornei* resistance the project aims to identify major QTL and to develop molecular markers linked to resistance. The population investigated was developed through crossing the synthetic wheat cultivar Sokoll, which has very strong resistance to *P. thornei*, to the South Australian adapted cultivar, Krichauff.

The specific aims are:

1. To investigate biological mechanisms of resistance to *P. thornei* by observing nematode penetration, migration and reproduction stages (Chapter 2 and 3).

2. To construct a genetic map of Sokoll x Krichauff and to identify *P. thornei* resistance QTL to establish linked molecular markers (Chapter 4).

3. To correlate biological resistance mechanism(s) to identified *P. thornei* resistance QTL (Chapter 5).

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Chapter 2

Characterisation of Resistance to *Pratylenchus thornei* in Wheat; Attraction, Penetration and Maturation

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ABSTRACT

Linsell, K.J., Riley, I.T., Davies, K.A., and Oldach K.H. 2012. Characterisation of Resistance to *Pratylenchus thornei* in Wheat – Attraction, Penetration and Maturation. Phytopathology

A selection of lines from a cross between two wheat cultivars with contrasting resistance phenotypes to *Pratylenchus thornei* were investigated to determine if resistance plays a role in impeding nematode attraction to and penetration of roots and nematode maturation within roots. There was no significant difference in the rate at which *P. thornei* was attracted towards resistant or susceptible roots when assessed in sand. However, on agar when both genotypes were present there was significantly higher movement towards susceptible roots. Despite this, no difference in penetration rates were observed in resistant or susceptible roots in sand or on agar. The maturation of *P. thornei* juveniles in resistant roots was suppressed. Time course studies found up to twice as many stage two juveniles in the susceptible than the resistant genotypes, 36 days after inoculation, with no stage two juveniles in roots of the resistant parent. Similar numbers of *P. thornei* juvenile stage two were still present within the resistant roots 10 days after inoculation, indicating that maturation was inhibited by resistance rather than causing nematodes to exit resistant roots.

KEY WORDS

Root lesion nematode, root exudates, moulting, repellent, attractant, juvenile development

Pratylenchus spp. are economically important endoparasites of many crops including wheat (30). In Australia, the two most prevalent and widespread *Pratylenchus* species affecting wheat are *Pratylenchus thornei* and *Pratylenchus neglectus* (43). *Pratylenchus* feeds and reproduces in the root cortex and migration through root tissue causes extensive damage resulting in the formation of necrotic lesions. The resulting damage to the root system impairs uptake of water and essential nutrients, causing reductions in growth and in turn yield (59; 60). Yield losses in Australia due to *P. thornei* have been estimated at more than $AUD 50 million each year (29).

The process by which *Pratylenchus* nematodes invade and develop can be separated into four main components: root recognition, penetration, feeding/migration and reproduction. Initially, the nematodes are attracted to the root and probe the surface for an acceptable penetration site. After a salivation period, the nematode may feed ectoparasitically on root hairs. Using stylet thrusting and body movement they penetrate the root epidermis (59), enter the root and migrate intracellularly while feeding endoparasitically on root cortex cells (60). While in the roots, adult *Pratylenchus* nematodes deposit eggs (1; 6). As the hatched juvenile matures, each subsequent moult produces an increase in size and allows for sexual development (26). The cycle from egg to adult is completed within 45 to 65 days, but is affected by host species and temperature (48).

Due to the wide host range of *Pratylenchus* spp. and the restrictions, costs and inefficiency of chemical pesticides, the development of resistant cultivars has become increasingly important as a control measure. Most studies of plant-nematode interactions have focussed on the inheritance of resistance (19). The identification of new genetic sources of *P. thornei* resistance and the identification of resistance quantitative trait loci (QTL) and associated molecular markers is important, due to the lack of resistance available in current commercial cultivars. However, understanding resistance at the biological and biochemical level can provide insights into the nature, timing and action of resistance genes. Common phenotypic resistance screening techniques are laborious, time consuming and inherently variable as they involve extracting and counting nematodes from the roots. DNA-based detection methods can achieve higher throughput (33) but are expensive. The identification of a resistance mechanism at a specific stage of invasion or within a particular tissue may enhance screening procedures by reducing these associated costs and time. In addition, finding more than one type of resistance mechanism would indicate the involvement of several different resistance genes amongst genotypes which could then be pyramided to provide more effective resistance.

Only a small number of studies have considered the effects of resistance on *Pratylenchus* biology. Talavera and Vanstone (42) demonstrated that *P. thornei* is able to penetrate

resistant cultivars and Farsi (12) observed equal root penetration of *P. neglectus* in both resistant and susceptible wheat cultivars. In this study, the parents of a cross between two wheat lines with contrasting resistance phenotypes to *P. thornei*, Sokoll (resistant) and Krichauff (susceptible) in addition to six selected lines from the population (three resistant and three susceptible) based on genotypic analysis by Linsell et al. (25) were investigated. The aim was to compare the early stages of the invasion process of *P. thornei* (root attraction, penetration and juvenile development) in these resistant and susceptible genotypes, in order to identify the stage(s) where and how resistance has an effect. The identification of biological resistance mechanisms and the development of techniques to allow effective screening will aid in the long term development of more efficient resistance breeding.

MATERIAL AND METHODS

Nematodes

Pratylenchus thornei was obtained from wheat at Nunjikompita, South Australia and were maintained on carrot callus described by Moody et al. (28). Cultures were stored at 22 °C and subcultured every 3 months. To collect the nematodes the carrot callus was placed in funnels in a misting chamber with an intermittent aqueous mist of 10 s every 10 min for 96 h (41). Nematodes extracted in the mister were counted in 250 µL aliquots in three replicates and diluted with water to the required inoculum concentration. To obtain inoculum of nematodes of a single developmental stage, suspensions were passed repeatedly through sieves. Adults and juvenile stage four (J4) were captured on a 40 µm sieve, juvenile stage three (J3) on 30 µm and juvenile stage two (J2) were retained on a 20 µm sieve. Eggs, but few nematodes, were able to pass through the 20 µm sieve. This sieving method provided relatively high purity; nevertheless, the presence of non-required stages was recorded.

Plant Material

The study investigated a doubled haploid (DH) population from a cross between the synthetic-wheat derived cultivar Sokoll (*P. thornei* resistant) and the Australian wheat cultivar Krichauff (*P. thornei* susceptible). This population was phenotyped using DNA quantification methods and genetically mapped to identify two highly significant resistant QTL on chromosomes 2B and 6D as described by Linsell et al. (25). The parents and six selected individual lines (three susceptible; S1, S2 and S3 and three resistant; R1, R2 and R3) were analysed. The six lines were selected based on genotypic and phenotypic data. The three resistant lines had both resistant QTL and the three susceptible lines had neither QTL. The chosen resistant and susceptible selected lines contained the highest and lowest amounts of quantified *P. thornei* DNA respectively. For all experiments, prior to germination seeds were surface-sterilised by submersion in 70% ethanol for 5 min, then hypochlorite (15%) for 20 min at room temperature (RT) of 21 to 22 °C, followed by four rinses with reverse osmosis (RO) water.

Nematode Inoculation/Extrapolation

For experiments on agar, 3 day old wheat seedlings with a primary leaf and a 20 mm seminal root were placed on 0.5% water agar in 90 mm Petri plates at RT. After 48 h, the plates were inoculated with *P. thornei* by pipetting suspensions onto the surface. Nematodes on the agar were counted using a stereomicroscope (M3C; Wild, Heerbrugg, Switzerland) and a grid template (10 mm block segments). To count nematodes that had entered the roots, seedlings were placed in a misting chamber for 96 h and were extracted and counted. Approximately 10 mL of nematode suspension was collected and stored at 4 °C until counted in 500 µL aliquots using a stereomicroscope, and the total number in the root system was estimated accordingly. Alternatively, nematodes that could no longer be seen on the agar

surface were assumed to have penetrated the roots (Initial inoculum number - number of nematodes remaining on agar plate = number of nematodes within roots). This was confirmed in a check experiment at 12 hours after inoculation (h.a.i.), where the total number of nematodes extracted from the agar via misting plus the total number that had entered the roots equalled the initial inoculum number.

For experiments in pots, plants were grown in a glasshouse maintained at 20 ± 3 °C. One DH seed was sown in steam-pasteurised sand in each seedling tube (55 × 120 mm) in a 5 x 5 randomised block arrangement. Seven days after planting, each seedling was inoculated with *P. thornei* by pipetting two 500 µL aliquots of nematode suspension into two 50-mm-deep holes on either side of the seedling. Inoculum densities were specific for each experiment and are described later. Two days after inoculation, slow-release fertiliser (Osmocote, Scotts Miracle-Gro, USA) was added (4 g/kg of sand) to the tubes. Plants were hand watered every two days to soil water holding capacity. Nematodes were extracted by placing the washed root systems into a misting chamber as described above.

Attraction

Attraction on Agar

Five replicates of 5-day-old seedlings of both parents were placed on 0.5% water agar. Plates were divided into 10 mm segments with roots occupying 30 mm at one end. In the first 20 mm at the opposite end to the roots, inoculum of 150 mixed stage *P. thornei* nematodes was added (Figure 1A). A no-root control plate was included with the same plate design but excluding the active seedling roots. The total number of *P. thornei* inoculated on the plate was counted. At 6, 9, 12, 27, 30, 33, 36 and 48 h.a.i. the number of nematodes in each segment was counted and their location on the plate grid recorded, reflecting the distance nematodes had moved towards the roots over time. For all experiments on agar, it was assumed that nematodes that could no longer be seen had penetrated the roots and were expressed as a percentage of the initial inoculation number. The assay was repeated twice under the same conditions.

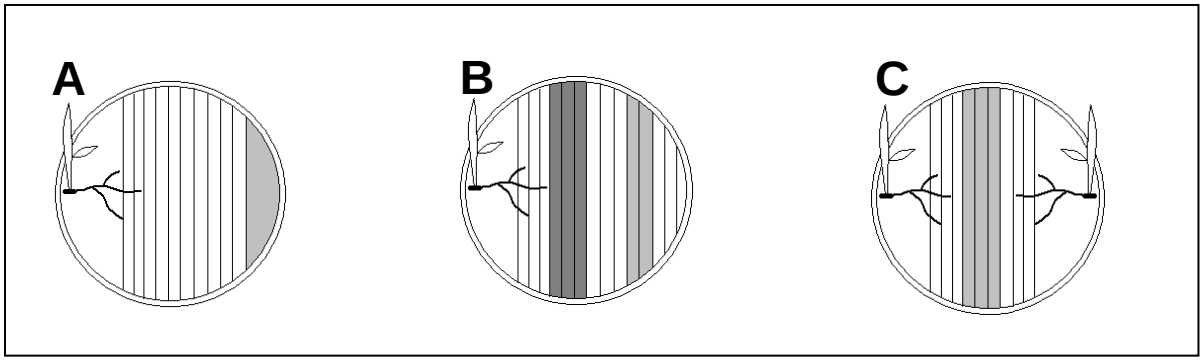

Figure 1: *Pratylenchus thornei* attraction assay plate designs. Plates divided into 10 mm segments with seedlings occupying the first 30 mm. Light grey shaded areas represent nematode inoculation blocks and dark grey shaded areas represent applied root exudates (RE).
A – Attraction on agar: Nematodes placed in distal 20 mm (shaded area).
B – Attraction on agar across RE: Root exudates in 30 mm space and nematodes in 20 mm space 30 mm from root exudates and edge of plate.
C - Attraction with both genotypes present: Resistant and susceptible seedlings at either end of plate with nematodes placed in centre 30 mm space.

Attraction on Agar Across Root Exudate

Six seedlings for both Sokoll and Krichauff were grown aseptically in 14 mL glass vials on nylon mesh in 8 mL of sterile RO water. Root exudate (RE) was collected after the roots had been submerged in the water for 7 days. Ten independent replications were collected and the experiment was repeated twice. A known susceptible seedling (Machete) was placed at one end of the 0.5% water agar plate to stimulate nematode movement. Root exudate was pipetted onto the plate in front of the root system so that in order to reach the roots the nematodes had to move across the RE solution. The same gridded plate setup as described above (Attraction on agar) was used but 1 mL of RE was placed in a 20 mm segment 30 mm from the edge of the plate (Figure 1B). Plates were inoculated with 500 mixed stage *P. thornei* nematodes 20 mm from the RE and 20 mm from the edge of the plate so the nematodes could either move towards or away from the exudates. After 48 h the number and location of individual nematodes on the plates was assessed. The numbers of nematodes that remained at the point of inoculation, which moved away from the root (repelled) and moved toward the root (attracted), were expressed as a percentage of the initial inoculation number (Table 1).

Attraction with Both Genotypes Present

Sixteen different resistant versus susceptible combinations made from the parents and the six DH lines were assessed either in sand or on agar. These same combinations were twice analysed independently both in sand and on agar. For experiments in sand, one resistant and one susceptible seedling was sown into the same pot (55 × 120 mm) and inoculated with

two 500 µL aliquots (500 nematodes per pot) into two 50-mm-deep holes in between the two seedlings. Roots were harvested after 14 days, freed from sand and placed on the mister for nematode extraction. For experiments on agar, resistant and susceptible 5 day old seedlings were placed at either end of a 90 mm plate with 0.5% water agar. The plate was inoculated with 225 mixed stage nematodes in a 30 x 10 mm rectangle near its centre (Figure 1C). After 9 days, the roots were placed on a mister for nematode extraction and collection. Attraction was assessed by measuring the percentage penetration of the resistant and susceptible root in each combination. Then, the number of combinations that had more penetration in the resistant or susceptible roots was calculated and presented as a percentage of the total number of combinations analysed (Table 2).

Penetration

Rates of Penetration in Sand and on Agar

The rate of *P. thornei* penetration was assessed both in sand and on agar systems. For experiments in pots, seedlings were grown in sand under glasshouse conditions (20 ± 3 °C) and were inoculated with 1500 mixed stage *P. thornei*/plant. Roots were harvested at 5,7,10 and 16 days after inoculation (d.a.i.), washed and placed on the mister. The parents and two lines from each of the resistant and susceptible lines were assessed with five replicates. For agar analysis, 3 day old seedlings that had been germinated on moistened sterile filter paper were placed at one end of a plate (0.5% water agar) and inoculated with 150 mixed stage nematodes/plant at the opposite end. Three independent analyses were conducted using three replicates of the parents and three lines of both resistant and susceptible genotypes. The nematodes visible on the plate were counted at 6, 9, 12, 30, and 36 h.a.i. The number of nematodes that had penetrated roots was calculated (Initial inoculum number – number visible on plate at timepoint) and means were expressed as a percentage of the initial inoculation number.

Penetration of Adults versus Juveniles on Agar

Differences in rates of penetration of adults and juveniles was assessed in sand and agar systems. For experiments in sand, seedlings were inoculated with 500 adult nematodes/plant or 500 J2 nematodes/plant. Roots were harvested at 3, 4, 6 and 8 d.a.i. Two resistant and susceptible lines and both parents were assessed with five replications. For experiments on agar, seedlings on 0.5% water agar were inoculated with 1000 nematodes/plant and the numbers of visible nematodes were counted at 6, 12, 24 and 30 h.a.i. Two independent analyses were conducted using two resistant and susceptible lines and both parents with five replications. The number of *P. thornei* that penetrated the roots was calculated and means at each timepoint were expressed as a percentage of the initial inoculation number.

Root Penetration Zones

Seedlings of the resistant and susceptible parents were grown on 0.5% water agar and inoculated with 1000 nematodes/plant. Nematodes were observed probing the root surface prior to penetration at various timepoints within 24 h.a.i. with a dissecting microscope (MZ6; Leica, Australia) and were imaged. At 4 and 10 d.a.i. roots were removed from the agar and stained with acid fuchsin (7). The roots and penetrated nematodes were visualised and imaged.

Reproduction and Development

Pratylenchus thornei development within resistant and susceptible roots was assessed in sand over 45 days (one generation). Five replicates of the parents and six DH lines (three susceptible and three resistant) were grown in glasshouse conditions as described above. Each plant was inoculated with 500 J2. Roots were harvested at 5, 10, 15, 24, 30, 36 and 45 d.a.i. and nematodes were extracted by misting. These times were chosen based on the timeline observed by Larson (24). The number of J2, J3 and J4/adult *P. thornei* present at each of these times was counted and means at each timepoint were calculated. Developmental stages were assessed by size after separation through sieving and counted with a dissecting microscope. The J4 and adults were counted as one group. The reproduction data set had a non-normal distribution and due to the large range of means for each developmental stage the data set was transformed using natural logs.

Statistical Analysis

Replicate means were calculated and were pooled for each sample from each independent assay to give one data set for each experiment. Normally distributed data sets were analysed by ANOVA with Fisher least significant difference (LSD) post-hoc test, where $P < 0.05$ was considered statistically significant using the statistical program Genstat (VSN International, USA). Correlation coefficients were calculated and used to interpret trends. Prior to ANOVA analysis, data sets were log-transformed where necessary to correct for deviations from normality and homogeneity of variances.

RESULTS

Attraction

Attraction on Agar

The rate at which *P. thornei* moved across the agar towards the roots over 36 h was not significantly different between genotypes. At each of the 7 intervals assessed (6, 9, 12, 27, 30, 33 and 36 h.a.i.) there were equal numbers of *P. thornei* in each grid (data not shown). After 36 h.a.i., the total number that had reached and entered the root was not significantly different. Root exudates were observed via microscopy being excreted from both resistant and susceptible root tips on the agar surface.

Attraction on Agar Across Root Exudate

This assay measured nematode movement in response to resistant and susceptible RE. To reach the root system of susceptible Machete (which acted to stimulate nematode movement) nematodes had to move through RE. Eight Sokoll, seven Krichauff and six no-root controls replications were analysed. At 48 h.a.i., there was no statistically significant difference between nematode movement away or towards the RE in each genotype or between genotypes. Generally, 23% (115 nematodes) were attracted to the roots and around 15% (78 nematodes) were repelled. The majority, with 65% or 325 nematodes, remained at the point of inoculation (Table 1). In the no-root control where there was no RE barrier a similar number moved away and towards the roots (Table 1).

Attraction with Both Genotypes Present

Across two repeated experiments sixteen different resistant and susceptible combinations including the parents and DH lines (S1, S2, S3 and Krichauff versus R1, R2, R3 and Sokoll) were assessed in sand and agar for their ability to attract *P. thornei*. Some replications were excluded due to poor plant growth leaving data from 13 combinations in sand and 14 on agar to be analysed. When both resistant and susceptible roots were available for penetration on agar, significantly more *P. thornei* were attracted to the susceptible roots (80%) 9 d.a.i., but in sand attraction to both genotypes 14 d.a.i. (50%) was the same (Table 2). On average in 11 of the 14 different resistant and susceptible root combinations tested, more *P. thornei* were attracted towards and penetrated the susceptible roots, and in three combinations more *P. thornei* entered the resistant roots.

Penetration

Rates of Penetration in Sand and on Agar

There was no relationship between the rate of *P. thornei* penetration and genotype on agar or in sand measured 36 h.a.i. and 16 d.a.i., respectively. However, there was a significant increase in penetration over time in both genotypes (Figure 2). Significantly higher penetration occurred in the agar system than in sand with over 50% penetration occurring after only 9 to 10 h.a.i.

Penetration of Adults versus Juveniles on Agar

As expected there was no difference in the rates of either J2 or Adult penetration between the genotypes. However, the comparison of adult and juvenile penetration showed a significant relationship between developmental stage and penetration rate with a correlation coefficient of 0.4 in both mediums. On agar, the rate of juvenile penetration was higher than adults but in sand it was reversed (Figure 3). At 30 h.a.i. on agar, J2 penetration was almost 40% more than adult penetration. In comparison at 8 d.a.i. in sand, adult penetration was 30% higher than J2, with only 7% total J2 penetration.

Root Penetration Zones

Pratylenchus thornei were observed probing the root surface in various root zones but were most frequently detected at the junction of the lateral and seminal roots (Figure 4A-D). In addition, after staining with acid fuchsin, large groups were often observed both in the zone of elongation and in seminal roots near the hypocotyl (Figure 4E). *Pratylenchus thornei* were seen within the dense root hair regions of both seminal (Figure 4F) and lateral roots but penetration in this zone was not observed. Once the nematodes had penetrated the root, large groups of *P. thornei* at various developmental stages, including eggs, were often seen aggregated in different regions of the root (Figure 4G).

Reproduction and Development

The times chosen to assay *P. thornei* development were based on the early study of Larson (1959) (24) and the observed nematode development, closely matched the reported timeline, with the moult of J2 to J3 taking 7 to 10 days and subsequent moults to J4 and Adult occurring within 18 to 28 days. Egg deposition occurred within 2 days after observing mature females and J2 hatched 7 to 10 days after.

At each developmental stage, there were significantly higher numbers of *P. thornei* in the susceptible Krichauff parent than the resistant Sokoll (Figure 5). Nematode numbers were log transformed (ln (x+1)) and are hereafter referred to as log units followed by (e^{x-1}). There was no second generation hatch of J2 at 36 d.a.i. (Figure 5A) in Sokoll or any of the resistant lines but in Krichauff a mean of 1.24 (13.3) juveniles and on average 3.4 (30) juveniles in the

susceptible lines resulted. The inoculated J2 moulted to J3 around 10 d.a.i. but no significant difference in numbers occurred (Figure 5B). However, at 45 d.a.i. the numbers of J3 in the second generation was significantly higher in Krichauff, 4.08 (52.3), and the susceptible lines C01 and C35 than in Sokoll, 0.9 (5.3) and the three resistant lines. The J3 moulted to J4 and adults at around 15 to 26 d.a.i. The number of J4 and adults measured at 24 d.a.i. (Figure 5C) was ten times higher in Krichauff, 2.58 (44.7) than in Sokoll, 0.64 (4.7). Thus, juvenile development was suppressed as early as the moult from J2 to J3.

Table 1: Direction of *Pratylenchus thornei* movement on agar in response to root exudates from the resistant (Sokoll) and susceptible (Krichauff) genotypes 48 hours after inoculation.

Genotype	n	Attracted (%)	Repelled (%)	Stationary (%)
Sokoll	8	22.6	16.6	70.7
Krichauff	7	23.3	14.3	60.8
No-Root Control	6	15.1	14.2	62.4
s.e.		9.2	7.7	7.6

Table 2: Attraction of *Pratylenchus thornei* (measured as penetration) to sixteen different resistant and susceptible combinations assessed in sand (14 days after inoculation) and on agar (9 days after inoculation) where roots of both genotypes were available for the nematode to penetrate.

		Resistant	Susceptible	n
Sand	Penetration (%)	52.2	47.8	13
	s.e.	9.1	5.2	
Agar	Penetration (%)	16.7	81.3	14
	s.e.	4.4	4.4	

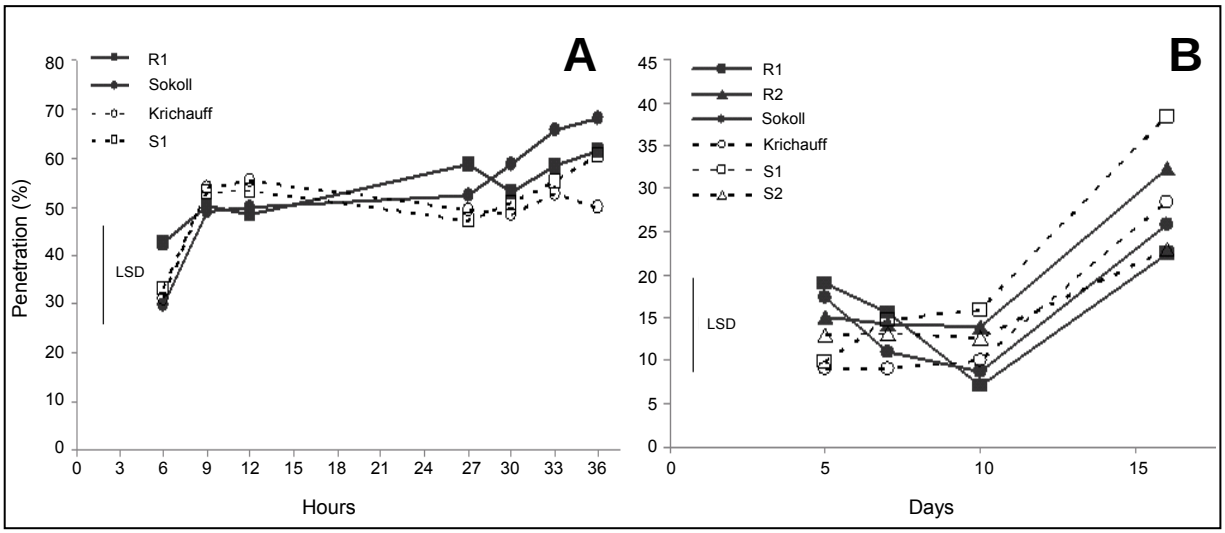

Figure 2: Penetration of *Pratylenchus thornei* on agar and in sand over 36 h and 16 d respectively. No statistical differences were found between resistant (Sokoll, R1 & R2) and susceptible (Krichauff, S1 & S2) genotypes for either (A) agar or (B) sand analysis with LSD of 21.26 and 11.76 respectively.

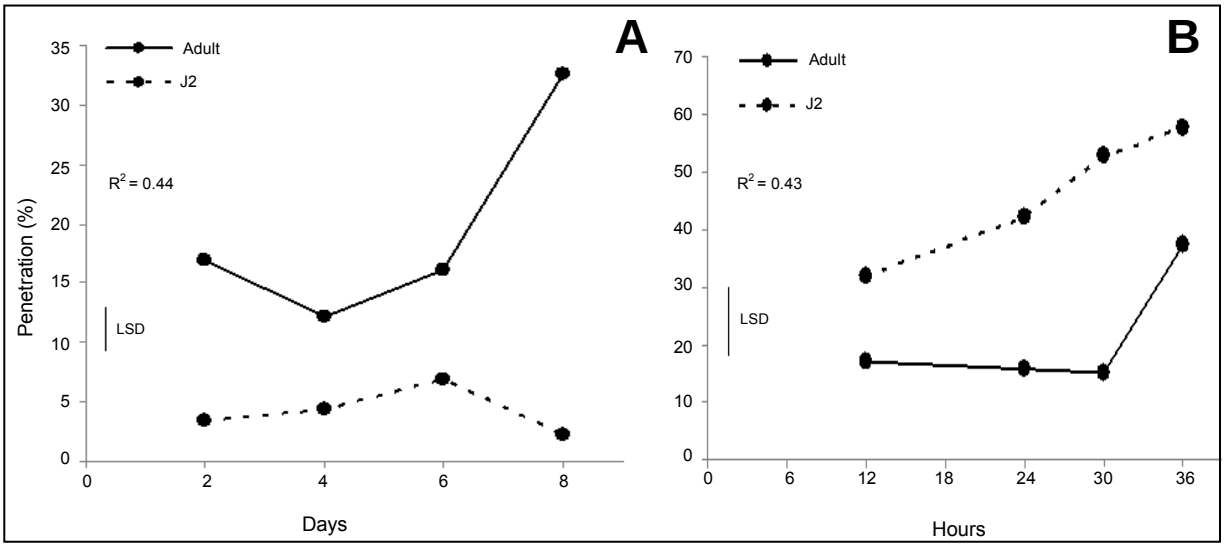

Figure 3: Penetration by different developmental stages of *Pratylenchus thornei* on agar and in sand over 36 h and 8 d respectively. A - Adults have a significantly higher penetration rate than juvenile stage two (J2) in sand, LSD = 12.94. B – Adults have a significantly lower root penetration rate grown on agar than J2, LSD 3.57. The correlation coefficient (R2) was 0.4 between developmental stages in both sand and agar.

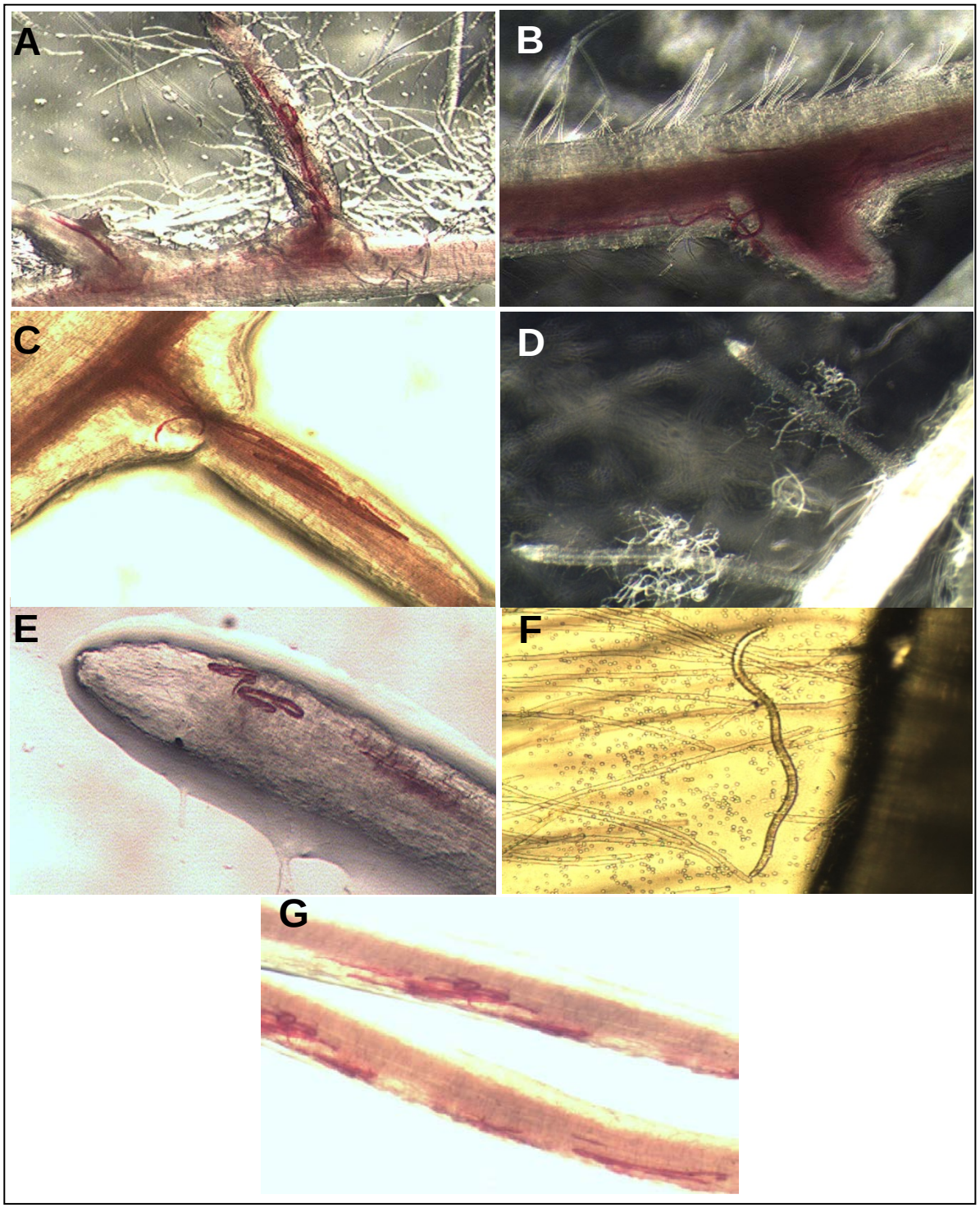

Figure 4: Root penetration zones *of Pratylenchus thornei* in wheat. Nematodes within the roots in A,B C, E and G are stained with acid fuchsin. A – C Several *P. thornei* at lateral root branches. D – Groups of *P. thornei* probing and penetrating lateral roots. E – Several *P. thornei* at zone of elongation. F – A single *P. thornei* in the dense root hair zone. G – Aggregations of *P. thornei* juvenile and adult developmental stages within root regions.

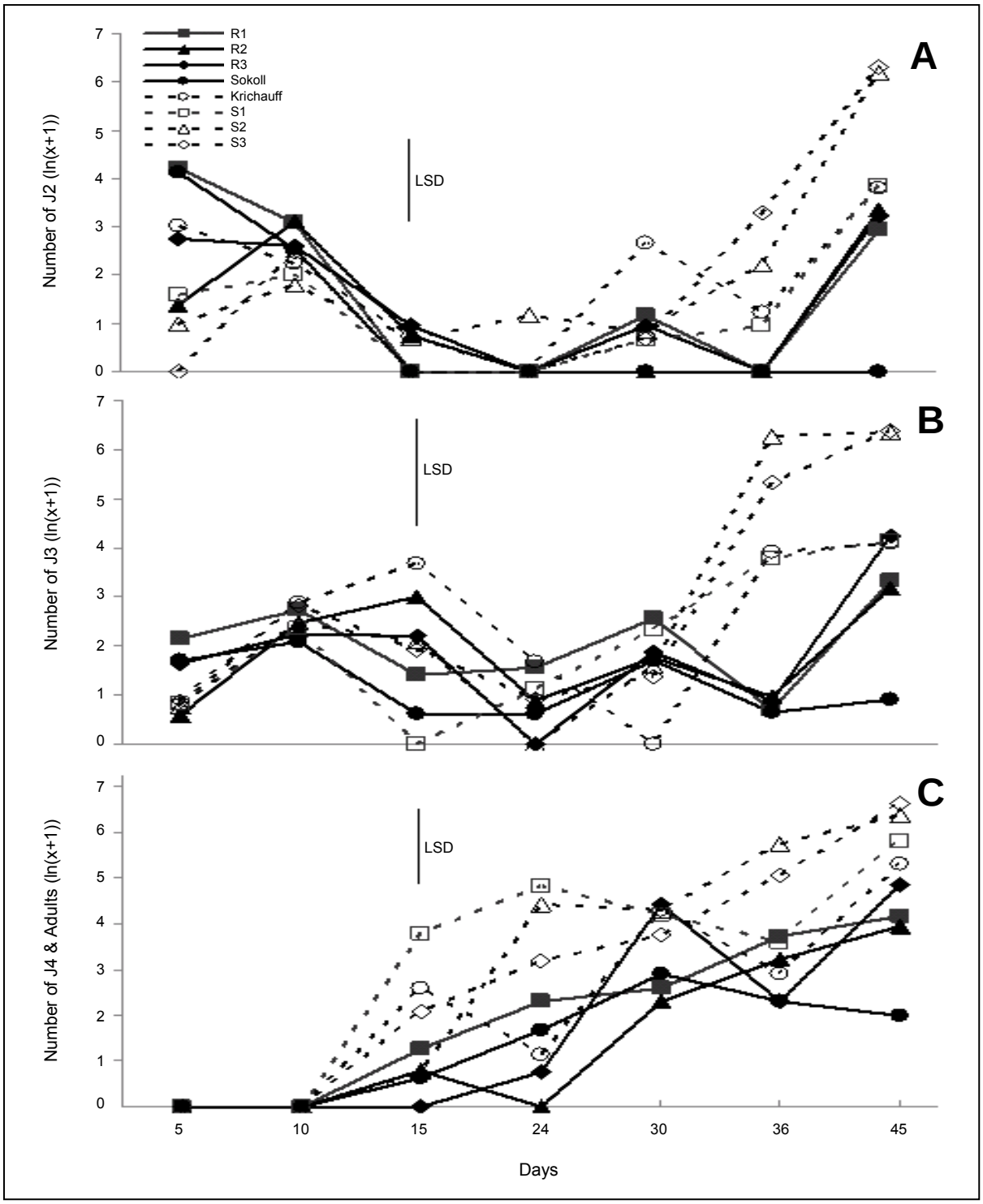

Figure 5: The number (log transformed) of *Pratylenchus thornei* developmental stages within resistant (Sokoll, R1, R2 & R3) and susceptible (Krichauff, S1, S2 & S3) roots at different times within one nematode life cycle, 45 days. A – Second generation juvenile stage two (J2) hatch at 36 days after inoculation (d.a.i.) LSD = 1.81. B – Juvenile stage three (J3) first generation moult at 10 d.a.i. and second generation at 45 d.a.i. LSD = 2.10. C – Juvenile stage four (J4) and adults first generation moult between 14-28 d.a.i. LSD = 1.76.

