Using targeted phenomics and environmental proxies to understand *Phytophthora medicaginis* and Phytophthora root rot resistance in Australian chickpea

Nicole Dron

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School of Agriculture, Food and Wine

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Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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List of publications and expected publications

1. **Dron N.**, Simpfendorfer S., Sutton T., Pengilley G. & Hobson K. 2022. Cause of Death: Phytophthora or Flood? Effects of Waterlogging on *Phytophthora medicaginis* and Resistance of Chickpea (*Cicer arietinum*). Agronomy, 12, 89.

2. **Dron N.**, Sutton T., Simpfendorfer S., Harden, S. & Hobson K. 2021. Phenotyping for waterlogging tolerance as a proxy for *Phytophthora medicaginis* resistance in chickpea. Plant Health Progress, 287-293.

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4. **Dron N.** 2020 Improving Phytophthora root rot resistance in chickpeas through breeding for waterlogging tolerance - implications for diagnosing root health and PREDICTA®B testing. GRDC update paper.

5. **Dron N.**, Ryan M., Forknall C., Hobson K., Sutton T. & Bithell S. 2022 Phytophthora root rot and waterlogging in chickpeas – minimising risk and management options. GRDC update paper.

6. **Dron N.**, Simpfendorfer S., Bithell S., Harden S. & Hobson K. 2020. Diagnostic Predicta®B testing for Phytophthora inoculum of chickpea during waterlogged soil conditions. NSW Department for Primary Industries, Northern NSW Research Results.

7. **Dron N.**, Simpfendorfer S., Sutton T., Pengilley G., & Hobson K. 2022. Phytophthora or flood? Effects of waterlogging on *Phytophthora medicaginis* and resistance of chickpea. NSW Department for Primary Industries, Northern NSW Research Results.

Chapter 1: General Introduction

1.1. Thesis abstract

Australian chickpea production is constrained by Phytophthora root rot (PRR), caused by the soil-borne oomycete *Phytophthora medicaginis*. Yield penalties are exacerbated by waterlogging resulting in partial or complete crop losses with few control options. Higher levels of PRR resistance have been identified in wild *Cicer* species but are associated with undesirable traits due to genetic linkage drag. Understanding the mechanisms involved in waterlogging tolerance and PRR resistance traits is necessary to determine if they can be un-coupled from poor agronomic traits when breeding improved varieties. The aims of this research were to; (1) investigate and characterise the response of both chickpea and the PRR causing organism, *P. medicaginis*, to waterlogging stress, (2) identify mechanisms of resistance using waterlogging as a proxy, (3) phenotype flavonoid phytoalexin metabolite accumulation associated with resistance following PRR infection, and (4) identify associated quantitative trait loci (QTL) with discoverable traits.

Under waterlogging conditions, oxygen levels in soil are reduced and the plant itself compromised through physiological and structural changes that increase the sensitivity of chickpea to PRR infection. This research found that late waterlogging in combination with PRR reduced total plant biomass in chickpea by an average of 94%; however, waterlogging alone accounted for 88% of loss. Further experimentation found that under hypoxic conditions associated with waterlogging, *P. medicaganis* did not proliferate as determined by zoospore counts and DNA quantification using qPCR, due to oxygen requirements of the pathogen. These results demonstrate that waterlogging alone can result in plant stunting, yield loss and a reduced ability to express resistance.

Chickpea genotypes demonstrated variability in phenotype, such as plant biomass and root parameters, when exposed to waterlogging stress. Following waterlogging conditions, the PRR

moderately susceptible chickpea variety, Yorker, had an eightfold increase in adventitious root growth when compared with the PRR moderately resistant interspecific backcross line, 04067-81-2-1-1. *Phytophthora* spp. are reportedly attracted to branch sites and leached exudates. It is proposed that compromised root barriers at emergence sites of adventitious roots under waterlogging increases chemotaxis and hastens hyphal entry, increasing susceptibility to PRR. Screening under waterlogging conditions may offer a novel proxy phenotyping method for PRR resistance traits at early stages of chickpea breeding.

This research explored the genetic relationship between waterlogging phenotype, metabolite accumulation, and their association with PRR disease resistance QTL. An F_6 bi-parental population of recombinant inbred lines derived from 04067-81-2-1-1 and Yorker was used to gather waterlogging response measures following 14 days of soil saturation, including dry root weight (DRW), dry shoot weight, plant height, primary root length (PRL) and adventitious root count (RC). Previously published QTL for field PRR resistance co-located closely with QTL mapped in this research for DRW, PRL and RC. The second component of this research explored the influence of *P. medicaginis* infection on the accumulation of flavonoid metabolites in chickpea root exudates following eight days of *P. medicaginis* infection. QTL were identified for formononetin, maackiain, biochanin A, morin and genistin (genistein-7-O-glucoside) biosynthesis. Two previously published QTL for field PRR resistance co-located closely with QTL for morin biosynthesis. *In vitro* tests demonstrated that this compound reduced *P. medicaginis* mycelial growth.

The genetic mapping of waterlogging tolerance and metabolite QTL to regions near to those reported for PRR resistance suggests that a single genetic mechanism may have pleiotropic effects on both waterlogging and PRR response. This information can be used in future to identify flanking markers to facilitate targeted breeding for waterlogging tolerance and PRR disease resistance in chickpea.

Schematic overview of thesis structure

Chapter 1	General Introduction	
Chapter 2	Review of the literature • Introducing Phytophthora root rot and waterlogging • Flooding/ waterlogging and Phytophthora interactions • Waterlogging tolerance and Phytophthora resistance mechanisms	
Chapter 3	Cause of death: Phytophthora or flood? Effects of waterlogging on *Phytophthora medicaginis* and resistance of chickpea (*Cicer arietinum*)	→ Published: Dron et al., (2022) *Agronomy*, **12**:1 p 89
Chapter 4	Phenotyping for waterlogging tolerance as a proxy for *Phytophthora medicaginis* resistance in chickpea	→ Published: Dron et al., (2021) *Plant Health Progress* **22** p 287-293
Chapter 5	QTL mapping of waterlogging tolerance traits in chickpea (*C. arietinum* & *C. echinospermum*) and the genetic relationship with QTL conferring Phytophthora root rot (*P. medicaginis*) resistance	→ Chapter in publication format
Chapter 6	QTL mapping flavonoid accumulation in chickpea (*C. arietinum* & *C. echinospermum*) infected with Phytophthora root rot (*P. medicaginis*)	→ Chapter in publication format
Chapter 7	General conclusion and future directions	

Chapter 2: Review of the Literature

Abbreviations

AMF	Arbuscular mycorrhiza fungi
GWAS	Genome wide association
H_2O_2	Hydrogen peroxide
hpi	Hours post infection
NSW	New South Wales
O_2^-	Superoxide anions
OH-	Hydroxyl radical
POD	Peroxidase
PRR	Phytophthora root rot
QLD	Queensland
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait locus/loci
ROS	Reactive oxygen species
SOD	Superoxide dismutase

2.1 Introduction

Chickpea (*Cicer arietinum*) was first domesticated 10,000 to 12,000 years ago in the arid to semi-arid Middle East and has since been integrated into many cropping areas internationally (Knights et al. 2007, Redden et al. 2007). Chickpea is an important source of plant protein and valuable rotation crop globally. Major production countries based on 2019-2021 data include India (32,926,490 t), Turkey (1,735,000 t), Myanmar (1,448,764 t), Ethiopia (1,370,724 t), Australia (1,362,798 t), Pakistan (1,178,126 t), United States of America (598,060 t), Canada (542,092 t) and Iran (532,677 t) (FAOSTAT 2022). Chickpea was first introduced into Australian cropping systems in the early 1980's with 10,000 to 70,000 hectares grown nationally (Siddique et al. 1997). In 2016, Australian chickpea production peaked with over 800,000 hectares sown, primarily driven by high export prices (FAO 2019). Since then, the area of chickpea in Australia has stabilised to an estimated 500,000 to 600,000 hectares with over 70% situated in the northern region comprising northern New South Wales (NSW) and southern and central Queensland (QLD). Southern and western growing regions of Australia have other profitable pulse and oil seed crop options (i.e., canola, lupin and lentil) that are preferred in their respective farming systems. Production in the northern growing region is constrained by Phytophthora root rot (PRR) that results in significant yield losses of an estimated $8.2 million AUD annually to industry (Murray et al. 2012). PRR of chickpea in this region is caused by the oomycete *Phytophthora medicaginis*, a long inter-host surviving soil-borne pathogen (Hansen et al. 1991). Oomycete species causing PRR in chickpea have been discovered across parts of Argentina, India, Pakistan, and Spain but are of considerably lower economic impact (Nene et al. 1996).

Most semi-arid chickpea growing regions of Australia, including the northern region, rely on rainfall during the season for yield. Both PRR and waterlogging independently have significant effects on chickpea productivity in seasons with above-average rainfall. The interaction of

chickpea with abiotic stress and disease is complex, especially in the case of PRR. In addition to *P. medicaginis*, there are a number of chickpea pathogens of economic significance in Australia which also proliferate on chickpea under wet conditions. High moisture results in an observed reduction in latent period and increase in disease severity of foliar diseases, including ascochyta blight (*Ascochyta rabiei*) and botrytis grey mould (*Botrytis cinerea*) (Trapero-Casas et al. 1992, Pande et al. 2006). The disease-causing pathogens have specific environmental requirements, that are influenced greatly by physiological growth stage of the host, duration of the soil saturation or leaf moisture, and temperature.

In 2010 and 2016, high rainfall occurred throughout the winter growing season (May-November) in the northern region, resulting in partial to complete chickpea crop loss. Losses were attributed to waterlogging, lodging, ascochyta blight, botrytis grey mould and PRR. In undulating paddocks with free-draining soil and where regular foliar fungicides could be strategically applied, growers suffered only minor yield penalties instead of complete crop loss. Table 1 demonstrates that the severity of PRR disease in chickpea increases with greater seasonal rainfall (Bithell 2018). Long-term average growing season rainfall for the northern grain region is typically 200 mm to 250 mm. In 2016, where soil was saturated for extended periods of time, growing season rainfall reached 450 mm and evaluation plots infected with *P. medicaginis* resulted in 90% yield loss in the moderately susceptible Australian variety PBA HatTrick. Lower rainfall resulted in considerably lower yield losses, with 33% and 68% following 137 mm and 194 mm in-crop rain, respectively (Table 1).

Table 1. Total in-crop rainfall (mm), yield averages of the PRR moderately susceptible chickpea variety, PBA HatTrick, and yield losses (%) due to PRR infection, for 2014-2016. Data taken from (Bithell 2018) (GRDC Update – Phytophthora in chickpea varieties 2016 and 2017 trials – resistance and yield loss).

Season	Total in-crop rainfall (mm)	PBA HatTrick yield (t/ha) in absence of PRR infection	PBA HatTrick % yield loss due to PRR infection
2014	137	2.94	33
2015	194	2.50	68
2016	450	4.02	90

The following review will discuss waterlogging and PRR independently and explore interactions between the two in chickpea. This is a relevant area as waterlogging can heighten chickpea sensitivity to PRR, through increased proliferation of PRR inoculum and waterlogging compromising plant resistance. Understanding this interaction may reveal waterlogging tolerance traits that confer novel PRR resistance as demonstrated previously in soybean (*Glycine max*) (Nguyen et al. 2012), where the incorporation of waterlogging tolerance improved quantitative resistance to soil-borne *Phytophthora sojae*. In chickpea, the potential to identify and incorporate superior waterlogging tolerance to improve PRR resistance is yet to be investigated. Given the high crop losses that are experienced from PRR, improved resistance conferred through waterlogging tolerance is worthy of further investigation and would improve yield stability and productivity of chickpea across the northern growing region.

2.2 Phytophthora

2.2.1 Taxonomy and ecology of *Phytophthora medicaginis*

Phytophthora spp. are a fungus-like oomycete with numerous host-specific species globally. Taxonomical characteristics of *P. medicaginis* include a thickened oospore wall, dominant convex apices of the zoospores, optimal survival temperature of 26 °C and a maximum

temperature range of 33 to 35 °C. (Hansen et al. 1991, Liew et al. 1994, Irwin 1997). *P. medicaginis* hosts outside of chickpea include lucerne (*Medicago sativa*), perennial and annual medics (*Medicago* spp.), and other legumes such as sulla (*Hedysarum* spp.) and sesbania (*Sesbania* spp.) (Knights et al., 2008).

The life cycle of most oomycetes including *Phytophthora* spp. consists of two phases which are polycyclic and driven by environmental surroundings (Figure 1) (Van West et al. 2003). The most prolific, indirect pathway is induced under high moisture and produces motile zoospores developed from sac-like sporangia which emerge from mycelium or oospore structures. Cytoplasmic cleavage results in the development of over 60 biflagellate zoospores in each sporangium, which are released when osmotic pressure is altered in flooded soils (Erwin et al. 1983). Zoospores will orientate and move towards plant roots, encysting and penetrating host tissue. The second, direct pathway is characterised by the production of a germ tube from encysted zoospores, oospores, or chlamydospores. Sexual oospores and asexual chlamydospores are thick-walled survival structures able to remain dormant for long periods in adverse dry soil conditions. Once the host has been successfully invaded, aseptate hyphae branch inter- and intra-cellularly, forming a mycelial mat and absorbing host nutrients that facilitate the further production of spores and mycelia. A susceptible host plant will succumb to disease, becoming an abundant source of inoculum. Symptoms of PRR disease include a reduction or cessation of shoot and root growth rate, leaf chlorosis, desiccation of foliage, premature senescence, wilt, decay of the lateral roots, a reddish-brown stem canker stemming from the tap root, and eventual plant death (Erwin et al. 1983, Erwin et al. 1996, Van West et al. 2003).

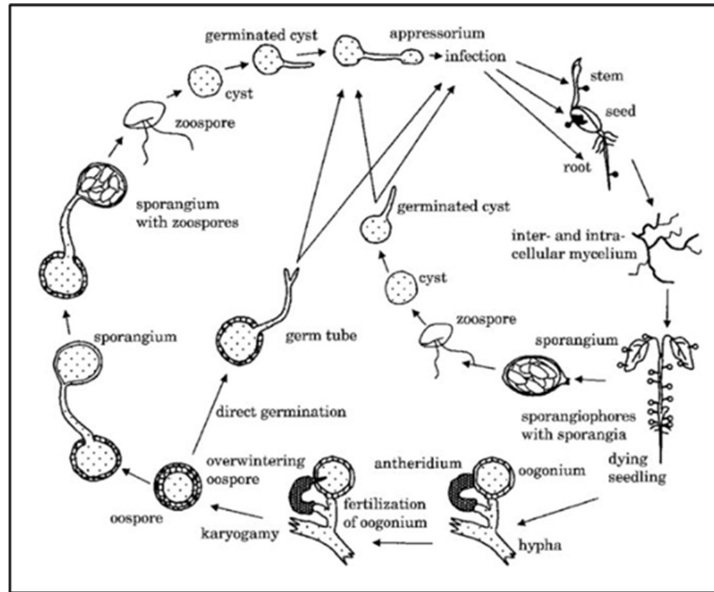

Figure 1. Life cycle of a typical root-infecting oomycete; *Pythium* and *Phytophthora* spp. (Van West et al. 2003).

The highly adapted *P. medicaginis* species are hemibiotrophic, initiating mycelial infection of the host during a biotrophic phase. Once established, secondary effectors kill host tissues leading to the destructive necrotrophic phase of infection (Irwin 1997, Vleeshouwers et al. 2000, Cahill et al. 2002). *P. medicaginis* acts similarly to other hemibiotrophic pathogens, where the biotrophic phase involving the colonisation of roots, haustoria development and cell death occurs during the first 24 hours post infection (hpi), followed by the necrotrophic phase from 36 hpi where increased cell damage, hyphal ramification, sporulation and necrosis occurs within chickpea roots (Coles et al. 2022). Greater understanding of both pathogen and host response to waterlogging, particularly with flood-sporulating *Phytophthora* spp., is key to unlocking higher levels of resistance in chickpea.

2.2.2 Phytophthora root rot in Australian chickpea

P. medicaginis and related *Phytophthora* spp. have been reported to have caused PRR of chickpea in Argentina, Spain, India, and Pakistan, based on limited taxonomic and economic evidence (Knights et al. 2008). The centre of origin of *P. medicaginis* is unknown, but is hypothesised to have been in Transcaucasia, co-evolving with lucerne (*Medicago sativa*) and chickpea (Irwin et al. 1995). *P. medicaginis* in Australia is thought to have originated from a single introduction event and was first discovered in QLD in 1956 (Irwin et al. 1995). *P. medicaginis* is now prevalent in chickpea and lucerne across the northern growing region (Knights et al. 2008). These areas are favorable to *P. medicaginis* due to poorly draining heavy clay soils, that increase inoculum survival across seasons and where chickpea and lucerne hosts are largely grown (Irwin et al. 1995, Knights et al. 2008).

