PATHOSYSTEM DEVELOPMENT, CHARACTERISATION AND GENETIC DISSECTION OF THE SOIL PATHOGEN *PHYTOPHTHORA MEDICAGINIS* AND THE MODEL LEGUME *MEDICAGO TRUNCATULA:* A VIEW TO APPLICATION OF DISEASE RESISTANCE IN SUSCEPTIBLE LEGUME SPECIES.

This thesis is presented for the degree of

Doctor of Philosophy

by

Nola Kim D'Souza BSc (Hons.)

2009

The Australian Centre for Necrotrophic Fungal Pathogens, Division of Health Sciences, State Agricultural Biotechnology Centre, Murdoch University, Perth, Western Australia

Supervised by:

Professor Richard P. Oliver and Dr Karam Singh

DECLARATION

I declare that this thesis is an account of my own research and contains as its main content, work which has not been previously submitted for a degree at any tertiary education institute.

Data that was collected from the research described in this thesis supported the publication of:

Kamphuis L.G., Williams A.G., D'Souza N.K., Pfaff T, Ellwood S.R., Groves E.J., Singh K.B., Oliver R.P. and Lichtenzveig J. (2007). The *Medicago truncatula* reference accession A17 has an aberrant chromosomal configuration. *New Phytologist* **174**: 299-303.

..……

Nola Kim D'Souza

ABSTRACT

Phytophthora medicaginis is an important soil-borne oomycete pathogen of lucerne (*Medicago sativa*) and chickpea (*Cicer arietinum*) within Australia and overseas. To understand the host/pathogen interaction, a pathosystem was developed using the model legume *Medicago truncatula*. Using the resources developed for genetics and molecular characterisation in this model plant, the aim of this research was to understand the interaction between *M. truncatula* and *P. medicaginis,* with a view to improving resistance to this important pathogen in related legumes.

To observe and characterise the interaction between *M. truncatula* and *P. medicaginis*, a pathosystem was developed by first screening a germplasm collection of 99 *M. truncatula* accessions. This revealed a continuous distribution in disease phenotypes with variable extremes in natural resistance to *P. medicaginis* culture UQ5750, isolated originally from *M. sativa.* *P. medicaginis* zoospore inoculation of 1-2 week-old seedlings in glasshouse experiments proved to be a robust and repeatable method to consistently confirm the responses observed for six key *M. truncatula* accessions; SA8618 and SA8623 exhibit high natural resistance to this pathogen, accession A17 is moderately resistant, A20 is moderately susceptible and accessions Borung and SA30199 are susceptible.

To characterise the genetic basis of resistance to *P. medicaginis*, two reciprocal F_2 populations from cross pollinations between A17 and Borung and SA8618 and SA30199 were produced and then phenotyped for disease symptoms. Genetic segregation patterns indicated the involvement of a gene with a major effect in both reciprocal populations. In particular, a 3:1 segregation ratio for resistance in the F_2

populations from cross pollinations between A17 and Borung indicated the possibility of a single dominant gene for moderate resistance. Further phenotyping of F_3 families is required to verify this.

A *M. truncatula* linkage map was constructed using 50 F_2 individuals of the A17 X Borung population and 49 F_2 individuals from the Borung X A17 population. The map, covering 519.3 cM, is comprised of 84 SSR markers with an average distance between markers of 8.7 cM. These are evenly spaced over 7 linkage groups, including a super linkage group conferred by a translocation event between LG4 and LG8 of accession A17.

Quantitative trait locus (QTL) analysis confirmed there was a QTL with a major effect in the A17/Borung reciprocal populations. A significant QTL was determined by quantifying two symptoms of *P. medicaginis* infection - proportion of dead/chlorotic leaves and root fresh weight. The trait loci for both symptoms were located on the same linkage group within the same region, supporting the putative position of the QTL and the authenticity of its involvement in resistance to *P. medicaginis*. This QTL was located on LG6 and accounted for 69.5% of the observed variation in proportion of dead/chlorotic leaves or 38.1% of the variation in root fresh weight within the inoculated populations. The effect of this QTL on resistance to *P. medicaginis* translated into 27.5% less dead/chlorotic leaves or 0.86 g more root fresh weight. Other QTLs with minor effects that are potentially involved in the interaction are located elsewhere on LG6 and LG2. However, the marker density of the linkage map and the population size need to be increased to verify this.