DISCUSSION

Root Attraction and Penetration

Recognition of and attraction to host roots are essential steps that precede nematode root invasion. Host recognition is thought to involve signals from the root that can stimulate egg hatch and/or attraction towards the roots (10; 35; 37). It is generally accepted that nematodes locate the roots through chemotactic factors diffused from the host (58). It has been suggested that signal elicitors from the host are water soluble so that they can move through soil towards the nematode (35). Root exudates are regarded as the most probable stimuli in attracting *Pratylenchus* nematodes to the root region (3; 51; 52). However, gradients in temperature, electrochemical potential, pH and carbon dioxide also play a role (37). Specific nematode repellents or attractants have not been identified but may include amino acids, sugars, phenolics and other secondary metabolites (13).

Root exudates contain a mix of positive and negative effectors which can attract, repel or be neutral in terms of nematode behaviour (39; 56; 57). Thus, the effects of RE depend on the combination of signals at a specific time (11; 15; 49; 53). In this study, there was no difference in the rate (both number and speed) at which *P. thornei* was attracted towards (23%) or away (15%) from resistant or susceptible roots when only one genotype was present. In addition, in sand medium, when both resistant and susceptible roots were available for penetration, no preference was observed for *P. thornei* attraction after 9 days. This suggests that wheat root exudates have a neutral effect on *P. thornei* attraction. Other studies, which utilised similar agar and sand biological systems, have also observed that resistance does not interfere with nematode root attraction. *Meloidogyne incognita* migration to resistant and susceptible cotton roots in sand and *Meloidogyne naasi* juveniles attraction to resistant and susceptible wheat and barley roots on agar were equal in both genotypes (2; 27). The presence of root exudates was confirmed in the agar system utilised in this study as secretions were observed from root tips of the live seedlings. Therefore, it is reasonable to assume that root exudates were present and the lack of attraction was not due to the random nature of *P. thornei* root attraction.

Contrasting observations have been made however, and preferential attraction has been observed to susceptible roots when both resistant and susceptible genotypes are present in other nematode plant interactions when analysed in soil. Bias towards the susceptible roots (72%) was observed in *Meloidogyne hapla* when juveniles hatched between the resistant and susceptible lucerne (alfalfa) roots grown in soil. However, when only one root was present there was equal attraction and penetration (14). Collectively these results show that in some interactions plants can influence nematode movement towards roots in positive and negative ways. Preferential attraction was also observed in this study but only when

attraction was investigated on agar. When both resistant and susceptible roots were present significantly higher *P. thornei* attraction (over 81%) occurred towards the susceptible roots 24 h.a.i. This indicates that chemicals might be exuded by the resistant and/or susceptible roots leading to preferential selection. The bias towards susceptible roots suggests that when given a choice of both resistant and susceptible roots either resistant root secretions deter *P. thornei* as they secrete repellent or toxic compounds early during pre-penetration as suggested by Berge (5) or the susceptible roots secrete compounds making them more attractive. The susceptible roots might not produce the repellent compound or produce it in lower concentrations than the resistant roots. When penetration on agar was measured again at 36 h.a.i., there was no difference between resistant and susceptible roots implying *P. thornei* may be able to tolerate the effects of resistant root chemicals by activating a protective mechanism/s. Similar to the observations here, in a resistant potato cultivar, few *Meloidogyne fallax* juveniles penetrated the roots 24 h.a.i. but at 60 h.a.i. penetration numbers were comparable to the susceptible cultivar (22). This indicates that the active compounds in resistant root secretions may decline in activity over time or that secretions may only influence nematode movement for a limited period. Alternatively there may be a potential adaptation by the nematode to overcome the activity of the resistant compounds.

The attraction bias observed only in agar systems in the presence of both resistant and susceptible genotypes may be due to the experimental design of the system. The greater attraction rates might be explained by the greater concentration of the active compounds on agar (350 mL) than in compact sand systems which have larger volumes (6600 mL). Thus, a more field realistic measure to investigate nematode attraction might be achieved using pot assays with sand or agar systems with larger volumes. In addition, due to the faster attraction/penetration on agar, analysis was performed on plants 3 to 4 days old. Whereas, plants in sand experiments were analysed between 12 and 22 days old. It is known that *Pratylenchus* attraction and penetration rates are greater with younger roots (18; 32). Thus, attraction variations between systems may also be attributed to differences in ages of the plants evaluated.

As plant feeding nematodes possess stylets and generally secrete cell wall degrading enzymes (40), they commonly successfully penetrate the host regardless of whether further development occurs. Thus, most nematodes seem capable of overcoming the physical protection barriers of plants. Resistance to root knot, cereal cyst and root lesion nematodes investigated in several crop species has never been associated with inability to penetrate roots. Juvenile *M. incognita* in cotton and lucerne and several *Heterodera* species in wheat, soybean and potato were able to successfully penetrate and migrate to the feeding site in both susceptible and resistant cultivars (9; 20; 54). In addition, similar penetration rates were observed in susceptible and resistant beans when inoculated with *Pratylenchus scribneri*, 24

h.a.i. Thus, it was hypothesised that penetration suppression plays no role in resistance of wheat to *Pratylenchus*. As expected, in this study juvenile *P. thornei* penetration rates did not significantly differ for resistant and susceptible roots both on agar and in sand even after 16 days. This is supported by Talavera and Vanstone (42) who observed *P. thornei* nematodes penetrating resistant wheat cultivars and Farsi (12) who found no difference in penetration rates of *P. neglectus* between resistant and susceptible wheat cultivars. As neither attraction to the roots nor penetration in the sand was negatively affected in the resistant lines investigated here, resistance to *P. thornei* seems to act post penetration.

Penetration of Developmental Stages

All *Pratylenchus* developmental stages are able to penetrate and invade roots. However, the rate of penetration by adults in this investigation was significantly higher than that of J2 penetration in sand. These results are supported by observations in lucerne where *Pratylenchus penetrans* adult females were more invasive than juveniles as they penetrated roots earlier, faster and over a wider range of soil temperatures. Eighty percent of adult females penetrated the roots within 96 h compared to 30% of the juveniles (44). Similarly, a higher number of adult *P. penetrans* (80%) penetrated celery roots than juveniles (45). It has been suggested that the greater ability of adults to penetrate is attributed to the size of the glands in the posterior subventral lobe as secretions from this gland are known to be important in penetration and feeding (17; 55). The larger size of these glands could allow them to produce greater quantities of enzymes and at a faster rate than in earlier life stages. Klinkenberg (21) suggested that the stylet of newly hatched juveniles was not fully developed and too weak to penetrate the strong cell walls, thus making them less invasive. In addition, differences in strength can be related to their respective sizes as *Pratylenchus* J2 are on average less than one third the size of adults (38). This developmental size difference could influence their ability to move through soil and or root cells impeding root penetration.

In this study, penetration by adults was significantly higher than J2 in sand assays, whereas the opposite occurred on agar. This indicates that differences in the three dimensional structures of these two substrates may affect the ability of the juvenile to move and penetrate roots. Thus, for *P. thornei*, it might not be the maturity and stylet development of juveniles that is important for penetration but the ability to move through three dimensional soil matrices. The higher penetration rates on agar may be due to their increased ability to move across this medium. It has been demonstrated that nematode movement in soil is directly dependent on the size of the soil pores in relation to nematode body diameter and the soil matrix potential (50). Zunke (60) observed that due to longer ectoparasitic feeding on root hairs than adults, *P. penetrans* juveniles penetrated the root later and suggested extended root hair feeding was essential for juveniles to obtain nutrients for further colonization. As juveniles on agar can more easily migrate towards the root perhaps less ectoparasitic

feeding is required as less energy will be spent in reaching the roots than in sand and therefore enabling quicker root entry than adults.

Root Penetration Zones

There are differences in the sites and mechanisms of root penetration by different species of *Pratylenchus* in various hosts. In lucerne and clover, *P. penetrans* preferred to penetrate the main roots where lateral roots ruptured the cortex and then migrated through the cortex of main roots into lateral roots (46). Other studies suggested that most *P. penetrans* aggregated and penetrated in the zone of root elongation (47; 59). In lucerne, strawberry and maize roots, the preferred zone of penetration for both adults and juveniles appeared to be the zone of dense root hairs, with 61% of the lesions developing on the root hairs of lucerne (44). Using high resolution video-enhanced contrast microscopy, Zunke (60) observed *P. penetrans* penetrating and feeding on root hairs of various hosts including rape, tobacco and potatoes. However, other studies indicated that *P. penetrans* explored and penetrated the root hairs but did not feed on them (23; 31). Castillo et al. (8) suggested that both females and juveniles of *P. thornei* penetrated the roots of chickpea without any site preference. In this current study, *P. thornei* were commonly observed at the junction where lateral roots branched from the seminal root. However, large groups were often also observed both in the zone of elongation and in seminal roots near the hypocotyl. Although penetration was not observed, *P. thornei* were seen within the dense root hair regions of both seminal and lateral roots. *Pratylenchus thornei* may feed on root hairs and then move elsewhere along the root to penetrate. These observations indicate that *P. thornei* does not have a preferred site of penetration in wheat. However, as RE act as attractants and are known to originate from regions where lateral roots emerge, from root tips and at sites that have been previously penetrated (56), it is expected that penetration will occur in these areas to which they are initially attracted.

Baxter and Blake (4) suggested that *P. thornei* does not invade roots randomly but is attracted to regions of the roots previously invaded. Thus, subsequent reproduction occurs in large groups at intervals within the roots. Once inside the root *P. penetrans* often move in tracks left by previously migrating nematodes (34). In this study, groups of *P. thornei* nematodes at various developmental stages, including eggs, aggregated in various root regions. Several studies observed that the proportion of nematodes present within the root decreased as the inoculum density increased (3; 16; 44). Townshend (1978) (44) suggested that if *P. penetrans* preferred to enter the root in a particular zone, the root surface area available for penetration would be limited, which could explain reduced penetration at high nematode density. In this study, as inoculum density increased from 500 to 1500 nematodes/mL the number of *P. thornei* nematodes within the roots increased accordingly (data not shown). Acedo and Rhode (1) also found that as the inoculum level rose there was

a corresponding increase in numbers of *P. penetrans* in *Brassica* roots, even at very high inoculum levels of 10,000 nematodes/plant. This correlation of increased penetration supports our observation that *P. thornei* has no initial preferential root penetration site. Rather than specific root region penetration where only a limiting number of *P. thornei* can penetrate at any one time, penetration occurs at different root regions simultaneously.

Development and Reproduction

The developmental stages of *P. thornei* collected from inoculated roots at multiple times across 45 days matched well to the life cycle observations made by Larson (24), the only other published report of *P. thornei* reproduction in wheat. Therefore, the development timeline reported in this study under glasshouse conditions can now be utilised in future studies of specific *P. thornei* reproductive stages.

In this study the analysis of development over time between the resistant and susceptible genotypes within the Sokoll x Krichauff population showed significantly less *P. thornei* nematodes at all stages in the resistant compared to the susceptible roots. Similarly, reduced reproduction was observed in a resistant durum wheat cultivar. Initially no difference was detected in penetration between the susceptible and resistant cultivars, but *P. thornei* numbers gradually decreased in the resistant roots and soil indicating failure of reproduction (42). Reduced reproduction indicates that either the nematodes exited the resistant roots or failed to develop and reproduce. This study showed that *P. thornei* do not exit roots as large numbers are still present in the roots even after 16 days, indicating that reproductive development is suppressed. In the second generation up to ten times as many J2 and J3 were detected in susceptible roots as in resistant roots, indicating that moulting is suppressed or delayed in the resistant genotypes. This is supported by Farsi (12) who observed that moulting of *P. neglectus* 10 d.a.i. was delayed in resistant wheat cultivars with only 1.7% moulting compared to 30% in the susceptible. Delayed maturation was also observed in *Pratylenchus goodeyi* in banana resistant roots. Adult and J4 development were delayed by 4 days in comparison to the susceptible cultivar, which effected optimum egg deposition and hatch (36). The current study shows that resistance expressed in the Sokoll/Krichauff population to *P. thornei* prevents juvenile maturation and thus reproduction. Additional analysis is necessary to establish whether reproduction itself (egg deposition and egg hatch) is also suppressed by resistance in this population.

In conclusion, in the Sokoll x Krichauff population investigated, no resistance to *P. thornei* was observed at attraction or penetration, however, maturation and thus reproduction was inhibited. Consistent with various other plant parasitic nematode species, there was no significant difference in the rate at which *P. thornei* was attracted towards resistant or susceptible roots in sand. However, when both genotypes were present on agar there was

an attraction bias towards the susceptible roots. This indicates secretion of repellent or toxic compounds by resistant roots during pre-penetration. There was no difference in *P. thornei* penetration rates between resistant and susceptible roots both on agar and in sand which is supported by similar observations of other *Pratylenchus* studies in wheat. The rate of penetration by adults was significantly higher than that of J2 in sand but lower on agar. Observations of other *Pratylenchus* species attributed this to the larger adult body strength and enzymic secretions required for root entry and movement in soil. No preferential root penetration zone was observed unlike other studies, but once inside the root, groups of *P. thornei* at various developmental stages moved in the tracks left by previously migrating nematodes. Nematode multiplication was inhibited by resistance as juvenile maturation was suppressed in resistant roots with very few developing past J3. As *P. thornei* J2 were still present within the roots 10 d.a.i., juvenile maturation is inhibited by resistance rather than causing the nematodes to exit resistant roots. Additional investigation is needed to determine whether egg deposition and egg hatch is also suppressed by resistance in this population. Further characterisation of biological resistance mechanisms and the development of robust screening protocols will enhance the development of *P. thornei* resistance breeding in wheat.

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Chapter 3

Characterisation of Resistance to *Pratylenchus thornei* in Wheat; Motility and Reproduction

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ABSTRACT

Linsell, K.J., Riley, I.T., Davies, K.A., and Oldach K.H. 2012. Characterisation of Resistance to Root Lesion Nematode *Pratylenchus thornei* in Wheat – Motility and Reproduction. Phytopathology

A selection of wheat lines from a cross between two cultivars with contrasting resistance phenotypes to *Pratylenchus thornei* were used to determine if resistance interferes with nematode reproduction and motility. Significantly higher numbers of *P. thornei* became non-motile when exposed to root exudates/extracts from resistant (65%) than susceptible (30%) roots after exposure for 3 days. Assays were also performed using *Pratylenchus neglectus*, but no effect on motility was observed. The inhibitory effect on *P. thornei* motility was partially reversible up to 2 days after exposure. In migration assays, within the roots themselves, *P. thornei* could only migrate 10 mm through the resistant root cortex from the point of inoculation, compared to 70 mm in the susceptible roots. In addition, *P. thornei* reproduction was affected by resistant genotypes. Egg deposition was up to 30% less, within/near the resistant lines after 10 days. About 40% less hatch occurred from eggs within and adjacent to roots of resistant versus susceptible seedlings grown on agar. Hatch increased after dilution of root exudates indicating the presence of hatching inhibitors.

KEY WORDS

Root lesion nematode, *Pratylenchus neglectus*, egg deposition, root exudates, migration, egg, hatching, inhibitor

Several *Pratylenchus* species occur in Australia on various hosts, with two dominant in cereals, *Pratylenchus thornei* and *Pratylenchus neglectus* (55). Necrotic lesions form as a result of cell death caused by *Pratylenchus* feeding on root cortical cells (32). Cortical degradation and reduced root branching diminishes the plant's ability to acquire nutrients and water from the soil, stressing the plant and reducing vegetative growth and yield. The value of wheat production lost in Australia due to *P. thornei* is estimated at over $AUD 50 million each year (36).

Once the nematode has located and penetrated a root, it migrates intracelluarly while feeding endoparasitically on root cortex cells (69). During migration, at each new cell the nematode punctures the cell wall with stylet thrusts and pushes through into the adjacent cells to continue feeding (25; 68; 69). While in the roots, adult *Pratylenchus* deposit eggs in the cavities created by migration (1; 4) and/or eggs are deposited in the soil in close vicinity to the roots (37). The juvenile hatches from the egg as a juvenile stage two and at each moult undergoes further sexual development (29). The full cycle from egg to adult is completed within 45 to 65 days (2), and is greatly influenced by host, temperature and *Pratylenchus* species.

Resistance is now the most economical and environmentally effective method of control against *P. thornei* due to the increasing costs, regulations and environmental concerns about nematicides (40). Several studies have identified various sources of resistance to *P. thornei* in wheat (50; 52; 56; 57; 60; 70-72). However, a simple understanding of the mechanisms of resistance and how they interfere with the nematode's biology is lacking.

Plants engage in two types of resistance responses to invading pathogens: passive and active. In post-infectional passive resistance the plant may for example, constitutively produce toxins that kill the pathogen or it may lack substances required by the pathogen for development and reproduction. In active post-infectional responses the plant reacts to contact with the pathogen by initiating distinct biochemical reactions that cause changes to tissues, such as necrosis by inducing gene expression of defense pathways (67) which includes the production of secondary metabolites. There are gaps in the knowledge of the biochemistry and molecular biology of plant defense responses to migratory endoparasitic nematodes, largely because they cause widespread cellular destruction and lack a fixed feeding site. Knowledge of the biological or biochemical resistance mechanisms is important as it could be used as improved phenotypic screening methods, to provide new sources of biochemical control and reveal the involvement of different resistance genes, which could be pyramided to provide more durable resistance (22).

The authors recently investigated the early stages of the invasion process and showed that resistance to *P. thornei* in a synthetic Sokoll x Krichauff population is not due to differential attraction or penetration but that nematode development is severely suppressed (26). Significantly fewer juveniles at all stages and adults were present in the roots of resistant (Sokoll derived) than susceptible lines (Krichauff derived). Other studies have shown that in other *Pratylenchus* species resistance is associated with reduced motility, egg deposition and egg hatch (3; 43; 46; 61). If the ability of the nematode to move and migrate through the root is suppressed and its feeding activity is limited, this will result in poor maturation and reduced reproduction.

In this study, the parents of a cross between two wheat lines with contrasting resistance phenotypes to *P. thornei*, Sokoll (resistant) and Krichauff (susceptible) in addition to six selected lines from the population were investigated. The chosen population lines were selected based on their resistant genotypes as determined by the quantitative trait loci (QTL) analysis conducted by Linsell et al. (27). The aim was to determine if the resistance within these genotypes is due to reduced nematode motility within the root and in root exudates, and whether egg deposition and egg hatch are affected by resistance. The investigation aimed to generate useful screening protocols, gain a better understanding of the biological resistance mechanisms, and provide a foundation for directed biochemical investigations into *P. thornei* resistant wheat responses.

MATERIAL AND METHODS

Nematodes and Plant Material

Pratylenchus thornei was obtained from wheat at Nunjikompita, South Australia and were maintained on carrot callus described by Moody et al. (33) and inoculum was collected as described by Linsell et al. (26). The study investigated a doubled haploid (DH) population from a cross between the synthetic-wheat derived cultivar Sokoll (*P. thornei* resistant) and the Australian wheat cultivar Krichauff (*P. thornei* susceptible). This population was phenotyped using DNA quantification methods and genetically mapped to identify two highly significant resistant QTL on chromosomes 2B and 6D as described by Linsell et al. (27). The parents and six selected individual lines (three susceptible; S1, S2 and S3 and three resistant; R1, R2 and R3) were analysed. The six lines were selected based on genotypic and phenotypic data. The three resistant lines have both resistant QTL and the three susceptible lines have neither. The chosen resistant and susceptible lines were then selected based on the highest and lowest amount of quantified *P. thornei* DNA respectively. Prior to germination seeds were surface-sterilised as described by Linsell et al. (26).

Impact of Crushed Root Suspensions and Root Exudates on Motility

The concentration of root exudates (RE) and crushed root (CR) suspensions were standardised to minimise variation between roots, as larger root systems are expected to produce more RE and contain more root compounds when crushed than smaller roots. Thus, all root exudates/suspension samples were adjusted to a set optimum concentration of 4 mg/mL of fresh weight root tissue in water as determined by testing motility suppression in a RE dilution series. The motility suppression of RE at 2, 4 and 100 mg/mL were assessed and the 4 mg/mL concentration was found to produce the best differentiation between the resistant and susceptible genotypes (Table 1).

Root Exudates

Seedlings were grown aseptically in Petri plates on sterile filter paper for 10 days at room temperature, 21 to 22 °C (RT). Three replicates of each of the six DH lines and the parents were analysed. One mL of reverse osmosis (RO) water was used to wash the roots and filter paper and the liquid was collected. Fresh roots were weighed and the collected RE were adjusted to a concentration of 4 mg/mL of fresh weight root tissue by addition of RO water. A 100 µL aliquot of RE was transferred to individual wells (350 µL) on a 96 well ELISA plate and inoculated with approximately 80 mixed stage nematodes. Three replicates of each sample were assessed. The numbers of motile and non-motile nematodes were counted at 1, 2 and 3 days after inoculation (d.a.i.) at RT. A nematode was considered non-motile if no movement was observed within 5 s. These assays were performed on both *P. thornei* and *P. neglectus*.

To determine whether the effect of resistant RE on *P. thornei* motility could be reversed, the nematodes were recovered from the resistant Sokoll (10 samples) RE after 1 day exposure into water and motility was re-assessed after 2 days.

Crushed Root Suspensions

One seed was sown in each seedling tube (55 × 120 mm) in steam-pasteurised sterilised sand. Three replicates of each of the four DH lines (two resistant and two susceptible) and the parents were analysed. Plants were grown for 1 week in a glasshouse maintained at 20 ± 3 °C and were hand watered every two days with 100 mL. Plants were then washed and fresh root weights were recorded. Roots were crushed in liquid nitrogen and immediately suspended in RO water. The CR suspensions were centrifuged and supernatant was removed and adjusted to a concentration of 4 mg/mL of fresh weight root tissue by addition of RO water. Experiments were conducted either in 6 mL of CR suspension in sample containers (40 mm x 45 mm) or 100 µL of CR suspension in wells on an ELISA plate. Each replicate was inoculated with approximately 80 mixed stage nematodes. The numbers of motile and non-motile nematodes were counted using a stereomicroscope (M3C; Wild, Heerbrugg, Switzerland) at 3 and 7 d.a.i. at RT.

Migration

Seedlings were germinated on sterile filter paper and then placed on 0.5% water agar for 3 to 4 days at RT. Three replicates of each of the six DH lines and the parents sample were analysed. Using strips of plastic all of the root system was blocked off except a 250 mm section of one root. Water agar (1%) was then used to cover the rest of the roots and the plastic strips acted as a wall so only the 250 mm root portion was exposed for nematode penetration (Figure 1). Approximately 100 mixed stage nematodes were inoculated near the exposed root. At 5 d.a.i., the seedling was removed from the plate and the roots were stained in acid fuchsin (5). Stained roots were placed in Petri plates and using a microscope the inoculation point and the distance nematodes had migrated from that point on the exposed root was marked on the plate and was measured.

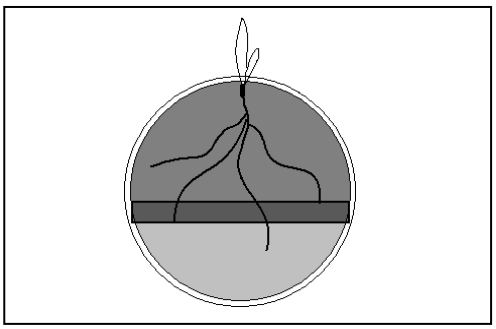

Figure 1: *Pratylenchus thornei* migration assay plate design. Using strips of plastic, all of the root system was blocked off with only a 250 mm section of one root exposed for nematode penetration. Nematodes were inoculated near the exposed root.

Egg Deposition

Five replicates of the parents and three DH resistant and susceptible lines were assessed. Seven day old seedlings on water agar (0.5%) were inoculated with 1000 stage four juveniles and adult *P. thornei* adjacent to the roots. No eggs were present. After 10 days at RT, the numbers of eggs on the agar were counted under a stereomicroscope. The roots were then stained in acid fuchsin and eggs deposited by adults that had entered the roots were counted.

Hatching

The same set of lines as for egg deposition was assessed for hatching assays in five replicates. For analysis on agar, seeds were germinated on moist filter paper and 6 day old seedlings were transferred to water agar (0.5%) plates and inoculated with 80 *P. thornei* eggs directly at the roots. At RT 8 d.a.i. the hatched juveniles on the agar were counted. To count the juveniles that had migrated into the roots, juveniles were extracted by misting (misting chamber) or roots were stained with acid fuchsin.

For analysis in RE, seedlings were grown aseptically in Petri plates on sterile filter paper for 12 days at RT. Root exudates were taken from young plants as *P. thornei* hatching was shown to be greater in RE from younger plants (44). One mL of RO water was used to wash the roots and filter paper and the liquid was then collected. Fresh roots were weighed and the collected exudates were adjusted to 4 mg/mL of root tissue. The RE (100 µL) was transferred to individual wells on a 96 well ELISA plate. Approximately 80 eggs were added to each well (350 µL) and the numbers of hatched stage two juveniles (J2) were counted at 3, 7 and 10 d.a.i. Hatching assays were also performed in dilutions of 4, 10 and 100 mg/mL of root tissue to determine the presence of hatching inhibitors. Five replicates of the parents and three DH resistant and susceptible lines were assessed twice. As *Pratylenchus* hatching is influenced by temperature experiments were performed at the optimum temperature of 21 to 22 °C.

Statistical Analysis

Replicate means were calculated and were pooled for each sample from each independent assay to give one data set for each experiment. Data sets were analysed by ANOVA. Least significant differences (LSD, $P = 0.05$) were calculated to compare means, where $P < 0.05$ was considered statistically significant using the statistical program Genstat (VSN International, USA).

RESULTS

Motility and Migration

There was greater *P. thornei* motility suppression in both resistant RE and CR suspensions after exposure for 3 days than in suspensions of susceptible genotypes (Figure 2A-B). A small amount of motility suppression occurred in Krichauff and the susceptible lines (30%) but was equal to the no-root control. The resistant Sokoll RE and CR suspensions caused significant loss of motility at 65 and 75%, respectively. In order to determine the specificity of the observed motility suppression, assays were also conducted on the closely related species, *P. neglectus*. There was no effect on *P. neglectus* motility when exposed to RE for 3 days as motility suppression was equivalent to the no-root control (25%) (Figure 3). The effect of resistant root compounds appeared partially reversible in Sokoll RE as recovery in water, after 2 days exposure, resulted in almost 50% of *P. thornei* regaining motility. In the resistant Sokoll RE after 1 day exposure motility suppression was 94%. The ability of *P. thornei* to migrate within resistant roots was also suppressed (Figure 4). On average, the migration of *P. thornei* from a set point of penetration was 10 mm in Sokoll compared to 68 mm in Krichauff.

Table 1: Motility of *Pratylenchus thornei* 1 day after inoculation in different concentrations of Sokoll (resistant) and Krichauff (susceptible) root exudates.

	Sokoll		Krichauff	
Root Exudate (mg/mL)	Non-Motile (%)	s.e	Non-Motile (%)	s.e.
100	46.55	4.76	32.59	7.89
4	35.76	1.42	16.25	4.74
2	25.79	2.02	22.38	2.62

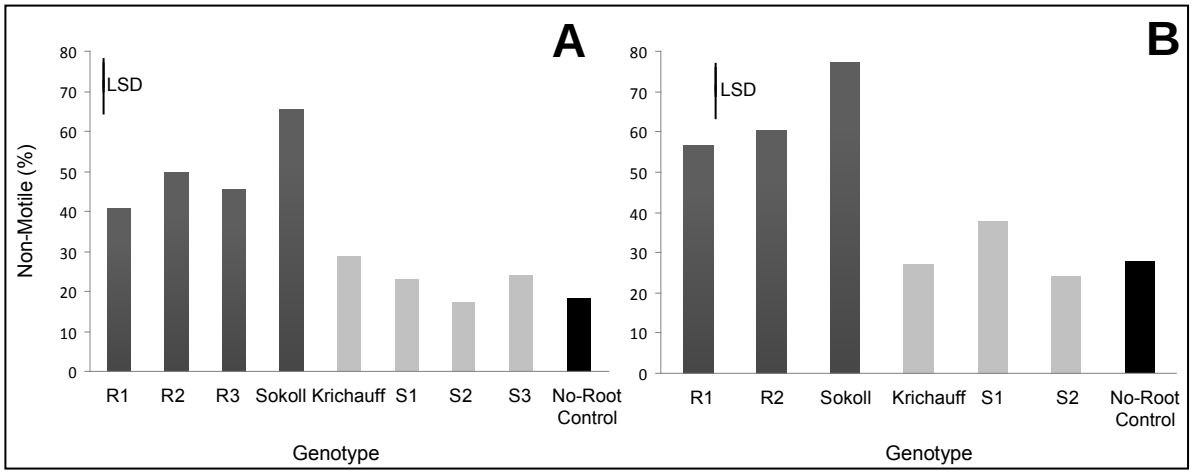

Figure 2: Motility of *Pratylenchus thornei* in (A) root exudates (RE) and (B) crushed root (CR) suspensions after exposure for 3 days. Significantly higher non motility in resistant (Sokoll, R1, R2 & R3) than susceptible (Krichauff, S1, S2 & S3) genotypes in both RE and CR with LSD = 13.98 and LSD = 13.85 respectively.

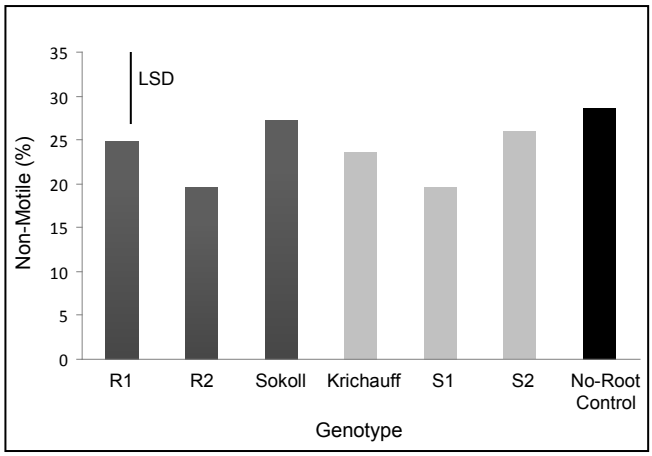

Figure 3: Motility of *Pratylenchus neglectus* in root exudates after 3 days exposure showed no significant differences between resistant (Sokoll, R1, R2 & R3) and susceptible (Krichauff, S1, S2 & S3) genotypes, LSD = 8.92.

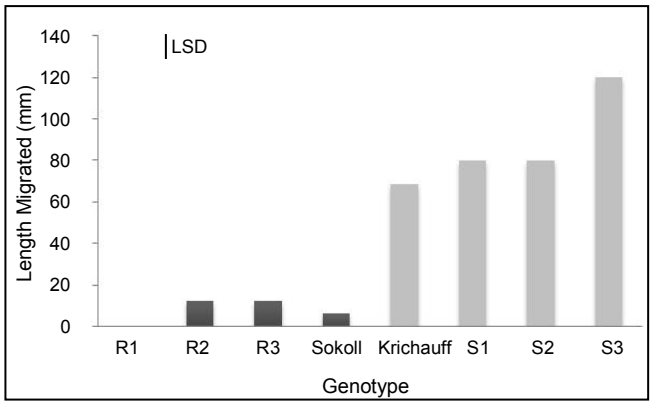

Figure 4: Migration of *Pratylenchus thornei* within roots 10 days after inoculation. The length *P. thornei* migrates from the point of inoculation within the root was significantly lower in resistant root genotypes (Sokoll & R1, R2 & R3) than the susceptible (Krichauff, S1, S2 & S3), LSD = 5.70. Root growth after inoculation does not confound results as the majority of penetration occurs within first 9 hours after inoculation.

Reproduction - Egg Deposition and Egg Hatch

The number of eggs deposited by adults placed directly adjacent to roots of seedlings on agar after 10 days was significantly higher in the presence of susceptible genotypes than resistant. The mean number of eggs deposited in or near Krichauff was 53 compared to 18 in Sokoll giving an egg deposition rate of 4.7 and 1.5 eggs/day, respectively (Figure 5A-B). Significantly more eggs were deposited within susceptible roots (83%) than outside (17%) while 50% of eggs were deposited both within and outside the resistant roots. Only 4 eggs were deposited in the absence of roots.

Resistant genotypes also stimulated significantly less hatching than susceptible. After 10 days the mean number of juveniles hatched from eggs placed adjacent to resistant Sokoll and susceptible Krichauff, roots was 4 and 48 respectively, giving respective hatching rates of 0.5 and 5 juveniles/day. There was no statistically significant difference in the position of juveniles (within or outside roots) after 10 days between genotypes (Figure 6A). There were high hatching rates (45%) in the no-root control, similar to that of Krichauff. Exposure of eggs to RE suppressed hatching in the resistant genotypes with 16.7% hatch in Sokoll and 24.6% in Krichauff after 3 days (Figure 6B). In the no-root water control only 8% hatch occurred. The dilution of RE corresponded to an increase in hatching in both genotypes. There was a statistically significant increase of hatch between the 100 and 4 mg/mL concentrations in all genotypes except S2 (Figure 7).

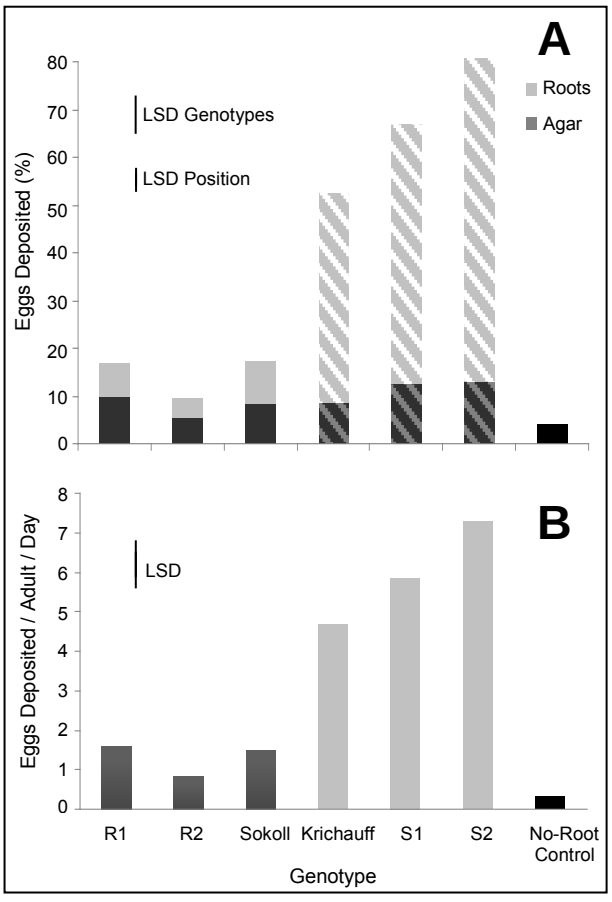

Figure 5: Egg deposition of *Pratylenchus thornei* in and near seedling roots propagated on agar.