2.2.3 Disease control

There is currently no cost-effective control for PRR disease in chickpea. Research has highlighted the control of PRR through seed dressing with fungicides (i.e., metalaxyl), and soil drenching plants with phosphoric acid and/or calcium compounds (Pegg et al. 1985, Tyler 2001, Sugimoto et al. 2005, Sugimoto et al. 2008). However, these methods of control are only able to provide short-term benefits and are cost-prohibitive in broadacre agriculture. Current recommendations in chickpea are largely agronomic, including variety choice and paddock selection, where extended periods between successive chickpea planting (minimum four years) provides a disease break. *P. medicaginis* has, however, been reported to survive dormant in the soil on average for four years with reports of up to ten years (Moore et al. 2011). Therefore, plant breeding for improved resistance is the most effective solution to prevent PRR-induced yield losses in chickpea.

2.2.4 Breeding for Phytophthora root rot resistance in chickpea

Extensive screening has shown that sources of PRR resistance in chickpea are scarce (Knights et al. 2008). The search is ongoing for novel sources of resistance that can be introgressed into new varieties adapted to the northern growing region of Australia. Brinsmead et al. (1985) screened over 200 chickpea lines from the Australian chickpea collection in fields naturally infected with *P. medicaginis*. The most resistant accession, ICC11870 (CPI56564), provided partial resistance following introgression into adapted varieties, contributing to early chickpea varieties including the moderately susceptible Yorker (Dale et al. 1991). More recent varieties with similar genetic backgrounds, such as PBA HatTrick and PBA Seamer, are also characterised by their moderately susceptible rating to PRR.

Wild *Cicer* species offer greater diversity in response to a range of biotic and abiotic stresses (Croser et al. 2003). Knights et al. (2008) tested 29 *C. reticulatum*, 21 *C. bijugum*, 9 *C. echinospermum*, 4 *C. judaicum*, and 3 *C. pinnatifidum* accessions that were provided by the International Center for Agricultural Research in the Dry Areas (ICARDA) and the International Center for Research in the Semi-Arid Tropics (ICRISAT). Higher levels of resistance were identified within *C. echinospermum* P.H. Davis wild *Cicer* species, specifically in accessions ILW00245 and ILW00246. Wild *Cicer* species are, however, non-adapted. They are typically prostrate, with poor seed quality. The non-adapted nature of accessions in these species makes introgression of novel sources of PRR a challenge in plant breeding. Accessions ILW00245 and ILW00246 were backcrossed extensively into cultivated *C. arietinum* material in an attempt to recover the adaptation and quality required of new varieties (Brinsmead et al. 1985, Knights et al. 2008). The narrow genetic base of cultivated chickpea in Australia dictates that interspecific crossing remains essential for increasing genetic diversity and the introduction of improved novel sources of PRR resistance (Abbo et al. 2003, Singh et al. 2021).

Additional accessions of wild *Cicer* and landraces are being made available for plant breeding efforts in Australia (Von Wettberg et al. 2018). Broad genetic diversity exists for many traits in wild *Cicer* species and evidence of successful introgression in response to various biotic and abiotic stresses is documented (Croser et al. 2003, Singh et al. 2005, Li et al. 2015). Sources of PRR resistance in chickpea have resulted in a reduction in PRR disease severity but not complete absence of disease, indicative of horizontal and quantitative resistance. PRR resistance quantitative trait loci (QTL) have been mapped in both intraspecific (*C. arietinum* (cultivated chickpea) × *C. arietinum*) and interspecific (*C. arietinum* × *C. echinospermum*) backcross populations and have been shown to differ in chromosomal regions (Amalraj et al. 2019). In a 2000 review of durable resistance in crops, Johnson stated that "…there is no single resistance phenotype and no single genetic basis for durable resistance". Therefore, to develop durable quantitative PRR resistance in chickpea, identification and pyramiding of major and minor functional QTL will require an ongoing exploratory pre-breeding effort (Johnson 2000, St Clair 2010). The advancement by Amalraj (2019) marks the beginning of the search for further QTL identification in chickpea breeding for PRR resistance, to facilitate rapid incorporation and stacking of novel resistance, and to better understand potential resistance mechanisms and whether they can be un-coupled from negative agronomic traits.

2.3 Waterlogging

Flooding is a common environmental stress worldwide and is documented extensively through history with flooded river systems and high precipitation. There are many types of waterlogging that affect cropping regions. These include sub-soil waterlogging that affects plant roots only and submergence that covers both plant aerial vegetation as well as roots in the sub-soil. Waterlogging can have varying degrees of impact on the plant, depending on duration, temperature, soil type, and presence of plants and/or microorganisms (Kozlowski 1984). Increased overall global rainfall of 5-15%, as well as greater frequency and severity of

El Niño and *La Niña* events, are projected due to effects of anthropomorphically induced climate change (Felton et al. 1997, Boye et al. 2010, Cai et al. 2023). Therefore, improving robustness of crop species to both drought and waterlogging remains a breeding priority globally.

Increased susceptibility to plant disease is often influenced by the level of waterlogging tolerance and physiological growth stage (Kozlowski 1984). The soil environment surrounding the roots plays a significant role. Soil structure, soil composition, level and duration of saturation all influence the tolerance capacity required for plants to overcome disease (Drew et al. 1980, Colmer et al. 2009). During a rainfall event, the soil profile will fill to field capacity and remain saturated depending on the quantity of rain, evapotranspiration, and soil structure. Under waterlogging conditions where water remains above field capacity, soil composition is altered and oxygen is rapidly depleted by the reduction of the soil and metabolism of plants/microorganisms over just a few days (Drew et al. 1980). It can take up to ten days upon draining for soil to return to pre-flooding conditions, and some soils may remain oxygen-deficient for longer periods due to poor structure. Hence, both transient and long-term waterlogging can have significant effects on dryland crop growth and yields (Leyshon et al. 1974, Jackson 1979, Malik et al. 2002).

2.3.1 Waterlogging in chickpea

Similar to most dryland species, chickpea is sensitive to waterlogging. The severity of damage has been shown to be greatly influenced by environmental conditions and physiological growth stage at flooding (Cowie et al. 1996). Increased waterlogging sensitivity in the late vegetative and reproductive phases of chickpea has been reported (Cowie et al. 1996, Dron et al. 2022). Waterlogging (13 days duration) experienced during flowering and pod set results in 35% and 65-100% yield penalty, respectively (Cowie et al. 1996). Subsoil waterlogging (10 cm below

soil surface) for 12 days at early vegetative stage results in yield penalties of between 44 and 50%, and was associated with a severe reduction in shoot biomass, root growth, and variation in root architecture (Palta et al. 2010). Root recovery is thought to be an indicative measure of waterlogging tolerance in chickpea, as a more vigorous plant will resume root proliferation soon after reaeration or develop adventitious roots in the non-waterlogged subsoil (Palta et al. 2010). Current levels of waterlogging tolerance in chickpea are minimal. Only a small number of lines have been screened for waterlogging tolerance, warranting further investigation (Chauhan 1987., Cowie et al. 1995, Palta et al. 2010).

2.3.2 Oxygen deficiency

Prolonged soil saturation and waterlogging results in the rapid depletion of soil oxygen, as diffusion is reduced 10^{-4}-fold, leaving a hypoxic (low oxygen, <18%) or anoxic (absence of oxygen) environment in the soil and rhizosphere. The reduction in oxygen availability of waterlogged soils results in suppressed root mitochondrial respiration, as oxygen is the final electron acceptor of electron transport chain, attributing to the decline of adenosine triphosphate (ATP) production (Bailey-Serres et al. 2008). Under anaerobic conditions plant metabolism shifts to the temporary ethanol fermentation or glycolysis pathways for energy production. Anaerobic respiration is an inefficient process that converts pyruvate to ethanol through the activity of the enzyme alcohol dehydrogenase, or lactate through activity of lactate dehydrogenase, resulting in production of toxic by-products including lactic acid, ethanol, and aldehydes (Colmer et al. 2009, Xuewen et al. 2014).

Damaging by-products of anaerobic respiration accumulate, along with the increased presence of cellular reactive oxygen species (ROS) due to low oxygen conditions (i.e., superoxide, radicals, hydroxyl radicals, and hydrogen peroxide). Low energy levels results in the deterioration of cell membranes and cell death in the root tissues (Pucciariello et al. 2012,

Tamang et al. 2014). Waterlogging tolerance in plant roots has been associated with the removal of excess by-products (i.e., ethanol) through transpiration and leakage, or alternative production of malate or lactate (Jackson et al. 2005); and the production of antioxidants and antioxidant pathway enzymes (e.g. catalase, ascorbate peroxidase, superoxide dismutase and polyphenol oxidase) to maintain ROS balance thus reducing the level of oxidative damage caused by waterlogging and anerobic respiration (Hasanuzzaman et al. 2020). Carbohydrate reserves support sustained ATP generation under anaerobic soil conditions and carbohydrate stored in the roots may potentially play an important role in plant survival. The use of this energy source results in inevitable carbohydrate deficiency over time, affecting overall metabolic functionality, including water and nutrient uptake, hormone balance, internal solute transport, and photosynthetic carbon fixation in sensitive plants (Malik et al. 2001, Jackson et al. 2005, Colmer et al. 2009).

2.3.3 Nutrient availability and toxicity

During waterlogging events, soil nutrient availability is influenced by duration and initial soil properties that can result in both toxicities and deficiencies. The reduction in oxygen levels in waterlogged soils decreases the plant's ability to take up some essential mineral nutrients (e.g., nitrogen, potassium). In some soils, chemical transformation through reduction and the accumulation of anaerobic microbial metabolites alters the soil environment. Consequences include denitrification (NO_3^- to N_2), and reduction of manganese (Mn^{4+} to Mn^{2+}), iron (Fe^{3+} to Fe^{2+}), and sulfate (SO_4^{2-} to H_2S, S^{2+} or HS^-, pH-dependent) rendering these essential nutrients unavailable to the plant. Fermentative by-products (ethanol, lactic acid, acetaldehyde, and aliphatic acids) also increase in concentration in the soil and can reach phytotoxic levels (Kozlowski 1984, Ponnamperuma 1984, Blom 1999, Pezeshki et al. 2012). Decomposing organic matter during soil saturation promotes reducing bacteria and fungi, further increasing

levels of phytotoxic compounds. As a result of nutritional imbalances and soil toxicities the plant often shows signs of poor vigour, chlorosis and in severe cases death (Kozlowski 1984).

2.4 Waterlogging and Phytophthora interactions

Symptoms of waterlogging are almost identical to those of PRR. However in the case for PRR, a characteristic stem canker is present and root rot is delayed (Erwin et al. 1996). Waterlogging damage is triggered by poor gas exchange, disruption to energy and carbohydrate provisions due to a change in soil conditions and the plant's inability to overcome or avoid damage. There is an inherent interaction between soil-borne *Phytophthora* spp., and soil moisture, aeration, and temperature. These factors are important considerations in understanding *Phytophthora* spp. population dynamics and predicting risk of disease under waterlogging conditions. Changes in soil conditions can rapidly manipulate the survivability and proliferation of *Phytophthora* during various morphological stages, which will influence the longevity, pathogenicity, and spread of the pathogen and disease. Each *Phytophthora* spp. has been shown to have specific oxygen requirements for optimal sporulation and growth (Moore 1975). It is often assumed that increased disease severity in the field is due to the presence of favourable soil conditions for the pathogen (Duniway 1983, Kong et al. 2014). However, hypoxic or anoxic soil waterlogging conditions are not always suitable for aerobic *Phytophthora* spp. and hence waterlogging may be more damaging than PRR initially, further supporting the need for research to improve waterlogging tolerance in chickpea.

2.4.1 Soil moisture and texture

Soil moisture or water potential is directly related to soil texture and has the greatest influence on *Phytophthora* spp. life cycle and disease development. Low lying areas or water courses maintain higher levels of soil moisture, providing a suitable environment for in-crop or inter-host crop development and survival of *P. medicaginis,* and are often the first sites of infection

following rainfall (Schwinghamer et al. 2011). Soil moisture at or above field capacity, during extended rain events and flooding, is required for oospore germination, sporangium and zoospore development, dispersal and disease establishment (Erwin et al. 1996).

High soil moisture increases free water and capillary action between soil particles, promoting zoospore motility. *Phytophthora* spp. zoospores have been shown to move between 5 and 65 mm in soil from the nearest sporangia. In *P. megasperma*, a 41 mm difference in distance travelled by zoospores was observed when comparing a sandy soil (65 mm) and a sandy loam (24 mm), with the difference attributed to soil porosity (Pfender et al. 1977, Duniway 1983). Topographical gradients in the paddock also affect the ability of the organism to spread within or above the soil surface in free water flow, transporting inoculum into low lying areas and resulting in high incidence of disease in these areas (Irwin et al. 1995).

In addition to zoospore movement, porosity factors such as soil particle and pore size directly influence soil water potential and *Phytophthora* survival. Larger pore spaces drain quickly while smaller soil particles, with reduced porosity, hold tightly to water for longer durations. Soil is rarely homogenous within the profile, and high spatial variability in soil type is often observed within paddocks. The variability in soil and water potential explains the discontinuous detection and variable concentration of *Phytophthora* spp. in field surveys (Meadows et al. 2011, Bithell 2017). Longevity of *Phytophthora* survival may occur at higher frequencies in pockets of soil with fine to medium texture. However, some very fine-textured soils have been found to hold onto water so tightly that they become anaerobic and inhibit spore movement (Erwin et al. 1983, Ponnamperuma 1984). Bithell et al. (2021) commented on the variation of soil quantification of *P. medicaginis* using qPCR-based soil DNA testing (Predicta®B - SARDI). Soil samples from the same site yielded a range of levels of *P. medicaginis* inoculum. This variability was attributed to the presence of diseased root tissue hosting large volumes of inoculum in some soil samples (Bithell et al. 2021). A key question which remains to be

definitively answered is whether the pathogen resides in soil pockets due to host presence, soil moisture, or both (Marks et al. 1975).

2.4.2 Soil oxygen

Soils free of compaction and waterlogging have sufficient oxygen concentration required for the development of PRR. After extended periods of extremely high-water potential at or above field capacity, hypoxia or anoxia results. *Phytophthora* spp. are restricted by its tolerance to the amount of oxygen and carbon dioxide in the soil and is therefore uncommon in deep or poorly aerated and anoxic soils (Erwin et al. 1983). Generally, *Phytophthora* spp. are able to survive and infect at relatively low levels of oxygen and high carbon dioxide levels, though this ability differs between species of *Phytophthora* (Mitchell et al. 1971, Moore 1975). Stolzy *et al* (1967) showed a lack of recovery of the oomycete after four days of waterlogging in soils after inoculation with zoospores, questioning the ability of zoospores to survive without first encountering a period of non-waterlogging environmental conditions, to facilitate development of more resistant structures. The rate of oxygen depletion appears to be critical to the development of *P. medicaginis* mycelia, zoospores and dormant structures (oospore chlamydospores). Under rapidly acquired waterlogging conditions, the plant would succumb to waterlogging and not PRR.

Zoospore survival rate generally decreases with increasing intensity of hypoxic conditions and exposure time, while numerous dormant oospore structures are produced (Erwin et al. 1983). This could be a survival mechanism of the species. Mycelia are less susceptible to hypoxia and can grow under a wider range of oxygen conditions (Moore 1975, Erwin et al. 1983). *P. megasperma* had decreased colony counts with increased exposure to hypoxia; whilst three related species (*P. nicotianae, P. pini* and *P. tropicalis*) showed greater colony counts (Erwin et al. 1983). Further to this, disease development was delayed in plants inoculated with *P.*

cinnamomi at low levels of oxygen in both aeroponic and hydroponic systems, when compared to an aerated system. Mycelia and oospore production for *P. medicaginis* has been shown to be significantly reduced under severe hypoxia (<0.02 atm oxygen) (Moore 1975). Therefore, the duration of waterlogging and oxygen level is crucial to the production and survivability of *P. medicaginis* spores, and inherent pathogenicity.

Oxygen-deficient soil results in indirect and direct physiological damage to plant roots which has been shown to predispose the host plant to PRR infection (Drew et al. 1980, Kuan et al. 1980, Li et al. 2016). Increased infection rates are attributed to easier hyphal penetration and a chemotaxic response to an increase in root exudates from leaky tissues. Root exudates can initiate a chemotactic response from germtubes and motile zoospores to the plant roots, increasing inoculum load (Kuan et al. 1980, Duniway 1983). Ultimately, the plant is overcome by *P. medicaginis* with an inability to maintain root integrity and regenerate healthy roots as metabolism is slowed in response to waterlogging-induced hypoxia and anoxia.