In parallel to this, an F_7 recombinant inbred line (RIL) population of chickpea (BG212 X Jimbour), developed by breeders at the New South Wales Department of Primary Industries (NSW DPI), was also assessed for the genetic basis of resistance to *P. medicaginis*. Variance component analysis of phenotype scores for this intraspecific RIL population indicated that 57.15% of the differences in between-family and within-family variance could be attributed to a genetic component. However, gene-based markers developed in *M. truncatula* and established simple sequence repeat (SSR) markers of chickpea were not sufficiently polymorphic in size to produce a linkage map for further QTL analysis.

An interspecific cross between *C. arietinum* and *C. echinospermum* (Howzat X ILWC246) was also performed by breeders at the NSW DPI to develop RILs. In the duration of this research these interspecific RILs were bred to generation F_3 and phenotyping assessment had not been performed. However, marker screening of the parents revealed 122 size polymorphic chickpea SSR markers. A sufficient linkage map could be produced for QTL analysis once field assessment of this population is performed. Initial screening of the *M. truncatula* gene-based markers on the parents of this interspecific cross also revealed that 50% show a sequence-identified base pair difference. A chickpea linkage map incorporating these markers could be comparatively mapped with *M. truncatula*.

Molecular investigations of the *M. truncatula/P. medicaginis* pathosystem were performed to elucidate the possible underlying defence mechanisms involved in the observed resistance. To determine the function of ethylene in the resistant response, the characterisation of defence associated mutants of *M. truncatula* and *Agrobacterium*

rhizogenes-mediated 'hairy root' transformations were employed. Comparison of response to inoculation of an ethylene insensitive mutant of *M. truncatula* (*sickle*) with the moderately resistant background genotype A17 showed that s*ickle* was hypersensitive to *P. medicaginis.* This indicated that ethylene insensitivity was not the source of resistance to this pathogen and importantly that ethylene is a key defence signalling molecule in the moderate resistance of A17 to *P. medicaginis*.

Agrobacterium-mediated 'hairy root' transformations of *M. truncatula* with 4GCC::Luc constructs, revealed that the production of ethylene and consequently ethylene response factors (ERFs) after inoculation by *P. medicaginis* was a general defence reaction by all accessions. The two susceptible *M. truncatula* accessions exhibited a much stronger and earlier response to inoculation than the highly resistant and moderately resistant accessions. This indicated that the resistant response may be directed by a transcriptional component governed by the host genotype, downstream of ethylene production. The *M. truncatula/P. medicaginis* 'hairy root' transformation assay has scope to be a powerful functional genomics tool for this pathogen interaction.

Reverse transcriptase quantitative polymerase chain reaction (RTqPCR) was employed to determine the general patterns of gene expression and function underlying the response to *P. medicaginis* infection. Relative changes in gene expression of key enzymes in each of the salicylic acid, jasmonic acid, ethylene and isoflavonoid defence pathways and in genes encoding downstream target proteins revealed potential genes involved in the resistance to *P. medicaginis*. There was a distinct molecular difference in the response between the high and moderately resistant *M. truncatula* phenotypes to this pathogen. Moderate resistance to *P. medicaginis* in *M. truncatula* is possibly

mediated by ethylene and involves the considerable induction of pathogenesis related protein 5 (*PR5*), which was not the same defence response that conferred the high resistance to *P. medicaginis.* Early and consistent expression of genes encoding key enzymes of the isoflavonoid pathway by the highly resistant accession indicated that phytoalexin response could be associated with the high resistance. Confirmation of the involvement of isoflavonoid phytoalexins in the high resistance response to *P. medicaginis* merits further investigation.

ACKNOWLEDGEMENTS

This research was made possible by the generous support of the Grains Research and Development Council (GRDC) of Australia (Project No. GRS82) and the collaborative interest of colleagues at the New South Wales Department of Primary Industry, (NSW-DPI) Australia. Particular thanks to Principal Plant Breeder Mr Ted Knights and Senior Plant Pathologist Dr Kevin Moore for imparting their valuable knowledge and for hosting me during my field visit. Also thanks to the many research officers at the NSW-DPI for their determined help in the limited time we had available to complete all the field and lab work.

Thankyou to Dr Rajeev Varshney (ICRISAT,India) Dr Chris Pittock (University of Melbourne, Australia) and Mr Nader Danehloueipour (University of Western Australia) for their generous contribution of primers used in this research.

