

**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

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by

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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.

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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 F₂ 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 F₂

populations from cross pollinations between A17 and Borung indicated the possibility of a single dominant gene for moderate resistance. Further phenotyping of F₃ families is required to verify this.

A *M. truncatula* linkage map was constructed using 50 F₂ individuals of the A17 X Borung population and 49 F₂ 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 F₇ 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 F₃ 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 *sickle* 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.

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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 <i>Avr</i> | 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 | <i>Medicago truncatula</i> 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 <i>R</i> | 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 | <i>Thermus aquaticus</i> 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 |