2.4.3 Other microbial interactions

The activities of other microorganisms in the soil, particularly in warm, wet conditions, offer the greatest threat to the survival of soil-borne *Phytophthora* (Duniway 1983). Under waterlogging conditions, aerobic microbes compete for and rapidly diminish remaining oxygen and other resources from the soil. Anaerobic microbial population growth is stimulated, further increasing the presence of phytotoxic, metabolic by-products in the soil (Kozlowski 1984). As described, both *P. medicaginis* and the chickpea host will be affected negatively under these increasingly hostile conditions.

Nitrogen (N) is a critical limiting element for plant growth and production. Symbiotic nitrogen fixation is an important feature of chickpea for improving the sustainability of farming practices by reducing the dependency on costly and environmentally harmful fertilisers. The

soil-borne bacteria, *Mesorhizobium ciceri,* converts atmospheric nitrogen (N_2) to plant-available ammonia (NH_3) in nodules that develop on the root system. Up to 85% of the crop's N requirements are met through N fixation when fertiliser is not applied and soil N is limited (Peoples et al. 1995). *M. ciceri* can fix an average of 58 kg of N per hectare in chickpea, with legacy N remaining in the soil to benefit the successive crops (Herridge et al. 2008). *M. ciceri* inoculants are used in-furrow at sowing to ensure adequate levels of bacteria are present for optimal N fixation. *Mesorhizobium* are mostly obligate aerobes and under waterlogging conditions, N fixation in many legumes is supressed (Thomas et al. 2005). It is unclear how current *M. ciceri* strains perform following extended periods of waterlogging.

Flavonoids are secondary metabolites that have numerous functions in plants, including UV protection, as pigments, signalling molecules, for defence and auxin transport inhibition for plant development. It is well documented that flavonoids play an important role in plant defence and nodulation in legumes (Aoki et al. 2000, Hassan et al. 2012, Weston et al. 2013). The effect of stress on flavonoid induction is varied and may be either positive or negative. During abiotic or biotic stress, a plant can encounter energy crisis and the ability to produce and/or transport compounds or their pre-cursors is restricted, thus limiting flavonoid biosynthesis. An alternative theory suggests that plants can divert resources to defence, with a caveat of limiting growth (Treutter 2006, Colmer et al. 2009). The response of the plant would be greatly affected by the type and level of stress a plant encounters. The function of flavonoids, specifically phytoalexin pterocarpans, in the chickpea defence response to *P. medicaginis* is not well understood. Similarly, the effect of waterlogging on flavonoid production is not documented.

2.5 Waterlogging tolerance mechanisms for Phytophthora resistance

Waterlogging tolerance involves the avoidance of damage through plant adaptation that includes the regulation of plant morphology, energy use, hormone biosynthesis, and signalling pathways (Pan et al. 2021). Characteristics of improved waterlogging tolerance include morphological and physiological changes to; (1) acquire gas, nutrients and water for respiration and photosynthesis, (2) maintain photosynthetic potential and respiration, and (3) reduce the effects of waterlogging-induced soil and plant cell toxicities (Colmer et al. 2009, Pan et al. 2021, Pedersen et al. 2021). Mechanisms to improve waterlogging tolerance are predominately controlled by hormonally induced pathways that affect root morphology and physiology to maintain the critical movement of gas, water, minerals and sucrose within the plant. Modification of root parameters within the plant include: Commencing anaerobic energy metabolism; production of antioxidant enzymes to protect root cells exposed to the hostile soil environment; formation of root exo- and endo-dermal barriers that reduce radial oxygen loss; and increased proliferation of roots adapted to waterlogging environments and/or thickening of roots to increase surface-to-volume ratio in roots near to the soil–surface, increasing gas movement from the atmosphere to deeper roots (Nishiuchi et al. 2012, Yamauchi et al. 2013, Takahashi et al. 2014, Steffens et al. 2016, Jitsuyama 2017, Kotula et al. 2017, Gill et al. 2019, Qamer et al. 2021). Modification of these waterlogging tolerance mechanisms could offer an opportunity to improve or maintain chickpea resistance to PRR under waterlogging stress through the maintenance of plant metabolism and dual-purpose protective barriers.

Root trait research is critical to improve both yield and yield stability. The heterogeneous nature of soils, plasticity of root systems, and demanding ‘shovel-omics’ techniques for exhuming root tissue in the field means that it is often difficult to keep these delicate tissues intact for characterisation. Progress towards the development of improved *in-situ* and *ex-situ* methods for root phenotyping has been a research priority (Meister et al. 2014, Atkinson et al. 2019).

These methods seek to improve the ease, accuracy, and capacity to characterise root traits of individual genotypes for improvement in plant breeding. QTL mapping and genome-wide association studies (GWAS) that follow on from accurate phenotyping of root physiology and architecture, have been used to identify genetic markers for favourable or un-favourable root phenotypes. For example, QTL associated with root traits have been identified for abiotic stress tolerance, especially for drought, waterlogging tolerance and yield improvement traits in rice, wheat, maize, millet, rapeseed, bean and soybean (Meister et al. 2014, Kuijken et al. 2015, Atkinson et al. 2019).

Some difficulty lies in the large environmental effects that compromise phenotyping data, thus reducing the heritability of many root traits. Traits with low heritability should therefore not be considered unusable and still be included in selection models for targeted phenotypes, as the underestimated heritability may be attributed to variability of root growth in heterogeneous soils. In wheat, for example, different waterlogging tolerance traits were identified between diverse waterlogging and soil conditions (Setter et al. 2009). Contrasting waterlogging tolerance phenotypes were attributed to the possible differentiation in primary cause of waterlogging damage, where it has been proposed that the level of hypoxia/anoxia and soil redox plus associated nutrition availability/toxicity differs locationally (Setter et al. 2009). Therefore, a suite of waterlogging tolerance traits may be required, depending on the genotype and the environment in which the plant is grown, that can improve general plant function under different waterlogging scenarios.

2.5.1 Adventitious roots

Variability in root architecture has been observed across agro-ecological regions that grow chickpea due to diversity in climate, including in-season rainfall, soil type and the associated capacity for storing soil moisture and drainage (Chen et al. 2017). Chickpea root systems are

ideal for scavenging residual soil moisture. They have been shown to have extensive tap roots that can reach depths of 90-140 cm, and/or have prolific networks of shallow secondary roots that extract water from the surface. Both traits are important and are being studied for improved drought tolerance (Ramamoorthy et al. 2017). Under waterlogging conditions, the ability of a plant to rapidly recover and produce new roots from the shallow upper root system is more important, as roots at depth are often compromised and cannot resume growth.

During waterlogging, oxygen zones can exist in soil extending from the soil surface to the root apex. In some soils, up to 5 to 10 cm from the surface will remain less reduced, with oxygen able to diffuse at a slow rate into the soil profile. This region offers a reprieve for plant growth (Patrick et al. 1972). Damage to the root apices inhibits growth and carbon is then re-allocated for shallow root initiation and growth for plant recovery, as described in Malik *et al* (2001). A plant's ability to recover roots is dependent on soil flooding level, duration of waterlogging and drainage of the soil post-waterlogging. For example, wheat showed minimal damage during periods of sub-soil waterlogging (>200 mm below soil surface), with adventitious root growth and root porosity preferentially increased in the free-draining zone at the soil surface (Malik et al. 2001). The capacity of chickpea to develop adventitious roots under waterlogging is not well understood. Further knowledge in this area may offer a breeding solution to increase survivability following waterlogging.

Research on the wetland plant species, *Solanum dulcamara*, under complete and partial submersion, revealed that access to the atmosphere was critical for the development of waterlogging escape mechanisms, including adventitious root growth. This was expected to be caused by the plants' limited accessibility to oxygen, and poor carbohydrate status (Zhang et al. 2017). The slow development of anoxia over a number of hours could feasibly allow regulation of gene expression to take place in response to the stress. This could involve up- or down-regulation of existing pathways, or the initiation of specific adaptive pathways to provide

protection or mechanisms for survival (i.e., glycolysis, fermentation, aerenchyma and adventitious root formation). As for the *Phytophthora* pathogen itself, it has been shown that the transition time is critical to plant survival even in waterlogging-tolerant plants (Subbaiah et al. 2003). Sudden and unnatural anoxia exposure without acclimatisation in root tip cells can lead to death within hours (Johnson et al. 1989). The severity and timing of application of waterlogging needs to be considered in evaluation of potential root phenotyping systems, as there are limits to a plant's ability to recover.

The partially underground epicotyl and hypocotyl, situated above and below the cotyledon respectively, are determinate structures with an established final number of cells. Secondary roots from this region are categorised as adventitious, and their growth is partly genotype-dependent (Verstraeten et al. 2014, Steffens et al. 2016). Adventitious root growth is stimulated following stress by the key plant growth regulator auxin (Verstraeten et al. 2014). In another hypogeal germinating species, mungbean (*Vigna radiata*), active growth of adventitious roots on the epicotyl is thought to play a critical role in recovery from damage due to waterlogging (Ahmed et al. 2002). In chickpea, decay of the root system was observed in a small, controlled environment sub-soil waterlogging study, where rapid root recovery was shown to be critical for yield advantage in the desi variety Rupali when compared to the kabuli variety Almaz (Palta et al. 2010). Further investigation of root recovery, specifically adventitious root growth, in chickpea is warranted to improve waterlogging tolerance through breeding.

In 1991, Dale and Irwin found that root tissues of PRR-resistant and susceptible chickpea genotypes showed no observable differences in *Phytophthora* colonisation, but hyphal growth was reduced in the resistant genotype. Chickpea is a hypogeal-germinating crop where the cotyledon remains below ground, and the PRR sensitive epicotyl region remains partially buried within the soil and at high risk of *Phytophthora* infection. The increase in PRR sensitivity at the epicotyl region was attributed to the presence of stomata in this region (Erwin

et al. 1983, Dale et al. 1991). However, zoospores have also been shown to preferentially accumulate at root hair zones and root apices. The epicotyl region in the presence of waterlogging in chickpea has an increased likelihood of adventitious and root recovery growth. This sensitive epicotyl region where root development is not predetermined but stimulated by nutrient requirements, wounding or stress (Steffens et al. 2016), could be a target region for breeding and agronomic improvement against PRR, as branch points, sites of elongation and stomata are all infection points for soil-borne *Phytophthora*. Limiting branching, adventitious root growth and potentially root vigour in some scenarios may assist the plant to maintain resistance to PRR. But what could this mean in terms of drought tolerance or yield potential? Are plant breeders indirectly selecting for poor drought tolerance and low potential yield when breeding for higher levels of PRR resistance?

2.5.2 Aerenchyma and root tissue ratio

Aerenchyma is the term given to the large, gas-filled chambers within root tissue, creating a low resistance pathway for gas movement and connecting the atmosphere to deep roots (Jackson et al. 1999, Drew et al. 2000). Aerenchyma facilitate the influx of oxygen and expulsion of plant- and soil-derived gases (carbon dioxide, methane, and ethylene) and toxic substances (ethanol and reduced metal ions) that are often present in waterlogged soils (Yamauchi et al. 2013). The increase in porosity can be innate in some dryland species or develop in response to waterlogging stress; including in rice (*Oryza sativa*), maize (*Zea mays*), barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*) (Armstrong 1980, Jackson et al. 1999, Evans 2004, Nishiuchi et al. 2012). There are two types of aerenchyma, termed schizogenous and lysigenous. Schizogenous aerenchyma are formed from the separation of adjacent file cells by the creation of gas spaces of cortical cells or differential cell enlargement, distending longitudinally to form intercellular spaces. Lysigenous aerenchyma are formed from

the spatially selective apoptosis of file or cortical cells, driven by the stress hormone ethylene under waterlogging stress (Das et al. 1977, Evans 2004).

Secondary aerenchyma can develop in the secondary cortex, stem phelloderm, epicotyl, hypocotyl and roots of some dicots, such as soybean (Takahashi et al. 2014). Under waterlogging, adventitious roots develop a greater proportion of aerenchyma tissue to enhance gas exchange and maintain aerobic cellular conditions (Kozlowski 1984, Jackson et al. 1985, Jackson et al. 1999, Gunawardena et al. 2001). For example, wheat adventitious roots can penetrate deeper (100-171 mm) into anoxic soils when supported by internal oxygen diffusion through aerenchyma (Thomson et al. 1992, Malik et al. 2001). Morphological features of roots which have been associated with increased aerenchyma or porosity include thicker roots (Armstrong 1980, Armstrong et al. 1982, Aguilar et al. 1999), small numbers of laterals (Armstrong et al. 1983), or if present, short laterals that emerge close to the aerated root base (Armstrong et al. 1990). Such types of roots have been observed in chickpea but have not been assessed for the presence of aerenchyma.

Pulses have limited waterlogging tolerance. Faba bean (*Vicia faba*) is the least waterlogging-sensitive pulse species, followed by narrow leaf lupin (*Lupinus angustifolius*), chickpea, lentil (*Lens culinaris*), and field pea (*Pisum sativum*) (Solaiman et al. 2007). Root traits that contribute to better faba bean performance include high relative growth rate of roots during recovery and the presence of aerenchyma in adventitious roots (Solaiman et al. 2007). These observations are supported by a difference in primary root porosity levels between chickpea (16.5%) and faba bean (29%) following hypoxia treatment (Munir 2016). In chickpea, there is little published literature investigating the presence or development of aerenchyma. Research is warranted across multiple chickpea genotypes, to search for high levels of root porosity and aerenchyma for improved waterlogging tolerance. Aerenchyma formed at depth would further benefit submerged nodules, maintaining the nitrogen fixation capacity of chickpea (Thomas et

al. 2005). The improved integrity of chickpea roots at depth through adequate atmospheric gas movement during a waterlogging event, would likely improve root function including resistance to PRR, and successive recovery post-waterlogging.

The outer cortex is more porous than the internal stele tissues of roots, and like aerenchyma, promotes the diffusion of oxygen to the roots at depth in anoxic soil conditions (Sundgren et al. 2018). Further to this, lysigenous aerenchyma develop in the root cortex, and if there is a greater root cortex-to-stele ratio either initially or induced under stagnant conditions, as observed in maize and rice, there is more potential for aerenchyma and gas diffusion to roots at depth in waterlogged soils (Yamauchi et al. 2019). Thicker roots with lower surface-area-to-volume also reduce the rate of radial oxygen loss, that would be particularly critical in crops such as wheat (Malik et al. 2001), maize (Abiko et al. 2012), or chickpea (Hartung et al. 2002) with no barrier to radial oxygen loss (Yamauchi et al. 2019). Chickpea is believed to have a limited capacity to create aerenchyma, but this is a trait worthy of further investigation. Alternatively, increased cortex-to-stele ratios and thicker root systems could deliver greater waterlogging tolerance capacity.

2.5.3 Apoplastic barrier

The apoplastic barrier separates root tissues from the external environment and neighbouring cellular tissues. These diffusive physical barriers are comprised of a complex of organic polymers, lipid-phenolic bio-polyesters, suberin and lignin. Deposition of suberin and lignin is highly regulated, varying in composition and quantity, both longitudinally and transversely in root tissues as determined by tissue type, age and species (Thomas et al. 2007, Kotula et al. 2009, Beisson et al. 2012, Andersen et al. 2015, Kotula et al. 2017). Increased suberisation is an adaptive mechanism to harsh environmental conditions such as anoxia, drought, salinity, heavy metal or nutrient stress, and mechanical damage (Enstone et al. 2002). Transcellular

transport is restricted by increased deposition of suberin in the outer membranes of endodermal cells, leaving non-suberised passenger cells to regulate metabolites and water through symplastic diffusion, transcellular aquaporins, and ion channels for cell function. In roots, Casparian strips and suberin lamellae are formed by the endodermis and exodermis of primary tissues and the periderm during secondary growth (Nawrath et al. 2013). The Casparian strip is a primary barrier for root protection that is comprised of suberin. It is found between the radial walls of endodermal cells, and in some species, the peridermal and exodermal cells also, greatly restricting apoplastic transport (Enstone et al. 2002, Ranathunge et al. 2011, Robbins et al. 2014, Andersen et al. 2015, Vetterlein et al. 2016).

For a plant to gain the most benefit from increased root porosity or aerenchyma, a reduction of radial oxygen loss (ROL) is required. Gasses are carried through the porous or arenchymatous tissues to and from the lower root system, maintaining root function at depth. The apoplastic barrier is thought to act synergistically, with root suberin and lignin increasing movement of radial oxygen to depth due to a reduced root permeability and consequential ROL (Kotula et al. 2009). A robust apoplastic barrier, whilst advantageous for carrying oxygen to roots at depth, has also been reported to reduce accessibility to water and nutrients (Colmer 2003). However, it is proposed that there is opportunity for improving suberisation without limiting plant productivity (Azaizeh et al. 1991, North et al. 1991, Colmer et al. 1998, Hose et al. 2001, Martre et al. 2001, Ranathunge et al. 2008, Kotula et al. 2009, Plett et al. 2016). There may be breeding opportunities to improve suberin deposition in chickpea and thus waterlogging tolerance.