To my principal supervisor Professor Richard Oliver (Australian Centre for Necrotrophic Fungal Pathogens (ACNFP), Murdoch University, Western Australia), thankyou for your enthusiasm, continued encouragement and support throughout my postgraduate studies. Your belief in my ability was invaluable. To my collaborative supervisor Dr Karam Singh (Plant Industries – CSIRO, Western Australia) thankyou for your enthusiasm and guidance and the opportunity to further develop my skills at CSIRO.

To my informal postdoctoral supervisors at the ACNFP and CSIRO, Doctors Jonathan Anderson, Simon Ellwood, Linling Gao, Judith Lichtenzveig, Theo Pfaff and Huyen Phan; thankyou all for your invaluable expertise and taking the time to help me

whenever I asked. Thanks go particularly to Judith for the extra supervision she provided to help me understand and appreciate the intricacies of all the statistics involved in scientific research.

To my colleagues and friends at the ACNFP and CSIRO, I couldn't have done this without your expertise, support and camaraderie. I hope all my future workplaces are as good to work in. Particular thanks to our research assistants because everything would grind to a halt without you! Especially to Emma Groves, Megan Jordan, Maryn Lord, Kasia Rybak, and Stephanie Whitehand for their help in my times of need. To my fellow medic PhD students Lars Kamphuis and Angela Williams, our discussions both scientific and not so scientific were much appreciated and I am honoured to have gone through this with people who understand.

Finally and especially to Sean for his belief and patience and his unending encouragement to start and to finish this PhD, even during those challenging years to complete his own MBA of which I am very proud. May the strength and values that have bound us together through this, help us in the next phase of our lives – parenthood.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACC	1-amino-cyclopropane carboxylic acid
ACC Oxidase	Aminocyclopropane carboxylic acid oxidase
ACNFP	Australian Centre for Necrotrophic Fungal Pathogens (Murdoch University Western Australia)
AFLP	Amplified fragment length polymorphism
ANOVA	One-way analysis of variance
ATP	Adenosine tri-phosphate
Avr or *Avr*	Avirulence
BAC	Bacterial artificial chromosome
BGL	β-1,3-glucanase
CAPS	Cleaved amplified polymorphic sequence
CBEL	Cellulose-binding, elicitor and lectin activity
CC	Coiled coil
cDNA	Complementary DNA
Chi III	Chitinase III
CHS	Chalcone synthase
cM	Centimorgan
CMA	Corn meal agar
CSIRO	Commonwealth Scientific and Industrial Research Organisation (Australia)
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
EMS	Ethylmethane sulfonate
ERF	Ethylene response factor
EST	Expressed sequence tag
ET	Ethylene
ETI	Effector triggered immunity
GST	Glutathione S-transferase
GTP	Guanosine tri-phosphate
HEL	Hevein-like protein
HR	Hypersensitive response
(HR)	Highly resistant
ICRISAT	International Crops Research Institute for the Semi Arid Tropics (India)
IFR	Isoflavone reductase
IFS	Isoflavone synthase
ITS	Internal transcribed spacer
JA	Jasmonic Acid
LOD	Logarithm of odds
LOX	Lipoxygenase
LRR	Leucine rich repeat
Mbp	Megabase pairs
mRNA	Messenger RNA
(MR)	Moderately resistant
(MS)	Moderately susceptible
Mt ERF	*Medicago truncatula* ethylene response factor
NBS	Nucleotide binding site

NSW	New South Wales
NSW DPI	New South Wales Department of Primary Industries
OPR	12-Oxophytodienoic acid
p.i.	post inoculation
PAL	Phenylalanine ammonia lyase
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PEV	Proportion of explained variability
PI	Protease inhibitor
PR	Pathogenesis related protein
PRR	Pattern recognition receptors
PTI	PAMP triggered immunity
QTL	Quantitative trait locus
QTLs	Quantitative trait loci
R or *R*	Resistance
(R)	Resistant
RAPD	Random amplified polymorphic DNA
RGA	Resistance gene analogue
RIL	Recombinant inbred line
RNA	Ribonucleic acid
RNAi	RNA interference
RTqPCR	Reverse transcriptase quantitative polymerase chain reaction
(S)	Susceptible
SA	Salicylic Acid
SAR	Systemic acquired resistance
SARDI	South Australian Research Development Industry
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat
TAQ Polymerase	*Thermus aquaticus* DNA polymerase
TC	Tentative consensus sequence
T-DNA	Transfer DNA
TILLING	Targeting induced local lesions in genomes
TIR	Toll interleukin receptor
UV light	Ultraviolet light
V8A	V8 Juice Agar
VSP	Vegetative storage protein