A – The number of eggs deposited in and directly adjacent to resistant root genotypes (Sokoll, R1 & R2) was significantly less than susceptible genotypes (Krichauff, S1 & S2), LSD Genotypes = 10.56, and significantly more eggs were deposited within susceptible roots than outside, LSD Position = 7.47.

B – The rate of eggs deposited per day was 1.50 and 4.70 in the resistant and susceptible parent genotypes respectively, LSD = 1.90.

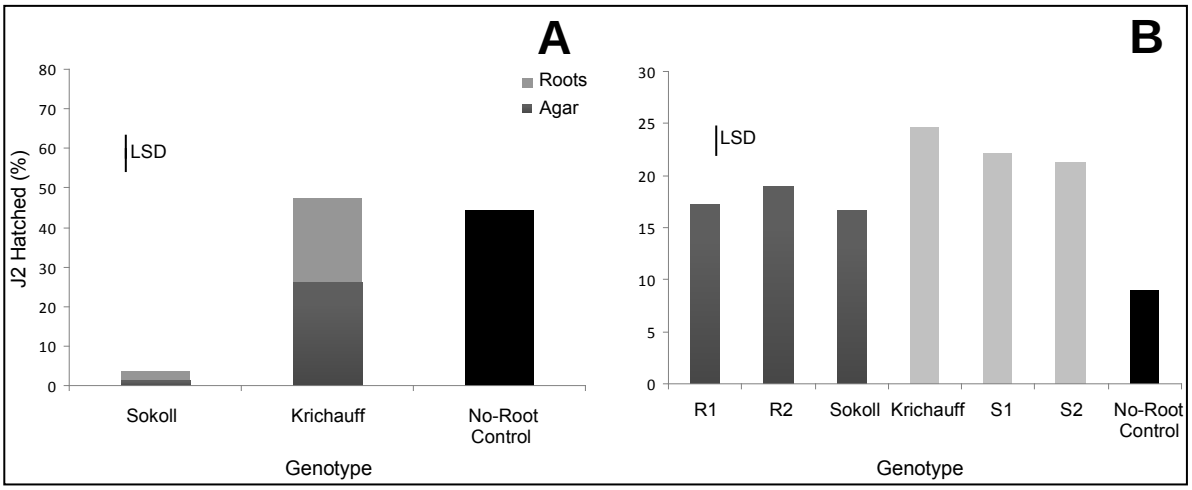

Figure 6: Hatching of *Pratylenchus thornei* near seedling roots propagated on agar and within root exudate solutions. A – The number of juveniles hatched near and within resistant roots (Sokoll) was significantly lower than susceptible roots (Krichauff), LSD = 11.48. There is no correlation between genotype and position hatched. B – The number of juveniles that hatched in resistant root exudates (Sokoll, R1 & R2) after exposure for 3 days was significantly lower than susceptible genotypes (Krichauff, S1 & S2), LSD = 4.04.

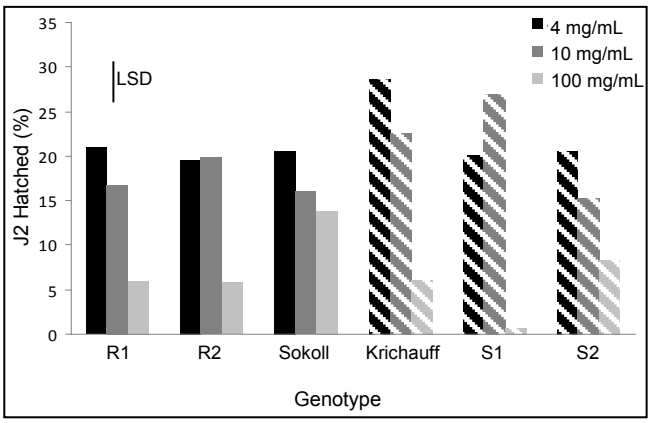

Figure 7: Number of *Pratylenchus thornei* juveniles hatched from eggs soaked in serial dilutions of root exudates. Higher hatching occurred in the 4 mg/mL than the 100 mg/mL concentration in both resistant (Sokoll, R1 & R2) and susceptible (Krichauff, S1 & S2) genotypes, LSD = 5.481.

DISCUSSION

Motility and Migration

When toxic or unfavourable conditions arise many organisms respond by becoming inactive, by arresting development (42), or become so damaged that they lose the ability to move. In this study, significantly higher numbers of *P. thornei* became non-motile when exposed to RE (65%) and CR (75%) from resistant roots than susceptible (30%) roots. Similarly, several other studies have reported suppressed motility in resistant roots and associated this response to accumulation of phytoalexins. For example, in response to invasion by *Meloidogyne incognita* and *Heterodera glycines*, soybean synthesised the isoflavonoid glyceollin in the stele of resistant cultivars within 3 d.a.i., which inhibited nematode respiration and motility (18; 23; 24). Increased production of isoflavonoids in response to *Pratylenchus* invasion was also observed in several plant species. Coumestrol inhibited *Pratylenchus scribneri* motility in resistant lima beans by 50% after exposure for 96 h (46). Similarly, high levels of medicarpin and coumestrol were found in resistant lucerne root tissue after invasion with *Pratylenchus penetrans*, and motility was inhibited on agar by medicarpin (3). Despite the common link between isoflavonoids and *Pratylenchus* motility inhibition, in order to confirm the role of phytoalexins in providing resistance in the Sokoll x Krichauff Wheat/*P. thornei* interaction, further work is required to identify specific compounds. Biochemical analyses such as chromatography and mass spectrometry will enable further characterisation of the complex mix of root chemicals responsible for wheat resistance.

In both the RE and CR motility assays, some loss of nematode motility (20 to 30%) occurred in the no-root water control, which was similar to that of the susceptible samples. In general, motility is never 100% in nematode populations; therefore this reflects the nematode motility suppression observed in the susceptible lines. The suppression of motility in the resistant lines was greater in the CR suspensions than in RE. When roots are crushed, compounds not normally excreted by growing roots from vacuoles, glycosides and other cellular compartments are released. Therefore, the CR suspensions are likely to contain a more complex and more concentrated mix of metabolites and thus have a greater effect on nematode motility suppression.

The effective RE and CR solutions were derived from seedlings not exposed to *Pratylenchus* or other plant pathogens. This indicates the presence of constitutively produced compound(s) in resistant roots that can suppress *P. thornei* motility. In a similar nematode species, *Radopholus similis*, temporary non motility was observed after exposure to RE from both susceptible and resistant banana cultivars that had not been infested with the nematode. Up to 50% were non-motile after exposure to exudates from susceptible plants

and the effects persisted for 1 to 3 days. However, in the case of exudates from resistant roots, over 80% of nematodes were non-motile for more than 6 days (65). Constitutive root defense fits well with resistance to the migratory nature of *Pratylenchus*, as fast accumulation of effective compounds will allow immobilisation of the nematode before significant tissue damage can occur.

Dual resistance to both *Pratylenchus* species is desirable as they commonly occur in wheat fields together (59). Generally, the investigated wheat sources of *Pratylenchus* resistance (AUS5205, AUS11984 and GS50a) do not contain resistance to both species at the same locus (11; 58). However, resistance quantitative trait loci to both *Pratylenchus* species from the same wheat source (CPI133872) have been identified at the same genetic region (71), suggesting that in some cases resistance mechanisms to both species may share the same biological mechanism. The specificity of the suppressive root compounds identified in this study were assessed by performing motility assays with both CR suspensions and RE on *P. neglectus*. Results showed low motility suppression (25%) but in both resistant and susceptible roots. Although some loss in motility was observed, the proportion between the samples and the no-root water control was not significantly different. This indicates that motility suppression caused by resistant root compounds investigated in this study is specific to *P. thornei* and play no role against *P. neglectus*. Similar species specificity was observed with the effect of coumestrol on *P. scribneri* as the motility of *Meloidogyne javanica*, a compatible nematode on lima beans, was not affected even at high concentrations (25 µg/mL) (46). The Sokoll x Krichauff population contains *P. neglectus* resistance as shown by the presence of the *Rlnn1* resistance gene (27). However, as shown in this investigation, this resistance does not appear to be associated with motility suppression.

In this study, the inhibitory effect on nematode motility of the resistant root compounds was partially reversible as 50% of the *P. thornei* recovered motility when washed with water up to 2 days after exposure. A similar motility effect was observed with *P. scribneri* in coumestrol at 25 µg/mL, where non motility was reversible after up to 2 days exposure. However, after 4 days few motile nematodes had recovered after washing (46). Further investigation is required into whether similar non reversible motility effects would occur with increased exposure to Sokoll x Krichauff RE. Based on the current results the compound(s) involved in *P. thornei* motility suppression appear to be nematostatic not nematocidal and affect *P. thornei* but not *P. neglectus*. As resistant genotypes caused inhibition of motility between 50 to 75%, the role of these toxic compounds as defense mechanisms is significant. The longer it takes for the nematode to move within the vicinity of RE or within the root itself, the more time the plant defense systems have to respond and thus limit damage. Therefore, breeding for intensified constitutive production of nematostatic compounds may be an alternative option for management of this nematode. However, before this can be achieved extensive

investigations of the complex biochemical pathways leading to the synthesis of the root compounds is required. In the mean time, the motility assays designed in this study may provide useful alternative phenotypic resistance screening protocols.

Since it was established that resistant roots were able to suppress nematode motility *in vitro*, an experiment was designed to measure nematode migration in roots, or essentially feeding within roots. The design meant that only a portion of one root was available for penetration, while the rest of the seedling's root system was physically blocked from the inoculated nematodes. These migration assays revealed that *P. thornei* did not migrate far through the root cortex from the point of inoculation in resistant roots. On average, nematodes were able to move more than 60 mm further in susceptible Krichauff than the resistant Sokoll. The observed effect on migration was attributed to resistance and was not influenced by different root structure or growth between genotypes (38). As over 50% penetration occurs within 9 hours after inoculation (h.a.i.) on agar (26), root growth after inoculation will not affect the migration measured. It was essential that motility and migration assays were performed under sterile conditions as fungi are known to be sources of anti-nematodal compounds that can suppress motility and hatching (62; 64).

A hypersensitive response involving programmed cell death in invaded tissues is associated with resistance to various nematodes (14) as it can suppress nematode migration (39) and feeding (48). Phenolic compounds and peroxidases are involved in programmed cell death (67) and in plant defense against nematodes. Phenols that accumulate in invaded tissues are directly synthesised or are released from bound forms, such as glycosides (48). In this study, resistant CR suspensions decreased motility indicating the possible release of bound compounds. Mountain and Patrick (34) suggested that phenolic compounds, associated with the formation of necrotic lesions, were released from glycosides via an interaction between the enzyme, β-glucosidase, secreted by *Pratylenchus* during feeding. Various phenolic compounds play a role in defense against *Pratylenchus* in other plant species and thus are candidates for further investigation of the inhibition of *P. thornei* root migration observed in this study. For example, oxidised phenols, including chlorogenic acid, reduce respiration and subsequently movement of *P. penetrans* in tomato (8). Chlorogenic acid is known to affect nematode coordination (19) and therefore may inhibit nematode root migration. To correlate the involvement of phenols with the Sokoll x Krichauff resistance response, the use of gene expression analysis, various staining methods and chromatography techniques will provide ways forward.

Reproduction - Egg Deposition and Egg Hatch

Linsell et al. (26) showed that resistance to *P. thornei* prevents juvenile development and thus inhibits reproduction. The study reported that stage two juveniles were absent and few stage three juveniles were present in the resistant lines 36 and 45 d.a.i. As juveniles failed to reach maturity, no egg deposition was initiated in the resistant lines. Thus, in this follow-up study, experiments were conducted on agar with mature females to test if egg deposition and development is affected by the presence of resistant roots.

Pratylenchus species deposit single eggs either in the soil or within roots (37), and in this study both were examined. Adult *P. thornei* nematodes did deposit eggs within and near resistant roots but egg deposition was up to thirty times less than in susceptible roots. Similar suppression was observed by Farsi (10) for *P. neglectus* in resistant wheat cultivars . In one resistant cultivar, egg deposition was delayed by 4 days compared to the susceptible cultivar, but in another resistant cultivar no eggs were observed even after 35 days. In *P. penetrans*, egg deposition occurs between 2 to 14 days at 20 to 22 °C (30; 43), and as *P. penetrans* and *P. thornei* have similar life cycles, comparisons can be drawn. Therefore, it is unlikely that *P. thornei* egg deposition is delayed by resistance in this study, as egg counts were recorded 10 d.a.i., a timeframe that would have been sufficient to observe any delay.

There is limited data on the capacity of *Pratylenchus* species to deposit eggs (20). However, the maximum egg deposition rate of *P. penetrans* on a conifer species was 2.7 eggs per day (30) and *Pratylenchus brachyurus* on maize was 4.8 eggs per day (15), both over 11 days at 20 °C. In this current study, mean deposition rates for *P. thornei* were 4.7 eggs per day, over 10 days at 21 °C in Krichauff. This suggests that temperature, plant and nematode species all affect the rate of egg deposition. In the current study the rate of egg deposition per female per day in the susceptible roots was almost three times higher than in the resistant. Thus, resistance appears to decrease the rate of egg deposition of *P. thornei* in the investigated wheat genotypes. In addition, fewer eggs were deposited on agar plates where no roots were present indicating *Pratylenchus* deposit eggs only when a food source is available. In susceptible plants, females prefer to deposit eggs within the roots (83%) rather than outside. In contrast, approximately half the numbers of eggs were deposited within and adjacent to the resistant roots. A lower deposition of eggs within resistant roots may be due to a higher concentration of suppressive compounds within the root which deter adults from depositing eggs within this toxic environment. It could also be suggested that due to the reduced migration and feeding, mature females simply do not have the food resources/energy to produce eggs. Reduced egg deposition has been associated with *Pratylenchus goodeyi* resistance in banana where in the susceptible roots *P. goodeyi* deposited 29.8 eggs per day compared to 18.8 in the resistant roots over the same time period (43). The identification of specific compounds that inhibit egg formation/deposition or the absence of required

compounds could be useful in the development of genetically modified resistant plants and bionematicides (31; 35).

Hatching is a complex biological process significantly affected by environmental factors such as temperature, soil type and moisture (9; 13; 47; 54) and in some cases by the host species (51; 53). Root exudates contain hatching factors (HF) that may stimulate hatching in some species of nematodes and at the same time RE can also contain hatching inhibitors (HI), which prevent juvenile emergence. It is known that most species of nematodes do not solely rely on host derived stimuli to initiate hatching and that many hatch freely in water (41), including *P. thornei* as observed in this study. However, host signals are vital to ensure that significant numbers of juveniles emerge within a close vicinity of the roots to increase rates of successful penetration (41), or to induce hatch after a period of nematode dormancy (21). Byrne et al. (6) showed that hatch in response to potato root leachate of *Globodera rostochiensis* on agar correlated well to in soil hatch. Thus, hatching in this study was performed *in vitro* both on agar and in RE.

In this investigation, significantly fewer *P. thornei* juveniles hatched from eggs within and adjacent to the roots of resistant versus susceptible seedlings grown on agar. Approximately 30% more J2 emerged from eggs near and within susceptible roots than resistant. Hatching was also decreased in resistant RE compared to susceptible but only by 8% after 10 days exposure. The smaller hatching suppression observed in resistant RE than compared to actively growing resistant roots on agar could be due to the presence of less HI in the 4 mg/mL concentration of RE used than that naturally exuded by live roots. In addition, it could be that the active compounds in RE are labile and become inactive over time (10 days). In the susceptible roots and in the no-root control, only 50% of *P. thornei* juveniles had emerged from deposited eggs after 10 days. Similarly, a maximum of 50% *P. penetrans* hatched even though 64% of the eggs already contained J1 or J2 during *in vitro* tests under optimum conditions (44). This indicates that despite uninterrupted juvenile maturation and deposition of eggs in favourable environmental conditions, not all juveniles will hatch. Similar suppression of hatching by resistant root exudates has been observed in other plant/nematode interactions. Root exudate from susceptible soybean cultivars stimulated more hatch and emergence of *H. glycines* than resistant cultivars (7; 51; 53). Similarly, partially resistant clones of *solanum* hybrids generally stimulated lower hatch of *Globodera pallida* than susceptible cultivars after several weeks exposure to RE (12). The flavonoids glyceollin and coumestrol, which suppressed *H. glycines* and *P. scribneri* motility as discussed earlier, also significantly affect egg hatch (61). Flavonoids and/or their derivatives such as ferulic, syringic and coumeric acid are known to be present in wheat roots (16; 17; 28; 49), and could explain the hatching suppression in resistant root exudates observed in

this investigation. Biochemical analyses are required to detect and characterise root chemicals linked specifically to the wheat resistance response to *P. thornei*.

This study has shown that resistance plays a role in *P. thornei* hatching but its mode of action is unknown. Host hatching stimulants are complex in makeup and mechanisms. Resistance at hatching could be explained by a loss of a HF or the presence of HI. In addition to HF, there are hatching factor stimulants, which are compounds that are hatch-neutral but can enhance the function of HF (6). Loss of any one of these compounds may reduce the effectiveness of the hatch. In the current study a lower hatch rate occurred in resistant roots than in the absence of roots indicating the presence of HI. The presence of HI was further investigated through dilution of the RE. As HI occur in low concentrations and HF in much higher, dilution of RE should predominantly affect HI without removing the activity of HF (6). As the RE became more diluted there was an increase in hatching in the resistant roots, thus further suggesting the presence of HI for *P. thornei*.

It has been suggested root branching patterns probably influence RE production and therefore hatching stimulation. It has been reported that main roots with many lateral roots stimulated greater hatch than those with fewer branches (45). Cereal roots invaded by *Pratylenchus* have impaired lateral root development in both number and size and have less root hair development (63). Thus, resistant roots may have more lateral roots and hence a larger number of actively growing root tips than susceptible roots. Root exudates are known to originate from regions where lateral roots emerge and from root tips (66). Therefore, due to extensive branching, *P. thornei* resistant roots may produce greater amounts of exudates and subsequently HI, which could explain the increased hatching suppression observed in the resistant lines investigated. However, a phenotypic root assessment of the 150 Sokoll x Krichauff DH lines showed there were no difference in the number, length and weight of roots between resistant and susceptible genotypes (38), which shows that in this population resistance is not linked to root morphology.

The observed decrease of hatching associated with the resistant wheat lines may also be explained by a delay of egg hatch. As the life cycle timelines for *P. thornei* and *P. penetrans* are similar, comparisons can be drawn. In various crops also exposed to RE, 50% of the maximum hatch of *P. penetrans* occurred between 5 to 7 days at 21 °C (44). Thus, if *P. thornei* hatching were delayed by 3 to 4 days, 50% emergence would be expected at 10 days. Hatched juveniles in this investigation were counted 12 days after exposure to RE, however, as only 4% of juveniles associated with Sokoll resistant roots had hatched after this period it indicates that hatching is severely suppressed or delayed much more than the 12 days of observation.

Although RE are vital for hatch in some cyst nematodes, most plant parasitic nematodes can hatch freely in water and RE only enhance the rate of hatching (41). In the agar no-root control, 45% of *P. thornei* juveniles hatched, which was similar to Krichauff, indicating that *P. thornei* hatch can occur in the absence of roots. It has only recently been shown that hatch in *Pratylenchus* species can occur in water and that RE simply promotes hatch. Pudasaini et al. (44) found that *P. penetrans* hatch (25%) occurred after 10 days exposure in soil leachate (root free systems) which was equal to and greater than some known host RE. Similarly in this study, after 10 days in distilled water, hatch of *P. thornei* eggs was observed. However, unlike the study of Pudasaini et al. (44), only a small percentage hatch, (8%) was observed in this study, most likely due to less oxygen transfer as a result of the sample container surface area.

In conclusion, migration and motility of *P. thornei* is suppressed in Sokoll x Krichauff resistant roots and exudates, suggesting resistant genotypes constitutively produce compounds that inhibit motility. The effects of these compounds are reversible and differentially affect *P. thornei* but not *P. neglectus*. In addition, egg deposition and hatch of *P. thornei* is significantly reduced in resistant roots and exudates. The increased hatch after dilution of RE and the lower hatch in resistant exudates versus the absence of roots, indicates the presence of hatching inhibitors. The actions of flavonoids and phenolics have been linked to *Pratylenchus* motility suppression in other resistant responses and thus are targets for future investigations into wheat resistance to *P. thornei*.

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Chapter 4

Quantitative Trait Loci for Resistance to Root Lesion Nematode, *Pratylenchus thornei*, from a Synthetic Hexaploid Wheat Source

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Abstract

Pratylenchus thornei are migratory nematodes that feed and reproduce within the wheat root cortex resulting in cell death (root lesions), causing severe reductions in yield. The identification of molecular markers closely linked to resistance genes will allow use of marker assisted selection to accelerate development of new resistant cultivars, eliminating laborious and costly resistance phenotyping. In this study, a doubled haploid wheat population (150 lines) from a cross between the synthetic derived cultivar Sokoll (*P. thornei* resistant) and the cultivar Krichauff (*P. thornei* moderately susceptible) has been investigated to identify quantitative trait loci (QTL) associated with *P. thornei* resistance. A high density map was constructed using Diversity Array Technology (DArT) and QTL regions identified were targeted with microsatellite markers. Highly significant ($P < 0.001$) *P. thornei* resistance QTL were detected on the distal ends of the short arms of chromosomes 2B and 6D. The QTL, *QRlnt.sk-6D*, was linked to the distal markers *gpw5182*, *gpw4357* and *barc183* (Likelihood Ratio Statistic (LRS) = 82.9) and explained 43% of the phenotypic variation. The other highly significant QTL on chromosome 2BS, *QRlnt.sk-2B.1*, was linked to *wmc382* and *gwm614* (LRS = 39.9) and explained 24% of the phenotypic variation. Two significant QTL ($P < 0.01$) for resistance were also identified on chromosomes 4A and 5A. The population was fixed for the highly significant QTL on 2BS and 6DS and further QTL were identified on chromosomes 2B, 2D, 3A, 5B and 6B.

Key Words

Synthetic hexaploid wheat, doubled haploid population, molecular markers, QTL mapping

Introduction

Root lesion nematodes (*Pratylenchus* spp.) are migratory endoparasitic nematodes that feed and migrate within root cortical tissue causing necrosis and reduced root lateral branching at high nematode densities (Vanstone et al. 1998). Water and nutrient uptake in infested plants is diminished, which leads to yield losses. Losses due to *Pratylenchus thornei* and *Pratylenchus neglectus*, the most widespread species in Australian wheat growing regions, is estimated at $AUD 50 million and $AUD 73 million each year (Murray and Brennan 2009).

Due to the wide host range of *Pratylenchus* spp. and the restrictions and inefficacy of chemical pesticides, the development of resistant cultivars has become increasingly important. Tolerant cultivars can survive and yield well within infested soils, but allow reproduction, thus leaving nematodes within the soil to attack subsequent crops. Resistant cultivars have the capacity to reduce nematode reproduction and nematode densities in the soil (Rohde 1972).

Considerable research efforts in Australia led to the identification of several sources of partial resistance, which have been mapped to reveal quantitative trait loci (QTL) for root lesion nematode resistance using different mapping populations in wheat. These *P. thornei* resistance QTL were identified over 10 years ago (Thompson et al. 1999), but despite this, currently there are no commercially available wheat cultivars with strong resistance to *Pratylenchus* (Sheedy and Thompson 2009). The GS50a resistance source identified by Thompson and Clewitt (1986) as a single plant selection from a severely affected field of the wheat cultivar Gatcher, has been used to improve the levels of resistance to some degree in local commercial varieties.

Due to the lack of genetic diversity in cultivated modern bread wheat, new sources of resistance are sought from wild wheat progenitors (Ogbonnaya 2008; Zwart et al. 2010). Hexaploid bread wheat originated in the Fertile Crescent in the Middle East through a few random crossings between wild wheat species (Nesbitt 2001). It has been suggested that in the original hybridisation, only a limited number of nematode resistance genes from the diploid *Aegilops tauschii* and the tetraploid *Triticum turgidum* wild progenitors were involved, thus hexaploid wheat lacks the diverse genetic sources of resistance genes that its ancestors possess (Breiman and Graur 1995). Initially, Thompson and Haak (1997) screened accessions of *Ae. tauschii* from Iran and found *P. thornei* resistance in all taxonomic subgroups of this species. They investigated over 200 accessions and identified many with lower *P. thornei* reproduction than GS50a. Resistance found in *Ae. tauschii* can be transferred to bread wheat by direct crossing (Gill and Raupp 1987) or by developing synthetic hexaploids through hybridisation with a durum, *T. turgidum*, which can then be crossed to bread wheats (Lagudah et al. 1993; Mujeeb-Kazi 1995). In recent years, effective

sources of resistance to *P. thornei* and *P. neglectus* have been identified and mapped in synthetic hexaploid wheat lines (Ogbonnaya 2008; Thompson et al. 2008; Toktay et al. 2006; Zwart et al. 2004, 2005; Zwart et al. 2010) and Middle Eastern landraces (Schmidt et al. 2005; Sheedy and Thompson 2009; Thompson et al. 2009). QTL for resistance to *P. thornei* have been identified on the B and D genomes on five different chromosomes in the investigated sources (Schmidt et al. 2005; Toktay et al. 2006; Zwart et al. 2005; Zwart et al. 2010; Zwart et al. 2006). The mode of resistance to *P. thornei* is multigenic and additive (Zwart et al. 2004) and thus makes it a suitable trait for marker assisted selection (MAS).

Genotypic selection using molecular markers closely linked to *Pratylenchus* resistance genes will accelerate the development of new resistant cultivars through reducing the problems associated with phenotypic selection. Due to the high throughput marker technologies available, phenotyping, especially with nematode resistance, is the limiting factor in terms of cost, reliability of data and sample size. The development of DNA quantification methods where the quantified nematode DNA in a plant line serves as an estimate of its resistance level, has made this task more efficient and allows higher throughput (Ophel-Keller et al. 2008). Although helpful, this technology is expensive for a breeding program as several repeats per genotype need to be tested. Instead, genotypic selection using molecular markers closely linked to *Pratylenchus* resistance genes would greatly accelerate the development of new resistant cultivars. The identification of novel sources of genetic resistance and understanding of their biological mechanisms will allow effective combinations of genes either to be used alternatively, or pyramided to generate effective and stable *Pratylenchus* resistance in wheat.

The study aimed to characterise the genetic resistance derived from a new synthetically derived *P. thornei* resistance source. A doubled haploid population originating from a cross between the synthetic hexaploid wheat Sokoll (*P. thornei* resistant) and the common South Australian wheat cultivar Krichauff (*P. thornei* susceptible) was investigated. Phenotypic analysis was conducted using nematode DNA quantification and a genetic map was constructed to identify QTL and associated molecular markers accounting for resistance to *P. thornei*.

Material and Methods

Plant Material

The population used consists of 150 doubled haploid (DH) lines derived from a cross between the synthetic cultivar Sokoll (pedigree: Pastor/3/Altar84/*Ae. squarrosa*(Taus)//Opata) developed by the International Maize and Wheat Improvement Centre Mexico (CIMMYT), and the cultivar Krichauff (pedigree: Wariquam//Kloka/Pitic62/3/Warimek/Halberd/4/3Ag3Aroona) developed by Tony Rathjen and the Waite Institute Wheat Breeding team. The DHs were generated by the South Australian Research and Development Institute (SARDI) doubled haploid and cell culture unit (Howes et al. 2003). Plants were grown in a glasshouse at 22 to 24 °C and leaves for DNA extraction were collected from 1 to 2 week old seedlings.

Nematodes

Pratylenchus thornei was obtained from the SARDI population (originally sourced from wheat at Nunjikompita, South Australia) maintained on carrot callus as described by Nicol and Vanstone (1993), modified from Moody et al. (1973). Cultures were stored at 22 ºC and subcultured every 3 months. To collect the nematodes, the carrot callus was placed in funnels in a misting chamber with an intermittent aqueous mist of 10 s every 10 min for 96 h (Southey 1986) at room temperature (22 °C). Nematodes extracted in the mister were counted in 250 µL aliquots in three replicates and adjusted to the required inoculum concentration by dilution with water.

Phenotypic Screening of *P. thornei* Resistance

The resistance response of the 150 DH lines to *P. thornei* was assessed in a glasshouse maintained at 20 to 23 °C. One pre-germinated seed was sown per tube (55 × 120 mm) in 350 g of steam-pasteurised sand. Tubes were arranged in a random block (5 x 5 blocks) design and placed in trays that were flooded for 4 min every 3 days to a depth of 100 mm. One week after the seedling emerged they were inoculated with two 500 µL aliquots (1500 nematodes per plant) into two 50-mm-deep holes on either side of the seedling. Waterings were suspended until 3 days after inoculation. Two days after inoculation a slow release fertiliser (Osmocote, Scotts Miracle-Gro, USA) was added (4 g/kg sand) and each tube was covered with plastic beads to reduce evaporation. Eight weeks after inoculation plants were removed and roots were washed free of soil and were dried. DNA was extracted from the roots and the amount of *P. thornei* reproduction within the roots was assessed via DNA quantification through the SARDI Root Disease Testing Service (Ophel-Keller et al. 2008). The amount of *P. thornei* DNA was quantified using a real-time TaqMan polymerase chain reaction (PCR) system with primers specific to the internal transcribed spacer (ITS) region of

P. thornei. The DNA extraction system and details of the *P. thornei* specific test has not been published and are protected by legal secrecy agreements. DNA was quantified using a standard curve that was established using known amounts of DNA. The amount of nematode reproduction was expressed as picograms (pg) *P. thornei* DNA per plant. The controls included a susceptible wheat cultivar, Machete, a moderately susceptible cultivar, Meering, and a moderately resistant cultivar, Chara. Resistance is scored based on correlations with DNA amounts in the check varieties. The trial included five replicates of each sample. The trial was independently replicated twice under the same experimental conditions one week apart in August/September 2006 and was conducted by SARDI Nematology staff. The means of the five replicates were calculated and used for phenotypic analysis. Significant differences between population individuals were assessed using one-way analysis of variance (ANOVA). The mean values of *P. thornei* DNA pg/plant in the parents, the population mean, and the minimum and maximum values were calculated. Broad sense heritability (H2) was estimated from the mean of the trials using the formula: H2 = Var(G)/Var(P), where Var(G) is the estimated variance of the genotypic effect and Var(P) is the estimated variance of the phenotypic effect expressed on a genotype mean basis.

Diversity Array Technology Map Construction and QTL Analysis

Leaf segments of about 30 mm in length were collected and were freeze-dried for 24 h. A ball bearing (4 mm diameter) was added to each tube (1.1 mL) and in a shaker leaf material was crushed to a fine powder (25 oscillations/second for 5 min). DNA was extracted using a DNA mini-prep method adapted from Rogowsky et al. (1991). The adapted method was as follows; DNA was extracted in 300 μL of DNA extraction buffer [100 mM Tris-HCl (pH 8.5), 100 mM NaCl, 10 mM EDTA, 1% sarkosyl and 2% polyvinylpoly-pyrolidone], and 300 μL phenol: chloroform:iso-amylalcohol (25:24:1), and the material was mixed for 20 min on an orbital shaker. The phases were separated by centrifugation and the upper aqueous phase was re-extracted as described above. The DNA was precipitated by the addition of 30 μL 3 M Na-acetate (pH 4.8) and 300 μL isopropanol. DNA was collected by centrifugation, washed with 70% ethanol, dried, and resuspended in 20 μL R40 buffer (4 mg RNase A in 100 mL TE Buffer).

Fifty mg/μL of DNA from each sample, which included the 150 DH lines and two replicates of the parents, were sent to Triticarte Pty Ltd (Yarralumla, ACT, Australia). The Diversity Array Technology (DArT) markers were scored on the DH population by Triticarte Pty Ltd, probing genomic DNA from individual DH lines against the wheat DArT array version 2.6 (5000 markers) (Akbari et al. 2006). A total of 889 DArT informative markers were scored and used to construct a high-density genetic linkage map of the Sokoll x Krichauff population. The map was constructed using MapManager QTXb20 (Manly and Olson 1999) with the Kosambi

mapping function (Kosambi 1944). For QTL analysis, single marker regression and composite mapping were performed using MapManager QTXb20. A permutation test was used to establish the threshold at which the logarithm of odds (LOD) score became suggestive ($P < 0.05$), significant ($P < 0.01$) and highly significant ($P < 0.001$) for QTL identification (Van Ooijen 2004) generated by the interval mapping procedures.

Genotyping and QTL Analysis

QTL analysis indicated highly significant *P. thornei* resistance QTL on chromosomes 6D and on 2B. The parent lines and six DH lines (three susceptible and resistant based on phenotype) were screened with 340 simple sequence repeat (SSR) markers previously mapped to chromosomes 6D and 2B (CMap, GrainGenes) to identify markers that were polymorphic between Sokoll and Krichauff. One hundred and sixty polymorphic SSR markers from 6D and 2B were screened against the 150 DH lines.

For marker screening, DNA was extracted as described by Palotta et al. (2003) from 30 mm leaf segments that had been freeze-dried and crushed as described earlier. Preheated DNA extraction buffer [100 mM Tris-HCl (pH 8.0), 500 mM EDTA, 1.25% sodium dodecyl sulfate], was added and the material was extracted by heating for 60 min at 65 °C. The samples were cooled before the addition of 150 µL 6 M Na-acetate (pH 4.8). Supernatant was recovered after centrifugation into 180 µL isopropanol. Precipitated DNA was collected by centrifugation, washed with 70% ethanol, dried, and resuspended in 50 µL R40 buffer.

For SSR marker screening, amplification was performed using a touchdown PCR profile as follows: initial denaturation step at 94 °C for 2 min, followed by cycles of 94 °C for 30 s, an annealing step for 30 s and 72 °C for 30 s. The initial annealing temperature was at 59 °C and was reduced by 0.5 °C for the next 8 cycles. The remaining 29 cycles had an annealing temperature of 55 °C, the program ended with 5 min extension at 72 °C. Amplification was performed in a total volume of 9.5 µL, using Taq polymerases from Qiagen (Victoria, Australia) and Bioline (Adelaide, Australia) with respective protocols. Polymerase chain reactions were performed in a PTC-225 thermocycler (MJ Research, Australia). SSR marker amplified products were separated on 8% SDS polyacrylamide gels (Sigma Aldrich, Australia) at constant 300 V for 180 min and viewed and photographed with ethidium bromide staining under UV light.

Sequences for eight DArT markers that map to the 2B and 6D QTL region were obtained from Triticarte and used to design PCR primers to amplify corresponding regions from the two mapping population parents. Homology searches were carried out using the BLAST algorithm and the EST (expressed sequence tag) databases at NCBI (National Centre for

Biotechnology Information). Resulting overlapping ESTs were assembled to larger contigs using ContigExpress, part of the DNA analysis software suite VectorNTI 11 (Invitrogen, Victoria, Australia). Primers were designed to amplify the largest contig. Six of the converted DArT markers were polymorphic and were mapped in the Sokoll x Krichauff population. The primers were 653 (CGCATCCTTTTAGGAGCAAG, GGCAGCACTTCAGAGTGGAT), 2864 (GAGCTCCAACCACAGCTCTT, ACTCCTCCATGAGCAGCTTG), 5114 (TGAGGCCGAGGAAGGTTCCA, TGCTCTTCGTAAGCTGAGCCGT), 3390 (TGTACAGGGAACTCCCAAGG, GTGGTCTCTCCTTACCAGCG) and 1634 (AGAAAACAGCCCCCAAATCT, GCACATTGTCAAGCTGCTGT). For converted DArT markers, amplification was performed as described above but using an initial denaturation step at 94 °C for 2 min, followed by 94 °C for 30 s, a 57 °C for 30 s and 72 °C for 30 s. Amplification products were separated on 2% agarose gels for 90 min at 100 V and viewed and photographed with ethidium bromide staining under UV light.