In addition to conferring abiotic stress tolerance, suberisation has been shown to be an important pathogen defence mechanism. Many pathogens, including the mycelium and appressoria of *Phytophthora* spp., travel between the cells of plants through apoplastic means. Travel is restricted by the deposition of suberin in the endodermis, disabling access to the

vascular tissues of the root. In soybean, younger root tissue towards the root tip contained less total suberin than older tissue (Thomas et al. 2007). In addition, the chemical composition varied when comparing suberin extracted from epidermal and endodermal layers of soybean roots. The epidermal tissue had less total suberin but a higher proportion of aliphatic ω-hydroxylated fatty acids over phenolic components when compared to endodermal tissues. The aliphatic component of suberin has been shown to negatively correlate with mortality rate in soybean when exposed to *P. sojae* (Thomas et al. 2007). This links with chemotaxis, and with preferential penetration of *Phytophthora* spp. at root tips and branch points over older, more suberised root tissues.

Partial resistance to *P. sojae* has been linked to early generation of suberin in the middle lamellae of the cortex during the infection process in soybean (Thomas et al. 2007, Ranathunge et al. 2008). Increased suberin levels impeded hyphal growth through the exodermis by up to three hours when compared with a non-resistant genotype, containing 50% less suberin. This delay may provide more time for the activation of plant defence responses. After ten hours, the non-resistant variety had hyphae entering the stele in abundance where those in the partially resistant genotype were coiled outside the endodermis. Interestingly, at eight days after infection the level of induced suberin levels in both genotypes were identical (Thomas et al. 2007, Ranathunge et al. 2008). Increasing the rate and quantity of suberin accumulation in chickpea through selection and breeding may improve both PRR resistance and waterlogging tolerance.

Most vascular plants have Casparian bands in the endodermal tissues, and most also have an additional Casparian band in the exodermal tissue of the roots. However, it has been noted that chickpea lacks exodermal suberisation and relies solely on the wall thickening of the endodermal tissues (Hose et al. 2001, Hartung et al. 2002). Knowledge in soybean, described above, supports a case for the involvement of endodermal suberin in PRR resistance; increased

suberisation and lignification in the endodermis alone could still be associated with partial tolerance to both waterlogging and PRR (Ranathunge et al. 2008). In addition to this, chickpea varieties resistant to the dry root rot causing fungi, *Macrophomina phaseolina* and *Fusarium oxysporum*, had a thick layer of suberin in the endodermis at the outer cortex of the root tissue, whilst susceptible varieties did not (Ayyar et al. 1936, Irulappan et al. 2022). Mycelium was observed to travel further over the root tissues to find a weak entry point in resistant varieties. Breeding for varieties with thicker and more complete apoplastic barriers on the exodermal and endodermal layers, or genetic modification, should be considered to improve levels of *P. medicaginis* resistance and waterlogging tolerance.

2.5.4 Reactive oxygen species

Reactive oxygen species (ROS), derived from cellular oxidative metabolism, play an important role in the modulation of cell survival, death, proliferation, differentiation and cell-to-cell signalling in plants (Tripathy et al. 2012, Noctor et al. 2018). The formation of ROS is attributed to changes in gene expression and protein synthesis of biological pathways triggered by the metabolic status of the plant. During homeostasis there is balance in redox between ROS, ROS-scavenging proteins, and ROS processing enzymes (superoxide dismutase (SOD), peroxidase (POD) and catalase). Biotic and abiotic stress can shift redox balance resulting from an increase in ROS production and/or a decrease in antioxidant enzymes, that leads to oxidative stress (Noctor et al. 2018). Under oxidative stress, ROS levels become phytotoxic and cause cellular injury via DNA damage, protein carbonylation, ion leakage, necrosis and apoptosis (Tripathy et al. 2012, Steffens 2014).

Surges of ROS can result in beneficial cell acclimation that improves tolerance to stresses such as heat, UV exposure, oxygen deficiency, and pathogen invasion (Tripathy et al. 2012, Steffens 2014, Qi et al. 2017). The stress hormone ethylene is linked to waterlogging tolerance response

in plants. Elevated ethylene led to increased ROS activity, in turn initiating a series of programmed cell death for the development of secondary aerenchyma, epidermal release of adventitious roots, and the activation of anaerobic metabolism (Mergemann et al. 2000, Fukao et al. 2004, Steffens et al. 2011, Steffens et al. 2012, Manzano et al. 2014, Liu et al. 2017, Basu et al. 2020, Chapman et al. 2021, Liu et al. 2021, Pan et al. 2022).

ROS can also induce oxidative burst or hypersensitive response following pathogen invasion. The plant hypersensitive response functions as a host and non-host resistance mechanism against many pathogens, including *Phytophthora* spp. (e.g., *P. infestans* and *P. sojae*) (Vleeshouwers et al. 2000, Shan et al. 2004). ROS restricts pathogen growth by killing cells surrounding the pathogen, preventing extensive proliferation of hyphae during the early infection stages. Hyphal growth that escapes this response can continue to proliferate into healthy plant tissues and commence infection. By this stage, however, the plant may be afforded time for alternative resistance mechanisms to be established (Vleeshouwers et al. 2000). For necrotrophic and hemibiotrophic pathogens, such as *Botrytis cinerea, P. sojae* and *P. infestans*, this resistance mechanism is usually unsuitable as the pathogen can continue to colonise with saprophytic growth in dead tissues, by disarming the response with a suite of antioxidant enzymes, or overcoming the response by escaping dead tissues to infect live tissues (Govrin et al. 2000, Vleeshouwers et al. 2000, Mayer et al. 2001, Cahill et al. 2002). Further investigation into the interaction between oxidative stress during waterlogging and the hypersensitive response in chickpea to *P. medicaginis* is required. A better understanding of the interplay between ROS formation, hypersensitive response, aerenchyma/adventitious root formation and antioxidant enzyme activity, is required to unpack waterlogging-PRR disease interactions, particularly when there is a high inoculum load.

2.5.5 Root exudates

Amino acids, organic acids and sugars exuded by actively growing roots provide important nutrients for oomycete survival and act as chemo-attractants. In eucalypts, for example, these substances stimulate the germination of *P. cinnamomi* spores and mycelial growth and are exuded by both resistant and susceptible hosts (Malajczuk et al. 1977). Further to this, when eucalypt seedlings and lucerne root tissues are compromised by experimental wounding, germ tubes from *Phytophthora* zoospores and oospores were found to orientate and grow towards the wounded tissue (Malajczuk et al. 1977, Kuan et al. 1980, Erwin et al. 1983). This suggests that plant exudates have a strong influence on the root-soil microbiome, including interactions with *Phytophthora* spp.

Following one week of waterlogging, non-specific accumulation of amino acids (30%) and sugars (10%) increased in root exudates of lucerne when compared to non-saturated controls (Kuan et al. 1980). This change was attributed to an increase in root damage and cell leaching. Waterlogged seedlings also had a 20% increase in death when exposed to *P. medicaginis* zoospores compared to the unsaturated control (Kuan et al. 1980). Other research has found that ethanol, the by-product of fermentative metabolism, is exuded by roots during waterlogging, and has a chemotactic effect on zoospore movement and germ tube orientation (Allen et al. 1973). Following waterlogging conditions, the increase in PRR disease severity would likely result from the predisposition of root tissue to oxygen stress and not increased inoculum concentration, as oxygen stress during waterlogging compromises cell integrity, resulting in leaching of solutes, and increased chemo-attraction and permeability of plant roots.

The specificity of *P. sojae* zoospore attraction to soybean has been attributed to legume-specific flavonoids, daidzein and genistein, found at high concentrations in soybean root exudates. These compounds promoted zoospore germination and encystment, highlighting the

advanced evolution of *P. sojae* soybean infection (Hua et al. 2015). The components were not attractants of any other PRR species. Interestingly though, when isoflavone synthase genes were silenced, reducing daidzein and genistein, the susceptibility of soybean to *P. sojae* increased, indicating an important role for these flavonoids or more likely their biosynthesis pathway, in partial resistance mechanisms (Subramanian et al. 2005).

Stevenson *et al* (1997) discovered that the flavonoids medicarpin and maackiain displayed phytoalexin (antifungal) activity towards the chickpea pathogen *Fusarium oxysporum*. Roots of fusarium wilt-resistant chickpea varieties generated 20 $\mu g\ g^{-1}$ of macerated root tissue as medicarpin and maackian. This amount was ten-fold greater ($P < 0.05$) than for susceptible varieties (Cachinero et al. 2002), suggesting a role for these antifungal chemicals in pathogen resistance. Chickpea flavonoid profile and response to *P. medicaginis* infection has not yet been tested. Phytoalexin flavonoid resistance pathways may be potential targets for breeding for chickpea resistance to PRR.

If flavonoid production and exudation is important for *P. medicaginis* resistance in chickpea, the possession of one or more waterlogging avoidance or response mechanisms would help to maintain plant function and metabolic responses to disease pressure in waterlogged soil conditions. Understanding the suite of traits in chickpea that may influence the interaction between waterlogging and PRR may identify opportunity for improvement of resistance to PRR and the breeding of improved varieties. Plett et al. (2016) identified a need for caution when identifying PRR resistance traits, as chickpea varieties of greater resistance also demonstrated reduced rhizobia and arbuscular mycorrhizal fungi (AMF) colonisation. This limits N fixation and increases the dependence on soil and fertiliser phosphorous and nitrogen. Transcript analysis revealed a total of 6,476 genes that were differentially expressed during microbial colonisation in chickpea root tissue (Plett et al. 2016). In the susceptible chickpea variety Sonali, 10.2% of the response was similar following colonisation testing of both the

pathogenic *P. medicaginis* and N fixing bacteria *M. ciceri*. In contrast, in the moderately susceptible variety PBA HatTrick, 49.7% of differentially expressed genes were oppositely regulated under the same conditions (Plett et al. 2016). This is an indication that regulatory genes involved in N fixation and PRR resistance may be interconnected, warranting further investigation in chickpea.

2.6 Thesis rationale

Understanding pathogen population dynamics and survival is integral for understanding the disease incidence risk and severity of PRR. Infection can spread quickly via rapid germination and population growth, followed by zoospore movement which is closely associated with inter- and intra-soil moisture and free water (Duniway 1983). Current levels of PRR resistance in Australian varieties is not sufficient to avoid severe yield losses in average to high rainfall seasons in the presence of *P. medicaginis* inoculum. This re-enforces the need for chickpea breeders to adopt selection methods that impose the most conducive flooding and/or waterlogging conditions practical for infection, to identify and incorporate the most robust level of resistance (Erwin and Ribeiro, 1996). This research aims to understand the relationship between PRR and waterlogging, and to further define the opportunity to improve PRR resistance by using targeted phenomics and environmental proxies to understand *P. medicaginis* and PRR resistance in Australian chickpea.

It is important to consider the variability of the field environment and plasticity of plant growth when phenotyping and making breeding selections. The ability to detect effects of minor quantitative resistance loci is dependent on accurate phenotyping methods that minimise variation due to environmental conditions. For phenotyping chickpea responses in the field, inoculum is usually applied as a uniform, oospore-mycelial slurry into furrows at sowing. However, soil characteristics and topography within plots or within a field will influence soil

moisture content. PRR disease severity is strongly correlated to inoculum levels, and soil moisture affects the distribution of *Phytophthora*. High moisture favours the germination of *P. medicaginis* relative to drier areas, introducing spatial variability and increasing the difficulty associated with the detection of minor effect QTL (Pfender et al. 1977, Duniway 1983). Moreover, prolonged periods of saturation can predispose a resistant variety to waterlogging and heightened *Phytophthora* disease as observed in chickpea and confirmed in other hosts of *Phytophthora* (Stolzy et al. 1967, Kuan et al. 1980, Wilcox et al. 1985, Bowers et al. 1990).

Further understanding of the environmental response of *P. medicaginis* is key to accurate phenotyping, and the discovery of genomic regions in chickpea associated with resistance, along with the underlying molecular and morphological characteristics of resistant genotypes. This study will provide breeders with suitable controlled environment screening and phenotyping techniques, to unravel the genotype-by-environment interaction and improve genetic tolerance to waterlogging and resistance to *P. medicaginis*. A major objective is to determine if less destructive and more repeatable waterlogging phenotyping methods can be used as a proxy for PRR resistance selection within early generation breeding material, thus significantly increasing genetic gain in breeding programs. Knowledge from this study will also inform strategies for reducing the risk of epidemics and minimising yield penalties from PRR in chickpea.

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Chapter 3: Cause of death: Phytophthora or flood? Effects of waterlogging on *Phytophthora medicaginis* and resistance of chickpea (*Cicer arietinum*)

Article

Cause of Death: Phytophthora or Flood? Effects of Waterlogging on *Phytophthora medicaginis* and Resistance of Chickpea (*Cicer arietinum*)

Nicole Dron [1,2,*], Steven Simpfendorfer [1], Tim Sutton [2,3], Georgina Pengilley [1] and Kristy Hobson [1]

3.1 Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Nicole Dron		
Contribution to the Paper	Performed analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	81%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/05/2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Steven Simpfendorfer		
Contribution to the Paper	Edited draft of manuscript (2% contribution)		
Signature		Date	26/10/2022

Co-Author Contributions cont.

Name of Co-Author	Tim Sutton		
Contribution to the Paper	5% Helped to evaluate and edit the manuscript. Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	22/5/2023

Name of Co-Author	Georgina Pengilley		
Contribution to the Paper	Edited draft of manuscript (2% contribution)		
Signature		Date	13/10/2022

Name of Co-Author	Kristy Hobson		
Contribution to the Paper	10% Helped to evaluate and edit the manuscript. Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	24/05/2023

Abbreviations

04067	04067-81-2-1-1
BC	Backcross
CI	Confidence interval
Exp 1	Experiment 1
Exp 2	Experiment 2
Exp 3	Experiment 3
FC	Field capacity
GLMM	general linear mixed model
kDNA	1000 DNA copies
ORP	Oxidation-reduction potential
P.med	*Phytophthora medicaginis*
PRR	Phytophthora root rot
qPCR	Quantitative Polymerase Chain Reaction
RO	Reverse osmosis
SARDI	South Australian Research and Development Institute
TAI	Tamworth Agricultural Institute
WL	Waterlogging

3.2 Abstract

Chickpea production in Australia is constrained by both waterlogging and the root disease Phytophthora root rot (PRR). Soil saturation is an important pre-condition for significant disease development for many soil-borne *Phytophthora* spp. In wet years, water can pool in low lying areas within a field, resulting in waterlogging, which, in the presence of PRR, can result in a significant yield loss for Australian chickpea varieties. In these circumstances, the specific cause of death is often difficult to discern, as the damage is rapid and the spread of PRR can be explosive in nature. The present study describes the impact of soil waterlogging on oxygen availability and the ability of *P. medicaginis* to infect chickpea plants. Late waterlogging in combination with PRR reduced the total plant biomass by an average of 94%; however, waterlogging alone accounted for 88% of this loss across three reference genotypes. Additional experiments found that under hypoxic conditions associated with waterlogging, *P. medicaginis* did not proliferate as determined by zoospore counts and DNA detection using qPCR. Consequently, minimizing waterlogging damage through breeding and agronomic practices should be a key priority for integrated disease management, as waterlogging alone results in plant stunting, yield loss and a reduced resistance to PRR.

3.3 Introduction

Chickpea (*Cicer arietinum*) was first domesticated 10,000 to 12,000 years ago in the arid to semi-arid Middle-East and has since been integrated into many cropping systems internationally (Redden et al. 2007). Australia has become the second-largest exporter of chickpea globally following its introduction in the 1970s (Rees et al. 1994, Rawal et al. 2019). The global population is increasing, and with environmental shifts induced by climate change, food security is being challenged. Chickpea is an important staple source of protein in south-east Asian countries, and demand for plant protein is increasing (Merga et al. 2019). The sustainable production of plant protein is dependent on plants' resilience to abiotic and biotic stress, and the frequency of severe weather events including flooding and drought is expected to increase, posing a threat to global food production into the future (Hirabayashi et al. 2013, FOA 2015).

The northern cropping region (northern New South Wales and southern Queensland) is the primary chickpea production region in Australia. Phytophthora root rot (PRR) caused by the soil-borne oomycete *Phytophthora medicaginis* is endemic to this region, and yield losses of over 70% can occur during seasons when above average rainfall is experienced (Bithell et al. 2021). Soil-borne *Phytophthora* spp. sporulate under high soil moisture conditions, releasing motile zoospore that rapidly infect roots, causing severe disease pressure across vast areas (Schwinghamer et al. 2011). *Phytophthora* spp. are obligate aerobes, and during periods of soil saturation in many circumstances it is difficult to decipher whether the crop damage is from PRR, waterlogging or a combination of both (Erwin et al. 1983, Davison et al. 1986). Extensive damage may be caused primarily by waterlogging, as most crop species exhibit a physical effect and become physiologically constrained, which in turn reduces the plants' ability to overcome stress (Colmer et al. 2009, Palta et al. 2010). Evidence from other pathosystems indicates that there is a possibility that PRR management could be improved though

minimizing the impact of waterlogging, as waterlogging contributes to disease development and yield loss in chickpea (Dron et al. 2021), as previously reported in soybean (*Glycine max*) (Nguyen et al. 2012).

P. medicaginis inoculum can survive for up to five years between chickpea crops, either dormant in the soil as oospore and chlamydospore structures or hosted on other medic weed species (Erwin et al. 1983). There is currently no economic control of PRR and growers are advised to avoid. fields prone to waterlogging and low-lying areas with a history of PRR (Schwinghamer et al. 2011). The level of PRR. infection can be reduced early through the repeated application of the short acting fungicide active metalaxyl, but this practice is uneconomical at the broadacre scale (Schwinghamer et al. 2011). Hence, improving the genetic resistance to PRR remains a high priority for chickpea breeding in Australia (Knights et al. 2008). Waterlogging is becoming a greater risk to crop production across Australia, with sporadic high rainfall events during the growing season and depreciating soil structure caused by prior flood events and farming practices inundating plants for days or weeks (Pagliai et al. 2004, Bakker et al. 2005). Breeding and agronomic practices to prevent severe PRR disease in high rainfall conditions may benefit from a shift in focus from plants' selection for PRR resistance alone to also include their genetic tolerance and the prevention of waterlogging for integrated disease management.

Genetic diversity in cultivated chickpea is narrow in comparison to other crops, and whilst there are many wild *Cicer* accessions, only *C. echinospermum* P.H. Davis and *C. reticulatum* Ladizinsky can successfully be crossed to cultivated chickpea (*C. arietinum*). Wild *Cicer* accessions can offer desirable agronomic traits for pyramiding and improved disease resistance but present major challenges to breeding that are associated with agronomic genetic lag (Croser et al. 2003). To date, little waterlogging tolerance has been discovered in cultivated or wild chickpea (Chauhan 1987., Cowie et al. 1995, Palta et al. 2010). Sourcing wild *Cicer* accessions

with beneficial root traits, which infer waterlogging tolerance and PRR resistance, is ongoing (Brinsmead et al. 1985, Dron et al. 2021). Agronomic practices such as soil amelioration and incorporating large deep rooting crops into the farming system can improve the soil structure and water filtration, thus also reducing the risk of waterlogging. Preventing the pooling of water and soil saturation within fields could in turn reduce *P. medicaginis* inoculum bursts near sensitive chickpea primary root systems (Erwin et al. 1983, Manik et al. 2019), whilst also allowing plants to maintain their normal metabolism (i.e., plant defence responses).

This study investigated the effect of waterlogging on *P. medicaginis* infection of chickpea in three controlled-environment experiments. Three hypotheses were tested: (1) waterlogging alone is as severely damaging to plants' growth as infection by *P. medicaginis* (Exp 1); (2) transient flooding increases *P. medicaginis* inoculum production, whilst waterlogging has an inhibitory effect (Exp 2); and (3) *P. medicaginis* inoculum production under waterlogged conditions is associated with hypoxic conditions (Exp 3).

3.4 Materials and Methods

Plant and oomycete material

Data are presented for three desi chickpea genotypes that differ in their susceptibility to PRR in all three experiments of this study: the moderately PRR resistant wild *C. echinospermum* backcross (BC), genotype 04067-81-2-1-1 (04067) (pedigree: Howzat/ILWC 245//99039-1013); the moderately susceptible *C. arietinum* Yorker (pedigree: 8507-28H)/946-31); and the very susceptible Rupali (pedigree: FLIP84-15C/ICCV88516//Amethyst) (Amalraj et al. 2019). The northern region *P. medicaginis* isolate TR4046 was used in each study, recovered from a PRR-infected chickpea plant near Moree, New South Wales in 2005. The isolate was cultured on V8 agar and a mycelial-oospore suspension prepared for inoculation as described by Dron et al., 2021.

Experiment 1: Effect of early and late waterlogging on Phytophthora root rot in chickpea

Design and conditions

Experiment 1 (Exp 1) was established in a shade house at the Tamworth Agricultural Institute, NSW (TAI) on 29 June 2020. The six treatments included: a nil control (no PRR or waterlogging); PRR infection; early waterlogging; late waterlogging; and combined treatments of PRR infection with early or late waterlogging. Each experiment consisted of four replicates in a factorial split plot design, with waterlogging (n = 3) × *P. medicaginis* inoculation (n = 2) as the main plot treatment level and chickpea genotypes (n = 8) the subplot treatment level. The three chickpea genotypes (04067, Yorker and Rupali) were tested against five other advanced chickpea genotypes randomized in each deep pot. Data from the advanced breeding genotypes were used in the analysis but are not presented in this study due to commercial in-confidence reasons.

In planta waterlogging and inoculation

Pre-moistened and sieved (10 mm) potting media (1:1:1 loam, sand and Greenlife® (Perth, Australia) with Pacific Fertiliser® (Tugun, Australia) Super Fine Ag lime (6.25% w/v) were packed into Handy® (Keysborough, Australia) 100 L wheelie bins used as deep pots (Figure 1). The potting media's pH of 7.6 ± 0.03 was determined by agitating 1:5 media to water for 1 h before measuring with a handheld pH meter (pH10, Oakton®, Vernon Hills, IL, USA). Potting mix used in all experiments was determined to be free of *P. medicaginis* and other pathogens of chickpea by testing soil media with PREDICTA® B (Adelaide, Australia) soil borne disease DNA test panel performed by the South Australian Research and Development Institute (SARDI), South Australia (https://pir.sa.gov.au/research/services/molecular_diagnostics/predicta_b, (accessed on 15 March 2021)). Soil moisture probes (EP100GL-80, Eviropro®, Golden Grove, Australia) were inserted into representative control, early and late waterlogging treatments to determine the

timing of manual watering (2 – 3 L rainwater) at approximately 80% field capacity 20 cm below the soil surface. Water holding capacity of potting media was determined using a modified version of the Schwenke et al. (2020) method to calibrate moisture probes. All bins were initially flooded for 24 h then drained for 48 h prior to sowing. Seed was sterilized with 0.04% (w/v) sodium hypochlorite for 2 min and triple rinsed prior to sowing at 10 cm alongside rhizobium inoculum using Nodulaid® (Southbank, Australia) peat slurry at the recommended rate. Plants were double sown and thinned post-emergence at the four-leaf stage. After eight weeks, all plants were fertilized fortnightly with 30 mL Yates® (Clayton, Australia) Thrive soluble all-purpose fertilizer at the recommended concentration. Plants were staked and clipped as required to ensure they remained upright.

Four weeks after sowing, PRR treatments were applied at a target rate of 500 oospores/plant in furrows adjacent to seedlings at the five-leaf stage. Early waterlogging treatments were applied after one week and late waterlogging treatments five weeks after PRR inoculation, during the early and late vegetative phase, respectively. Waterlogging treatments were sustained for five weeks by maintaining water level to the soil surface daily. Visual chlorosis scores (1 green and healthy – 9 chlorotic and dead) were taken two weeks after the late waterlogging treatment was completed. Once the control treatment had senesced, the experiment was desiccated with glyphosate (RoundUp® Baulkham Hills, Australia) at 7.2 g/L. All other measurements, including plant height, adventitious root count as described in Dron et al. (2021), root disease score (1 healthy–9 dead), nodulation scored using a modified method from Corbin et al. (1977) (1 no nodulation–5 extensive nodulation), reproductive node count, total biomass (root and shoot) and seed weight, were taken at harvest on 19 October 2020. Biomass (root and shoot) measurements were taken after drying at 70 C for 48 h. Ambient temperature was recorded continuously (Tinytag Plus 2) and averages were reported across time-periods (day and night). Soil oxygen reduction potential (mV), pH and soil temperature were measured using a

handheld, pH, temperature and oxidation-reduction potential (ORP) meter (TPS, WP-90 Pty Ltd., Brendale, QLD, Australia). Soil temperature and ORP data were captured pre-waterlogging, mid waterlogging and post-waterlogging by measuring all field capacity, early waterlogging and late waterlogging treatment replicates for 2 min at a depth of 10 cm four times throughout the duration of the experiment.

Figure 1: Wheelie bins with volume 100 L used as deep pots in Experiment 1 to investigate the effects of waterlogging and *P. medicaginis* infection on eight chickpea genotypes.

Experiment 2: Detection of P. medicaginis DNA in chickpea root and soil media under transient and long-term waterlogging using qPCR

Design and conditions

Experiment 2 (Exp 2) was conducted on seedlings of three chickpea genotypes (04067, Yorker and Rupali) in a temperature-controlled growth chamber where minimum and maximum temperatures were set diurnally (12 h) at 18 C to 25 C at relative humidity of 30–50% (environmental control room, Percival Scientific®, Perry, USA). The experiment was a randomized complete block design with six replicates of each genotype (n = 3) × water and *P. medicaginis* treatment (n = 5).

In planta waterlogging and inoculation

Seed was prepared as per Exp 1 and sown at 3 cm into 200 mL plastic cups containing 160 g pre-moistened sieved (5 mm) potting media. Seedlings were grown out to the two-leaf stage and *P. medicaginis* inoculum was applied at a concentration of 2,000 oospores/ plant adjacent to the base of the seedling. Five treatments were then applied: (1) nil control (no PRR or waterlogging); (2) PRR infection and watering to 80% field capacity (FC) (non-waterlogged); (3) initial transient waterlogging for 48 h post inoculation; (4) transient waterlogging for 48 h post inoculation and again once potting media returned to 80% FC; and (5) long-term waterlogging for 12 days flooding to soil surface. Pots were watered to weight and the long-term waterlogging level was maintained every 2–3 days. Data were collected from all treatments after the 12 day waterlogging treatment was completed. Fresh shoot and root weight measurements were collected along with chlorosis and root disease scores, as described in Exp 1. Roots were carefully brushed to remove loose soil prior to visual scoring and weighing and then added back to their corresponding potting mix before drying down at 40 C prior to submitting to SARDI (South Australia) for total (root + potting mix) *P. medicaginis* DNA quantification via qPCR.

Experiment 3: Detection of P. medicaginis zoospores and PRR in chickpea under hypoxic hydroponic conditions

Design and conditions

Experiment 3 (Exp 3) was completed in a growth room at TAI, as described in Exp 2, using the same three genotypes 04067, Yorker and Rupali. A split plot design was used with aeration and *P. medicaginis* as the main plots and chickpea genotypes as the subplot treatment. The main treatments included: (1) aerated control (no PRR inoculation); (2) aeration plus PRR inoculation; and (3) hypoxia plus PRR inoculation. There were three replicates of each main

treatment (n = 3) and a further two replicates of each genotype (n = 3) subplot within each main plot. Two seedlings of the three genotypes were randomized in 2.3 L buckets with holes in their lids and covered in black plastic to keep out light.

In planta flooding & infection

Seeds were washed in 0.04% (W/V) sodium hypochlorite for two minutes then triple rinsed before pre-germinating in darkness with 0.25 nutrient solution in reverse osmosis (RO) water over mesh. Full-strength nutrient formulae (mM): 5.0 Ca^{2+}, 5.0 K+, 0.625 NH^{4+}, 0.4 Mg_2+, 0.2 Na^+, 5.4 SO_4^{2-}, 4.4 NO^{3-}, 0.2 $H2PO^{4-}$, 0.1 SiO_3^{2-}, 0.1 Fe-sequestrene, 0.05 Cl^-, 0.025 BO_3^{3-}, 0.002 Mn^{2+}, 0.002 Zn^{2+}, 0.0005 Cu^{2+}, 0.0005 MoO_4^{2-} and 0.001 Ni^{2+}. The solution was buffered with 1.0 mM MES (2-[N-morpholino] ethane sulfonic acid) (Corbin et al. 1977, Samineni et al. 2011). The pH was adjusted to 6.5 using KOH at commencement of the experiment using a pH meter (Oakton®, pH10). After six days, seedlings were transplanted into full-strength hydroponic media in 2.3 L buckets and light was introduced. Seedlings were held in place with polyethylene disks and buckets topped up with RO water and agitated every 2-3 days.

Treatments were applied to seedlings at the two-leaf stage, two days after seedlings were transferred to buckets. Mycelial-oospore suspension was added to the inoculated treatments to provide a final solution concentration of 30 oospores/mL and agitated. Nitrogen gas was bubbled through the nutrient solution of the hypoxia treatment at 170 mL/h for three days to reduce oxygen levels (0.3 ppm $\pm$ 0.1) and remained unaerated for the duration of the experiment. Control and aerated PRR treatments were continually aerated with bubbling of external air through the nutrient solution. Following three days of imposed hypoxia, nutrient solution was subsampled three times from each replicate across all treatments post agitation. Zoospores were counted from each sub-sample under a compound microscope (Leitz-Wetzlar Dialux Microscope, Germany) at 40× magnification using a haemocytometer. Chlorosis and

root disease scores; along with fresh root and shoot weights were measured at harvest, ten days after treatment application.

Statistical analysis

Data (shoot weight, biomass, root weight, root count, height, reproductive node count, nodulation, chlorosis and root disease scores) were analysed with a general linear mixed model (GLMM) using the statistical software ASReml (Butler et al. 2018) using the biometry training package (Nielsen et al. 2021) on R (R Core Team 2021). Biomass and reproductive node data were square root transformed to ensure homogeneity of variance prior to analysis. The fixed terms in the model were the factors treatment (control, PRR and waterlogging treatments), genotype (eight genotypes for Exp 1 and three genotypes for Exp 2 and Exp 3) and their interaction. Random terms in the model were replicate and plant within replicate. *P. medicaginis* DNA concentrations and zoospore concentrations were also analysed using a linear mixed model, with kDNA and zoospore data transformed using a $\log_{10}$ transformation to ensure homogeneity of variance. Means and standard deviations for temperature and redox measures for Exp 1 over time were analysed using Microsoft Excel (2010). All data are reported as means and 5–95% confidence intervals (CI) or ± standard deviation.

3.5 Results

Experiment 1: Effect of early and late growth stage waterlogging on and Phytophthora root rot in chickpea

Results showed that the waterlogging treatment had the largest main effect on most parameters measured in the experiment, particularly biomass ($F = 236$, $P < 0.001$) and seed weight ($F = 305$, $P< 0.001$) when compared with chickpea line ($F = 18$, $P < 0.001$ & $F = 33$, < 0.001) and PRR ($F = 9$, $P < 0.001$ & $F= 10.2$. $P< 0.05$), respectively (Table 1). Reductions in biomass and seed weight were observed to be associated with plant stunting and early senescence, with early waterlogging and plant death following late waterlogging treatments.

Table 1: Wald test of GLMM effects for growth and disease parameters in a three-way interaction for chickpea genotypes (*C. echinospermum* BC PRR moderately resistant 04067, *C. arietinum* PRR moderately susceptible Yorker and PRR very susceptible Rupali); water treatment (field capacity, early and late vegetative waterlogging); and uninoculated or *P. medicaginis* inoculated (PRR) status. F-values and the corresponding p-values are displayed.

	Df	Adventitious root count	Root disease	Nodulation	Reproductive node*	Biomass *	Seed weight
Water (W)	2	†NS	33.2***	34.7***	145.4***	235.7***	304.5***
PRR (P)	1	5.9*	10.3*	6.3*	15.1***	9.0***	10.2*
Genotype (G)	7	5.5***	3.0*	2.9*	13.5***	18.4***	32.7***
P × G	7	†NS	†NS	†NS	†NS	†NS	2.5*
W × P	2	5.6*	†NS	†NS	6.3*	†NS	†NS
W × G	14	†NS	†NS	3.2***	2.9***	†NS	10.0***
W × P × G	14	2.6*	2.8***	†NS	†NS	2.1*	2.1*

*Significant at the .05 probability level. ***Significant at the .001 probability level. †NS, non-significant.

None of the three genotypes examined were able to fully recover their seed weight following early and late waterlogging treatments, with late waterlogging having a more detrimental effect than early waterlogging (Table 2, Figure 2a) due to a significantly greater root disease incidence under waterlogging conditions (Table 2, Figure 2b).