In MapManager QTXb20 the marker allele distribution among the 150 DH lines were integrated into the DArT map using the Links report function. In conjunction with the ripple function and published maps, an order of markers was established, with the aim of minimising double recombinations and chromosome length. Marker order was finalised using RECORD (Van Os et al. 2005). Segregation ratios of the two genotypes were tested using a chi-square (χ^2) test to determine the extent of linkage disequilibrium. Markers that showed distorted segregation from the expected 1:1 ratio were excluded from QTL analysis. A χ^2 value exceeding 3.84 (df =1) indicated distortion at P = 0.05. The proportion of phenotypic variation explained by each locus was estimated by the coefficient of determination (R^2). A permutation test (1000 permutations) was used to determine the critical LOD threshold.

Results

Phenotypic Assessment

There was a high level of correlation between the two trials (R^2 = 0.7) (Fig. 1), which for nematode resistance phenotypic data is very high (J. Thompson personal communication) and thus data from the two trials were pooled and QTL analysis was performed on trial means. As a check, QTL analysis was performed on each individual trial and QTL location and significance matched that of the pooled data. The population displayed a continuous distribution with the average nematode count (pg DNA/plant) for the population of 9153, for the resistant parent Sokoll, 3766, and for the moderately susceptible Krichauff, 15816. The phenotypic nematode quantification data showed a regression (Figs. 1 and 2) that was significantly skewed, 1.27 ± 0.2013. ANOVA showed highly significant differences between individual genotypes for *P. thornei* DNA pg/plant (average of trials 1 and 2). The broad sense heritability (H2) was high (0.971) and shows this resistance is largely controlled by genetic variation (Fig. 2).

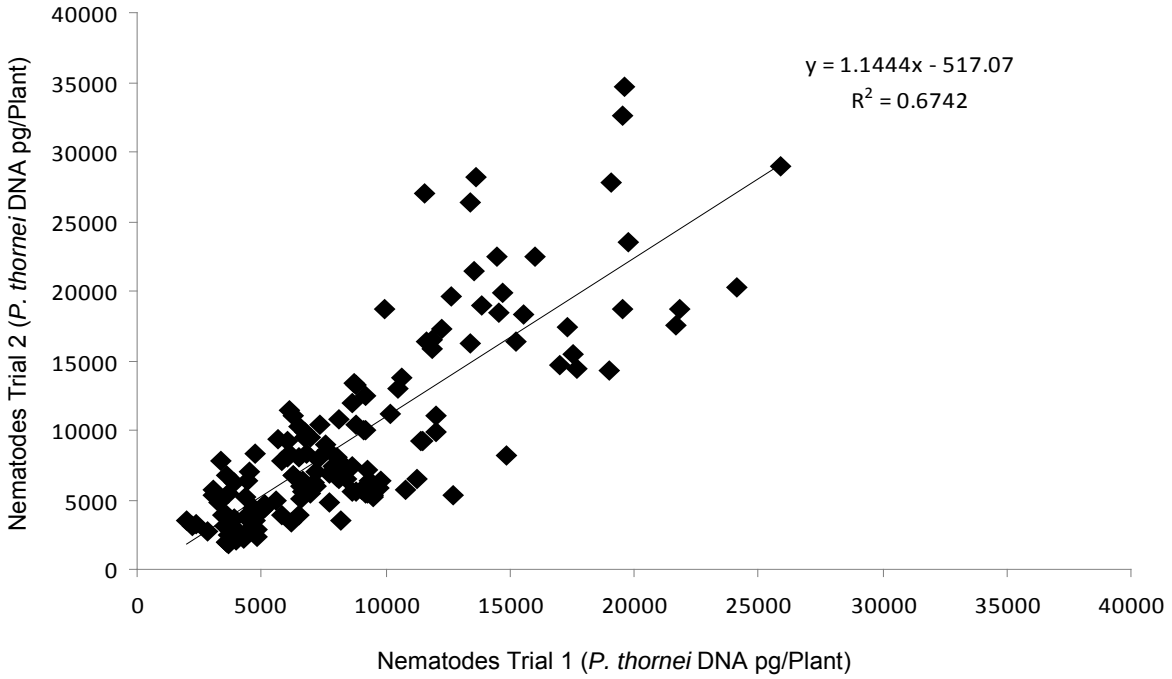


Fig. 1 *Pratylenchus thornei* nematode DNA count data from trial 1 and 2 shows a strong linear correlation. The trend line fitted has a coefficient of determination of 0.67

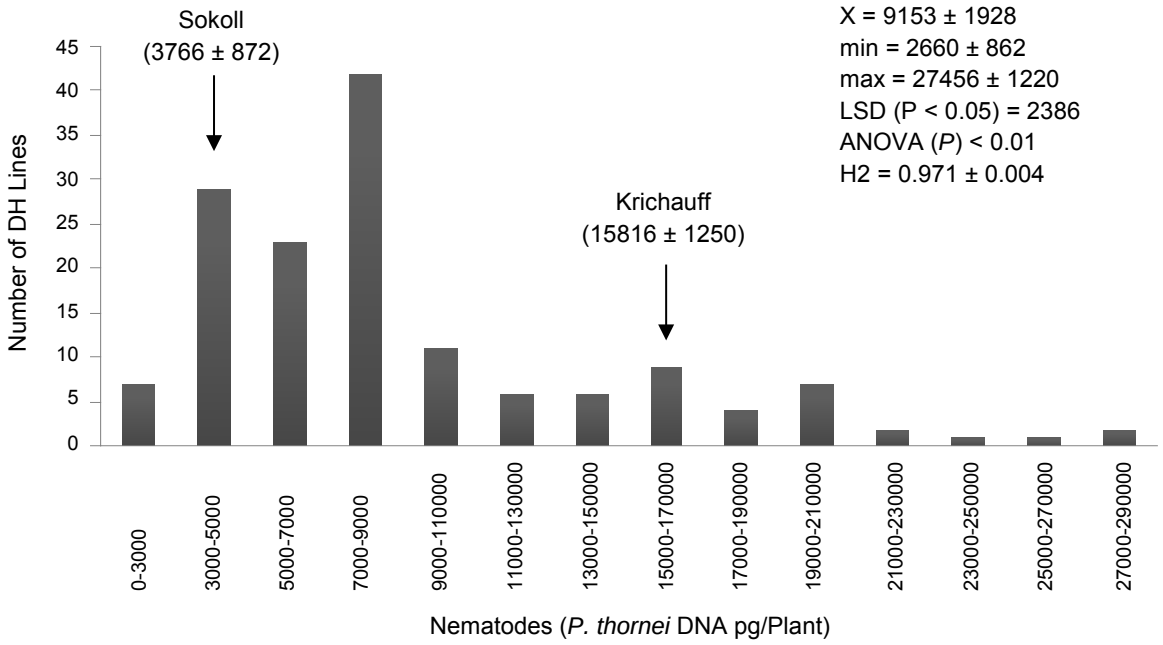
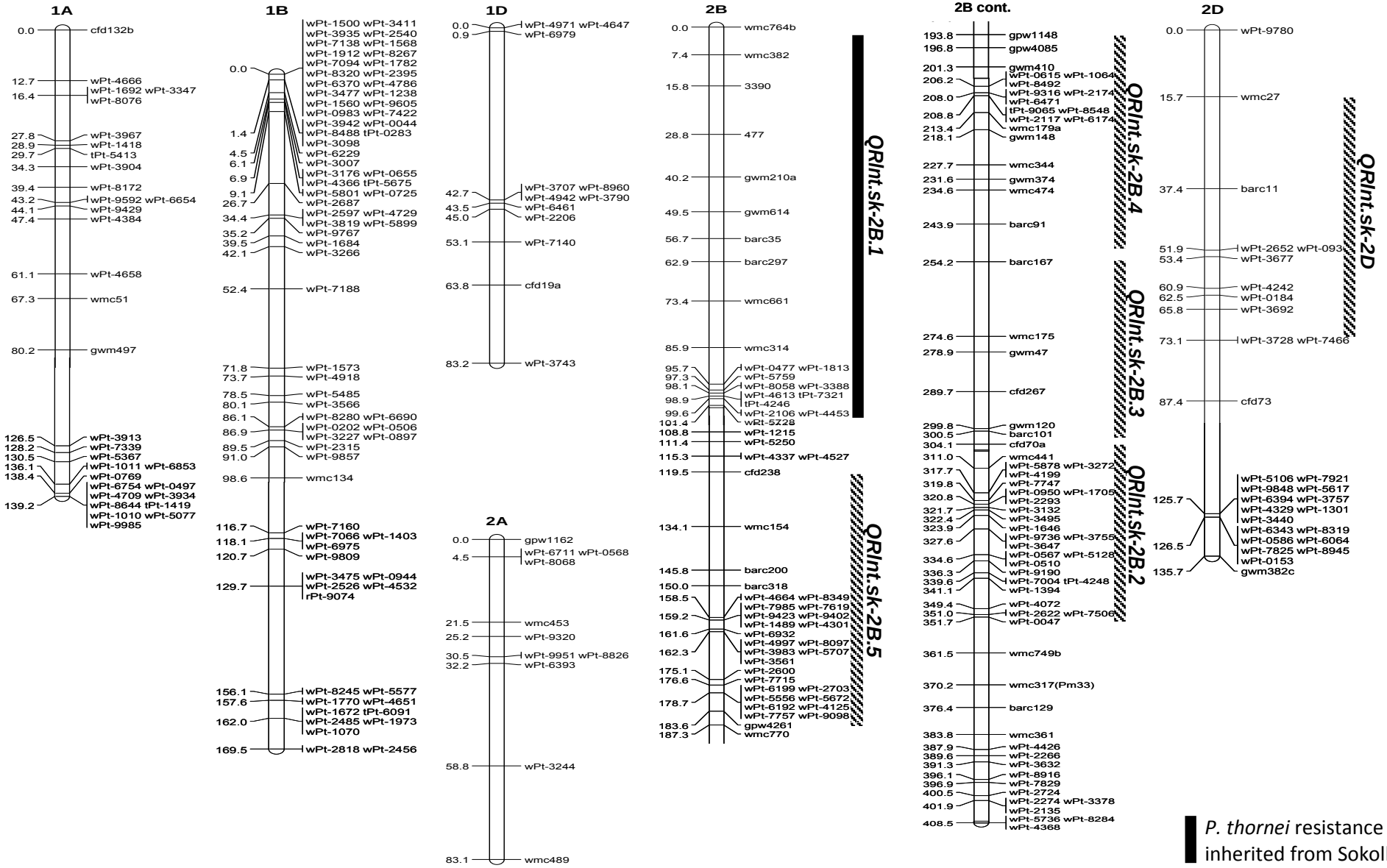


Fig. 2 *Pratylenchus thornei* nematode DNA count data (average of trials 1 and 2) shows a normal continuous distribution in the Sokoll x Krichauff DH population. The mean (± standard error) values of *P. thornei* DNA pg/plant in the parents are indicated. In addition, the population mean (X), the minimum (min) and the maximum (max) values (± standard error), least significant difference (LSD), analysis of variance (ANOVA) and the broad sense heritability (H2)

Genotyping and Map Construction

In addition to the 860 DArT markers, 340 SSR markers were screened for polymorphisms between the parental alleles with 209 appearing polymorphic. Sixty five markers were unlinked and could not be assigned to any of the 21 linkage groups. Thirty five were assigned to chromosome 2B and 33 to chromosome 6D. The final Sokoll x Krichauff map was generated with 860 DArT markers and 111 microsatellites arranged across 21 linkage groups (Fig. 3). The map covers a total of 3477 centiMorgans (cM) of the wheat genome with an average marker density of 3.56 cM. Segregation distortion was observed in 61 markers (6.3%) of the mapped loci ($P < 0.05$) from the expected 1:1 ratio as determined by a chi-square analysis and were deleted from the dataset. The frequency of Sokoll alleles was high at loci on chromosomes 2B, 6D and 7A (3 loci, 79%) while frequency of Krichauff alleles was high at loci on chromosomes 4A and 5A (2 loci, 21%). When the population was fixed for the 2B and 6D QTL the frequency of Sokoll alleles was high at loci on chromosomes 3A, 5B, 6B, 7A and 7B (5 loci, 39%) while frequency of Krichauff alleles was high at loci on chromosomes 2A, 2B and 2D (3 loci, 61%).

Vernalisation markers *Vrn1A*, *1B* and *1D* and the *P. neglectus* resistance gene *Rlnn1* marker *uat0001* [developed by Australian Wheat and Barley Molecular Marker Program (AWBMMP), University of Adelaide] were also mapped. The height genes *Rht1* and *Rht2* were analysed but were not segregating in this population.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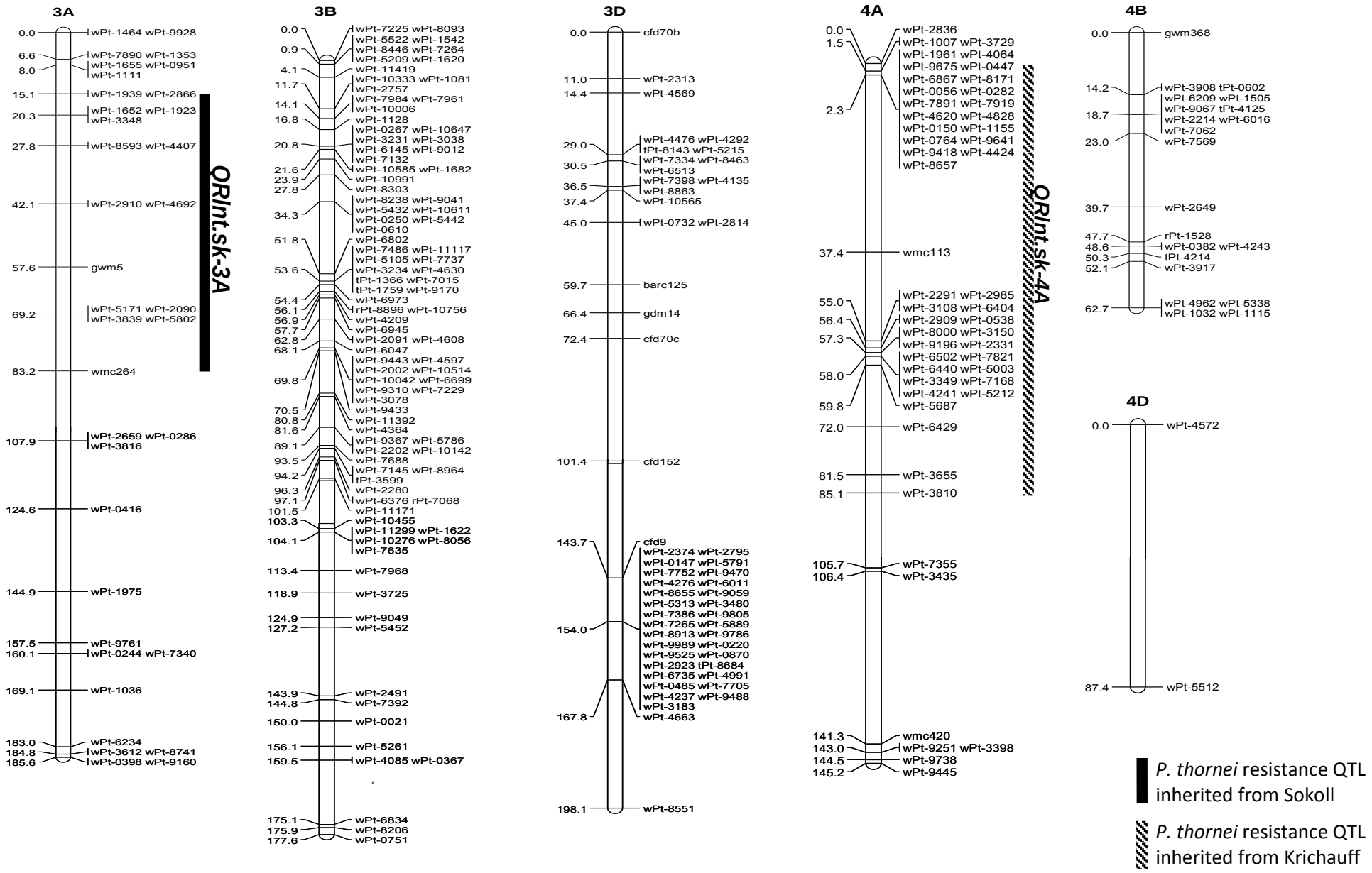
29.7 tPt-5413
34.3 wPt-3904
39.4 wPt-8172
43.2 wPt-9592 wPt-6654
44.1 wPt-9429
47.4 wPt-4384
61.1 wPt-4658
67.3 wmc51
80.2 gwm497
126.5 wPt-3913
128.2 wPt-7339
130.5 wPt-5367
136.1 wPt-1011 wPt-6853
138.4 wPt-0769
wPt-6754 wPt-0497
wPt-4709 wPt-3934
139.2 wPt-8644 tPt-1419
wPt-1010 wPt-5077
wPt-9985
1B
wPt-1500 wPt-3411
wPt-3935 wPt-2540
wPt-7138 wPt-1568
wPt-1912 wPt-8267
0.0 wPt-7094 wPt-1782
wPt-8320 wPt-2395
wPt-6370 wPt-4786
wPt-3477 wPt-1238
wPt-1560 wPt-9605
wPt-0983 wPt-7422
1.4 wPt-3942 wPt-0044
wPt-8488 tPt-0283
4.5 wPt-3098
6.1 wPt-6229
wPt-3007
6.9 wPt-3176 wPt-0655
9.1 wPt-4366 tPt-5675
26.7 wPt-5801 wPt-0725
wPt-2687
34.4 wPt-2597 wPt-4729
wPt-3819 wPt-5899
35.2 wPt-9767
39.5 wPt-1684
42.1 wPt-3266
52.4 wPt-7188
71.8 wPt-1573
73.7 wPt-4918
78.5 wPt-5485
80.1 wPt-3566
86.1 wPt-8280 wPt-6690
86.9 wPt-0202 wPt-0506
wPt-3227 wPt-0897
89.5 wPt-2315
91.0 wPt-9857
98.6 wmc134
116.7 wPt-7160
118.1 wPt-7066 wPt-1403
wPt-6975
120.7 wPt-9809
129.7 wPt-3475 wPt-0944
wPt-2526 wPt-4532
rPt-9074
156.1 wPt-8245 wPt-5577
157.6 wPt-1770 wPt-4651
wPt-1672 tPt-6091
162.0 wPt-2485 wPt-1973
wPt-1070
169.5 wPt-2818 wPt-2456
1D
0.0 wPt-4971 wPt-4647
0.9 wPt-6979
42.7 wPt-3707 wPt-8960
wPt-4942 wPt-3790
43.5 wPt-6461
45.0 wPt-2206
53.1 wPt-7140
63.8 cfd19a
83.2 wPt-3743
2A
0.0 gpw1162
4.5 wPt-6711 wPt-0568
wPt-8068
21.5 wmc453
25.2 wPt-9320
30.5 wPt-9951 wPt-8826
32.2 wPt-6393
58.8 wPt-3244
83.1 wmc489
2B
0.0 wmc764b
7.4 wmc382
15.8 3390
28.8 477
40.2 gwm210a
49.5 gwm614
56.7 barc35
62.9 barc297
73.4 wmc661
85.9 wmc314
95.7 wPt-0477 wPt-1813
97.3 wPt-5759
98.1 wPt-8058 wPt-3388
wPt-4613 tPt-7321
98.9 tPt-4246
99.6 wPt-2106 wPt-4453
101.4 wPt-5728
108.8 wPt-1215
111.4 wPt-5250
115.3 wPt-4337 wPt-4527
119.5 cfd238
134.1 wmc154
145.8 barc200
150.0 barc318
158.5 wPt-4664 wPt-8349
159.2 wPt-7985 wPt-7619
wPt-9423 wPt-9402
161.6 wPt-1489 wPt-4301
wPt-6932
162.3 wPt-4997 wPt-8097
wPt-3983 wPt-5707
wPt-3561
175.1 wPt-2600
176.6 wPt-7715
wPt-6199 wPt-2703
178.7 wPt-5556 wPt-5672
wPt-6192 wPt-4125
wPt-7757 wPt-9098
183.6 gpw4261
187.3 wmc770
QRInt.sk-2B.1
QRInt.sk-2B.5
2B cont.
193.8 gpw1148
196.8 gpw4085
201.3 gwm410
206.2 wPt-0615 wPt-1064
wPt-8492
208.0 wPt-9316 wPt-2174
wPt-6471
208.8 tPt-9065 wPt-8548
wPt-2117 wPt-6174
213.4 wmc179a
218.1 gwm148
227.7 wmc344
231.6 gwm374
234.6 wmc474
243.9 barc91
254.2 barc167
274.6 wmc175
278.9 gwm47
289.7 cfd267
299.8 gwm120
300.5 barc101
304.1 cfd70a
311.0 wmc441
317.7 wPt-5878 wPt-3272
wPt-4199
319.8 wPt-7747
320.8 wPt-0950 wPt-1705
wPt-2293
321.7 wPt-3132
322.4 wPt-3495
323.9 wPt-1646
327.6 wPt-9736 wPt-3755
wPt-3647
334.6 wPt-0567 wPt-5128
wPt-0510
336.3 wPt-9190
339.6 wPt-7004 tPt-4248
341.1 wPt-1394
349.4 wPt-4072
351.0 wPt-2622 wPt-7506
351.7 wPt-0047
361.5 wmc749b
370.2 wmc317(Pm33)
376.4 barc129
383.8 wmc361
387.9 wPt-4426
389.6 wPt-2266
391.3 wPt-3632
396.1 wPt-8916
396.9 wPt-7829
400.5 wPt-2724
401.9 wPt-2274 wPt-3378
wPt-2135
408.5 wPt-5736 wPt-8284
wPt-4368
QRInt.sk-2B.4
QRInt.sk-2B.3
QRInt.sk-2B.2
2D
0.0 wPt-9780
15.7 wmc27
37.4 barc11
51.9 wPt-2652 wPt-093
53.4 wPt-3677
60.9 wPt-4242
62.5 wPt-0184
65.8 wPt-3692
73.1 wPt-3728 wPt-7466
87.4 cfd73
125.7 wPt-5106 wPt-7921
wPt-9848 wPt-5617
wPt-6394 wPt-3757
wPt-4329 wPt-1301
wPt-3440
wPt-6343 wPt-8319
126.5 wPt-0586 wPt-6064
wPt-7825 wPt-8945
wPt-0153
135.7 gwm382c
QRInt.sk-2D
P. thornei resistance QTL inherited from Sokoll
P. thornei resistance QTL inherited from Krichauff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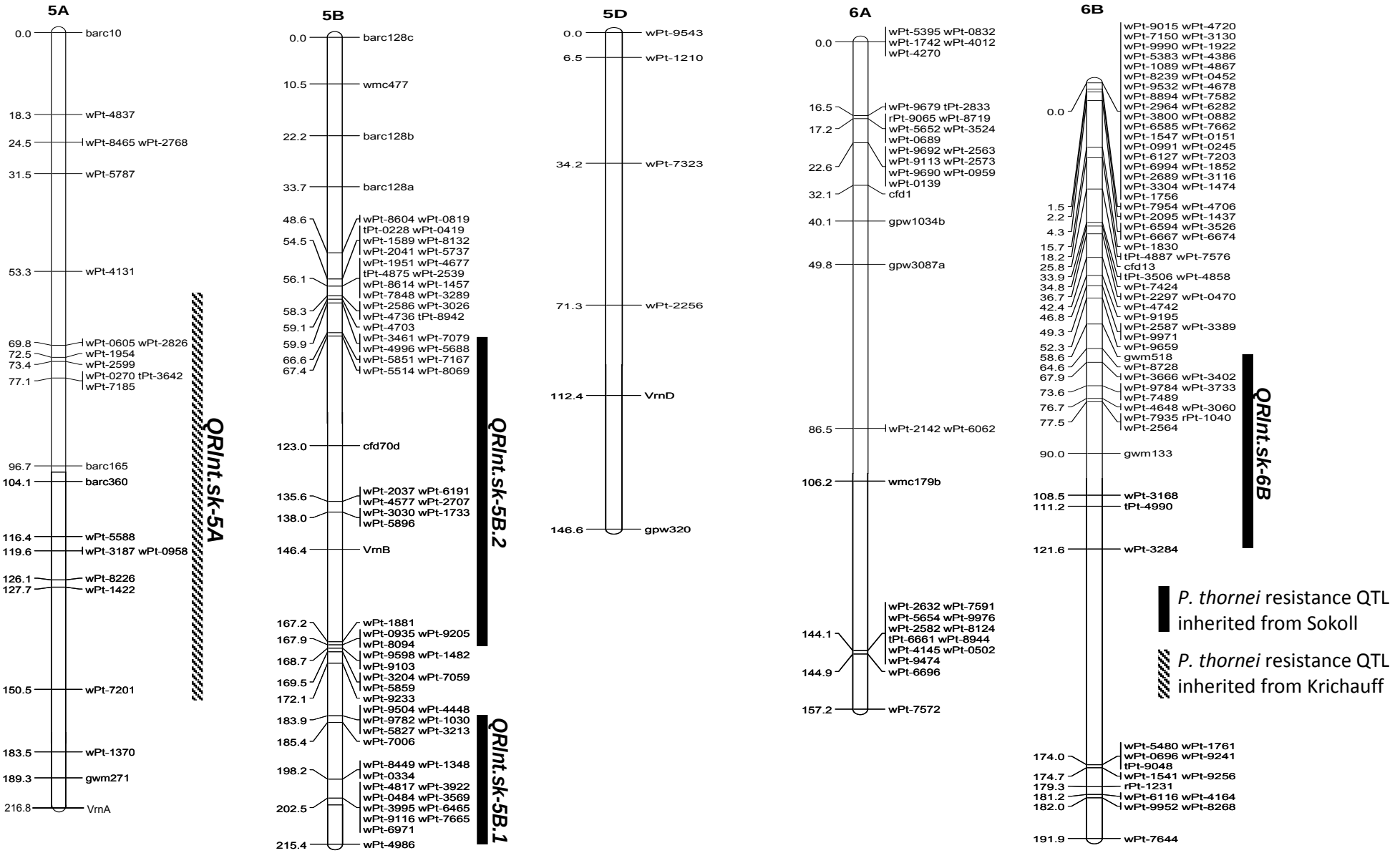
6.6 wPt-7890 wPt-1353
8.0 wPt-1655 wPt-0951
wPt-1111
15.1 wPt-1939 wPt-2866
20.3 wPt-1652 wPt-1923
wPt-3348
27.8 wPt-8593 wPt-4407
42.1 wPt-2910 wPt-4692
57.6 gwm5
69.2 wPt-5171 wPt-2090
wPt-3839 wPt-5802
83.2 wmc264
107.9 wPt-2659 wPt-0286
wPt-3816
124.6 wPt-0416
144.9 wPt-1975
157.5 wPt-9761
160.1 wPt-0244 wPt-7340
169.1 wPt-1036
183.0 wPt-6234
184.8 wPt-3612 wPt-8741
185.6 wPt-0398 wPt-9160
QRlnt.sk-3A
3B
0.0 wPt-7225 wPt-8093
0.9 wPt-5522 wPt-1542
wPt-8446 wPt-7264
wPt-5209 wPt-1620
4.1 wPt-11419
11.7 wPt-10333 wPt-1081
wPt-2757
14.1 wPt-7984 wPt-7961
wPt-10006
16.8 wPt-1128
wPt-0267 wPt-10647
20.8 wPt-3231 wPt-3038
wPt-6145 wPt-9012
wPt-7132
21.6 wPt-10585 wPt-1682
23.9 wPt-10991
27.8 wPt-8303
wPt-8238 wPt-9041
34.3 wPt-5432 wPt-10611
wPt-0250 wPt-5442
wPt-0610
51.8 wPt-6802
wPt-7486 wPt-11117
53.6 wPt-5105 wPt-7737
wPt-3234 wPt-4630
tPt-1366 wPt-7015
tPt-1759 wPt-9170
54.4 wPt-6973
56.1 rPt-8896 wPt-10756
56.9 wPt-4209
57.7 wPt-6945
62.8 wPt-2091 wPt-4608
68.1 wPt-6047
wPt-9443 wPt-4597
wPt-2002 wPt-10514
69.8 wPt-10042 wPt-6699
wPt-9310 wPt-7229
wPt-3078
70.5 wPt-9433
80.8 wPt-11392
81.6 wPt-4364
89.1 wPt-9367 wPt-5786
wPt-2202 wPt-10142
93.5 wPt-7688
94.2 wPt-7145 wPt-8964
tPt-3599
96.3 wPt-2280
97.1 wPt-6376 rPt-7068
101.5 wPt-11171
103.3 wPt-10455
104.1 wPt-11299 wPt-1622
wPt-10276 wPt-8056
wPt-7635
113.4 wPt-7968
118.9 wPt-3725
124.9 wPt-9049
127.2 wPt-5452
143.9 wPt-2491
144.8 wPt-7392
150.0 wPt-0021
156.1 wPt-5261
159.5 wPt-4085 wPt-0367
175.1 wPt-6834
175.9 wPt-8206
177.6 wPt-0751
3D
0.0 cfd70b
11.0 wPt-2313
14.4 wPt-4569
29.0 wPt-4476 wPt-4292
tPt-8143 wPt-5215
30.5 wPt-7334 wPt-8463
wPt-6513
36.5 wPt-7398 wPt-4135
wPt-8863
37.4 wPt-10565
45.0 wPt-0732 wPt-2814
59.7 barc125
66.4 gdm14
72.4 cfd70c
101.4 cfd152
143.7 cfd9
wPt-2374 wPt-2795
wPt-0147 wPt-5791
wPt-7752 wPt-9470
wPt-4276 wPt-6011
wPt-8655 wPt-9059
wPt-5313 wPt-3480
wPt-7386 wPt-9805
154.0 wPt-7265 wPt-5889
wPt-8913 wPt-9786
wPt-9989 wPt-0220
wPt-9525 wPt-0870
wPt-2923 tPt-8684
wPt-6735 wPt-4991
wPt-0485 wPt-7705
wPt-4237 wPt-9488
wPt-3183
167.8 wPt-4663
198.1 wPt-8551
4A
0.0 wPt-2836
1.5 wPt-1007 wPt-3729
wPt-1961 wPt-4064
wPt-9675 wPt-0447
wPt-6867 wPt-8171
wPt-0056 wPt-0282
2.3 wPt-7891 wPt-7919
wPt-4620 wPt-4828
wPt-0150 wPt-1155
wPt-0764 wPt-9641
wPt-9418 wPt-4424
wPt-8657
37.4 wmc113
55.0 wPt-2291 wPt-2985
56.4 wPt-3108 wPt-6404
wPt-2909 wPt-0538
57.3 wPt-8000 wPt-3150
wPt-9196 wPt-2331
wPt-6502 wPt-7821
58.0 wPt-6440 wPt-5003
wPt-3349 wPt-7168
wPt-4241 wPt-5212
59.8 wPt-5687
72.0 wPt-6429
81.5 wPt-3655
85.1 wPt-3810
105.7 wPt-7355
106.4 wPt-3435
141.3 wmc420
143.0 wPt-9251 wPt-3398
144.5 wPt-9738
145.2 wPt-9445
QRlnt.sk-4A
4B
0.0 gwm368
14.2 wPt-3908 tPt-0602
wPt-6209 wPt-1505
18.7 wPt-9067 tPt-4125
wPt-2214 wPt-6016
wPt-7062
23.0 wPt-7569
39.7 wPt-2649
47.7 rPt-1528
48.6 wPt-0382 wPt-4243
50.3 tPt-4214
52.1 wPt-3917
62.7 wPt-4962 wPt-5338
wPt-1032 wPt-1115
4D
0.0 wPt-4572
87.4 wPt-5512
P. thornei resistance QTL inherited from Sokoll
P. thornei resistance QTL inherited from Krichauff


5A
0.0 barc10
18.3 wPt-4837
24.5 wPt-8465 wPt-2768
31.5 wPt-5787
53.3 wPt-4131
69.8 wPt-0605 wPt-2826
72.5 wPt-1954
73.4 wPt-2599
77.1 wPt-0270 tPt-3642
wPt-7185
96.7 barc165
104.1 barc360
116.4 wPt-5588
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126.1 wPt-8226
127.7 wPt-1422
150.5 wPt-7201
183.5 wPt-1370
189.3 gwm271
216.8 VrnA
QRlnt.sk-5A
5B
0.0 barc128c
10.5 wmc477
22.2 barc128b
33.7 barc128a
48.6 wPt-8604 wPt-0819
tPt-0228 wPt-0419
54.5 wPt-1589 wPt-8132
wPt-2041 wPt-5737
56.1 wPt-1951 wPt-4677
tPt-4875 wPt-2539
wPt-8614 wPt-1457
wPt-7848 wPt-3289
58.3 wPt-2586 wPt-3026
59.1 wPt-4736 tPt-8942
wPt-4703
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wPt-4996 wPt-5688
66.6 wPt-5851 wPt-7167
67.4 wPt-5514 wPt-8069
123.0 cfd70d
135.6 wPt-2037 wPt-6191
wPt-4577 wPt-2707
138.0 wPt-3030 wPt-1733
wPt-5896
146.4 VrnB
167.2 wPt-1881
167.9 wPt-0935 wPt-9205
wPt-8094
168.7 wPt-9598 wPt-1482
wPt-9103
169.5 wPt-3204 wPt-7059
wPt-5859
172.1 wPt-9233
183.9 wPt-9504 wPt-4448
wPt-9782 wPt-1030
185.4 wPt-5827 wPt-3213
wPt-7006
198.2 wPt-8449 wPt-1348
wPt-0334
202.5 wPt-4817 wPt-3922
wPt-0484 wPt-3569
wPt-3995 wPt-6465
wPt-9116 wPt-7665
wPt-6971
215.4 wPt-4986
QRlnt.sk-5B.2
QRlnt.sk-5B.1
5D
0.0 wPt-9543
6.5 wPt-1210
34.2 wPt-7323
71.3 wPt-2256
112.4 VrnD
146.6 gpw320
6A
0.0 wPt-5395 wPt-0832
wPt-1742 wPt-4012
wPt-4270
16.5 wPt-9679 tPt-2833
17.2 rPt-9065 wPt-8719
wPt-5652 wPt-3524
wPt-0689
22.6 wPt-9692 wPt-2563
wPt-9113 wPt-2573
wPt-9690 wPt-0959
32.1 wPt-0139
cfd1
40.1 gpw1034b
49.8 gpw3087a
86.5 wPt-2142 wPt-6062
106.2 wmc179b
144.1 wPt-2632 wPt-7591
wPt-5654 wPt-9976
wPt-2582 wPt-8124
tPt-6661 wPt-8944
wPt-4145 wPt-0502
144.9 wPt-9474
wPt-6696
157.2 wPt-7572
6B
0.0 wPt-9015 wPt-4720
wPt-7150 wPt-3130
wPt-9990 wPt-1922
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wPt-8239 wPt-0452
wPt-9532 wPt-4678
wPt-8894 wPt-7582
wPt-2964 wPt-6282
wPt-3800 wPt-0882
wPt-6585 wPt-7662
wPt-1547 wPt-0151
wPt-0991 wPt-0245
wPt-6127 wPt-7203
wPt-6994 wPt-1852
wPt-2689 wPt-3116
wPt-3304 wPt-1474
wPt-1756
1.5 wPt-7954 wPt-4706
2.2 wPt-2095 wPt-1437
4.3 wPt-6594 wPt-3526
15.7 wPt-6667 wPt-6674
18.2 wPt-1830
25.8 tPt-4887 wPt-7576
33.9 cfd13
34.8 tPt-3506 wPt-4858
36.7 wPt-7424
42.4 wPt-2297 wPt-0470
46.8 wPt-4742
49.3 wPt-9195
52.3 wPt-2587 wPt-3389
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64.6 wPt-9659
67.9 gwm518
73.6 wPt-8728
76.7 wPt-3666 wPt-3402
77.5 wPt-9784 wPt-3733
wPt-7489
90.0 wPt-4648 wPt-3060
wPt-7935 rPt-1040
wPt-2564
gwm133
108.5 wPt-3168
111.2 tPt-4990
121.6 wPt-3284
QRlnt.sk-6B
P. thornei resistance QTL inherited from Sokoll
P. thornei resistance QTL inherited from Krichauff
174.0 wPt-5480 wPt-1761
wPt-0696 wPt-9241
tPt-9048
174.7 wPt-1541 wPt-9256
179.3 rPt-1231
181.2 wPt-6116 wPt-4164
182.0 wPt-9952 wPt-8268
191.9 wPt-7644