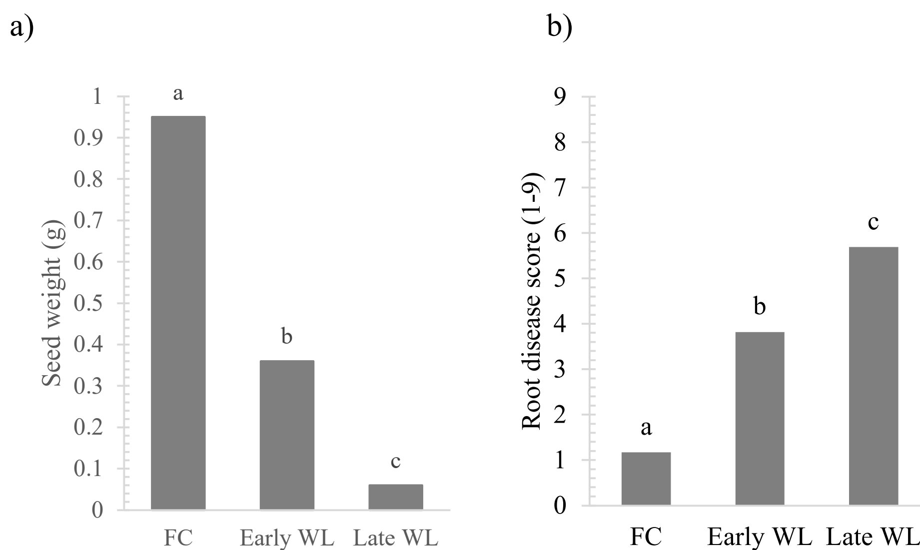

Figure 2. effects of waterlogging (WL) treatments (field capacity (FC), early WL and late WL) on (a) seed weight ($p < 0.001$) and (b) root disease (1 good – 9 poor) ($p < 0.001$) in a three-way analysis with three chickpea genotypes with or without *Phytophthora medicaginis* infection. Letters indicate significant differences.

Table 2 : Mean values and 5–95% CI in parentheses for the three-way GLMM for early and late vegetative waterlogging (WL) and *P. medicaginis* infection (PRR) for disease and growth parameters of three chickpea genotypes (Geno) *C. echinospermum* BC PRR moderately resistant 04067, *C. arietinum* PRR moderately susceptible Yorker and PRR very susceptible Rupali.

Water	PRR	Geno	Root disease	Adventitious root count	Biomass (g)*	Seed weight (g)
Field Capacity	−	04067	1 (-0.2)	24.5 (17.1-31.9)	3.7 (3.2-4.2)	0.26 (0.14-0.38)
		Yorker	1 (-0.2)	34.0 (26.6-41.4)	5.4 (5.0-5.9)	1.20 (1.08-1.33)
		Rupali	1 (-0.2)	31.0 (23.6-38.4)	6.4 (5.9-6.8)	1.27 (1.15-1.39)
	+	04067	1 (-0.2)	19.5 (12.1-26.9)	3.8 (3.3-4.2)	0.34 (0.34-0.21)
		Yorker	1.5 (0.3-2.7)	29.3 (21.8-36.7)	4.6 (4.1-5.1)	1.08 0.96-1.21)
		Rupali	2.8 (1.5-4.0)	27.5 (20.1-34.9)	6.7 (6.2-7.2)	1.31 (1.19-1.43)
Early WL	−	04067	2.8 (1.5-4.0)	25.8 (18.3-33.2)	2.0 (1.5-2.4)	0.06 (-0.06-0.19)
		Yorker	3.0 (1.8-4.2)	28.5 (21.1-35.9)	3.1 (2.6-3.5)	0.65 (0.53-0.77)
		Rupali	3.5 (2.3-4.7)	25.0 (17.59-2.41)	3.0 (2.5-3.5)	0.49 (0.37-0.61)
	+	04067	3.3 (2.0-4.5)	34.3 (26.8-41.7)	2.3 (1.8-2.7)	0.04 (-0.08-0.16)
		Yorker	3.5 (2.3-4.7)	42.0 (34.6-49.4)	3.1 (2.6-3.6)	0.43 (0.31-0.55)
		Rupali	4.3 (3.0-5.5)	36.5 (29.1-43.9)	3.1 (2.6-3.6)	0.57 (0.45-0.69)
Late WL	−	04067	4.0 (2.8-5.2)	28.3 (20.8-35.7)	1.7 (1.2-2.2)	0.00 (-0.12-0.12)
		Yorker	4.0 (2.8-5.2)	49.3 (41.8-56.7)	2.2 (1.7-2.7)	0.25 (0.25-0.12)
		Rupali	5.3 (4.0-6.5)	24.5 (17.1-31.9)	1.6 (1.1-2.0)	0.06 (-0.07-0.18)
	+	04067	6.8 (5.5-8.0)	10.8 (3.3-18.2)	0.9 (0.5-1.4)	0.00 (-0.12-0.12)
		Yorker	7.0 (5.8-8.2)	18.8 (11.3-26.2)	1.4 (1.0-1.9)	0.01 (-0.11-0.13)
		Rupali	7.3 (6.0-8.5)	13.3 (5.8-20.7)	1.4(1.0-1.9)	0.00 (-0.12-0.12)

*Square-root transformed mean predictions.

PRR significantly reduced the seed weight (Figure 3a) and increased the root disease (Figure 3b). In this study, the PRR treatment without waterlogging displayed no foliar evidence of infection or biomass reduction (Table 2); root disease was, however, significantly higher in the very susceptible genotype Rupali, indicating the effectiveness of the PRR inoculation (Table 2).

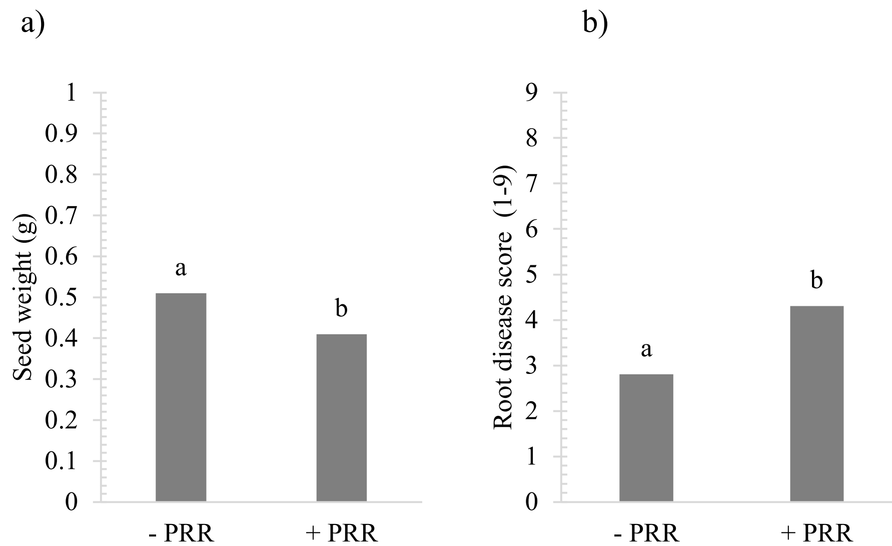

Figure 3. Main effects of *Phytophthora medicaginis* infection (PRR) on (a) seed weight ($p < 0.001$) and (b) root disease (1 good–9 poor) ($p < 0.001$) in a three-way analysis for three chickpea genotypes and waterlogging (WL) treatments (field capacity (FC), early WL and late WL). Letters indicate significant differences.

Reproductive node count was significantly reduced in all genotypes following both waterlogging and PRR treatments (Table 2); and was most severe following late waterlogging (3.0), when compared with early waterlogging (6.8) and FC (10.1) treatments, respectively (Figure 4). Wild *Cicer* BC PRR resistant 04067 had lower biomass and seed weight reductions across all treatments compared with the other two genotypes examined, including the control, indicating that 04067 suffered the least damage when compared to Rupali and Yorker (Table 2). Interestingly, 04067 had a significantly higher reproductive node count in control and PRR treatments but set the least amount of seed when compared to Yorker and Rupali (Table 2, Figure 4).

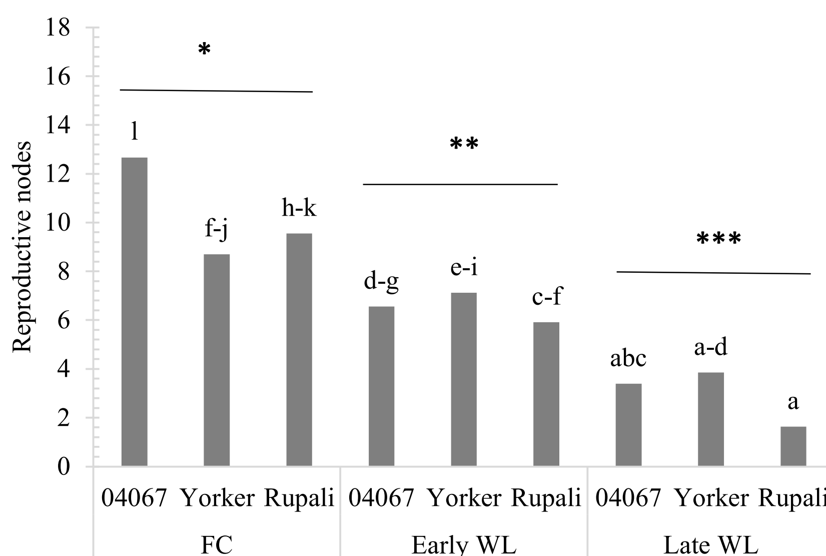

Figure 4. Main waterlogging (WL) and two-way genotype by WL effects on reproductive node count following WL treatments (field capacity (FC), early WL and late WL) for three chickpea genotypes. Letters indicate the significant difference in the two-way interaction ($p < 0.001$). *, ** and *** indicate significant differences at the main effect level WL ($p < 0.001$).

Late waterlogging (2) resulted in the greatest reduction in rhizobium root nodulation, which was significantly lower than that in the early waterlogging (3) and field capacity (4) treatments (Figure 5). Nodulation in the genotype Rupali was significantly reduced following the late waterlogging and early waterlogging, as well as in Yorker following late waterlogging, when compared with plants maintained at field capacity (Figure 5). No significant reduction in nodulation was evident in 04067, with it having a lower nodulation score under the non-waterlogged FC treatment compared with the other chickpea entries (Figure 5). Adventitious root counts were significantly lower in PRR inoculation (25.7) treatments compared to non-inoculation (31.4) treatments (Figure 6). Moreover, the adventitious root count was significantly reduced in the PRR and late waterlogging combination treatment, due to PRR root disease (Figure 6, Table 2). The adventitious root count was significantly increased under the late waterlogging PRR treatment in Yorker (31%), but not in 04067 (13%) or Rupali (20%), when compared to the control (Table 2).

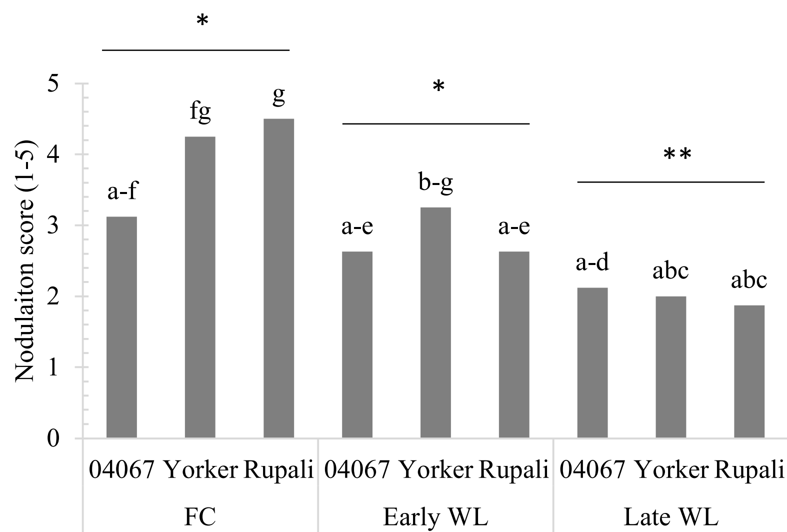

Figure 5. Main waterlogging (WL) and two-way genotype by WL effects on nodulation (1 poor–5 extensive) in three chickpea genotypes following WL treatments (field capacity (FC), early WL and late WL). Letters indicate the significant difference in the two-way interaction ($p < 0.001$). * and ** indicate significant differences at the main effect level WL ($p < 0.001$).

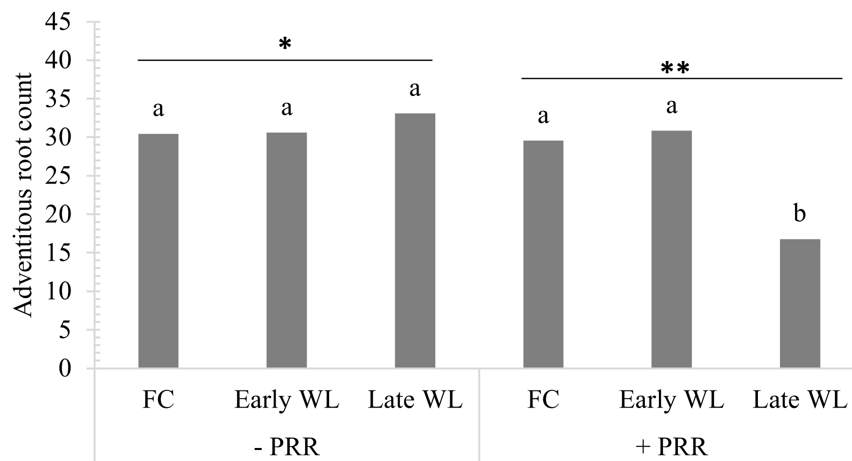

Figure 6. Main *Phytophthora medicaginis* infection (PRR) and two-way PRR by waterlogging (WL)(field capacity (FC), early and late WL) effects on adventitious root counts in three chickpea genotypes. Letters indicate the significant difference in the two-way interaction ($p < 0.05$). * and ** indicate significant differences at the main effect level PRR ($p < 0.05$).

Final redox measures (mV) were not significantly different between early and late waterlogging treatments; however, the soil temperature and ambient temperature were 12.1 C and 7.8 C warmer during the late waterlogging treatment than they were during the early waterlogging treatment, respectively (Figure 7).

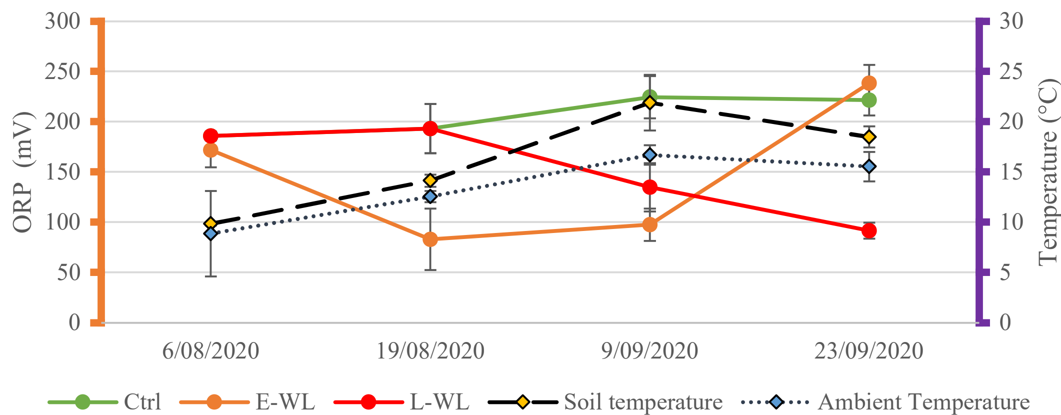

Figure 7: Soil oxidation-reduction potential (ORP), ambient and soil temperatures during non-waterlogged (ctrl), early- (E- WL) and late-waterlogging (L-WL) treatments in Experiment 1; pH of 7.6 ± 0.03 remained consistent for the duration of experiment.

Experiment 2: Detection of P. medicaginis DNA in chickpea root and soil media under transient and long-term waterlogging using qPCR

There was no significant difference in *P. medicaginis* kDNA after either the transient waterlogging or long-term waterlogging treatments in the moderately susceptible Yorker and moderately resistant 04067 genotypes (Table 3). An increase in detected inoculum was observed in the very susceptible genotype Rupali after long-term waterlogging when compared to the treatment with no transient flooding (Table 3). All three genotypes suffered significantly greater root disease, foliar chlorosis, reductions in shoot and root weights under the longer-term waterlogging in the presence of *P. medicaginis* treatment (Table 3).

Table 3: Mean values and 5%–95% CI in parentheses for the two-way GLMM for qPCR detection of *Phytophthora medicaginis* (kDNA copies) growth and disease parameters for three chickpea genotypes: *C. echinospermum* moderately resistant BC 04067, *C. arietinum* moderately susceptible Yorker and very susceptible Rupali in potting media infected with *P. medicaginis* (PRR) under differing flooding regimes.

Treatment	Genotype	Shoot weight (g)	Root weight (g)	Chlorosis Score (1-9)	Root disease Score (1-9)	*P. med* kDNA copies/g soil (Log_{10})*
Control (FC)	04067	2.5 (2.1-2.9)	0.7 (0.6-0.8)	1.0	1 (0.5-1.5)	0
	Yorker	4.0 (3.6-4.4)	0.9 (0.8-1.0)	1.0	1 (0.5-1.5)	0
	Rupali	4.6 (4.2-5.0)	0.9 (0.8-1.0)	1.0	1 (0.5-1.5)	0
PRR (FC)	04067	2.9 (2.5-3.3)	0.8 (0.7-0.9)	1.0 (0.2-1.8)	1.0 (0.5-1.5)	1.5 (1.4-1.6)
	Yorker	3.8 (3.4-4.3)	1.1 (1.0-1.2)	1.0 (0.2-1.8)	1.0 (0.5-1.5)	1.5 (1.4-1.6)
	Rupali	4.7 (4.3-5.1)	1.0 (0.9-1.1)	1.3 (0.6-2.1)	2.5 (2.0-3.0)	1.5 (1.4-1.6)
PRR + Transient WL (48 h)	04067	2.4 (1.9-2.8)	0.7 (0.6-0.8)	1.0 (0.2-1.8)	1.2 (0.7-1.6)	1.6 (1.5-1.7)
	Yorker	3.3 (2.9-3.8)	0.9 (0.8-1.0)	1.3 (0.6-2.1)	1.5 (1.0-2.0)	1.6 (1.5-1.7)
	Rupali	3.8 (3.4-4.3)	0.9 (0.8-1.0)	2.2 (1.4-3.0)	2.2 (1.7-1.6)	1.6 (1.5-1.7)
Inoculated + repeated Transient WL (48 h + 48 h)	04067	2.2 (1.8-2.6)	0.7 (0.5-0.7)	1.0 (0.2-1.8)	2.5 (2.0-3.0)	1.6 (1.5-1.7)
	Yorker	3.0 (2.6-3.5)	0.9 (0.8-1.0)	1.8 (1.1-2.6)	2.8 (2.4-3.3)	1.7 (1.6-1.8)
	Rupali	3.1 (2.7-3.5)	0.9 (0.8-1.0)	2.3 (1.6-3.1)	3.8 (3.4-4.3)	1.7 (1.6-1.8)
PRR + WL (240 h)	04067	1.6 (1.2-2.0)	0.5 (0.4-0.7)	2.7 (1.9-3.4)	3.8 (3.4-4.3)	1.6 (1.5-1.7)
	Yorker	1.9 (1.5-2.3)	0.6 (0.5-0.7)	5.0 (4.2-5.8)	5.2 (4.7-5.6)	1.5 (1.4-1.6)
	Rupali	1.6 (1.2-2.0)	0.5 (0.4-0.6)	6.8 (6.1-7.6)	6.8 (6.4-7.3)	1.8 (1.7-1.9)
	df			*F-value*		
Treatment (T)	4	36.3***	10.8***	42.1***	112.1***	†NS
Genotype (G)	2	36.9***	19.0***	14.6***	30.6***	†NS
T × G	14	2.9*	†NS	3.4*	4.0*	†NS

FC- Field capacity, WL – Waterlogging. * Significant at the .05 probability level. ***Significant at the 0.001 probability level. †NS, non-significant.

Experiment 3: Detection of P. medicaginis zoospores and PRR in chickpea under hypoxic hydroponic conditions

P. medicaginis zoospore counts showed that proliferation under hypoxic conditions was not as high as that under the aerated infected treatments when compared to the control (Table 4, Figure 8). Under the combination of hypoxia and *P. medicaginis* infection, the level of root disease was greater in the very susceptible genotype Rupali but not in the moderately susceptible Yorker or moderately resistant 04067 genotypes. Chlorosis was more severe in all three genotypes in the hypoxic plus PRR infected treatment than it was in the aerated infected or aerated non-infected control (Table 4). Shoot and root weights were significantly reduced in

the hypoxic plus PRR infected treatment and aerated infected, consecutively, when compared to the control. Hypoxia appeared to have a greater effect on the severity of PRR's development in the three chickpea genotypes but not on the proliferation of *P. medicaginis*.

Table 4: Mean values and 5-95% CI in parentheses for the two-way GLMM of hypoxia and *P. medicaginis* treatments on parameters for three chickpea genotypes *C. echinospermum* moderately resistant 04067, *C. arietinum* moderately susceptible Yorker and very susceptible Rupali grown in hydroponic solution.

Treatment	Genotype	Shoot weight (g)	Root weight (g)	Chlorosis Score (1-9)	Root disease Score (1-9)
Aerated Control	04067	1.8 (1.5-2.0)	1.8 (1.5-2.3)	1 (0.5-1.5)	1 (0.5-1.5)
	Yorker	2.2 (1.9-2.4)	2.8 (2.5-3.2)	1 (0.5-1.5)	1 (0.5-1.5)
	Rupali	2.6 (2.4-2.6)	3.6 (3.2-4.0)	1 (0.5-1.5)	1 (0.5-1.5)
Aerated PRR	04067	1.1 (0.9-1.4)	1.9 (1.5-2.3)	1 (0.5-1.5)	3.2 (2.7-3.7)
	Yorker	1.2 (1.0-1.5)	2.4 (2.0-2.7)	1 (0.5-1.5)	4.2 (3.7-4.7)
	Rupali	1.3 (1.1-1.52)	2.8 (2.4-3.2)	1 (0.5-1.5)	4.3 (3.9-4.8)
Hypoxic PRR	04067	0.5 (0.2-0.7)	0.8 (0.4-1.2)	2.3 (1.9-2.8)	3.7 (3.2-4.2)
	Yorker	0.4 (0.2-0.7)	1.1 (0.7-1.4)	2.3 (1.9-2.8)	4.2 (3.7-4.7)
	Rupali	0.6 (0.4-0.8)	1.2 (0.8-1.6)	3.3 (2.9-3.8)	6.8 (6.4-7.3)
	Df		*F-value*		
Treatment (T)	2	109.0***	53.3***	34.9***	267.3***
Genotype (G)	2	5.5*	16.5***	†NS	36.5***
T x G	8	†NS	†NS	†NS	23.7***

*Significant at the 0.05 probability level. ***Significant at the 0.001 probability level. †NS, nonsignificant.

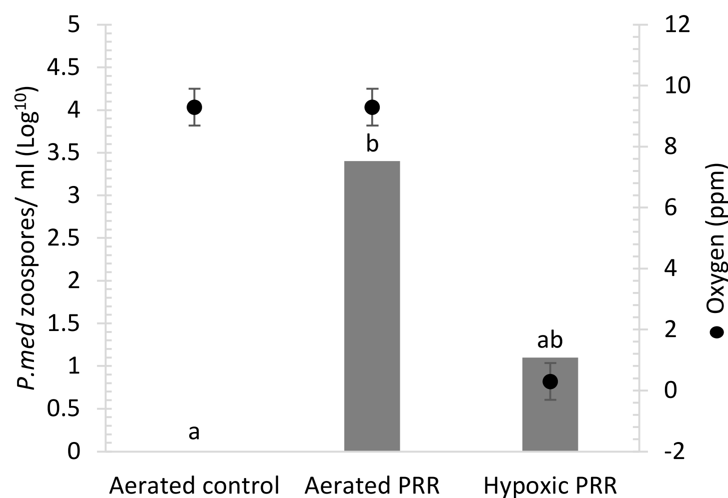

Figure 8. Predicted mean *Phytophthora medicaginis* (*P. med*) zoospore counts (F = 4.2, p < 0.05) for three aeration × Phytophthora root rot (*P.med*) inoculated treatments and the associated oxygen with CI (ppm) (F = 540.0, p < 0.001) for a hydroponic chickpea experiment with three genotype sub-treatments listed in Experiment 3. Letters indicate significant differences.

3.6 Discussion

This study demonstrated that waterlogging in the absence of *P. medicaginis* infection caused severe damage to chickpea plants, including genotypes with a moderate resistance to PRR. Furthermore, late waterlogging had a greater impact than early-season waterlogging, both with and without PRR. Hence, limiting the impact of waterlogging should be a research priority for chickpea breeding and integrated disease management in regions where *P. medicaginis* is prevalent. The present study found that under long-term waterlogging and in the associated hypoxic conditions, *P. medicaginis* proliferation and secondary infection (zoospore) failed due to a lack of oxygen, as observed previously in the cases of other *Phytophthora* spp. (Moore 1975, Erwin et al. 1983). Waterlogging increased the likelihood of severe PRR infection, as shown across all experiments and previously reported in the case of lucerne infected with *P. medicaginis* (Kuan et al. 1980) and oak and avocado infected with *P. cinnamomi* (Curtis et al. 1949, Jacobs et al. 1997). Kuan et al. (1980) attributed the greater damage to breaks in the root surface membranes and an increase in the chemotaxis of *P. megasperma* f. sp. *medicaginis* zoospores.

Environmental effects such as soil properties and temperature alter the speed at which hypoxia or anoxia is achieved (Ponnamperuma 1984). This study demonstrated that temperature in early and late season waterlogging did not significantly alter the final level of soil reduction and the associated oxygen level in the long-term experiment (Figure 7). This study, however, did not capture the change over time to maximal redox reduction, which may have affected the total amount of time for which both the plant and *P. medicaginis* were hypoxic in the early and late waterlogging treatments. Soil and ambient temperatures were significantly higher during late waterlogging compared to early waterlogging. Increased soil and ambient temperatures were reported to be important factors in many plant waterlogging systems and are linked to reduced photosynthesis potential, respiration rates and the induction of stress responses, which result in

damage and pre-mature senescence (Xu et al. 2019). Higher soil and ambient temperatures in late waterlogging may be attributed to severe damage compared to the early waterlogging. In addition, Cowie et al. (1996) found that waterlogging at any age of chickpea plant reduced the yield, but the likelihood of a recovery reduced with the plant's physiological age. This observation is supported by results from this study, i.e., that late waterlogging had a greater effect than early waterlogging.

Environmental factors directly influence the polycyclic soil-borne *Phytophthora* spp. and the associated disease aggressiveness. In drier environments, the direct germination of oospores results in localized root infections, leading to a low disease pressure. Conversely, soil flooding promotes a rapid oospore germination and the production of sporangia that release large numbers of motile zoospores, initiating multiple root infections and a high disease pressure (Erwin et al. 1983, Van West et al. 2003). However, under severe hypoxia (<0.02 atm O_2), as commonly observed under waterlogging conditions, both the mycelial and oospore production is significantly reduced in *P. medicaginis* (Moore 1975). The high disease pressure in the late waterlogging infected treatment may have been associated with the greater hyphal growth prior to late waterlogging or the increase in temperature speeding up the production of zoospores prior to the effects of waterlogging and hypoxia; or, as previously described, it may have been due to the inability of the plant to maintain its defence responses due to the waterlogging damage (Stolzy et al. 1984).

Adventitious root counts are reduced significantly following waterlogging in the presence of *P. medicaginis* due to root rot (Dron et al. 2021). Following late waterlogging alone, the adventitious root count increased significantly in Yorker and not in 04067. Previously, it was reported that Rupali also demonstrated an increase in adventitious root proliferation (Dron et al. 2021) however this was not observed in this study. At sites of new root development, the remodelling of the dermal layers provides a temporary entry point for soil-borne *Phytophthora*

spp. along with an attraction to the apex of the new root tissue (Erwin et al. 1983). Therefore, encouraging root proliferation earlier in the crop season, rather than in response to stress (i.e., waterlogging) or late in the season, may reduce the risk of soil-borne diseases such as PRR and is worthy of further investigation.

Genotype 04067 had a lower vigour, as reflected in its adventitious root count and biomass, and under the dry finish in the control conditions it did not yield or recover sufficiently from the earlier waterlogging damage. High-vigour genotypes (i.e., Yorker and Rupali) were able to set seed; however, these genotypes are less disease resistant and their recovery was not sufficient to restore their yield potential. The lack of plant and root vigour in 04067 may be linked to the reduced ability of the wild *Cicer* BC to set seed, due to its poor adaption and indeterminate growth, which has been reported previously as an obstacle when introgressing wild *Cicer* (Knights et al. 2008). Under more moderate PRR conditions, in the absence of waterlogging the moderately susceptible genotype Yorker displays a higher level of PRR resistance compared with the very susceptible genotype Rupali. This further strengthens the concept that there is a difference in the resistance gene mechanisms between the *C. arietinum* and *C. echinospermum* genotypes (Amalraj et al. 2019). Pyramiding these resistance mechanisms is possible for providing incremental gains in PRR resistance, but it is important to consider how these low-vigour root growth ideotypes might affect the yield potential and stability of commercial varieties.

Nodulation was lower in the wild *Cicer* BC 04067 genotype than in Yorker and Rupali in the control treatment, further demonstrating a potential difference in their basal metabolic activity. The metabolites involved in nodulation and root disease resistance among these genotypes are being further investigated in a recombinant inbred line population (data not published). This is an important area of research, which will build on this study, as under late, long-term waterlogging, nodulation is decreased, indicating that the whole root microbiome is altered,

such that beneficial and pathogenic microorganism populations are reduced. Incorporating waterlogging tolerance is important to be able to maintain plant metabolism during periods of waterlogging. Traits to consider include an increased lignification and suberisation of the epidermal root tissue to reduce the radial oxygen loss, and an increased aerenchyma to move oxygen into the lower root tissue, as seen in wheat and rice (Jackson et al. 1999, Colmer 2003, Kotula et al. 2009), thus also removing the need for the production of new adventitious roots that are more vulnerable to PRR infection (Kotula et al. 2009, Nishiuchi et al. 2012).

The pre-breeding effort for chickpea in Australia is currently focused on pyramiding known mechanisms of resistance to PRR with a tolerance of waterlogging. In chickpea there is limited genetic diversity, especially in recognized waterlogging tolerance traits, most likely due to the species origin in semi-arid environments (Chauhan 1987., Cowie et al. 1995, Palta et al. 2010). An opportunity now exists to engineer, via gene editing, additional waterlogging traits identified as beneficial in other plant species into chickpea. Gene editing technology could be used to introduce variation in known waterlogging tolerance genes, such as those shown to be involved in aerenchyma (Casto et al. 2018) and/or epidermal and endodermal barriers (Ejiri et al. 2021). The level of seedling waterlogging tolerance previously described by Dron et al. (2021) in the genotype 04067 appears to be too low to have an industry benefit under late waterlogging conditions, especially in the presence of *P. medicaginis*. Extending current agronomic controls for waterlogging and PRR beyond the sole recommendation to avoid low-lying paddocks with histories of PRR should be considered if the chickpea industry is to make significant progress towards reducing losses from waterlogging and PRR. The broadscale conservation of the soil structure to reduce waterlogging's occurrence through farming practices including controlled traffic farming, direct drilling, crop choice and stubble retention should be recommended (Brinsmead et al. 1985). Other more novel agronomic solutions such as deep ripping, the incorporation of organic matter and other soil amelioration practices should

be investigated to understand their efficacy in reducing waterlogging, whilst taking into consideration the legacy effects within farming systems.

3.7 Conclusions

This study investigated the effects of waterlogging on *P. medicaginis* infection of chickpea, both of which are common occurrences in the northern grain region of Australia. Previous research indicated that severe PRR disease development resulted from the extreme proliferation of inoculum following flooding events. This study supports the consideration that waterlogging itself compromises chickpea plant defences and *P. medicaginis* growth is restricted by its inability to obtain oxygen requirements under hypoxic conditions during waterlogging. The inability of *P. medicaginis* to proliferate under low-oxygen environments associated with waterlogging and the severity of plant damage incurred by chickpea from early and late season waterlogging alone indicates that waterlogging should be considered a key priority for development and the adoption of improved management strategies. Furthermore, with an expected increase in extreme climatic events and the limited ability to forecast the timing and volumes of rainfall, research should be conducted on both breeding and agronomic practices to reduce the impact of both waterlogging and PRR on chickpea.

3.8 Author Contributions

ND, KH, and TS developed the phenotyping experiments excluding oospore production methods. ND conducted the Experimentation and drafted the manuscript. All authors reviewed and revised the manuscript. KH, SS, GP and TS supervised the project.

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Chapter 4: Phenotyping for waterlogging tolerance as a proxy for *Phytophthora medicaginis* resistance in chickpea

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Research

Phenotyping for Waterlogging Tolerance as a Proxy for *Phytophthora medicaginis* Resistance in Chickpea

Nicole Dron,[1,†] Tim Sutton,[2,3] Steve Harden,[1] Steven Simpfendorfer,[1] and Kristy Hobson[1]

[1] NSW Department of Primary Industries, 4 Marsden Park Road, Tamworth, NSW 2340, Australia
[2] South Australian Research and Development Institute, GPO Box 397, Adelaide, SA 5001, Australia
[3] School of Agriculture, Food and Wine, University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia

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Principal Author

Name of Principal Author (Candidate)	Nicole Dron		
Contribution to the Paper	Performed analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	78%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/05/203

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

iv. the candidate's stated contribution to the publication is accurate (as detailed above);

v. permission is granted for the candidate in include the publication in the thesis; and

vi. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Tim Sutton		
Contribution to the Paper	5% Helped to evaluate and edit the manuscript. Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	22/5/2023

Co-Author Contributions cont.