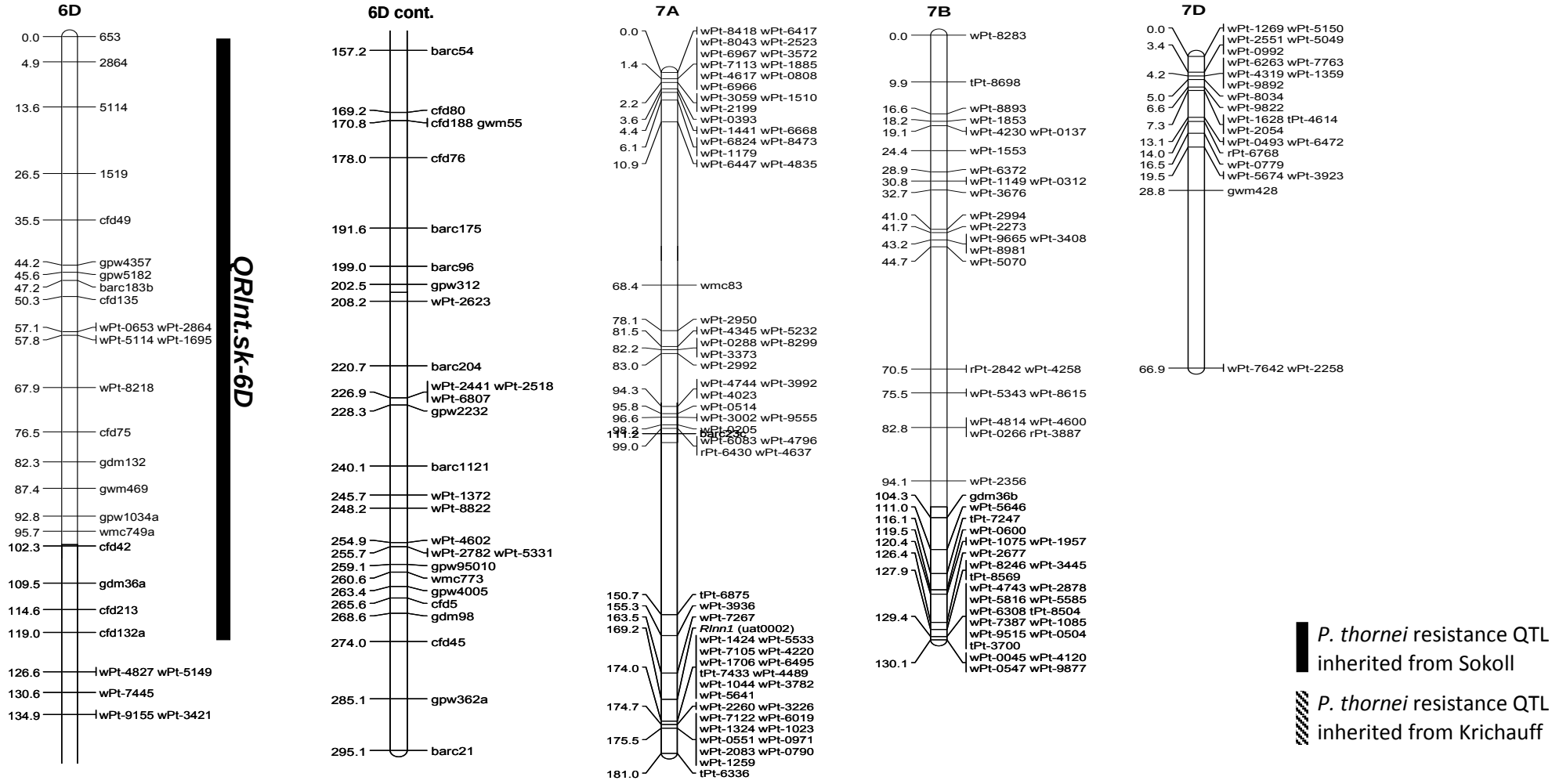


Fig. 3 Genetic map of Sokoll x Krichauff DH population. The map comprises 974 markers arranged across 21 linkage groups. The map covers a total size of 3477 centiMorgans (cM) of the wheat genome with an average density of 3.56 cM. Highly significant QTL for *Pratylenchus thornei* resistance are indicated on chromosomes 2B (*QRlnt.sk-2B.1*) and 6D (*QRlnt.sk-6D*). Significant QTL for *P. thornei* resistance were identified on chromosomes 4A (*QRlnt.sk-4A*) and 5A (*QRlnt.sk-5A*). After fixing the population for *QRlnt.sk-2B.1* and *QRlnt.sk-6D*, significant QTL for *P. thornei* resistance were further revealed with *QRlnt.sk-2B.2*, *QRlnt.sk-2B.*3, *QRlnt.sk-2D*, *QRlnt.sk-3B*, *QRlnt.sk-5B.1* and *QRlnt.sk-6B*

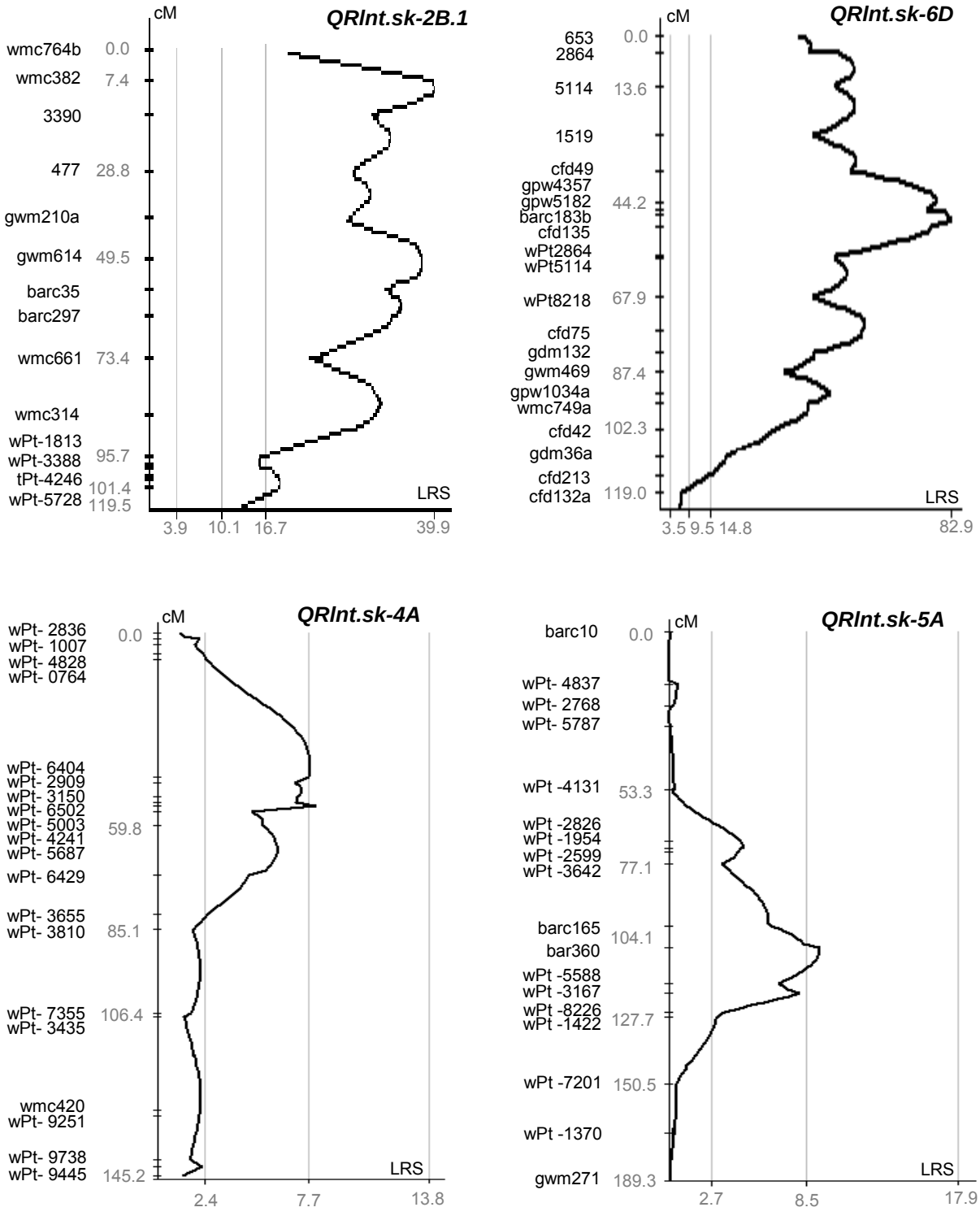
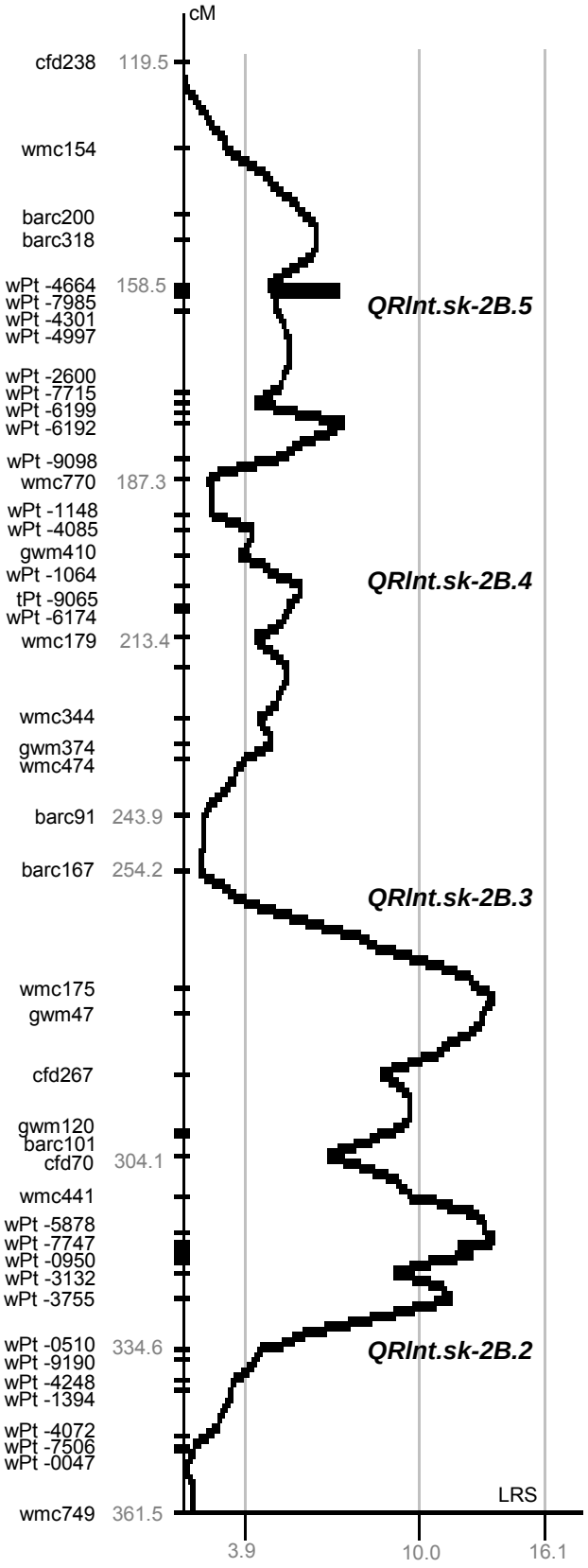


Fig. 4 Composite interval mapping showing QTL for resistance to *Pratylenchus thornei* in the Sokoll x Krichauff population. Highly significant QTL were identified on the short arms of chromosomes 2B and 6D and significant QTL on chromosomes 4A and 5A. The numbers on the x-axis represent the threshold lines as set by 1000 permutations shown as likelihood ratio statistics (LRS). The first line represents suggestive QTL at $P < 0.05$ the second, significant QTL at $P < 0.01$ and the third, highly significant QTL at $P < 0.001$. The y-axis represents distances in centiMorgans (cM) from the distal end of the short arms of the chromosomes. These figures represent only the chromosome region which contains the QTL


cM
cfd238 119.5
wmc154
barc200
barc318
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wPt -7985
wPt -4301
wPt -4997
wPt -2600
wPt -7715
wPt -6199
wPt -6192
wPt -9098
wmc770 187.3
wPt -1148
wPt -4085
gwm410
wPt -1064
tPt -9065
wPt -6174
wmc179 213.4
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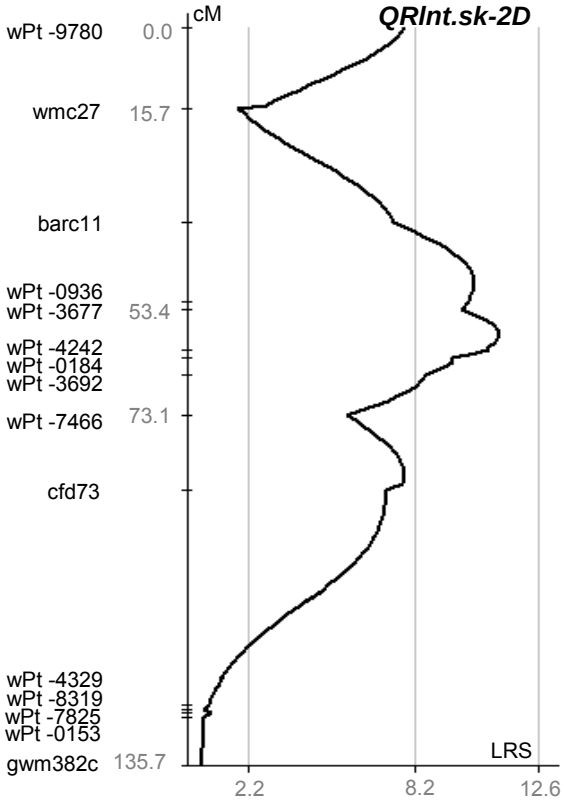
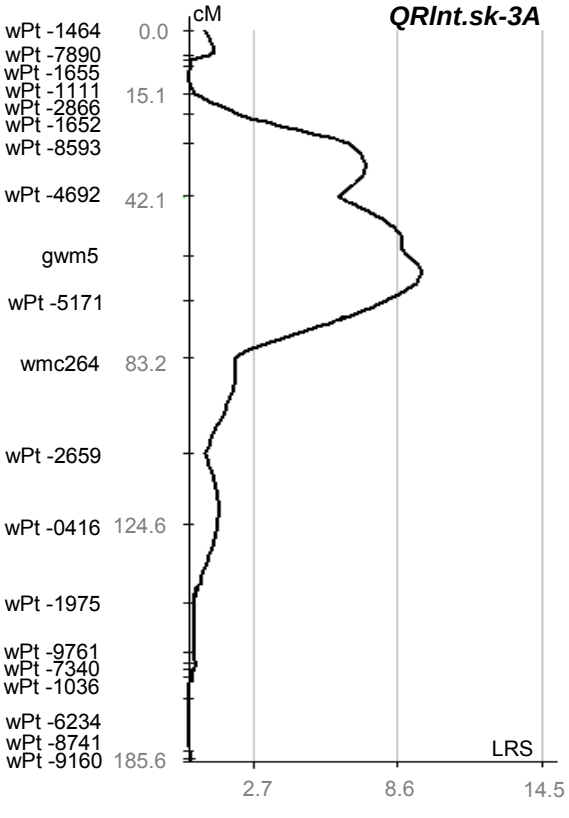
gwm374
wmc474
barc91 243.9
barc167 254.2
wmc175
gwm47
cfd267
gwm120
barc101
cfd70 304.1
wmc441
wPt -5878
wPt -7747
wPt -0950
wPt -3132
wPt -3755
wPt -0510 334.6
wPt -9190
wPt -4248
wPt -1394
wPt -4072
wPt -7506
wPt -0047
wmc749 361.5
QRInt.sk-2B.5
QRInt.sk-2B.4
QRInt.sk-2B.3
QRInt.sk-2B.2
LRS
3.9
10.0
16.1


cM
QRInt.sk-2D
wPt -9780 0.0
wmc27 15.7
barc11
wPt -0936
wPt -3677 53.4
wPt -4242
wPt -0184
wPt -3692
wPt -7466 73.1
cfd73
wPt -4329
wPt -8319
wPt -7825
wPt -0153
gwm382c 135.7
LRS
2.2
8.2
12.6


cM
QRInt.sk-3A
wPt -1464 0.0
wPt -7890
wPt -1655
wPt -1111 15.1
wPt -2866
wPt -1652
wPt -8593
wPt -4692 42.1
gwm5
wPt -5171
wmc264 83.2
wPt -2659
wPt -0416 124.6
wPt -1975
wPt -9761
wPt -7340
wPt -1036
wPt -6234
wPt -8741
wPt -9160 185.6
LRS
2.7
8.6
14.5

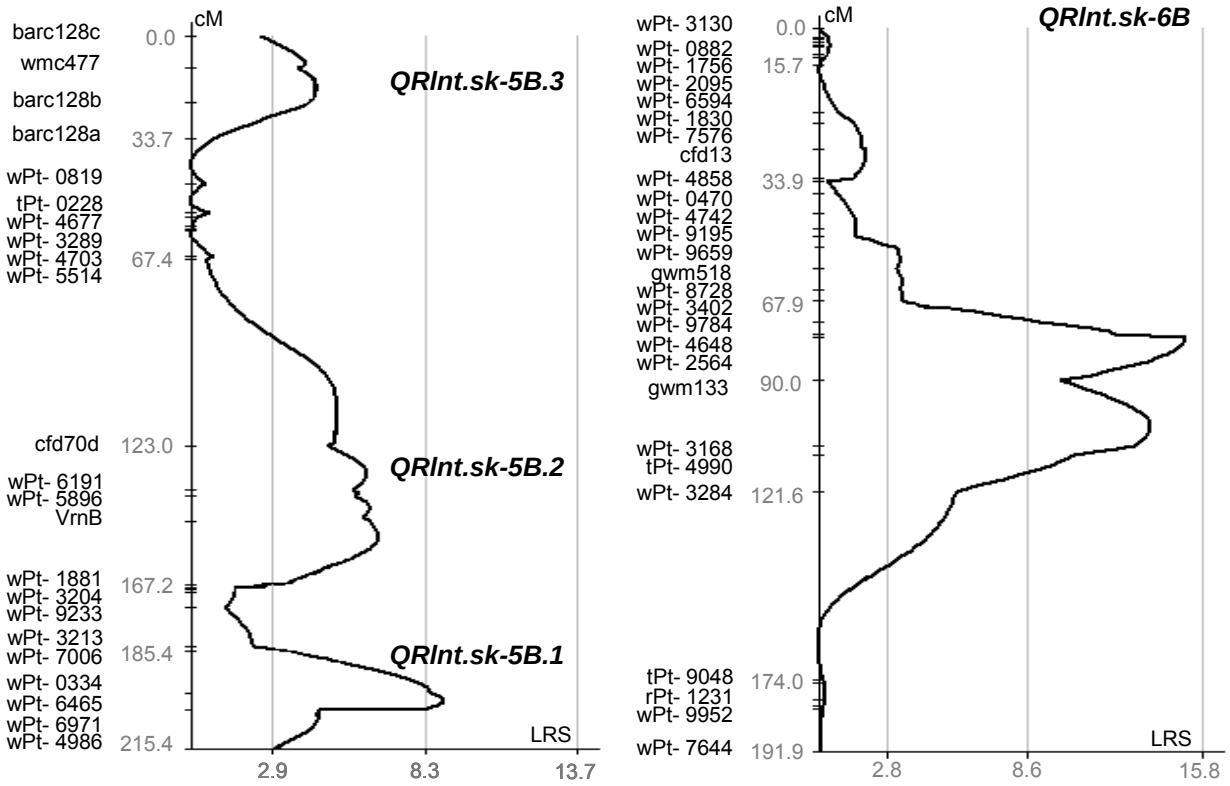


Fig. 5 Composite interval mapping showing QTL for resistance to *Pratylenchus thornei* in the Sokoll x Krichauff population after fixing the 2BS and 6DS QTL. Significant QTL were identified on chromosomes 2B, 2D, 3A, 5B and 6B and suggestive QTL on 2B and 5B. The numbers on the x-axis represent the threshold lines as set by 1000 permutation tests shown as likelihood ratio statistics (LRS). The first line represents suggestive QTL at $P < 0.05$, the second, significant QTL at $P < 0.01$ and the third, highly significant QTL at $P < 0.001$. The y-axis represents distances in centiMorgans (cM) from the distal end of the short arms of the chromosomes

Marker Regression and QTL Analysis

QTL analysis revealed 75 markers with linkage to *P. thornei* resistance. Nine chromosome regions were identified with markers associated to *P. thornei* resistance located on chromosomes 1A, 2B, 3A, 4A, 4B, 5A, 6B, 6D and 7A. Markers on the two chromosomes 4A and 5A were significantly linked to resistance, while markers on chromosomes 2B and 6D showed highly significant linkage to *P. thornei* resistance.

Highly significant QTL associations were detected on the short arms of two chromosomes and in both cases the marker allele was inherited from the synthetic-derived parent, Sokoll (Fig. 4). A resistance QTL was identified on chromosome 6DS (*QRInt.sk-6D*) linked to the distal markers *gpw5182*, *gpw4357* and *barc183b* (likelihood ratio statistic (LRS) = 82.9) explaining 43% of the phenotypic variation for *P. thornei* resistance. The QTL spans 119 cM within the 295 cM 6D chromosome and is flanked by the SSR *cfd132a* and the converted DArT marker *653* (Fig. 4). A second highly significant QTL for resistance to *P. thornei* was detected on chromosome 2BS (*QRInt.sk-2B.1*) linked to *wmc382* and *gwm614* (LRS = 39.9), explaining 24% of the phenotypic variation. The QTL spans 101 cM within the 409 cM 2B

chromosome and is flanked by the SSR *wmc764b* and the converted DArT marker *5728* (Fig. 4). Two significant QTL for resistance were identified to be contributed by the more susceptible parent, Krichauff, on chromosomes 4A and 5A. The *QRlnt.sk-4A* was most closely linked to the marker *wPt-6502* (LRS = 8.2) explaining 6% of the phenotype and spanning 59.8 cM of the 145.2 cM chromosome. The *QRlnt.sk-5A* was most closely linked to the marker *wPt-5588* (LRS = 9.1) also explaining 6% of the phenotype and spanning 97.2 cM of the 189.3 cM chromosome (Fig. 4). The *QRlnt.sk-5A* does not include the *Vrn1A* marker in this map.

The *Rlnn1* marker *uat0001* was mapped to 7AL, where the resistance locus to *P. neglectus* was originally mapped (Williams et al. 2002). The *Rlnn1* QTL, donated by Krichauff, did not contribute to improved *P. thornei* resistance. A suggestive QTL was detected on 7A (LRS = 5.9) contributed by Sokoll but was not located near the *Rlnn1* corresponding chromosome region on 7AL.

As a result of fixing the population for the 2BS and 6DS, single marker regression identified 100 markers with linkage to *P. thornei* resistance. Fifteen chromosome regions were identified with markers associated to *P. thornei* resistance located on chromosomes 1B, 2A, 2B, 2D, 3A, 3D, 4A, 5A, 5B, 5D, 6B, 6D, 7A, 7B and 7D. Markers on the five chromosomes, 2B, 2D, 3A, 5B and 6B were significantly linked to resistance. Marker regression did not identify resistance associations to the *Vrn1A*, *Vrn1B* or *Vrn1D* markers on chromosomes 5A, 5B and 5D.

QTL analysis on the population fixed for the highly significant *QRlnt.sk-6D* and *QRlnt.sk*-2B, identified an additional six significant and four suggestive QTL associated with *P. thornei* resistance on 5 different chromosomes (Fig. 5). Two significant QTL on chromosome 2B with *QRlnt.sk-2B.2* linked to *wPt-0950* (LRS = 11.3) and *wPt-9736* (LRS = 11) and *QRlnt.sk-2B.3* linked to *gwm47* (LRS = 10.1) with both explaining 2% of the phenotypic variation and spanning 50 cM. Both *QRlnt.sk-2B.2* and *QRlnt.sk-2B.3* were inherited from Krichauff. A significant QTL on 2D linked to *wPt-0184* (LRS = 11.1) explaining another 2% of the phenotypic variation and spanning 57 cM was also inherited from Krichauff. A significant QTL on 3A, *QRlnt.sk-3A*, linked to *wPt-5171* (LRS = 8.6) explaining 2% phenotypic variation and spanning 68 cM and a significant QTL on 6B linked to markers *wPt-4648* (LRS = 15.8) and *wPt-2564* (LRS = 15) explaining 4% phenotypic variation and spanning 60 cM were inherited from Sokoll. A significant QTL inherited from Sokoll was also identified on chromosome 5B, *QRlnt.sk-5B.1*, linked to *wPt-0334* (LRS = 9.7) explaining 2% phenotypic variation and spanning 32 cM (Fig. 5).

Two suggestive QTL on 2B, *QRlnt.sk-2B.4 and QRlnt.sk-2B.5* were both inherited from Krichauff and span 50 cM and 53 cM respectively. *QRlnt.sk-2B.4* is linked to *tPt-9065* (LRS = 6.2) and *QRlnt.sk-2B.5* to *wPt-4997* (LRS = 7.2) and *wPt-9098* (LRS = 7.2) explaining 1% and 2% of the phenotypic variation, respectively. Two suggestive QTL were also identified on chromosome 5B. *QRlnt.sk-5B.2* is linked to *wPt-5896* (LRS = 7.3) and *QRlnt.sk-5B.3* to *barc128b* (LRS = 4.2) explaining 2% and 1% of phenotypic variation, respectively (Fig. 5). A summary of identified *P. thornei* resistance QTL and their closely linked and flanking markers is summarised in Table 1.

Table 1 QTL associated with *Pratylenchus thornei* resistance identified in the Sokoll x Krichauff population. The donor parent (Inherited Origin), the chromosomal location of QTL, the species (*Pt – P. thornei*), flanking and most closely linked markers, percentage of phenotypic variation (% Var) explained, likelihood ratio statistic (LRS) and QTL width (cM) is presented

QTL	Inherited Origin	Chromosome	Species	Flanking Markers	Linked Markers	% Var	LRS	QTL Width (cM)
QRlnt.sk-6D	Sokoll	6DS	*Pt*	*653* *gpw5182* *cfd132*	*gpw5182* *gpw4357* *barc183*	43	82.9	119.0
QRlnt.sk-2B.1	Sokoll	2BS	*Pt*	*wmc764* *wPt-5728*	*wmc382* *gwm614*	24	39.9	101.4
QRlnt.sk-4A	Krichauff	4A	*Pt*	*wPt-2836* *wPt-3810*	*wPt-6502*	6	8.2	59.8
QRlnt.sk-5A	Krichauff	5A	*Pt*	*wPt-4131* *wPt-3187* *wPt-1422*	*wPt-5588*	6	9.1	97.2
*QRlnt.sk-7A**	Sokoll	7A	*Pt*	*wPt-3992*	*wPt-3992*	4	5.9	-
QRlnt.sk-2B.2[f]	Krichauff	2B	*Pt*	*cfd70* *wPt-0047*	*wPt-0950* *wPt-9736*	2 2	11.3 11.0	47.6
QRlnt.sk-2B.3[f]	Krichauff	2B	*Pt*	*barc167* *cfd70*	*gwm47* *wmc175*	2 2	10.1 9.3	49.9
*QRlnt.sk-2B.4**[f]	Krichauff	2B	*Pt*	*wPt-1148* *barc91*	*tpt-9065*	1	6.2	50.1
*QRlnt.sk-2B.5**[f]	Krichauff	2B	*Pt*	*cfd238* *wmc770*	*wPt-4997* *wPt-9098*	2 2	7.2 7.2	53.2
QRlnt.sk-2D[f]	Krichauff	2D	*Pt*	*wmc27* *wPt-7466*	*wPt-0184*	2	11.1	57.4
QRlnt.sk-3A[f]	Sokoll	3A	*Pt*	*wPt-2866* *wmc364*	*wPt-5171*	2	8.6	68.1
QRlnt.sk-5B.1[f]	Sokoll	5B	*Pt*	*wPt-7006* *wPt-4986*	*wPt-0334*	2	9.7	44.9
*QRlnt.sk-5B.2**[f]	Sokoll	5B	*Pt*	*cfd70* *wPt-1881*	*wPt-5896*	2	7.3	32.0
*QRlnt.sk-5B.3**[f]	Sokoll	5B	*Pt*	*barc128* *wmc477*	*barc128*	1	4.4	33.7
QRlnt.sk-6B[f]	Sokoll	6B	*Pt*	*wPt-3402* *wPt-3284*	*wPt-4648* *wPt-2564*	4 4	15.8 15.0	59.7

* Below significance threshold
[f] Fixed for 2Bs and 6DS

Table 2 Previously mapped *Pratylenchus* resistance QTL in wheat. The table shows the donor parent (Inherited Origin), the chromosomal location of the resistance QTL (Chromosome/QTL), the *Pratylenchus* species (*Pt* - *Pratylenchus thornei* or *Pn* -*Pratylenchus neglectus*), the flanking markers, the percentage of phenotypic variation (% Var) explained by the QTL and the likelihood ratio statistic (LRS)

Reference	Inherited Origin	Chromosome/QTL	Species	Flanking Markers	% Var	LRS
Thompson et al. 1999 & Vicars et al. 1999	GS50a	6D	*Pt*	np	np	np
Williams et al. 2002	Excalibur	7A	*Pn*	*AGC/CCT179* *cdo347* *psr121* *psr680* *schfc3*	8	20.8
Zwart et al. 2005	CPI133872	*QRlnt.lrc-6D.1*	*Pt*	*barc183* *barc173*	22-24	23.9-42.5
	Janz	*QRlnn.lrc-6D.1*	*Pn*	*barc183* *barc173*	11-14	17.3-17.8
	CPI133872	*QRlnt.lrc-2B.1**	*Pt*	*wmc25* *wmc154*	7	15.2
	CPI133872	*QRlnn.lrc-2B.1**	*Pn*	*wmc25* *wmc154*	7-11	9.4-12.4
	CPI133872	*QRlnt.lrc-6D.2**	*Pt*	*gdm98* *gpw95010* *barc21*	8-13	16.5-24.6
	CPI133872	*QRlnt.lrc-6A.1**	*Pt*	*psp3029* *gwm459*	9	11.3-11.7
	CPI133872	*QRlnt.lrc-3D.1**	*Pn*	*gwm161* *gwm183* *gwm664*	11	12.6
	CPI133872	*QRlnt.lrc-4B.1**	*Pn*	*gwm66* *wmc47*	10	17.2
	Janz	*QRlnt.lrc-4D.1*	*Pn*	*wmc52* *wmc331* *barc98*	10-15	11.6-15.8
Schmidt et al. 2005	AUS13124	2B	*Pt*	*gwm319* *gwm494.2* *ACT/CTC.1* *gwm191.2*	6-13	9.4-13.7
	AUS13124	6D*	*Pt*	*gwm469* *gdm36* *gwm518* *gdm132*	4-6	4.9-9.3
	AUS13124	3B*	*Pt*	*gwm133* *gwm340.2*	7-24	5.2-20.1
	AUS13124	1B*	*Pt*	*gwm153.1* *gwm153.2*	3-6	3.3-3.6
	AUS4926	3B	*Pt*	*gwm112.2* *gwm66.1* *gwm213.2* *gwm133.1* *AGC/CAT.1*	36	10.3
	AUS4926	2B*	*Pt*	*wmc25.5* *wmc25.4* *gwm428*	2-3	2.7-3.4
	AUS4926	6D*	*Pt*	*gdm98.1* *gdm98.2* *gdm132*	2-2	2.6-2.7

Table 2 Continued

Reference	Inherited Origin	Chromosome/QTL	Species	Flanking Markers	% Var	LRS
Zwart et al. 2006	W-7984	6DS	*Pt*	*psr964* *psr889* *barc183*	11-23	4.4-8.5
	W-7984	2BS	*Pt*	*cdo447* *bcd348* *gwm210*	19-5	2.0-7.4
Zwart et al. 2010	CPI133872	6DS	*Pt*	*barc183* *cfd49* *cfd135*	18-27	28.1-46.5
	Janz	6DS	*Pn*	*barc183* *cfd49* *cfd135*	9	10.6-17.9
	CPI133872	6DL	*Pt*	*gdm98* *gpw95010* *barc21*	6-15	11.0-23.5
	CPI133872	2B	*Pt*	*wPt-2410* *wPt-6706* *wPt-6311* *wPt-8737*	13-22	19.8-36.3
	CPI133872	2B	*Pn*	*wPt-2410* *wPt-6706* *wPt-6311* *wPt-8737*	11-16	12.4-27.6
	CPI133872	3D	*Pn*	*gwm2* *gwm664* *gwm314*	8	15.6
	CPI133872	4B	*Pn*	*gwm368* *gwm66* *wmc47*	13	25.3
	CPI133872	4D	*Pn*	*wPt-5809* *wPt-431*	13	24.4

np - not provided

* - below the significance threshold

Table 3 Wheat populations with previously mapped *Pratylenchus* resistance QTL. The parents of the mapping population (Population), its size and the number and type of markers are shown. The genomic coverage of each map and the map resolution (average marker density as markers per cM) is provided

Reference	Population	Size	Number of Markers	Type of Markers	Map coverage (cM)	Marker Density (cM/marker)
Schmidt et al. 2005	AUS13124 x Janz	126 DH	114	108 Microsatellite 6 AFLP	1987	17.0
	AUS4926 x Janz	126 DH	148	135 Microsatellite 13 AFLP	3229	22.0
Zwart et al. 2005	CPI133872 x Janz	100 DH	169	148 Microsatellite 21 AFLP	2570	15.9
Zwart et al. 2006	(ITMI) W-7984 x Opata85	150 RIL	537	Microsatellite RFLP	np	np
Zwart et al. 2010	CPI133872 x Janz	111 DH	384	242 DArT 125 Microsatellite 17 AFLP	1521	3.9
Linsell et al. 2011c	Sokoll x Krichauff	150 DH	971	860 DArT 111 Microsatellite	3477	3.6

np - not provided

Table 4 Common markers linked to *QRlnt.sk-6D* and *QRlnt.sk-2B* and other wheat pathogen resistance genes. The table shows the disease and its causal pathogen, the resistance gene, the associated chromosome (Chr) and the most closely linked molecular markers

***P. thornei* Resistance QTL**	**Disease**	**Pathogen**	**Gene**	**Chr**	**Linked Markers**	**Reference**
QRlnt.sk-6D	Hessian Fly	*Mayetiola destructor*	*H13*	6DS	*cfd132*	Liu et al. 2005
					gdm36	Gill et al. 1987
	Hessian Fly	*Mayetiola destructor*	*H23*		*ksuG48*	Ma et al. 1993
	Septoria tritici Blotch	*Mycosphaerella graminicola* (Anamorph: *Septoria tritici*)	*Stb3*	6DS	*gdm132*	Adhikari et al. 2004
	Wheat Curl Mite	*Aceria tosichella*	*Cmc*	6DS	*ksuG48*	Malik et al. 2003
					gdm141	
	Stem Rust	*Puccinia graminis*	*SrCad*	6DS	*cfd49*	Hiebert et al. 2011
			Sr42	6DS	*na*	McIntosh et al. 1995
			Sr5	6DS	*barc183*	Prins et al. 2011,
					wpPt-3879	Sears 1957
QRlnt.sk-2B.1	Stem Rust	*Puccinia graminis*	*Sr40*	2BS	*wmc661*	Wu et al. 2009
			Sr23	2BS	*wmc764*	McCartney et al.
					gwm210	2005
					gwm614	
					wmc489	
					barc35	
	Leaf Rust	*Puccinia triticina*	*Lr16*	2BS	*wmc661*	McCartney et al.
					wmc764	2005
					gwm210	
			Lr35/Sr39	2BS	*na*	Gold et al. 1999
	Common Bunt	*Tilletia tritici*	*Bt10*	2BS	*FSD_RSA*	Menzies et al. 2006
						Laroche et al. 2000
	Orange Wheat Blossom Midge	*Sitodiplosis mosellana*	*Sm1*	2BS	*gwm210*	Thomas et al. 2005
					barc35	
QRlnt.sk-2B.3	Leaf Rust	*Puccinia triticina*	*Lr50*	2B	*gwm382*	Brown-Guedira et
					gdm87	al. 2003
	Stem Rust	*Puccinia graminis*	*Sr36*	2B	*gwm319*	Tsilo et al. 2008
					wmc477	
					gwm429	
	Stem Rust/	*Puccinia graminis/*	*Sr28*	2B	*na*	McIntosh 1978
	Stripe Rust	*Puccinia striiformis*	*Sr16*	2B	*na*	McIntosh 1978
			Sr9/Yr5/Yr7	2B	*gwm47*	Tsilo et al. 2007
					gwm120	
					barc101	
					wmc175	
	Stripe Rust	*Puccinia striiformis*	*QYrlu.cau-2BS2*	2B	*wmc148*	Smith et al. 2007
					barc167	
			QYrac.cau-2BL	2B	*wmc175*	Smith et al. 2007
					wmc332	
	Stagonospora nodorum blotch	*Stagonospora nodorum*	*QTL*	2B	*gwm120*	Czembor et al. 2003

Table 4 Continued

***P. thornei* Resistance QTL**	**Disease**	**Pathogen**	**Gene**	**Chr**	**Linked Markers**	**Reference**
QRlnt.sk-2B.3	Black Point	*Stagonospora nodorum*	*QTL*	2B	*gwm319 gwm271 gwm501*	Lehmensick et al. 2004
	Powdery Mildew	*Blumeria graminis*	*Pm6*	2B	*bcd135 bcd266 bcd301*	Tao et al. 2000
			QPm.vt-2B/ QPm.inra.2B	2B	*gwm47 cfd267*	Liu et al. 2001, Bougot et al. 2006
QRlnt.sk-2B.5	Stripe Rust	*Puccinia striiformis*	*QYrlu.cau-2BS1*	2B	*wmc154 barc200*	Smith et al. 2007
QRlnt.sk-2B.4	Stripe Rust	*Puccinia striiformis*	*Yr27/Yr31*	2B	*cdo405*	McDonald et al. 2004
			Yr41	2B	*gwm410 gwm374*	Lou et al. 2008
			Qyr.sgi-2B	2B	*gwm148*	Ramburan et al. 2004
			Qyr.ipk-2B	2B	*cdo405 bcd152*	Boukhatem et al. 2002
			Qyr.inra-2B	2B	*gwm148*	Mallard et al. 2005
	Leaf /Stem Rust	*Puccinia triticina/ Puccinia graminis*	*Lr23/Lr13/Sr19*	2B	*tam72/ gwm630*	Nelson et al. 1997, Seyfarth et al. 2000
	Stem Rust	*Puccinia graminis*	*Sr36*	2B	*gwm271 stm773 gwm148 barc55*	Parker 1998
	Powdery Mildew	*Blumeria graminis*	*Pm42*	2B	*gwm148 wmc154*	Hua et al. 2009

Discussion

The 6D and 2B resistance loci are widespread in both Middle Eastern landraces and synthetic hexaploid wheats, as in six out of the seven previous *P. thornei* resistance mapping studies, significant resistance QTL were identified on chromosome 6D and in four studies also on chromosome 2B (Schmidt et al. 2005; Thompson et al. 1999; Zwart et al. 2005; Zwart et al. 2010; Zwart et al. 2006). Two highly significant *P. thornei* QTL identified in this study co-located to the same 2BS and 6DS chromosomal regions previously linked to *P. thornei* resistance. This validates their robustness as useful sources of resistance in different germplasm but also indicates a possible common biological/biochemical resistance mechanism. The sizes of these QTL are currently quite large (40 to 60 cM) and this needs to be considered when comparing QTL locations.

Through the comparison of common markers in the different published genetic maps, the 6DS QTL identified in this study, *QRlnt.sk-6D*, appears to map to the same location as identified by Zwart et al. (2005; 2006; 2010) and Schmidt et al. (2005), as one of the most closely linked markers in this study, *barc183*, was also significantly linked to *P. thornei* resistance (Table 1). The 6DS QTL identified by Zwart et al. (2006) in the synthetic hexaploid International Triticeae Mapping Initiative (ITMI) population, derived from a cross between the synthetic W-7984 and the spring wheat Opata 85, resides near RFLP *bcd1821* (Table 2) and SSR *barc183b* (Zwart et al. 2006), and thus appears to coincide with *QRlnt.sk-6D*. The major *P. thornei* resistance QTL on 6DS identified by Zwart et al. (2005; 2010) was also linked to *barc183*, in the synthetic hexaploid wheat population CPI133872 crossed with the susceptible Australian bread wheat cultivar Janz. This QTL was also associated with *P. neglectus* resistance, where *barc183* was inherited from Janz (Table 2). A link between this marker locus and *P. neglectus* resistance was not detected in the Sokoll x Krichauff population. In addition, the segregating *Rlnn1* locus did not contribute to *P. thornei* resistance. These findings imply that the *P. neglectus* resistance allele or gene near *barc183* found in CPI133872 x Janz (Zwart et al. 2005) is different to the resistance gene present in Sokoll or Krichauff. Furthermore, the resistance mechanism controlled by *Rlnn1* must be different to the *barc183*-linked resistance gene as *Rlnn1* appears to have no contribution to *P. thornei* resistance in our study. Zwart et al. (2005) reported a second *P. thornei* resistance QTL on the long arm of 6D that explained up to 14% of the phenotypic variation (Table 2), although, the corresponding region in the Sokoll x Krichauff population was not associated to *P. thornei* resistance.

Schmidt et al. (2005) also identified *P. thornei* resistance QTL in the same location as the *QRlnt.sk-6D* identified in this study, when investigating two Middle Eastern landraces with

superior *P. thornei* resistance, AUS13124 and AUS4926 (Seymour and Thompson 2001) crossed with Janz (Schmidt et al. 2005). The map generated with less than 150 AFLP and SSR markers identified a 6D QTL but it did not pass the significance threshold in either population. However, its presence was confirmed using single marker regression with SSR markers *gwm469*, *gwm518* and *gdm132* all showing significant linkage (Table 2). In Sokoll x Krichauff, the markers *gwm469* and *gdm132* were closely linked to the *QRlnt.sk-6D* QTL (Fig. 4). In addition, the screening of the Middle Eastern synthetic AUS4930 x Pastor (Toktay et al. 2006) also revealed the presence of a 6DS QTL in a similar region to the *QRlnt.sk-6D* in Sokoll x Krichauff as determined by linkage with marker *gwm469*. The microsatellite markers linked to resistance QTL identified in this study and in other genetic backgrounds highlights their usefulness for marker-assisted selection of *P. thornei* resistance.

All of these previously identified 6DS QTL regions explained less than 25% of phenotypic variation (Table 3), but in this current investigation the *QRlnt.sk-6D* explains nearly 50% of the variation. Multiple alleles of the same gene or different genes clustered at the *QRlnt.sk-6D* QTL locus may be associated with resistance in the different resistance sources. The presence of different alleles or genes at this locus and the small number of markers in earlier studies could explain the different level of phenotypic variations demonstrated by the apparent same locus.

Previous mapping studies have also identified significant *P. thornei* resistance QTL on 2BS from three different sources (Table 2). The location of the highly significant 2BS QTL, *QRlnt.sk-2B.1* identified in this study co-locates with the 2BS QTL identified by Zwart et al. (2006) when comparing the locations of common markers. In the synthetic W-7984 x Opata85 (ITMI) population, a 2BS QTL explaining up to 19% of the variation, enclosed SSR marker *gwm210* (Table 2), which in this investigation lies within the *QRlnt.sk-2B.1* QTL (Fig. 4). Toktay et al. (2006) also showed the presence of this 2BS QTL in the Middle Eastern synthetic AUS4930 x Pastor with linkage to *gwm614*.

Two significant QTL for *P. thornei* resistance were also identified on chromosomes 4A and 5A (Table 1) in this investigation. No previous studies have identified QTL on these chromosomes through QTL mapping. However, Zwart et al. (2005) and Schmidt et al. (2005) identified markers linked to 5A with single marker regression analysis. The marker *gwm304* associated with *P. thornei* resistance on 5A (Zwart et al. 2005) maps within 2 cM to both *barc360* and *barc165* (Appels 2004), which fall within the 5A QTL in this investigation. Schmidt et al. (2005) identified linkage to 5A through marker *gwm126*, which maps 4 cM from *barc360* (Song et al. 2005). This suggests previous studies also identified resistance associated with the 5A QTL identified here. A suggestive QTL, contributed by Sokoll, was

detected on 7A (LRS = 5.9). Zwart et al. (2005) and Schmidt et al. (2005) also identified markers linked to 7A through marker regression analysis. However, no comparisons could be drawn on chromosome location as there were no common markers.

Several QTL identified after fixing the population for the highly significant QTL on 2B and 6D also co-locate to previously identified *P. thornei* resistance QTL. The *QRlnt.sk-2B.3* inherited from Krichauff is linked to the *P. thornei* resistance QTL identified by Schmidt et al. (2005), from the landrace AUS13124, on chromosome 2B. The SSR markers linked to this QTL, *gwm191* and *gwm319* (Table 2), were not polymorphic when scored on Sokoll x Krichauff. However, the markers *barc167* and *gwm47*, positioned within *QRlnt.sk-2B.3*, (Fig. 5) reside 2 and 4 cM, respectively, from *gwm191* and *gwm319* (Somers et al. 2004). Although only suggestive in the Sokoll x Krichauff population, *QRlnt.sk-2B.5* appears to map in the same location as the two other 2BS QTL identified in CPI133872 (Zwart et al. 2005) and AUS4926 (Schmidt et al. 2005). The CPI133872 QTL on 2BS had resistance to both *P. thornei* and *P. neglectus* in the CPI133872 x Janz population, but it fell below the significance threshold. Single regression analysis showed that the SSR marker *wmc25* was significantly associated with *P. thornei* resistance and mapped to the QTL region (Zwart et al. 2004) (Table 2). Through single marker regression, Schmidt et al. (2005) also identified a 2BS QTL linked to *wmc25* from the Middle Eastern landrace AUS4926, but the QTL was not detected by interval mapping. The SSR marker *wmc25* in this investigation was polymorphic between the parents Sokoll and Krichauff but was not part of any linkage group. However, the *QRlnt.sk-2B.5* linked markers *wmc154*, *barc200* and *barc318* (Fig. 5) reside within a 2.5 cM interval of *wmc25* according to other published maps (Sourdille et al. 2004). Therefore, both *QRlnt.sk-2B.5* and *QRlnt.sk-2B.3* reside within 2BS regions previously identified as linked to *P. thornei* resistance. The *P. thornei* resistance QTL on chromosomes 2D, 3A, 5B and 6B in addition to the *QRlnt.sk-2B.2 and QRlnt.sk-2B.4* identified after fixing the population for the 2BS and 6DS QTL have not been previously reported. The identification of multiple QTL all contributing small amounts to the resistance phenotype further confirms the complexity of *Pratylenchus* resistance.

Diversity Array Technology has become the marker method of choice for constructing maps, due to the density of markers and the high data quality it generates. For example, the Sokoll x Krichauff map generated in this investigation using DArT gave greater genome coverage and marker density than compared to other maps constructed using more traditional marker systems (Table 3). However, marker density can only be increased if DNA polymorphisms exist between elite lines. For example, the analysis of a Berkut x Krichauff DH population for salinity tolerance using the wheat DArT array version 3.0. identified 311 DArT polymorphisms (Genc et al. 2010). In the investigation reported here, 889 polymorphisms were detected

using a similar array on Sokoll x Krichauff. The differences in the number of polymorphisms suggests a lack of genetic diversity in modern hexaploid wheats and the benefits of synthetics using crosses with wild wheat relatives to identify unique sources of resistance. Although DArT technology has helped increase the resolution of genetic maps, it is a dominant marker system relying on the presence or absence of a hybridisation signal, and thus cannot distinguish homozygous and heterozygous individuals. DArT markers tend to form clusters and thus are not evenly distributed across the genome which could potentially be an issue (Francki et al. 2009). In addition, before these markers can be utilised they need to be converted to user friendly PCR based markers which is not always successful and can be both timely and costly. DArT has the potential to overcome several restrictions through the development of codominant scoring by taking into account the strength of the signal for each DArT marker and through the generation of new algorithms (James et al. 2008). This improvement will make this technology more adaptable and informative.

Transgressive segregation refers to the presence of phenotypes that exceed those of either parental line. It is thought to result from combinations of alleles of both parents that provide complementary gene action. The inheritance of *P. thornei* resistance is conditioned by additive gene action, shown by Zwart et al. (2004) with crosses between the most resistant parents producing even more resistant progeny. In this study, the cross between Sokoll and Krichauff resulted in transgressive segregation in both directions with lines with greater and weaker resistance than either parent. Transgressive segregation is supported by the presence of other significant QTL - *QRlnt.sk-2B.*2, *QRlnt.sk-2B.3*, *QRlnt.sk-2B.4*, *QRlnt.sk-2B.5*, *QRlnt.sk*-2D, *QRlnt.sk-4A* and *QRlnt.sk-5A* from Krichauff - in addition to the highly significant 6DS and 2BS QTL. Due to the additive nature of *P. thornei* resistance, the 20 lines with superior resistance to Sokoll contain the 6D and 2B loci from Sokoll and/or the resistance loci from Krichauff.

Despite the development of DNA quantification methods which can more easily and reliably detect and quantify *Pratylenchus* within soil and roots, phenotypic resistance analysis remains cumbersome in comparison to genetic selection. For example, while there was good correlation between the *P. thornei* DNA means of each line between the two trials, there was large variation between the sample replicates themselves. This variability is most likely incurred due to the difficulties associated with *Pratylenchus* phenotyping/inoculation methods (Barloy et al. 2000; Proctor and Marks 1974; Williams et al. 2002). Variation was minimised through using five replicates for each line, the more objective DNA quantitative technique and through the use of permutation testing at significant thresholds. Resistance markers linked to the major 2B and 6D resistance QTL through marker assisted selection (MAS) eliminates the need to select progeny lines based on their phenotypes, enhancing resistant wheat breeding programs. MAS can be used to help identify resistant progeny lines when

this resistance is being introgressed into commercial cultivars through selective backcrossing but also helps identify new and valuable wild alleles in tested germplasm. MAS can also be applied in the selective pyramiding of several different *P. thornei* resistance genes. Minor *P. thornei* resistance QTL can also be useful for breeding if they are linked to and contribute to resistance and are stable across genotypes. Genotypic selection with closely linked markers is valuable as it can minimise the linkage drag effect, which prevents unfavourable alleles being introgressed along with the desired resistance.

To successfully implement MAS in a plant breeding program, the identified molecular markers need to be tightly linked, reliable across genotypes and cost-efficient. Currently, the QTL intervals of the *QRlnt.sk-2B.*1 and *QRlnt.sk-6D* span over 100 cM and thus require further refinement to establish more closely linked markers. This could be achieved through fine mapping a recombinant inbred line population which has already been established for Sokoll x Krichauff. Although these two QTL have been previously identified in different mapping populations, (Schmidt et al. 2005; Thompson et al. 1999; Toktay et al. 2006; Zwart et al. 2005; Zwart et al. 2010; Zwart et al. 2006), all phenotypic data has been generated from glasshouse trials. A subset of the Sokoll x Krichauff DH lines (ten lines) from each of the four genotypic groups based on whether they contained both resistant QTL (2B and 6D), only the 2B QTL, only the 6D QTL and lines with neither QTL were assessed in the field. The 40 lines were screened for *Pratylenchus* resistance in the field in 2010 at two sites in Australia (South Australia and Victoria). The mean amount of *P. thornei* DNA was quantified using the SARDI Root Disease Testing Service. All lines reduced *P. thornei* numbers below the initial soil population level but lines with both resistance QTL supported the least amount of nematode reproduction followed by the lines with 6D and 2B QTL only. All of these had significantly less reproduction than lines with neither QTL (A. Mckay, personal communication).Therefore, the resistance of Sokoll x Krichauff observed in the glasshouse trials is upheld in the field, further validating the usefulness of these identified resistant QTL.

Resistance genes have been shown to cluster (Williamson and Hussey 1996) and particularly at the ends of chromosomes in many plants (Li et al. 1999), which are gene rich regions (Gill et al. 1993). For example, the root knot nematode resistance gene *Mi1.2* and the potato cyst nematode resistance gene *HeroA* are found in clusters of seven and fourteen homologous copies, respectively. Both highly significant QTL in this study are located at the distal chromosome ends. The SSR markers which are linked to *QRlnt.sk-6D*, *cfd49*, *gpw4357*, *cfd135*, *gpw5182*, *gwm469*, *gpw1034*, *cfd213*, *gdm36* and *cfd132* have been physically mapped using deletion lines to the deletion bin 6DS-6-0.99-1.00 at the very end of 6D (Sourdille et al. 2004). This region is known to contain five putative resistance gene analogues (Dilbirligi et al. 2004). The SSR marker *gwm210* linked to *QRlnt.sk-2B.1* was

mapped to the 2BS-4-0.84-1.00 deletion bin (Sourdille et al. 2004), which contains eight putative resistance gene analogues (Dilbirligi et al. 2004).
Clustering of resistance genes (and linked markers) implies that they may share a common mechanism regarding their evolution (Kanazin et al. 1996) and thus the co-location of QTL/genes can identify plant pathways to help characterise the biological and biochemical resistance. The co-location of QTL for resistance to various pathogens may indicate that the locus has pleiotropic effects due to a common resistance mechanism or that multiple resistance genes are located at the same locus. Wheat resistance genes have been shown to cluster on chromosomes in the highly recombinant subtelomeric ends (Botella et al. 1998; Halterman et al. 2001; Meyers et al. 1999; Sakamoto et al. 1999) and thus many marker loci linked to known resistance genes for various plant pathogenic diseases have been mapped in the short arms of the 2B and 6D chromosomal regions as summarised in Table 4. For example, the *H13* gene controlling resistance to hessian fly of wheat is completely linked to *cfd132* and very closely linked to *gdm36* (Gill et al. 1987; Liu et al. 2005), which flank the *QRlnt.sk-6D* QTL in this study. The physical location of *H13* has been mapped proximal and close to the breakpoint of bin 6DS-6-0.99-1.00. The *H13* resistance is conferred by antibiosis where first stage larvae die after feeding on toxic compounds within resistant plants (El Bouhssini et al. 1999; Gill et al. 1987; Hatchett and Gill 1981; Hatchett et al. 1981; Ratcliffe and Hatchett 1997). This phenomenon is similar to the presence of toxic nematostatic compounds and hatching inhibitors linked to reduced *P. thornei* motility, moulting and hatching observed in resistant roots of the Sokoll x Krichauff population (Linsell et al. 2012b).

In addition, several other defense response and resistance genes including peroxidases and phenolic compounds also co-locate to the *QRlnt.sk-6D* and *QRlnt.sk-2B.1* QTL. The genes, *Ppo* for polyphenol oxidase and the *Rip*, ribosome inactivating protein map closely to RFLP *ksuG48a* (Li et al. 1999; Malik et al. 2003), which lies between *gdm141* and *gdm132* (Pestsova et al. 2000) that fall within the *QRlnt.sk-6D* QTL. Polyphenol oxidase catalyses the oxidation of polyphenols in wheat to quinines, through non-enzymatic oxidation and polymerization, to produce brown coloured melanins (Stauffer 1987). Increased production of peroxidases has been observed in resistant cultivars in response to various different *Pratylenchus* species and in some cases were associated with suppression of nematode feeding and migration (Acedo and Rhode 1971; Andres et al. 2001; Nithya et al. 2007). Increased production of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase has been associated with wheat resistance to the cyst nematode *Heterodera avenae* (Deepika et al. 2009). In addition, the gene *Sm1* that confers resistance to orange wheat blossom midge (Thomas et al. 2005) is associated with the marker locus *gwm210a* which falls within *QRlnt.sk-2B.1*. The *Sm1* resistance has been linked to increased levels of ferulic acid in the outer layer of the wheat grain to provide a physical barrier against insect invasion

through covalently binding to cell wall polysaccharides. Similarly, resistance to the migratory nematode, *R. similis* was associated with ferulic acid (Wuyts et al. 2007), acting as a physical mechanism to protect cell walls from nematode-secreted cell wall degrading enzymes (Hartley and Jones 1977) and thus prevent nematode migration and feeding. Therefore, based on co-location with the *QRlnt.sk-6D* and *QRlnt.sk-2B.1* identified in this study, the production and/or the action of phenolic compounds may be involved in resistance to *P. thornei* and explain the reduced motility and migration observed by Linsell et al. (2012b).

Several stripe, stem and leaf rust resistance genes map to the 2B *P. thornei* resistance QTL identified in this study (Bariana et al. 2001; Dundas et al. 2007; Kolmer 1996; Leonova et al. 2007; Luo et al. 2008) (Table 4). For example, the *Sr40* stem rust gene (Wu et al. 2009) and the leaf rust, *Puccinia triticina*, resistance locus *Lr16* (McCartney et al. 2005) map closely to *Rlnt.sk-2B.1*. The *QRlnt.sk-2B.5* chromosome region contains a stripe rust resistance QTL linked to *wmc154* and *barc200* (Smith et al. 2007). The *QRlnt.sk-2B.3* further along the 2B chromosome is also densely mapped with rust resistance including the stripe rust genes *Yr5* and *Yr7* (Tsilo et al. 2007), the stem rust genes *Sr9* and *Sr36* (Tsilo et al. 2007, 2008) and the leaf rust gene *Lr50* (Brown-Guedira et al. 2003). Although to date little is known about the resistance mechanisms of these rust loci, future investigations could reveal important linkages to *P. thornei* resistance. In addition, if these co-located QTL are robust across environments then they will be useful for the introgression of multiple pathogen resistance.

In summary, two highly significant *P. thornei* resistance QTL were identified on chromosomes 2BS and 6DS, *QRlnt.sk-2B.1 and QRlnt.sk-6D*, which mapped to locations previously identified to be associated with *Pratylenchus* resistance (Schmidt et al. 2005; Zwart et al. 2005; Zwart et al. 2010; Zwart et al. 2006). These QTL account for a large portion of the resistance observed, which shows that in this population the Sokoll derived resistance to *P. thornei* is very strong and is controlled by a few loci with large effects. The phenotypic variation explained by these resistance QTL is much larger than previously reported for other *Pratylenchus* resistance QTL in wheat. Several other QTL on chromosomes 2B, 2D, 3A, 4A, 5A, 5B and 6B all accounted for a small percentage (1 to 6%) of *P. thornei* resistance, indicating the complexity of resistance. Comparison of maps using common markers revealed more than ten SSR markers within and flanking the *QRlnt.sk-2B.1* and *QRlnt.sk-6D* QTL that were associated with known resistance genes to other plant pathogens. Investigation of resistance from a functional point of view will identify how these resistance genes play specific roles in the suppression of nematode development. The linkage of SSR marker locus *barc183* to *QRlnt.sk-6D* has previously been reported to be associated with *P. thornei* resistance in other mapping studies in populations of different genetic backgrounds. This highlights the potential benefit of this marker for use in

locus/marker assisted selection. Fine mapping to delimit the resistance QTL identified in this study will enable the development of tightly linked markers which through MAS, will greatly accelerate the development of new *P. thornei* resistant cultivars.

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Chapter 5

Identification of QTL and Characterisation of Compounds Associated with *Pratylenchus thornei* Motility and Hatching Inhibition

5.1 Introduction

It was established previously (Chapter 3) that *P. thornei* migration and motility is suppressed in resistant roots and exudates from the Sokoll x Krichauff wheat population, suggesting that resistant lines constitutively produce compounds that inhibit motility. In addition, resistant root exudates significantly reduced egg deposition and egg hatch of *P. thornei* and indicated the presence of compounds inhibiting hatch. In Chapter 4, several resistant QTL were identified to be linked to *P. thornei* resistance in the Sokoll x Krichauff population. The research summarised in this chapter aimed to identify whether these resistant QTL correlate to the observed motility and/or hatching suppression. A larger subset of the population was phenotyped for motility and hatching, using the efficient and economical phenotypic screening protocols developed in Chapter 3. The identification and characterisation of resistance gene(s) and their mechanism(s) would allow genes to be pyramided to provide more effective resistance, by combining genes that control different biological mechanisms. A further aim of this chapter was to investigate the biochemical characteristics of the motility and hatching suppressive compounds. The identification of resistance at a specific stage of invasion and the biochemicals involved may enhance resistance phenotyping screening procedures by reducing the costs and time associated with current methods and could provide chemicals for novel control.

The effective root exudates against motility and hatching were derived from seedlings not exposed to *Pratylenchus* or other plant pathogens, indicating the presence of preformed defense compound(s) in resistant roots. Preformed compounds, known as phytoanticipins, are defined as low molecular weight antimicrobial compounds that are present in plants prior to challenge by pathogens or are produced after invasion entirely from pre-existing components (VanEtten et al. 1994). These compounds may be present in their biologically active forms. They more commonly occur as inactive precursors sequestered in cellular compartments and are activated by enzymes due to cellular breakdown (Osbourn 1996). As discussed in Chapter 3, resistant CR suspensions increased motility suppression, indicating the possible release of phenolic bound compounds during crushing. The release of toxic compounds from glucosides has been observed previously after the secretion of the enzyme β-glucosidase by *Pratylenchus* during feeding in peach roots (Mountain and Patrick 1959). Phytoanticipins are generally located at sites where defense signalling can be instigated

rapidly or play a direct role in pathogen defense, and this is essential in the case of migratory nematodes, which cause tissue damage soon after root entry.

The formation of plant defense compounds commonly arise from the products of secondary metabolism including the phenylpropanoid pathway, which produces phenylalanine derivatives. The deamination of phenylalanine leads to cinnamic acid, which after a series of hydroxylations results in the hydroxycinnamates including coumaric and ferulic acids. The reduction of hydroxycinnamates leads to the production of alcohols or monolignols, which when polymerised via peroxidases leads to the formation of lignin. These simple phenols can then be further modified to produce polyphenolic compounds such as flavonoids, flavonoid derivatives and condensed tannins (Iriti and Faoro 2009). Many phenylpropanoids have been implicated in resistance to *Pratylenchus*. Ferulic acid was associated with necrosis in cabbage after *Pratylenchus penetrans* invasion (Acedo and Rhode 1971). The flavonoids medicarpin and coumestrol were found in resistant lucerne roots after invasion with *P. penetrans* and inhibited motility (Baldridge et al. 1998). In addition, *P. penetrans* invasion increased the synthesis of lignin-like substances in resistant lucerne roots (Acedo and Rhode 1971). Although none of these phenylalanine derivatives have been implicated in the Sokoll x Krichauff/*P. thornei* interaction, their common link to *P. penetrans* resistance, which has a very similar invasive root approach, indicates they may play a role in the observed motility and hatch suppression.

The structures of some compounds are sensitive to physical and chemical treatments, which can induce changes in the molecule structure causing loss of the required function. Physical treatments, including temperature extremes, were used to assess the stability of the resistant compounds within root exudates.

This chapter tied together the outcomes of Chapters 2, 3 and 4 which identified (1) the stages where resistance provided suppression to nematode invasion, (2) the possible role of motility and hatching suppressive compounds and (3) QTL linked to *P. thornei* resistance. The work described in this chapter aimed to establish possible links between the observed motility and hatch suppression and the identified *P. thornei* resistance QTL in Sokoll x Krichauff, using the phenotyping methods developed in Chapter 3. In addition, to further characterise the resistance compounds associated with motility and hatching suppression. Therefore, this work aimed to correlate a biological resistance mechanism to the genetic resistance.

5.2 Materials and Methods

5.2.1 Nematodes

Pratylenchus thornei was obtained from wheat at Nunjikompita, South Australia and were maintained on carrot callus described by Moody et al. (1973). To collect the nematodes the carrot callus was placed in funnels in a misting chamber and inoculum was prepared as described in Chapters 2 to 4.

5.2.2 Plant Material and Phenotypic Analysis of *P. thornei* Motility and Hatching

The responses of the DH lines to *P. thornei* were assessed using the motility and hatching assays designed in Chapter 3 in order to generate phenotypic data to analyse linkage to the QTL identified in Chapter 4. Forty DH lines were selected and phenotyped for motility with two replicates for each sample. The 40 lines were selected based on genotypic data generated in Chapter 4. Ten lines had both resistance QTL (2B and 6D), ten lines had only the 2B QTL, ten lines had only the 6D QTL and ten lines had neither QTL. For hatching analysis, thirty and thirty five lines (some lines were excluded due to fungal contamination) were phenotyped using RE and CR suspensions respectively, with four replicates for each sample. The RE and CR suspensions were collected as described in Chapter 3 and were made to the concentration of 4 mg/mL and 100 µL was transferred to individual wells on a 96 well ELISA plate. Motility and hatching assays were then conducted as described in Chapter 3.

5.2.3 Marker Regression and QTL Analysis of *P. thornei* Motility and Hatching

Single point regression was performed using the genotypic map data generated in Chapter 4 using MapManager QTXb20 (Manly and Olson 1999). A permutation test was used to establish the threshold at which the LOD score became suggestive ($P < 0.05$) and significant ($P < 0.01$) for QTL identification (Van Ooijen 2004). Significant differences between genotypes were assessed using one-way analysis of variance (ANOVA). Broad sense heritability (H2) was estimated within each motility and hatching trial using the formula: $H2 = Var(G)/Var(P)$, where Var(G) is the estimated variance of the genotypic effect and Var(P) is the estimated variance of the phenotypic effect expressed on a genotype mean basis.

5.2.4 Temperature and Oxidation Treatment of Roots on Motility

Roots were grown and crushed and *P. thornei* motility was assessed in the CR suspensions in the plate motility assays as described in Chapter 3. For heat treatment, directly after crushing, the root suspension was heated to 60 °C for 45 min and then assayed for motility 1 d.a.i. For freezing treatment, 1 week old seedlings were harvested and immediately freeze-dried (Christ, Alpha 1-2 LD, Germany) and then crushed and assayed for motility 1 d.a.i. Each treatment group, which included the resistant and susceptible parents and three susceptible and resistant lines, were analysed twice, each with five replicates. The three resistant lines had both resistant QTL and the three susceptible lines had neither QTL. A no-

root control and an untreated fresh CR control of each sample was analysed simultaneously also with five replicates. Data sets were analysed by ANOVA. Least significant differences (LSD, $P = 0.05$) were calculated to compare means, where $P < 0.05$ was considered statistically significant using the statistical program Genstat (VSN International, USA).

5.3 Results

5.3.1 Phenotypic Assessment

The hatching suppression data obtained from the Sokoll x Krichauff population had a continuous unimodal distribution, skewed towards lines with high suppression (Figure 1.1 and 1.2). The percentage of hatching suppression in RE in Sokoll is 89.98 and in Krichauff, 80.65 in trial 1 and in trial 2, 94.34 and 89.26, respectively. The percentage of hatching suppression in CR suspensions in Sokoll is 90.31 and in Krichauff, 74.01 in trial 1 and in trial 2, 89.51 and 77.09, respectively. ANOVA showed highly significant differences between individual genotypes for *P. thornei* hatching suppression in both RE and CR in trials 1 and 2 (Figure 1.1 and 1.2). The motility suppression data showed a unimodal normal distribution and ANOVA showed highly significant differences between genotypes. The percentage of motility suppression in RE in Sokoll is 76.65 and in Krichauff, 60.31 in trial 1 and in trial 2, 62.34 and 54.06, respectively (Figure 1.3). The broad sense heritability (H2) of hatch suppression in RE is 0.159 and 0.415 and in hatch suppression in CR, 0.158 and 0.016 for trial 1 and 2 respectively. The H2 in motility suppression in RE is higher at 0.458 for trial 1 and 0.589 for trial 2.

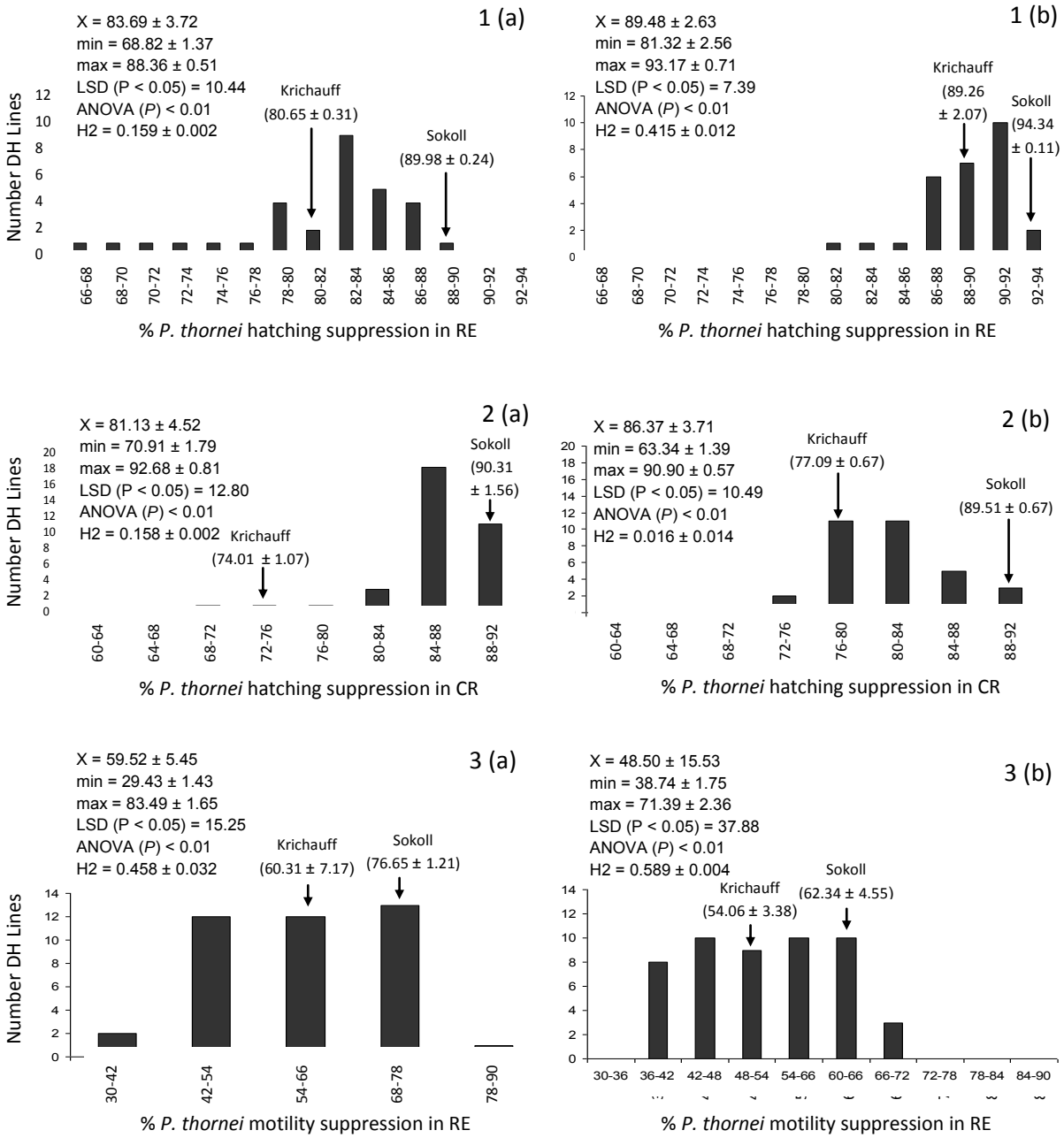

Figure 1: Phenotypic distributions of the percentage of *Pratylenchus thornei* hatching and motility suppression in the forty Sokoll x Krichauff DH lines. **1.** - Distribution of *P. thornei* hatching suppression in root exudate (RE) in trial 1(1a) and in trial 2 (1b) **2** - Distribution of *P. thornei* hatching suppression in crushed root (CR) suspensions in trial 1 (2a) and in trial 2 (2b). **3** - Distribution of *P. thornei* motility suppression in RE (3a) in trial 1 and in trial 2 (3b). The mean (± standard error) values of *P. thornei* DNA pg/plant in the parents are indicated. In addition, the population mean (X), the minimum (min) and the maximum (max) values (± standard error), least significant difference (LSD), analysis of variance (ANOVA) and the broad sense heritability (H2).

5.3.2 QTL Analysis of *P. thornei* Hatching and Motility

Single marker regression analysis identified 88 markers that showed linkage (P < 0.05) to *P. thornei* hatching suppression in RE. The frequency of Sokoll alleles was high at markers on chromosomes 1B, 2D, 3B, and 6D (30%) while frequency of Krichauff alleles was high at markers on chromosomes 2B, 5B, 6B and 7A (70%) . Single marker regression identified 58 markers linked to *P. thornei* hatching suppression when exposed to CR suspensions. The markers were distributed on seven chromosomes with the frequency of Sokoll alleles high at markers on chromosomes 1A and 6B (52%) while frequency of Krichauff alleles were high at markers on chromosomes 2B and 3B (48%). Single marker regression identified 31 markers linked to motility suppression. The frequency of Sokoll alleles was high at markers on chromosomes 1B, 3A and 6A (58%) while frequency of Krichauff alleles was high at markers on chromosomes 2B and 3B (42%).

QTL analyses revealed suggestive QTL on chromosomes 1B, 2B, 3B, 6B, 6D and 7A for RE hatch suppression and 2B, 3B, 6B and 6D for CR hatch suppression. The suggestive QTL on 1B, 3B and 7A spanned large sections (>100 cM) of the chromosomes as there was no distinct peak, and thus were not presented. Suggestive QTL associated with motility suppression were identified on chromosome 2B and 6B.

Two suggestive QTL that are located close together on chromosome 2BL were identified in both trials of hatching in CR and were inherited from Krichauff. As QTL are in the same locations, data is presented only from trial 1. The *QRlnt.hatCR.sk-2B.*1 (LRS = 8.0) and *QRlnt.hatCR.sk-2B.2* (LRS = 8.5), explaining 21 and 22% of the phenotypic variation respectively, are both inherited from Krichauff (Figure 2, Table 1). These same QTL were also associated with motility suppression, but had lower LRS scores. The *QRlnt.motRE.sk-2B.1* (LRS = 4.9) and the more distal QTL, *QRlnt.motRE.sk-2B.2* (LRS = 5.2), both explaining 12% of the phenotypic variation were also both inherited from Krichauff (Figure 2, Table 1).

Two suggestive QTL on chromosome 2B were identified in trial 1 only of hatching in RE, *QRlnt.hatRE.sk-2B.1* (LRS = 4.7), explaining 16% of the phenotypic variation, inherited from the synthetic hexaploid parent Sokoll, and *QRlnt.hatRE.sk-2B.2* (LRS = 5.8) explaining 19% of the phenotypic variation, inherited from Krichauff (Figure 2, Table 1). Suggestive QTL associated with hatching suppression in RE were identified on 6DS in trial 1 only with *QRlnt.hatRE.sk-6D.1* (LRS = 4.6), explaining 15% of the phenotypic variation, inherited from Krichauff and in both trials on 6DL, *QRlnt.hatRE.sk-6D.*2 (LRS = 5.5), explaining 18% of the phenotypic variation, inherited from Sokoll (Figure 2, Table 1).

A significant QTL, *QRlnt.hatRE.sk-5B.1* (LRS = 8.4) and a suggestive QTL, *QRlnt.hatRE.sk-5B.2* (LRS = 6.8), explaining 27% and 22%of the phenotypic variation respectively, were associated with suppressed hatch in RE in trial 1 and both were inherited from Krichauff. The

QRlnt.hatRE.sk-5B.1 is linked to the *Vrn1B* marker indicating this QTL is linked to vernalisation. A suggestive QTL also associated with suppressed hatch in RE was identified on chromosome 6B in trial 2, *QRlnt.hatRE.sk-6B* (LRS = 6.8) explaining 22% of the phenotypic variation (Figure 3, Table 1). Another suggestive QTL in the same 6B chromosome region, *QRlnt.motRE.sk-6B* (LRS = 6.9), explaining 16% of the phenotypic variation, was associated with motility suppression in trial 1 (Figure 3, Table 1). Many of these identified hatching and motility suppression QTL are inherited from the moderately susceptible parent Krichauff.

Table 1: QTL associated with *Pratylenchus thornei* hatching and motility suppression mapped in this study on a subset of the Sokoll x Krichauff DH population. The QTL shown indicate the donor parent (Inherited Origin) which chromosome (Chr) they map to, markers flanking the QTL and the most closely linked markers, the percentage of phenotypic variation explained (% Var), the likelihood ratio statistic (LRS) and the width the QTL spans in centiMorgans (cM).

QTL	Inherited Origin	Chr	Flanking Markers	Linked Markers	% Var	LRS	QTL Width (cM)
QRlnt.motRE.sk-2B.1	Krichauff	2B*	*barc167* *cfd267*	*gwm47*	12	4.9	35.5
QRlnt.motRE.sk-2B.2	Krichauff	2B*	*wmc441* *wPt-510*	*wPt-7747*	12	5.2	23.6
QRlnt.hatCR.sk-2B.1	Krichauff	2B*	*barc167* *wmc441*	*gwm47*	21	8.0	56.8
QRlnt.hatCR.sk-2B.2	Krichauff	2B*	*wmc441* *wPt-0047*	*wPt-1705*	22	8.5	30.7
QRlnt.hatRE.sk-2B.1	Sokoll	2B*	*wPt-1215* *barc200*	*wPt-4337*	16	4.7	37.0
QRlnt.hatRE.sk-2B.2	Krichauff	2B*	*wPt-1394* *wmc749*	*wPt-4072*	19	5.8	20.4
QRlnt.hatRE.sk-6D.1	Krichauff	6DS*	*1519* *barc183*	*cfd49*	15	4.6	20.7
QRlnt.hatRE.sk-6D.2	Sokoll	6DL*	*gpw2232* *barc21*	*wPt-4602* *wPt-5331*	18	5.5	86.9
QRlnt.hatRE.sk-5B.1	Krichauff	5B	*cfd70* *wPt-1881*	*wPt-4557* *wPt-5896*	27 26	8.4 8.1	46.7
QRlnt.hatRE.sk-5B.2	Krichauff	5B*	*wPt-3204* *wPt-4986*	*wPt-1030* *wPt-0334*	22 15	6.8 4.8	38.2
QRlnt.hatRE.sk-6B	Krichauff	6B*	*wPt-9195* *wPt-3284*	*wPt-9784*	22	6.8	74.8
QRlnt.motRE.sk-6B	Sokoll	6B*	*wPt-9659* *wPt-3284*	*wPt-3168*	16	6.9	69.3

* Below the significance threshold

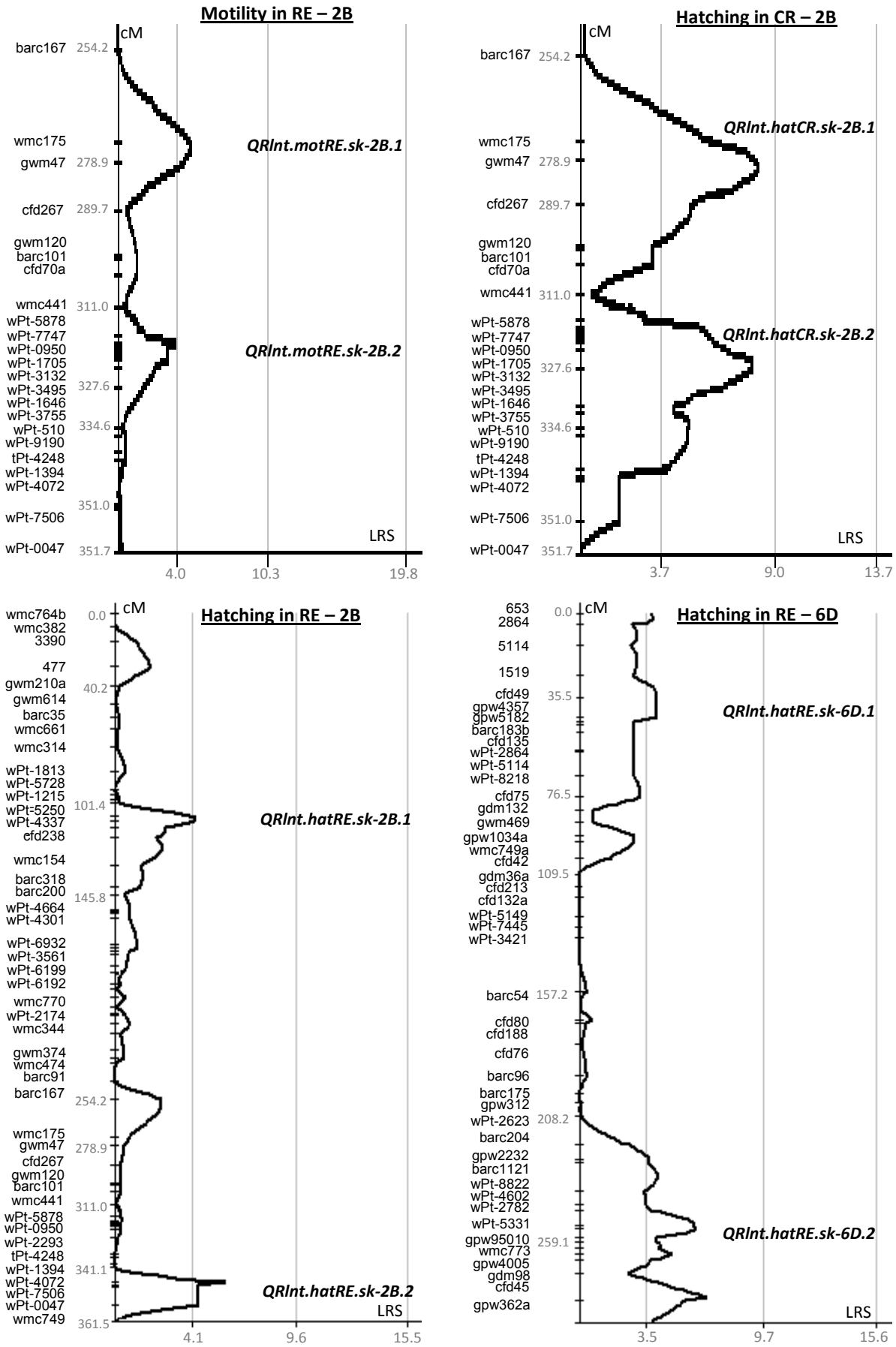

Figure 2: Composite interval mapping showing QTL for *Pratylenchus thornei* hatching and motility suppression in crushed root (CR) suspensions and root exudates (RE) in the Sokoll x Krichauff population. Suggestive QTL were identified on chromosome 2B for both motility and hatching suppression and two suggestive QTL on chromosome 6D for hatching suppression in RE. The numbers on the x-axis represent the threshold lines as set by 1000 permutation tests shown as likelihood ratio statistics (LRS). The first line represents suggestive QTL at $P < 0.05$ the second, significant QTL at $P < 0.01$ and the third, highly significant QTL at $P < 0.001$. The y-axis represents distances in centiMorgans (cM) from the distal end of the short arms of the chromosomes.

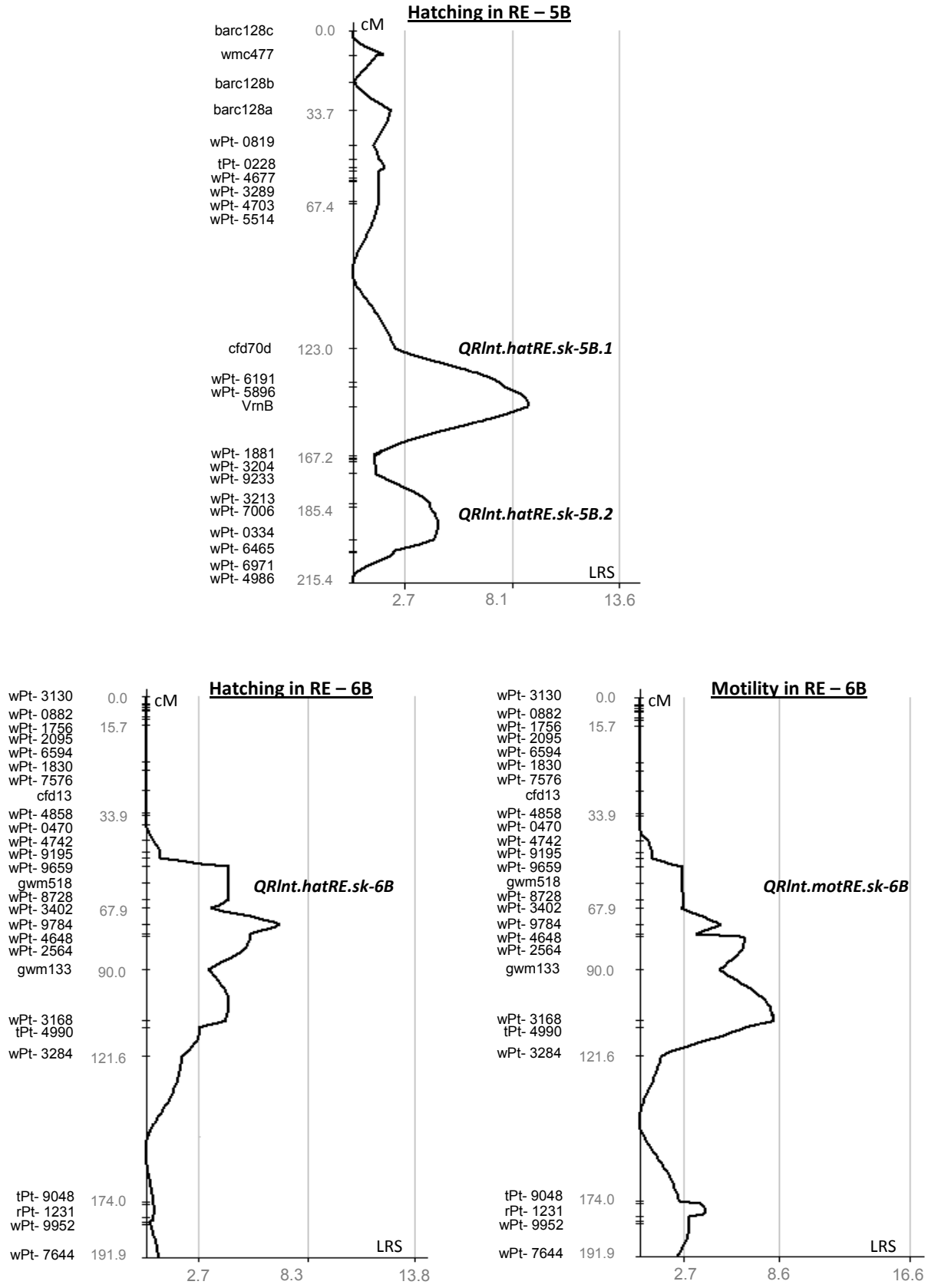

Figure 3: Composite interval mapping showing QTL for *Pratylenchus thornei* hatching and motility suppression in crushed root (CR) suspensions and root exudates (RE) in the Sokoll x Krichauff population. A suggestive QTL was identified on chromosome 6B for motility suppression. In addition, a significant QTL on 5B and a suggestive QTL on 5B and 6B for hatching suppression in RE. The numbers on the x-axis represent the threshold lines as set by 1000 permutation tests shown as likelihood ratio statistics (LRS). The first line represents suggestive QTL at $P < 0.05$ the second, significant QTL at $P < 0.01$ and the third, highly significant QTL at $P < 0.001$. The y-axis represents distances in centiMorgans (cM) from the distal end of the short arms of the chromosomes.

Table 2: *Pratylenchus thornei* resistance QTL mapped in this study that co-locate with *P. thornei* resistance QTL identified in previous studies and/or with *P. thornei* hatching and motility suppression QTL mapped in this study based on common markers. The table shows the donor parent (Inherited Origin), the chromosomal location (Chromosome), the flanking markers, the percentage of phenotypic variation (% Var) explained by the QTL and the likelihood ratio statistic (LRS).

Reference	QTL	Inherited Origin	Chromosome	Flanking Markers	% Var	LRS
Linsell, Chapter 4	*QRlnt.sk-6D*	Sokoll	6DS	*653*		
				barc183	43	82.9
				gpw4357		
				gpw5182		
				cfd132		
Linsell, Chapter 5	*QRlnt.hatRE.sk-6D.1*	Krichauff	6DS*	*1519*		
				cfd49	15	4.6
				barc183		
Schmidt et al. 2005		AUS13124	6D*	*gwm469*	4-6	4.9-9.3
				gdm36		
				gwm518		
				gdm132		
Zwart et al. 2005 & 2010	*QRlnt.lrc-6D.1*	CPI133872	6DS	*barc183*	18-27	28.1-46.5
				cfd49		
				cfd135		
Zwart et al. 2006		W-7984	6DS	*psr964*	11-23	4.4-8.5
				psr889		
				barc183		
Linsell, Chapter 5	*QRlnt.hatRE.sk-6D.2*	Sokoll	6DL*	*gpw2232*		
				wPt-4602	18	5.5
				wPt-5331		
				barc21		
Zwart et al. 2005	*QRlnt.lrc-6D.2*	CPI133872	6DL	*gdm98*	8-13	16.5-24.6
				gpw95010		
				barc21		
Schmidt et al. 2005		AUS4926	6D*	*gdm98.1*	2-2	2.6-2.7
				gdm98.2		
				gdm132		
Linsell, Chapter 4	*QRlnt.sk-2B.1*	Sokoll	2BS	*wmc764*		
				gwm614	24	39.9
				wmc382		
				wPt-5728		
Zwart et al. 2006		W-7984	2BS	*cdo447*	5-19	2.0-7.4
				bcd348		
				gwm210		
Linsell, Chapter 4	*QRlnt.sk-2B.2*	Krichauff	2B	*cfd70*		
				wPt-0950	2	11.3
				wPt-9736	2	11.0
				wPt-0047		
Linsell, Chapter 5	*QRlnt.hatCR.sk-2B.2*	Krichauff	2B*	*wmc441*		
				wPt-1705	22	8.5
				wPt-0047		
Linsell, Chapter 5	*QRlnt.motRE.sk-2B.2*	Krichauff	2B*	*wmc441*		
				wPt-7747	12	5.2
				wPt-510		
Linsell, Chapter 5	*QRlnt.hatRE.sk-2B.2*	Krichauff	2B*	*wPt-1394*		
				wPt-4072	19	5.8
				wmc749		

Table 2: continued.

Reference	QTL	Inherited Origin	Chromosome	Flanking Markers	% Var	LRS
Linsell, Chapter 4	*QRInt.sk-2B.3*	Krichauff	2B*	*barc167*		
				gwm47	2	10.1
				wmc175	2	9.3
				cfd70		
Linsell, Chapter 5	*QRInt.hatCR.sk-2B.1*	Krichauff	2B*	*barc167*		
				gwm47	21	8.0
				wmc441		
Linsell, Chapter 5	*QRInt.motRE.sk-2B.1*	Krichauff	2B*	*barc167*		
				gwm47	12	4.9
				cfd267		
Schmidt et al. 2005		AUS13124	2B	*gwm319*	6-13	9.5-13.7
				gwm494.2		
				ACT/CTC.1		
				gwm191.2		
Linsell, Chapter 4	*QRInt.sk-2B.4*	Krichauff	2B*	*wPt-1148*		
				tpt-9065	1	6.2
				barc91		
Schmidt et al. 2005		AUS13124	2B	*gwm319*	6-13	9.5-13.7
				gwm494.2		
				ACT/CTC.1		
				gwm191.2		
Linsell, Chapter 4	*QRInt.sk-2B.5*	Krichauff	2B*	*cfd238*		
				wPt-4997	2	7.2
				wPt-9098	2	7.2
				wmc770		
Linsell, Chapter 5	*QRInt.hatRE.sk-2B.1*	Sokoll	2B*	*wPt-1215*		
				wPt-4337	16	4.7
				barc200		
Schmidt et al. 2005		AUS4926	2B*	*wmc25.5*	2-3	2.7-3.4
				wmc25.4		
				gwm428		
Zwart et al. 2005	*QRInt.lrc-2B.1*	CPI133872	2B*	*wmc25*	7.4	15.2
				wmc154		
Linsell, Chapter 4	*QRInt.sk-2D*	Krichauff	2D	*wmc27*		
				wPt-0184	2	11.1
				wPt-7466		

Table 2: continued.

Reference	QTL	Inherited Origin	Chromosome	Flanking Markers	% Var	LRS
Linsell, Chapter 4	*QRlnt.sk-3A*	Sokoll	3A	*wPt-2866*		
				wPt-5171	2	8.6
				wmc364		
Linsell, Chapter 4	*QRlnt.sk-4A*	Krichauff	4A	*wPt-2836*		
				wPt-6502	6	8.2
				wPt-3810		
Linsell, Chapter 4	*QRlnt.sk-5A*	Krichauff	5A	*wPt-4131*		
				wPt-3187	6	9.1
				wPt-5588		
				wPt-1422		
Linsell, Chapter 4	*QRlnt.sk-5B.1*	Sokoll	5B	*wPt-7006*		
				wPt-0334	2	9.7
				wPt-4986		
Linsell, Chapter 5	*QRlnt.hatRE.sk-5B.2*	Krichauff	5B*	*wPt-3204*		
				wPt-1036	22	6.8
				wPt-0334	15	4.8
				wPt-4986		
Linsell, Chapter 4	*QRlnt.sk-5B.2*	Sokoll	5B*	*cfd70*		
				wPt-5986	2	7.3
				wPt-1881		
Linsell, Chapter 5	*QRlnt.hatRE.sk-5B.1*	Krichauff	5B	*cfd70*		
				wPt-1881		
				wPt-4557	27	8.4
				wPt-5896	26	8.1
Linsell, Chapter 4	*QRlnt.sk-6B*	Sokoll	6B	*wPt-3402*		
				wPt-4648	4	15.8
				wPt-2564	4	15.0
				wPt-3284		
Linsell, Chapter 5	*QRlnt.hatRE.sk-6B*	Krichauff	6B*	*wPt-9195*		
				wPt-9784	22	6.8
				wPt-3284		
Linsell, Chapter 5	*QRlnt.motRE.sk-6B*	Sokoll	6B*	*wPt-9659*		
				wPt-3168	16	6.9
				wPt-3284		

* Below the significance threshold

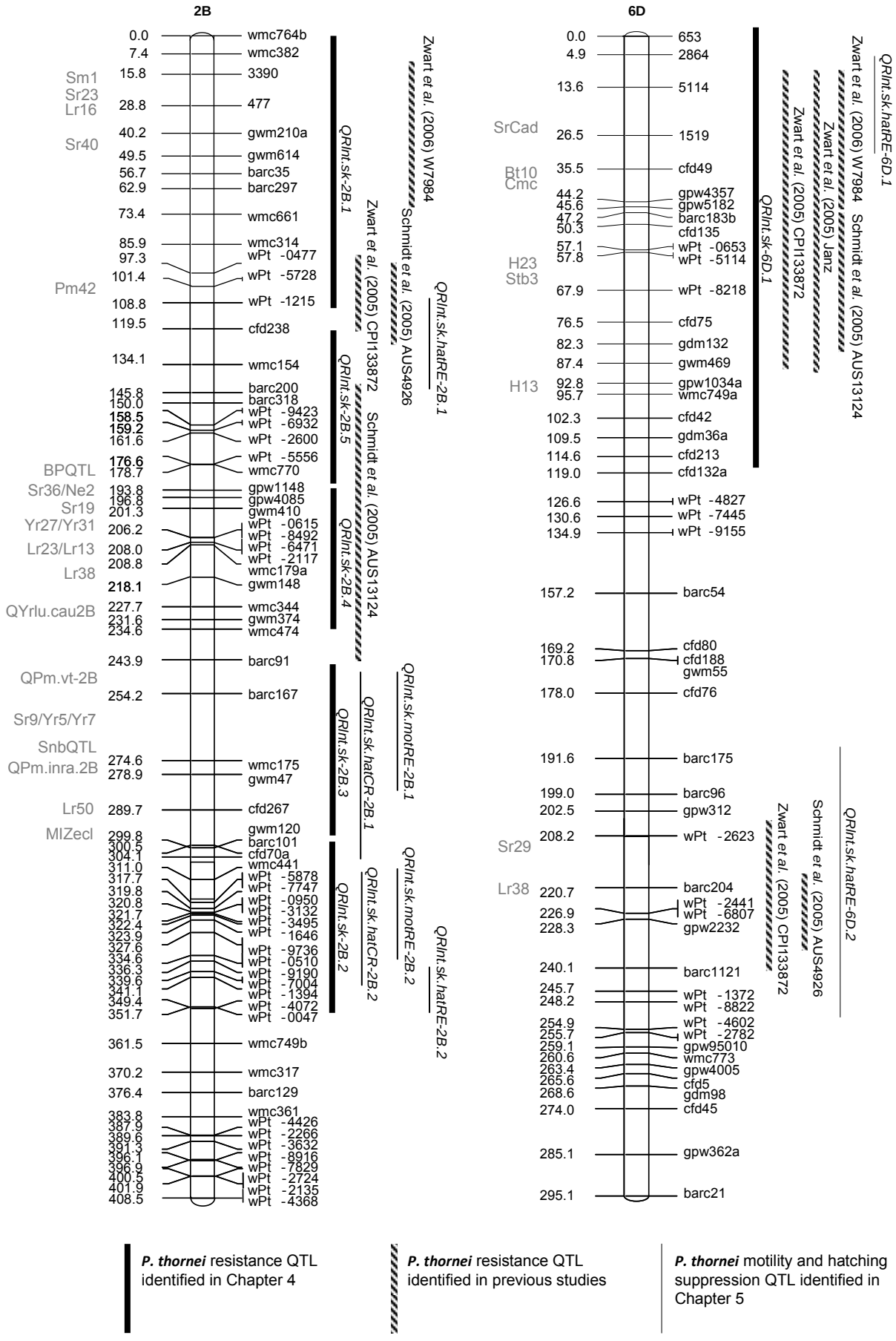

Figure 4: Co-location of *Pratylenchus thornei* QTL and known wheat pathogen resistance genes on chromosomes 2B and 6D. The location of *P. thornei* hatching and motility suppression QTL are shown with respect to the resistance QTL identified in Chapter 4 and previously identified in other studies and with characterised wheat pathogen resistance genes.

5.3.3 Temperature Stability of Root Exudate and Effects on Motility

To investigate the biochemical characteristics of the root compounds causing suppressed *P. thornei* motility and hatch, CR suspensions were subjected to temperature treatments (Figure 5). For heat treatment, directly after crushing the root suspension was heated to 60 ºC for 45 min. There was a significant decrease in motility suppression in both resistant and susceptible genotypes in comparison to the fresh crushed root suspension. More suppression was observed in Sokoll than Krichauff even after heat treatment. Compared to the other treatments, despite the harsh conditions, heat treated CR had the highest motility suppression. CR suspensions obtained from roots that had been freeze-dried immediately after harvesting also showed decreased motility suppression than compared to the untreated control. Suppression levels of freeze-dried treatments were similar to that observed in the water control. The fresh CR suspension showed greater motility suppression in Sokoll and the three DH resistant lines compared to Krichauff and the three susceptible DH lines.

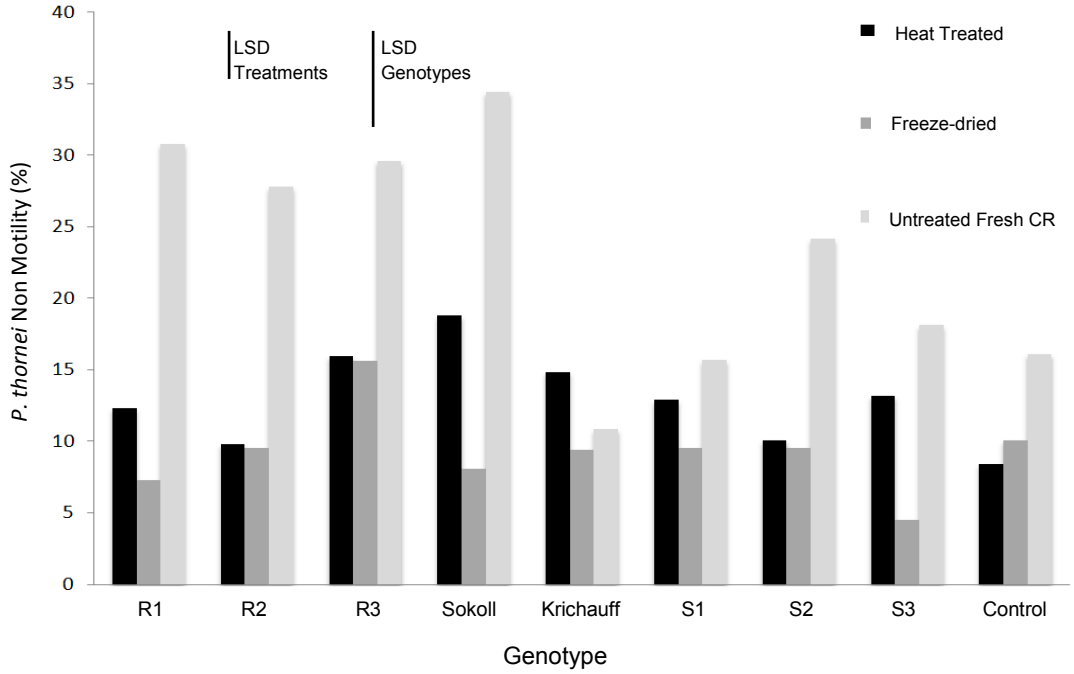

Figure 5: Motility inhibition of *Pratylenchus thornei* within crushed root (CR) suspensions subjected to heat and freeze-drying in resistant and susceptible genotypes. The first LSD vertical bar represents LSD for treatments (P = 0.05) of 2.8 and the second represents LSD for genotypes (P = 0.05) of 6.0.

5.4 Discussion

5.4.1 *Pratylenchus thornei* Hatching and Motility Suppression QTL

In Chapter 3, it was shown that *P. thornei* migration and motility is suppressed in Sokoll roots and exudates, indicating resistant genotypes constitutively produce compounds that inhibit motility. In addition, egg deposition and hatch of *P. thornei* was reduced in resistant roots and exudates. The motility and hatching assays described in Chapter 3 were relatively fast (11 to 14 days), simple and cheap and were utilised here to determine whether hatching and/or motility suppression played a role in the resistance observed in the Sokoll x Krichauff population. QTL linked to hatching and motility suppression were identified and their location was compared to the QTL associated with resistance to *P. thornei* (based on reproduction numbers) identified in Chapter 4 and to other published *P. thornei* resistance QTL.

Two suggestive QTL identified on 2B associated with both hatching and motility suppression, *QRlnt.hatCR.sk-2B.1* and *QRlnt.motRE.sk-2B.1*, flanked by *barc167* and *wmc441*, are closely linked to *wmc175* and *gwm47*, which are also linked to the *QRlnt.sk-2B.3 P. thornei* resistance identified in Chapter 4 (Table 2, Figure 4). Using other publicly available maps (Appels 2004) *wmc175* maps 5 to 6 cM from *gwm319* and *gwm191*, respectively, and these markers are closely linked to a 2BL QTL associated with *P. thornei* resistance in the Middle Eastern landrace AUS13124 (Schmidt et al. 2005). The second set of suggestive QTL directly proximal to *QRlnt.hatCR.sk-2B.1*, are also associated with both hatching and motility suppression, *QRlnt.hatCR.sk-2B.2* and *QRlnt.motRE.sk-2B.2*, and are linked to *wPt-0950* (Table 2, Figure 4). This DArT marker is closely linked to the *QRlnt.sk-2B.2 P. thornei* resistance locus identified in Chapter 4. The co-location of these 2B QTL associated with hatching and motility suppression, with *P. thornei* resistance QTL in other investigations, suggests that the *QRlnt.sk-2B.2* and *QRlnt.sk-2B.3* may play a role in resistance by containing gene(s) that suppress *P. thornei* motility and hatching. As the plants from which RE and CR suspensions were collected were not exposed to nematodes, this resistance must be preformed or constitutively active. Despite linking other 2B QTL to possible roles in hatching and motility suppression, the role of the highly significant QTL *QRlnt.sk-2B.1* on 2BS identified in Chapter 4 remains uncharacterised. In this study, the methods used to obtain root compounds only extracted water soluble compounds. Thus, less polar compounds may be linked to the resistance expressed by the *QRlnt.sk-2B*, and require extraction with other solvents.

Although the motility and hatching suppression 2B QTL (*QRlnt.hatCR.sk-2B.*1 / *2B.2* and *QRlnt.motRE.sk-2B.1* / *2B.*2) are only suggestive their presence in two completely different Sokoll x Krichauff phenotypic data sets and in other synthetic populations re-enforces their genuine linkage to resistance and indicates that these QTL are in fact two distinct regions. As only a subset of the population was phenotyped, it is expected that the significance of these QTL would increase as more lines are phenotyped. Now that a link between resistance and

hatching and mobility suppression has been made, the next logical step would be to phenotype the entire mapping population.

A suggestive QTL on 2B associated with hatching suppression in RE, *QRlnt.hatRE.sk-2B.1*, included the SSR marker *wmc154*, which is also closely linked to the *QRlnt.sk-2B.5 P. thornei* resistance identified in Chapter 4 and the *P. thornei* resistance QTL identified by Zwart et al. (2005) in the synthetic CPI133872 (Table 2, Figure 4). A second QTL found on the long arm of 2B, *QRlnt.hatRE.sk-2B.2*, was also identified with CR hatching suppression and with motility suppression, and co-locates the loci *QRlnt.hatCR.sk-2B.2* and *QRlnt.motRE.sk-2B.2* (Table 2, Figure 4). A suggestive QTL linked to *cfd49*, *QRlnt.hatRE.sk-6D.1*, on 6DS associated with suppressed *P. thornei* hatching in RE, is in the same 6DS region as the *QRlnt.sk-6D*, associated with *P. thornei* resistance detected in Chapter 4 (Table 2, Figure 4). A second suggestive QTL on 6DL, *QRlnt.hatRE.sk-6D.2*, which contains the SSR markers *barc21*, *gdm98* and *gpw95010* is closely linked to *P. thornei* resistance in the synthetic CPI133872 (Zwart et al. 2005, Zwart et al. 2010) and in AUS4926 (Schmidt et al. 2005) (Table 2, Figure 4). This indicates a possible role of hatching suppression in resistance observed in Sokoll x Krichauff at *QRlnt.sk-2B.5* and *QRlnt.sk-6D*, but also in the 6DL resistance identified by Schmidt et al. (2005).

A suggestive QTL on chromosome 6B flanked by *wPt-9659* and *wPt-3284* was identified for both hatching and motility suppression, *QRlnt.hatRE.sk-6B* and *QRlnt.motRE.sk-6B* (Table 2). These QTL, closely linked to *wPt-3168*, co-locate to the *QRlnt.sk-6B P. thornei* resistance QTL identified in Chapter 4. The significant *QRlnt.hatRE.sk-5B.1* associated with hatching suppression is linked to *wPt-5896*, which is also closely linked to the *QRlnt.sk-5B.2 P. thornei* resistance identified in Chapter 4 (Table 2). Similarly the suggestive *QRlnt.hatRE.sk-5B.2* co-locates to the *QRlnt.sk-5B.1 P. thornei* resistance linked to *wPt-0334* (Table 2). Therefore, these 5B and 6D *P. thornei* resistance QTL identified in chapter 4 may also play a role in hatching and motility suppression.

5.4.2 Biochemical Characterisation of *P. thornei* Hatching and Motility Suppression

The defense response of plants to nematode invasion typically involves a hyper sensitive reaction that limits the invasion and leads to a systemic response (Rich et al. 1977, Keen 1992, Lindgren et al. 1992). These responses typically include the production of secondary metabolites such as flavonoids, isoflavonoids, amino-acid-derived compounds and soluble esters such as lignin and wall-bound phenolics (Keen 1992, Lindgren et al. 1992). In response to hostile and toxic environmental conditions within plant tissues that have initiated chemical defense, many organisms including nematodes adopt an inactive state as a mechanism of survival (Evans and Perry 1976). However, the non-motility observed in the resistant lines in Chapter 3 due to the toxic compounds secreted in these lines may enforce involuntary nematode motility. Several approaches were taken to establish the nature of the compound(s) that suppress *P. thornei* motility.

The structures of some compounds are sensitive to physical and chemical treatments, which can lead to their denaturation. Physical treatments such as heating, cooling, pressure changes and interactions with chemicals may induce changes in the molecule structure causing loss of the desired function. To determine whether protein(s) within resistant roots and root secretions had a role in the suppressed motility of *P. thornei*, CR suspensions were heated. When compared to the untreated control samples it was evident that this heat treatment significantly reduced the effect of nematode motility suppression (Figure 5). Heating can lead to changes in proteins causing functional changes including inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, or decreased uptake by cells (Shacter 2000). Thus, the loss of motility suppression may be due to root compounds losing activity by structural modification or by aggregation causing inactivity and/or preventing entry into the nematode. However, the level of suppression by heating was less than compared to the lyophilisation treatment and more suppression was still observed in the CR suspensions of the resistant Sokoll than the susceptible Krichauff. The conditions of this assay were severe and most proteins heated at this temperature for this period would denature. Thus, the presence of some motility suppression indicates these resistant root compounds are fairly heat resistant.

Lyophilisation (freeze-drying) generally preserves the structures of proteins as solvents are transferred to the vapour state, without first passing through an intermediate liquid phase. However, CR suspensions obtained from freeze-dried roots had significantly less motility suppression compared to the untreated CR control (Figure 5). This indicates that lyophilisation removed the activity of some root enzymes or secondary metabolites involved in motility suppression. During lyophilisation, enzyme folding may be distorted causing conformational protein changes and a loss activity. During the lyophilisation process the solutes in water reach concentrations much higher than their initial concentration, which can lead to changes in pH and ionic strength affecting the activity of enzymes (Jammel et al.

1997, Heller et al. 1999). Motility suppression was generally similar in both the resistant and susceptible roots following freeze-dry treatments and levels were equal to that observed in the no-root control indicating little to no suppression. These results suggest that chemical or structural changes induced by temperature extremes change the function of proteins present within the resistant roots that affect the ability of *P. thornei* to move.

Roots secrete both low and high molecular weight molecules, primarily carbon containing compounds, into the rhizosphere (Uren 2000). These exudates alter the physical and chemical properties of the soil and its community to improve plant growth including defense against pathogens (Bertin et al. 2003). To further characterise the compounds involved in motility suppression, size fractionation could be conducted to separate compounds in the root suspension by their molecular weights to provide some insights into their physical properties. The nature of a molecule's structure is determined by atoms and their arrangement in functional groups. Using the chemical properties of the nature and location of their functional groups, molecules can be separated based on their polarity. Reverse phase C18 chromatography can help measure the polarity of a compound which can help determine the location and role it may play during biochemical interactions within the plant.

Defense compounds secreted by plant roots have antibiotic properties against various pathogens (Paxton 1981) and have been linked with suppressed motility in several plant-nematode interactions. The defense action of these compounds is normally due to greater synthesis or accumulation in resistant roots, or the pathogen in the susceptible root can metabolise the compound, and/or avoid eliciting defense pathways (Bell 1981, Kuc 1995). In this investigation, root exudates were collected from resistant roots not exposed to nematodes and thus the motility and hatching suppression observed must come from preformed defense compounds, which are synthesised during normal plant development. These preformed defense compounds commonly are located at sites where they can quickly initiate defense signalling or play a direct role in pathogen defense (Treutter 2006). This fits well with resistance to the migratory nature of *Pratylenchus*, as an effective compound should accumulate quickly to sufficient levels to immobilise the nematode before significant tissue damage can occur. In this study and in other *in vitro* motility suppression studies, motility was reduced as early as 24 h.a.i. (Rich et al. 1977, Baldridge et al. 1998), further indicating a preformed motility resistance mechanism.

5.4.3 Co-located Defense Response and Resistance Genes and Possible Secondary Metabolites Involved in *Pratylenchus* Hatching and Motility Suppression

A gene conferring resistance through the action of flavonoids co-locates to the *QRlnt.sk-2B.1* region suggesting a possible role of flavonoids in resistance to *P. thornei*. Flavonoids are either synthesised in response to pathogen invasion or are 'preformed' and constitutively synthesised (Treutter 2006). Flavonoids have antimicrobial properties and play important roles in plant defense, especially in the development of the early accumulation of phenolic compounds and peroxidases at the entry site (Zinov'eva et al. 2004) to limit invasion and migration (Fernandez and Heath 1989, 1989). QTL mapping of resistance to orange wheat blossom midge (OWBM), *Sitodiplosis mosellana*, positions the corresponding resistance gene *Sm1* in the same chromosome region as the *P. thornei* resistance *QRlnt.sk-2B.1* (Table 4, Chapter 4 and Figure 4, Chapter 5). This *Sm1* resistance confers an antibiotic form of resistance by impeding larval development through the activity of flavonoids and phenolics. Analysis of seed extracts revealed that the most resistant wheats had higher constitutive levels and more rapid induction after midge invasion of ferulic acid and p-coumaric acid than susceptible wheat (Ding et al. 2000). The *Sm1* resistance blocks development of the midge at the first or second larval stage resulting in stunting and eventual death (Lamb et al. 2000, Berzonsky et al. 2003). Similar early *P. thornei* juvenile developmental suppression was observed in the resistant roots in this study, where juveniles were unable to moult to second or third stage juveniles. The co-location of this gene with *P. thornei* resistance QTL indicates a possible role of flavonoids in resistance, however, further fine mapping is required to determine whether this *Sm1* locus is in fact the gene controlling *P. thornei* resistance.

The production and role of flavonoids inhibiting *Pratylenchus* motility is known from several plant species further strengthening their role as potential candidates in the *P. thornei* motility suppression observed in the Sokoll x Krichauff derived resistance. For example, resistant lima beans produced the toxic isoflavonoid coumestrol, 1 d.a.i. with *Pratylenchus scribneri*, and *in vitro* assays found concentrations between 10 to 15 µg/mL inhibited *P. scribneri* motility by 50% after exposure for 96 h (Rich et al. 1977). Baldridge et al. (1998) found higher messenger ribonucleic acid (mRNA) levels of isoflavone reductase, a key enzyme in the isoflavonoid medicarpin synthesis, in resistant than in susceptible lucerne roots in the absence of *P. penetrans*. An *in vitro* assay showed *P. penetrans* motility was inhibited on agar by medicarpin. Increased flavonoid levels in addition to other phenylpropanoid pathway enzymes transcripts in resistant roots may form components of a constitutive plant defense response that suppresses nematode motility. The involvement of flavonoids in other *Pratylenchus* resistance interactions provides rationale for further investigation as prospective compounds involved in *P. thornei* motility suppression.

The action of flavonoids on nematode motility suppression has shown species specificity. Soybean resistant cultivars synthesise the isoflavonoid glyceollin within 3 d.a.i. with *Meloidogyne incognita* and *Heterodera glycines*. Glyceollin specifically inhibits site I of the mitochondrial electron transport system (Boydston et al. 1983) and affects motility by inhibiting nematode respiration (Kaplan et al. 1980, 1980, Huang and Barker 1986). After exposure to 15 g/mL of glyceollin for 24 h, 70% of the *M. incognita* juveniles became non-motile. Although it was expected that glyceollin would inhibit electron transport of all soybean infecting nematodes, a specificity effect was observed as even application of 60 g/mL glyceollin had no effect on *Meloidogyne javanica* motility (Kaplan et al. 1980). A similar specificity was observed in Chapter 3 where motility suppression observed in resistant RE was restricted to *P. thornei* and did not affect the closely related species, *P. neglectus*. This suggests different nematodes may have differential uptakes or abilities to degrade or overcome the effects of these flavonoids.

Plant phenolic compounds are either synthesised in invaded cells or are released from preformed glycosidic compounds and play an important roles in resistance, especially to migratory nematodes which cause tissue damage immediately upon penetration (Treutter 2005, 2006). Preformed phenolic compounds stored in specialised cells and released into invaded tissue fits well with the resistance observed in this study where motility and hatching suppression occurred with unchallenged resistant roots. The released preformed phenolic compounds can be subsequently oxidised to form compounds toxic to both the plant but more importantly the nematode. Oxidised toxic compounds have been linked to *Pratylenchus* resistance. Mountain and Patrick (1959) suggested that *Pratylenchus* induced peach root lesion formation resulted from the release of plant phenolic compounds from glycosides due to the action of the enzyme, β-glucosidase, secreted by *Pratylenchus* during feeding. Although these observations have not been described in wheat, the hydrolysis of phenolic compounds by nematode secretions has been observed in other nematode-plant interactions. The potato cyst nematode, *Globodera rostochiensis*, injected a β-glucosidase that hydrolysed a glycoside to release phenolic aglycones that caused necrosis only in resistant roots (Giebel 1982). The presence of phenolics or the secretion of β-glucosidase were not identified in this study, but given their involvement in numerous other plant-nematode resistant responses and their effects on *Pratylenchus* motility, their role in suppression of *P. thornei* motility cannot be ruled out.

Phenolics not only play a role in resistance by inhibiting motility through toxic oxidised compounds but also through forming physical cell wall protection mechanisms. The ferulic acid, produced as a result of *P. penetrans* cabbage invasion, was found covalently bound to cell wall polysaccharides (Wuyts et al. 2007) leading to the formation of lignin-like polymers (Fry 1986). The synthesis of lignin-like substances also occurred in lucerne roots invaded with *P. penetrans* (Townshend and Stobbs 1981) and was associated with the phenolic

compound hydroxybenzaldehyde (Pridham 1960). This cross-linking may act as a physical mechanism to protect cell walls from enzymatic attack by cell wall degrading enzymes secreted by nematodes (Hartley and Jones 1977). An increase in activity of certain peroxidase enzymes has been correlated to nematode resistance responses due to associated increased cell wall lignification (Zinov'eva et al. 2004) (Hiraga 2001, Kawano 2003). Increased peroxidase activity, about five times higher, was observed in resistant cabbage roots invaded with *P. penetrans* compared to susceptible roots (Acedo and Rhode 1971). In addition, an increase in peroxidase activity in *H. avenae* resistant wheat was associated with increased lignin in the cells where the nematode was localised (Andres et al. 2001). Peroxidases have a role in lignification as they produce free radicals of monomeric lignin precursors which spontaneously polymerise to form lignin networks (Harkin and Obst 1973, Grisebach 1981). Lignification may act as a physical and chemical protection mechanism, as it hardens cell walls preventing entry of nematode enzymes to cell wall polysaccharides (Ride 1978) and makes cell walls more resistant to the diffusion of pathogen toxins. This cell wall strengthening may explain the reduced *P. thornei* migration/motility in the Sokoll x Krichauff resistant roots observed in Chapter 3. In addition, the prevention of movement through the cortex due to reduced entry into lignified cells may lead to the suppression of feeding and development (as discussed in Chapter 2 and 3) due to reduced access to host nutrients. In order to determine whether lignification occurs as a *P. thornei* resistance mechanism, simple root staining techniques could be utilised.

Co-located resistance genes to the hatching and motility QTL identified in this study have also been associated with the action of peroxidises, and their role in the generation of reactive oxygen species (ROS) (Hiraga 2001, Kawano 2003). Reactive oxygen species, generated as part of the hypersensitive response, are involved in the induction of necrotic cell death, directly or indirectly causing damage to proteins, lipids and DNA, which ultimately disrupts cellular integrity and causes cell death. The *Sr5* stem rust resistance gene maps within the *P. thornei* resistance *QRlnt.sk-6D* and *QRlnt.hatRE.sk-6D.1* chromosome regions (Table 4, Chapter 4 and Figure 4, Chapter 5). The *Sr5* resistance response is correlated with rapid increases in peroxidase activity, lignification and rapid cell death (Beardmore et al. 1983, Tiburzy 1984, Reisener et al. 1986, Moerschbacher 1988). The gene for resistance to hessian fly (*Mayetiola destructor*), *H13*, is linked to *cfd132*, a marker that resides within the *P. thornei* resistance *QRlnt.sk-6D* and *QRlnt.hatRE.sk-6D.1* chromosome region (Table 4, Chapter 4 and Figure 4, Chapter 5). The *H13* Hessian fly antibiosis resistance (Hatchett and Gill 1981, Hatchett et al. 1981, Gill et al. 1987) is due to increased peroxidase activity. Resistance causing larval death is thought to involve a localised oxidative burst-associated hypersensitive response with rapid and prolonged accumulation of hydrogen peroxide (H_2O_2) and superoxide. Both ROS species were detected at the attack site during resistant interactions but not in the case of susceptible (Liu et al. 2010). Based on co-located markers, the defense response genes *Ppo*, encoding polyphenol oxidase (peroxidase) lies within the

QRlnt.sk-6D and *QRlnt.hatRE.sk-6D.1* region and the defense response genes, *Per2* for peroxidase, and *Sod* for superoxide dismutase, linked to the RFLP *mwg950* (Li et al. 1999) which is closely linked to *gwm410* (Appels 2004) that resides in the same region as the *QRlnt.sk-2B.2*, *QRlnt.hatCR.sk-2B.2* and *QRlnt.motRE.sk-2B.2*. The co-location of these peroxidase defense response genes strongly suggests that the production of peroxidases leading to ROS could play an integral role in the Sokoll x Krichauff resistance. ROS may induce necrosis and death of cortical cells rapidly in advance of penetrated areas, to serve as a physical barrier to prevent further *Pratylenchus* migration (Moldenhauer et al. 2006).

Chitinase genes that co-locate with the hatching suppression QTL may explain the reduced *P. thornei* hatch observed in this study. In addition to lignification, the co-located *Sr5* resistance gene has also been associated with the accumulation of mRNA encoding 1,3-glucanase and chitinase in resistant lines after invasion (Munch-Garthoff et al. 1997). Chitinases involved in fungal resistance cause lysis of hyphal tips (Mauch et al. 1988, Leah et al. 1991) as they break the chitin polymer linkages in fungal cell walls. Chitinases are induced locally at invasion sites, accumulate in other tissues following invasion or are constitutively expressed (Pan et al. 1992). Chitin is found in nematodes as a component of egg shells (Bird and Bird 1991) but generally not as a component of the cuticle (Spiegel and McClure 1995). Nematode hatching involves a change in membrane permeability of the eggshell (Clarke et al. 1978), causing an influx of water into the egg to rehydrate and activate the juvenile (Perry 1978). The presence of chitinases in resistant roots may act on egg shell chitin to induce egg shell permeability leading to eclosion before the juvenile has developed to hatching maturity (Mercer et al. 1992). For example, in response to *M. incognita*, resistant soybean roots had increased (2 units/mg) and earlier production of chitinases than in susceptible lines (Qiu et al. 1997). Exposure of *M. hapla* eggs to microbial chitinase solutions resulted in enhanced hatching but increased mortality of juveniles released, suggesting chitinases degraded egg shell chitin to the degree where juveniles were released prematurely. The expression and thus presence of chitinases in resistant roots could explain the reduced *P. thornei* hatching observed in the resistant lines in this study. Hatching may have been induced before juvenile development was completed causing the release of juveniles from eggs not ready to survive in the environment outside the egg.

In addition, chitin binding protein genes that co-locate with the hatching suppression QTL could also promote premature *Pratylenchus* hatch and in turn induce resistance. The defense gene *Cbp1*, a chitin binding protein linked to the RFLP *cdo678* (Li et al. 1999), maps closely to *gwm47* (Appels 2004), which resides within the *QRlnt.sk-2B.3*, *QRlnt.hatCR.sk-2B.1* and *QRlnt.motRE.sk-2B.1* regions. Chitin binding proteins have also been shown to have antifungal properties, most likely inhibiting growth through interfering with cell wall synthesis, by preferentially binding to sites where cell wall synthesis takes place (Suetake et al. 2000). Thus, the chitin in nematode egg shells may be a target for these defense proteins

to cause the weakening egg cell walls making them more susceptible to damage. The expression of chitin binding proteins could also lead the premature release of juveniles causing the *P. thornei* hatching suppression observed in Chapter 3.

The genomic position of a ribosome inactivating protein (RIP) is closely linked to *cfd49* and *KsuG48* (Sourdille et al. 2004), which reside within the *QRlnt.sk-6D* and *QRlnt.hatRE.sk-6D.1*. RIPs are widely distributed in higher plants (Barbieri et al. 1993) and inactivate eukaryotic ribosomes which inhibits protein synthesis and thus play important roles in plant defense systems (Nielsen and Boston 2001, Peumans et al. 2001). RIPs are active against a wide range of pathogenic fungi including *Fusarium oxysporum*, *Rhizoctonia solani*, *Erysiphe graminis* and *Pythium irregular* (Logemann et al. 1992, Vivanco et al. 1999 , Bieri et al. 2000). They also have defensive roles against certain insects including Coleopteran and Lepidopteran species (Gatehouse et al. 1990). The nematode cuticle, covered with glycoproteins, may serve as binding sites for RIPs, as in fungi, which may disrupt protein synthesis within migrating nematodes causing the motility and hatching suppression observed in the resistant Sokoll lines investigated.

No one compound or mechanism for the control of plant resistance has been reported (Kuc 1995) and thus a complex mix of compounds and responses are likely required to provide resistance against nematodes. Due to the complexity of the plant's defense system, one or several of the above described resistance pathways/compounds may play a role in the suppression of *P. thornei* motility, feeding and hatching observed in Chapters 2 and 3. Further investigation is required to narrow down the likely candidates. For example, various staining techniques, mass spectrometry, chromatography and gene expression analyses can be utilised to detect, quantify and localise flavonoids, phenolics, lignin, peroxidises and ROS in resistant and susceptible root tissue. Once the *Pratylenchus* resistance has been further characterised genetically in Sokoll x Krichauff, which will require extensive fine mapping, it can be exploited and/or introgressed into other wheat varieties. Resistance can also be engineered through the transgenic expression of defense-related genes in heterologous species or by disrupting biological processes in nematodes. Through the regulation of gene expression, production of plant defense compounds can be manipulated to synthesise phytoalexins for disease control. However, the pathways for phytoalexin synthesis are quite complex and vary in different plant species (Kuc 1995) and thus this approach may be difficult. Some success has come from the production of elicitors to stimulate phytoalexin pathways after invasion at the invasion site (Yoshikawa et al. 1993). An alternative approach would be to genetically engineer plants with a key enzyme in the biosynthetic pathway to synthesise phytoalexin stereoisomer's (Kuc 1995) to produce resistant compounds when and where defense is required.

5.5 Conclusion

The QTL linked to hatching and motility suppression co-locate to the *P. thornei* resistance QTL identified in this study (Chapter 4) and in previous studies (Schmidt et al. 2005, Zwart et al. 2005, Zwart et al. 2006) on chromosomes 2B, 5B, 6B and 6D. This indicates that these resistance QTL play a role in inhibiting *P. thornei* motility or juvenile hatching and that the simple and inexpensive assays designed in this study are able to detect *P. thornei* resistance. To further define and confirm these QTL, phenotypic analysis needs to be performed on the entire DH population. Heat treatments suggested that the preformed resistant root compounds causing motility and hatching suppression are moderately heat resistant. Flavonoids, oxidised phenols, lignifications and peroxidases associated with insect resistance genes that co-located with the hatching and motility suppression QTL and the *P. thornei* resistance QTL regions have been implicated in other *Pratylenchus*-plant resistance interactions. This indicates a potential role for these compounds in the *P. thornei* resistance observed in Sokoll x Krichauff. Further investigation of these resistant root compounds is needed to define their chemical nature and their specific role in suppression of nematode development. Additional characterisation of these compounds will lead to the correlation of a biological resistance mechanism to an identified *P. thornei* resistance QTL**.**

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Chapter 6
General Conclusions and Future Directions

Methods for control of *Pratylenchus* in wheat are restricted due to the wide host range preventing use of rotations and the inefficiencies associated with chemical control. Although nematicides have been effective in the past, due to their poor target specificity, high cost and damaging effects on animals and the environment, research focus has turned away from the synthesis and design of new synthetic nematicides toward the development of alternatives such as biological control and resistant cultivars. Several studies have identified various sources of resistance to *P. thornei* in wheat and developed molecular markers for use in resistance breeding. However, a simple understanding of the mechanisms of resistance and how they interfere with the nematode's biology and how they could be utilised as biochemical control alternatives is lacking. Prior to this investigation, the biological mechanisms of *P. thornei* resistance were not well understood. The resistance to *P. thornei* observed in the Sokoll x Krichauff wheat population is complex and under the control of several loci which suppress all nematode developmental stages. Through analysing invasion of roots by each life stage, it was shown that resistance in the Sokoll x Krichauff population occurs post penetration to suppress *P. thornei* motility/migration and juvenile development causing reduced reproduction.

This investigation showed that migration and motility of *P. thornei* was suppressed in resistant roots when exposed to root exudates from unchallenged plants, indicating that resistant genotypes constitutively produce compounds that inhibit motility which are specific to *P. thornei* and not *P. neglectus*. Egg deposition and hatch of *P. thornei* were also significantly reduced in resistant roots which may be explained by the presence of hatching inhibitors observed in root exudates. Biological control of *Pratylenchus* can be achieved through manipulating these complex chemical resistance interactions. Temperature treatments indicated that these resistant root compounds are generally stable in nature. However, further characterisation of these resistance root compounds is needed to define their chemical nature and specific role in suppression of *P. thornei* development.

Secondary metabolite candidate compounds within resistant roots linked to *Pratylenchus* mobility and hatching suppression were identified in this study as sugars, organic acids and amino acids (flavonoids, cinnamic acids, phenolics and phytoecdysteroids). Various histochemical techniques could be utilised to detect, quantify and localise compounds such as flavonoids, lignin and wall-bound phenolics. However, metabolomics analysis using gas chromatographies coupled with mass spectrometry would be the best ways forward to characterise resistant exudates. Gas chromatography enables separation of the components of the root exudate mixture based on their chemical properties. Downstream mass

spectroscopy can characterise each of the components individually by breaking each molecule into ionised fragments and detects these fragments using their mass to charge ratio. Combination of the two techniques allows both qualitative and quantitative evaluation of each component of the complex exudate mix to characterise differences in susceptible and resistant root secretions at different points during nematode invasion. Chromatography methods have been successfully used to characterise resistant compounds from root exudates that prevent nematode motility. For example, chromatography techniques identified dopamine and ferulic acid as resistant compounds in roots of banana cultivars against *R. similis* (Valette et al. 1997, Wuyts et al. 2007) and liquid chromatography identified chlorogenic acid in resistant tomato root extracts which inhibited *Meloidogyne* motility (Pegard et al. 2005). The generation of near-isogenic lines (NIL) for each of the major QTL (2B and 6D) would be useful for comparative physiological and biochemical studies of the function of these QTL. The NIL lines, generated by crossing lines with the QTL of interest to the recurrent parent and then repeated selfing or backcrossing the F1 plants to the recurrent parents, produces lines identical to the parent except in the region around the QTL, and thus any differences observed would be due to the resistance QTL.

Characterisation of resistance compounds will provide new sources of biochemical and biological control. The turn in the focus from chemical to biocontrol agents has led to some success with the development of several commercially available bionematicide products. These products are based on natural predators such as nematophagous fungi and bacteria, by their direct involvement as predators or biochemically by the production of toxic metabolites that interfere with nematode physiology inhibiting parasitic activities. For example, the spores of the bacterial pathogen *Pasteuria*, can be applied to the soil and attach to the nematode's cuticle, germinate, penetrate and parasitise the nematode causing death (Jatala 1986). Biochemical control can also be achieved through use of natural plant extracts as nematicides themselves when applied directly, as organic soil amendments or through rotational crops. For example, the polyacetylene, thiarubrine C, from *Asteraceae* (sunflower) roots inhibited *P. penetrans* and *M. incognita* in a motility bioassay and when used as a soil treatment decreased *M. incognita* infection of tomato seedlings by 95% (Sanchez de Viala et al. 1998). The resistant wheat root compounds identified in this study could provide *Pratylenchus* resistance through application to the soil or as seed treatments. In addition, these natural plant compounds can serve as models for the development of chemically synthesised derivatives.

Alternatively, the plants existing defenses can be enhanced through genetic engineering. The wheat biochemical pathways involved in *P. thornei* resistance could be manipulated *in vivo* to produce or enhance production of these motility and hatching suppressive compounds. For example, Arabidopsis plants transformed with the *F5H* gene had increased

lignin content (50% higher) which decreased *M. incognita* reproduction (Wuyts et al. 2006), probably due to the prevention of a feeding site. Alternatively, genetic engineering can be used to target the nematode directly or interfere with the nematode-plant interactions. For example, resistance to *G. pallida* in potatoes was achieved through feeding inhibition. The cysteine proteinases of *G. pallida* are thought to be important in digestion of plant material during feeding and potatoes genetically modified to express cysteine proteinase inhibitors (cystatins) showed resistance to *G. pallida* under field conditions (Urwin et al. 2001). Essential nematode genes can be silenced, preventing nematode invasion and/or development. For example, the secreted protein encoded by the parasitism gene *16D10* expressed in *Meloidogyne* esophageal gland cells is essential for giant-cell establishment (Hussey 1989, Hussey et al. 1989, Huang et al. 2003, Davis et al. 2004). RNA interference (RNAi) approaches silenced this gene and reduced nematode infectivity by the four major *Meloidogyne* species (Huang et al. 2006). In addition, the silencing of two *P. thornei* genes, calponin and troponin C, resulted in paralysis and uncoordinated movements which caused up to 80% reduction in reproduction on carrot mini discs probably due to suppressed root feeding/migration (Tan et al. 2013). Thus, RNAi is another possible approach to engineer *Pratylenchus* resistance as silencing of genes to disrupt the parasitic process could create novel, durable and broad resistance.

Although *P. thornei* penetration, migration and juvenile development were assessed within the roots in addition to *in vitro* analysis, destructive staining methods and extraction techniques utilised did not allow observations in real time. While *in vitro* assays can closely resemble the root or soil environment, critical factors absent from these systems or unrealistic conditions may produce artificial results. For example, in this study, less hatching suppression was observed in resistant RE than near actively growing roots. Most likely due to lower concentrations of hatching inhibitors in collected RE than in exudates continually produced by live roots. Emerging technologies now enable the fluorescent tagging of nematodes so that in combination with microscopy, nematode development within the root can be visualised in real time during all invasive stages. Examples include the use of green fluorescent protein transformation, fluorescent dyes (fluorescein isothiocyanate/diacetate) or quantum dots (Hashmi et al. 1997, Huang et al. 2007 , Goto et al. 2010).

Increasing importance has been placed on the development and deployment of natural sources of resistance to *P. thornei* within commercial wheat cultivars. Despite the identification and investigation of several resistant sources and resistance QTL, no *P. thornei* resistant commercial cultivar has been released to date that carries *P. thornei* resistance genes. The identification of novel sources of genetic resistance and understanding of their biological mechanisms will allow effective combinations of genes either to be used alternatively or pyramided to generate effective and stable *Pratylenchus* resistance. Due to

the extensive costs and labour associated with *Pratylenchus* phenotypic screening methods the development of molecular markers and their employment through marker-assisted selection will accelerate the development and thus availability of resistant cultivars to the grower. In this study two highly significant *P. thornei* resistance QTL were identified on chromosomes 2BS and 6DS, *QRlnt.sk-2B.1* and *QRlnt.sk-6D*, which mapped to locations previously identified to be associated with *Pratylenchus* resistance and accounted for a large portion (24% and 43%) of the resistance observed. This shows that in this population much of the resistance to *P. thornei* is controlled by a few loci with large effects. The linkage of SSR marker locus *barc183* to *QRlnt.sk-6D* is also associated with *P. thornei* resistance found in other mapping studies in different genetic backgrounds. This highlights the potential benefit of this marker for use in marker-assisted selection. This marker and other linked markers will allow gene pyramiding to provide effective resistance combinations. When incorporating resistance into new cultivars through backcrossing, use of resistant markers through marker assisted selection will eliminate the need to select progeny lines based on their resistance phenotypes. Thus, molecular markers eliminate the need for large scale phenotyping and facilitate the rapid identification of resistance loci, accelerating the development of new resistant cultivars (Schmidt et al. 2005, Toktay et al. 2006). As more than one allele may be responsible for the resistance at *QRlnt.sk-6D* map based cloning may be a good approach to further characterise *Pratylenchus* resistance genes and their functions.

The Sokoll x Krichauff genetic map constructed with over 900 molecular markers has a high marker density covering a total of 3477 cM of the wheat genome with an average marker density of 3.56 cM. This map is a valuable resource which could be used to map other agronomically important traits such as quality (protein content, hardiness, seed colour), abiotic stresses (drought, salinity, cold and heat) and biotic stresses including resistance to other pathogens.

The limiting factor in identifying new sources of *Pratylenchus* resistance and the development of resistant cultivars has been the phenotyping of resistance due to the laborious nature of screening methods and large variation associated with reproducibility. However, with the development of PCR diagnostic approaches, phenotypic resistance data is more easily and reliably obtained. However, this developing technology is expensive and protocols are currently proprietary. The biological assays designed in this study to investigate motility and hatching suppression are simple and inexpensive and allow high throughput. They have the potential use as an alternative resistance phenotypic screening methods. In addition, knowledge of the biochemicals involved in resistance could also be utilised to improve phenotypic resistance screening methods.

For a molecular marker to be useful in a breeding program it needs to be tightly linked to resistance, polymorphic in different germplasm and have high heritability. In this study the *QRlnt.sk-2B.1* and *QRlnt.sk-6D* span large chromosomal regions of 101 and 119 cM within 409 and 295 cM chromosomes, respectively. Therefore, before markers flanking these QTL can be utilised for MAS, fine mapping is required to delimit the QTL interval and to establish tightly linked markers. This could be achieved through using a large recombinant inbred line population and the development of near-isogenic lines for each of the QTL. Once the distance in the region between the *P. thornei* resistance gene/s and the cosegregating markers is considered to be within a useful range, a map-based cloning approach would be the next step in characterising resistance and would allow development of allele specific markers. Markers based on the gene sequences would allow allele mining in different germplasms to identify new and superior alleles, or perfect markers, for resistance.

To establish whether linked markers are polymorphic in different germplasm and have high heritability, validation in diverse germplasm and environments is required. The genetic variation detected in the mapping population around the QTL region may not be shared by other genetic and breeding populations due to allelic diversity (Nicholas 2006). Although markers linked to the *Pratylenchus* resistance QTL identified in this study explain a large percentage of the phenotypic variation they need to be validated in representative parental lines or breeding populations before being utilised in routine MAS. Despite the 6D and 2B resistance being upheld in field trials (A. Mckay, personal communication), it is important these trials are replicated in phenotypic extremes including varied environmental and climatic conditions (soil type, rainfall, temperature, disease pressures) to ensure resistance is stable. The synthetically derived Sokoll is unadapted and therefore could contain undesirable gene linkages and/or lack important agronomic traits present in adapted varieties. Repeated backcrossing to cultivated wheat is required to remove/add these traits before Sokoll can be used commercially. Although the root compounds identified provide *P. thornei* resistance, they may have undesirable effects such as being toxic to non target beneficial organisms, which could affect plant nutrition and growth. It would be beneficial to investigate differences and changes in plant and root morphology and in the nematode soil community associated with the resistant population lines.

The ultimate aim of this project was to correlate a biological role with an identified *P. thornei* resistance QTL. Suggestive QTL linked to both hatching and motility suppression were identified and co-located to the *P. thornei* resistance QTL identified in Sokoll x Krichauff and in previous studies (Schmidt et al. 2005, Zwart et al. 2005, Zwart et al. 2006) on chromosomes 2B, 5B, 6B and 6D. This indicates that these resistance QTL play a role in inhibiting *P. thornei* motility or juvenile hatching. The motility and hatching suppression QTL were analysed only in a subset (40 lines) of the population due to the time constraints of the

study. To further define and confirm these QTL, phenotypic analysis needs to be performed on the entire DH population using the hatching and motility assays developed in this study.

Changes to the global climate are making agricultural regions more variable and drier and have placed emphasis on the development of higher-yielding cultivars which can grow in marginal environments. In particular, resistance to root diseases and thus better root systems are critical to ensure maximum access to limited soil moisture. As resistance to *Pratylenchus* continues to gain attention as a priority trait for wheat breeding, the identification, characterisation of deployment of resistance genes/QTL will become increasingly important. Knowledge of the biological or biochemical resistance mechanisms is important as it could be used to develop improved phenotypic screening methods, provide new sources of biochemical control and reveal the involvement of different resistance genes, which could be pyramided to provide more durable *P. thornei* resistance.

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