Name of Co-Author	Steven Harden		
Contribution to the Paper	5% Assisted with the experimental designs, principal component plots, and analysis of results		
Signature		Date	19/10/2022

Name of Co-Author	Steven Simpfendorfer		
Contribution to the Paper	Edited draft of manuscript (2% contribution)		
Signature		Date	22/10/2022

Name of Co-Author	Kristy Hobson		
Contribution to the Paper	10% Helped to evaluate and edit the manuscript. Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	24/05/2023

Abbreviations

AR	Adventitious root counts
BC	Backcross
CFR	Chlorophyll fluorescence ratio
CTRL	Control
DRW	Dry root weight
DSW	Dry shoot weight
Ex1	Experiment 1
Ex2	Experiment 2
L	Line
PAM	Partitioning around medoids
PCA	Principal component analysis
PRL	Primary root length
PRR	Phytophthora root rot
PRR:WL	*P. medicaginis* infection and waterlogging in combination
RO	Reverse osmosis
T	Treatment
WL	Waterlogging

4.2 Abstract

Phytophthora root rot (PRR) caused by the soilborne oomycete *Phytophthora medicaginis* is a significant constraint to chickpea (*Cicer arietinum*) production across the northern grains region of Australia. In flooded soil, which is conducive to PRR disease development, up to 70% yield loss can occur in the most resistant Australian cultivars. Incorporating waterlogging tolerance in soybean (*Glycine max*) has been shown to improve quantitative resistance to *Phytophthora sojae*. Root growth of three chickpea genotypes was assessed at the seedling stage under waterlogging, PRR, and the combination of these abiotic and biotic constraints. Levels of waterlogging tolerance in chickpea are inherently low, yet selected genotypes displayed variability in root traits linked to improved waterlogging tolerance. The PRR moderately susceptible chickpea cultivar Yorker and PRR very susceptible Rupali demonstrated an eightfold increase in early adventitious root growth from the epicotyl region under waterlogging stress, compared with the PRR resistant interspecific backcross genotype 04067-81-2-1-1 (*C. echinospermum* × *C. arietinum**2). Selection for primary root depth, which was significantly greater in 04067-81-2-1-1 under waterlogging, appears to improve PRR resistance compared with root replacement traits. Soilborne *Phytophthora* spp. are reportedly attracted to branch sites and leached exudates. We propose that compromised root barriers at emergence sites of adventitious roots under waterlogging conditions hasten hyphal entry, potentially increasing susceptibility to PRR. Hence, screening for root depth and absence of adventitious root development under waterlogged conditions may offer a novel proxy phenotyping method for PRR resistance traits at early stages of chickpea breeding.

4.3 Introduction

Chickpea (*Cicer arietinum*) is an important global source of plant protein within human diets and a valuable rotation crop in broadacre systems. Countries including India, Pakistan, Turkey, Iran, Myanmar, Iraq, Ethiopia, Mexico, Spain, Canada, and Australia are major producers of chickpea (Knights et al. 2007, Boye et al. 2010). Chickpea was first introduced into Australian cropping systems in the early 1980s; production peaked in 2017 with 1.1 million hectares sown in response to rising export prices. Over 70% of the area sown to chickpea is located in the northern grains region (northern New South Wales and southern Queensland) (ABARES 2018). Phytophthora root rot (PRR) caused by the oomycete *Phytophthora medicaginis* (E. M. Hansen and D. P. Maxwell) is prevalent across this northern grains region, with yield losses estimated at AUD 8.2 million annually (Murray et al. 2012). Various *Phytophthora* spp. have been associated with PRR of chickpea crops across parts of Argentina, India, Pakistan, and Spain, but they are of considerably lower economic impact compared with in Australia (Nene et al. 1996).

Flooding has been identified as an important precondition to severe PRR development (Kuan et al. 1980, Schwinghamer et al. 2011). Extreme weather events, including flooding, are predicted to increase globally as a result of climate change, so understanding the links between PRR and waterlogging is becoming increasingly relevant (Arnell 2004, Hirabayashi et al. 2013). Under dryland conditions in the northern region of Australia, yield losses of 30 to 50% are typical for the moderately susceptible PRR chickpea cultivar Yorker, yet under extended periods of waterlogging up to 70% yield loss from PRR has been recorded in this cultivar (Bithell et al. 2021). There are two established concepts that explain the increase in PRR pressure under waterlogged conditions: an increase in *Phytophthora* inoculum (Pfender et al. 1977, Erwin et al. 1983) and a breakdown of plant resistance due to physiological damage,

restricted metabolic function, and reduced capacity to overcome stress (Stolzy et al. 1984, Jackson et al. 2005). In soybean (*Glycine max*), four chromosomal regions associated with both waterlogging tolerance and *P. sojae* resistance additively reduced yield losses from PRR (Cornelious et al. 2005, Nguyen et al. 2012). Therefore, it is likely that incorporating waterlogging tolerance into chickpea could improve the durability of resistance to *P. medicaginis* under high soil moisture conditions favorable to the development of PRR.

Disease pressure induced by *Phytophthora* spp. is directly associated with their polycyclic lifecycle, which is driven by environmental conditions. In drier environments, the homothallic *P. medicaginis* oospores undergo direct germination resulting in localized root infections, causing low inoculum pressure. In environments with greater moisture, oospores germinate rapidly, favoring the production of sporangia, which release large numbers of motile zoospores, initiating multiple root infections and resulting in high inoculum pressure (Pfender et al. 1977, Erwin et al. 1983, Van West et al. 2003). In the field, symptoms of PRR include a reduction or cessation of growth rate, leaf chlorosis, desiccation of foliage, premature senescence, wilting, decay of the lateral roots, reddish-brown stem canker, and yield loss. Symptoms of waterlogging are similar to PRR, with the exclusion of characteristic stem canker and root lesions (Erwin et al. 1983, Chen et al. 2011).

Phytophthora inoculum in soil has been reported to survive up to 5 years after a host crop, so rotation with alternative nonhost crops is largely impractical and ineffective (Erwin et al. 1983). Application of fungicides, such as short-acting metalaxyl, has been shown to reduce PRR, but they are uneconomical at the broadacre scale. Hence, selection of improved levels of PRR resistance remains a high priority for chickpea breeding in Australia. Sources of PRR resistance within the cultivated chickpea species *C. arietinum* are scarce. Moderate levels of resistance have been incorporated from a landrace (ICC11870) into the cultivars Yorker, PBA HatTrick, and PBA Seamer (Brinsmead et al. 1985). Wild *Cicer* accessions (*C. echinospermum* P.H.

Davis and *C. reticulatum* Ladizinsky) offer high levels of resistance but present major challenges for breeding because of the prostrate growth habit affecting harvestability, low yields, and poor seed quality (Knights et al. 2008). Linkage drag of agronomically undesirable characteristics is therefore a major challenge when introgressing PRR resistance loci from these wild *Cicer* sources. High levels of waterlogging tolerance in chickpea have not yet been found (Chauhan 1987., Cowie et al. 1995, Palta et al. 2010). However, waterlogging tolerance may have been indirectly selected for in the Australian breeding strategy through efforts to increase PRR resistance.

PRR resistance in chickpea is predominantly identified through field screening and selection, where the process is highly dependent on achieving the required soil moisture for plant and disease establishment. More so, field heterogeneity generates spatial variability in disease pressure, making it difficult to uncouple genotypic and environmental effects. In order to overcome this problem, breeding programs have successfully utilized controlled-environment phenotyping systems in parallel to field screening. Greenhouse soil-based and hydroponic methods have been developed in which seedlings are inoculated using a *P. medicaginis* mycelial-oospore and zoospore inoculum (Knights et al. 2008, Amalraj et al. 2019b). Both field and controlled environments were able to identify the best sources of PRR resistance, although in some cases genotypes with moderate resistance shuffled in their resistance rankings (Knights et al. 2008, Amalraj et al. 2019a). Alternatively, phenotyping for PRR resistance in a wet environment (e.g., hydroponics or under flooding in the field) and focusing on specific root traits closely associated with waterlogging tolerance may identify additional genetic regions associated with higher levels of genetic resistance to PRR. Furthermore, less destructive and more repeatable waterlogging phenotyping methods could be used as a proxy for PRR resistance selection within early generation material, significantly increasing genetic gain in breeding programs.

The objective of this study was to determine if root traits related to waterlogging tolerance could be identified in chickpea using a rapid waterlogging phenotyping method and investigate whether these traits are associated with improved resistance to PRR. We describe a novel rapid waterlogging phenotyping method and measurable root traits of chickpea that could be used to select for improved resistance to PRR.

4.4 Materials and Methods

Plant and oomycete material

Three chickpea lines that differ in PRR resistance were used to develop the controlled-environment phenotyping protocols (Amalraj et al. 2019a). The PRR-resistant wild *C. echinospermum* backcross (BC) genotype 04067-81-2-1-1 (pedigree: Howzat/ILWC 245//99039-1013), the moderately susceptible *C. arietinum* Yorker (pedigree: 8507-28H/946-31), and the very susceptible Rupali (pedigree: FLIP84-15C/ICCV88516//Amethyst).

The *P. medicaginis* isolate TR4046 used in this study was recovered from a PRR-infected chickpea plant near Moree, New South Wales, in 2005. The isolate was cultured on V8 agar with 2.5% $CaCO_3$ for 4 weeks at 25°C in darkness until mycelium had spread across the plate and oospores within the agar had matured. Mycelial mats and agar were flooded with reverse osmosis (RO) water and macerated for 130 s with a stick blender. Oospores in the resulting liquid mycelial-oospore suspension were counted using a hemocytometer.

Experimental design and conditions

This study comprised two experiments (experiment 1 and experiment 2) with both conducted in a glasshouse located at the Tamworth Agricultural Institute in Tamworth, New South Wales. Diurnal temperatures were set to 25°C/18°C; a higher temperature range was selected to accelerate waterlogging damage to the seedlings (Cowie et al. 1995). Experiment 1 was conducted during summer and experiment 2 in the winter months of 2018. A split-plot design

was used with waterlogging × *P. medicaginis* inoculation at the main plot treatment level and chickpea lines the subplot treatment level. Main treatments included a control, *P. medicaginis*-inoculated (PRR), waterlogging (WL), and the combination of both waterlogging and *P. medicaginis* inoculation (PRR:WL). There were six replicates of each main treatment combination. Sixteen seedling tubes were held in place in a plastic container (22 × 22 × 12 cm) with five drainage holes; this container was inserted into a second fully sealed plastic container of the same size to hold water within waterlogging treatments (Fig. 1).

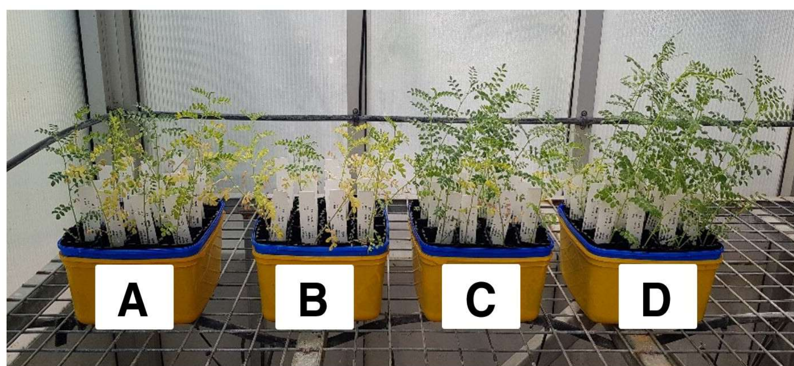

Figure 1. Growth of chickpea plants at 12 days following four treatments: A, inoculated with *Phytophthora medicaginis* only; B, inoculated with *P. medicaginis* and subjected to waterlogging; C, subjected to waterlogging only; and D, control (no inoculation and no waterlogging).

In experiment 1, five or six tubes of the three chickpea genotypes (04067-81-2-1-1, Yorker, and Rupali) were randomized and held in each treatment container. Experiment 2 had a similar design, except 13 advanced chickpea breeding genotypes were evaluated alongside the three genotypes examined in experiment 1. Data from the advanced breeding genotypes were used in the analysis of experiment 2 but are not presented in this study due to being commercial in confidence.

In planta flooding and infection

Chickpea seeds were washed in 0.01% (w/v) sodium hypochlorite for 2 min and triple rinsed in RO water. A covered 10-liter container of 0.1-strength nutrient solution was used to germinate the seedlings on mesh. The composition of the nutrient solution in RO water as full-strength was as follows (mM): 5.0 Ca^{2+}, 5.0 K+, 0.625 NH^{4+}, 0.4 Mg2+, 0.2 Na^{+}, 5.4 SO_4^{2-}, 4.4 NO^{3-}, 0.2 $H2PO^{4-}$, 0.1 SiO_3^{2-}, 0.1 Fe-sequestrene, 0.05 Cl^{-}, 0.025 BO_3^{3-}, 0.002 Mn^{2+}, 0.002 Zn^{2+}, 0.0005 Cu^{2+}, 0.0005 MoO_4^{2-} and 0.001 Ni^{2+}. The solution was buffered with 1.0 mM 2-(N-morpholino) ethane sulfonic acid (Samineni et al. 2011). After 3 days in darkness, seedlings of the same size were transplanted into free-draining potting tubes (50 × 50 × 100 mm) containing 130 g of premoistened sieved (5-mm) potting medium (1:1:1 loam, sand, and Greenlife potting mix). Following planting, each seedling received 30 mL of RO water every 2 to 3 days.

Treatments were applied to the seedlings at the two-leaf stage. First, *P. medicaginis* inoculum was applied by the flood application of mycelial-oospore suspension at a concentration of 30 oospores/mL in RO water. This rate was chosen because field screening showed that 1,500 oospores per plant differentiated levels of PRR resistance and was calculated based on the 2.5 litres of water applied at plant inoculation across treatments. Flooding on the surface of the potting medium remained for 48 h. During this time all treatments, including the control, were flooded and then drained to regulate leaching of nutrients and *P. medicaginis* inoculum between waterlogged and nonwaterlogged treatments. After 1 h of free draining, waterlogging treatments were then refilled with RO water level with the surface of the potting medium, and flooding was maintained daily for 12 days in experiment 1 and for 15 days in experiment 2. Experimental treatments were extended in experiment 2 because the visual PRR symptoms were not evident after the 10 days, due to the cooler winter conditions and shortened day length

in the glasshouse. Watering continued in non-waterlogged treatments with 30 ml of RO water added to the surface of the potting medium every 2 to 3 days and allowed to freely drain.

All plants were individually assessed after completion of waterlogging treatments (12 days for experiment 1 and 15 days for experiment 2). Chlorophyll fluorescence ratio (CFR) readings were taken using a handheld chlorophyll content meter (CCM-300, Opti-sciences, U.S.A.). CFR is the ratio of chlorophyll fluorescence at 735 nm/700 nm and is linearly proportional to chlorophyll content (R2 ≥ 0.95) (Gitelson et al. 1999). One measurement per plant was taken in the middle of the youngest fully expanded leaflet on the leaf closest to the stem at a temperature of 23 ± 5°C for experiment 1 and 20 ± 5°C for experiment 2 in the glasshouse. Plants were then removed from potting tubes and lightly washed to remove potting mix from the roots prior to measuring adventitious root counts (AR) and primary root length (PRL). AR count data were collected from the epicotyl and hypocotyl regions of chickpea seedlings. Finally, dry shoot weights (DSW) and dry root weights (DRW), with the cotyledon detached, were recorded for each seedling after drying at 60°C for 72 h.

Statistical analysis

For experiment 1, a principal component analysis (PCA) and cluster analysis were performed using the partitioning around medoids (PAM) package for R (Maechler et al. 2019) to look at the association between the measured variables and grouping of the data. Most data (DRW, DSW, PRL, and CFR) were analyzed with a linear mixed model using the statistical software asreml (Butler 2020). The fixed terms in the model were the factors treatment (control, PRR, WL, and PRR:WL), genotype (three genotypes for experiment 1 and 16 genotypes for experiment 2), and their interaction. Random terms in the model were replicate and container within replicate. For count data (AR), a generalized linear mixed model with a “log” link was used. Least significant differences at the 5% level and predicted means for the three lines used

in both experiments were calculated using the "predictPlus" command from the R package asremlPlus (Brien 2020).

4.5 Results

The PCA (experiment 1) showed four of the phenotyping data variables (PRL, DRW, DSW, and CFR) to be positively correlated. The AR variable had low correlation with the other four. Treatments clustered in three groups, which represented the control, WL, and PRR:WL treatments (Fig. 2, left panel). The PRR treatment demonstrated greater spread across measured traits, as expected due to the underlying differences in the levels of PRR resistance between genotypes. Interestingly, the WL-only cluster separated based on AR, in which *C. arietinum* genotypes Yorker and Rupali outperformed the *C. echinospermum* BC 04067-81-2-1-1 (Fig. 2, right panel). A tight cluster was observed for the PRR:WL treatment with little genotypic effect between lines except for the PRL trait.

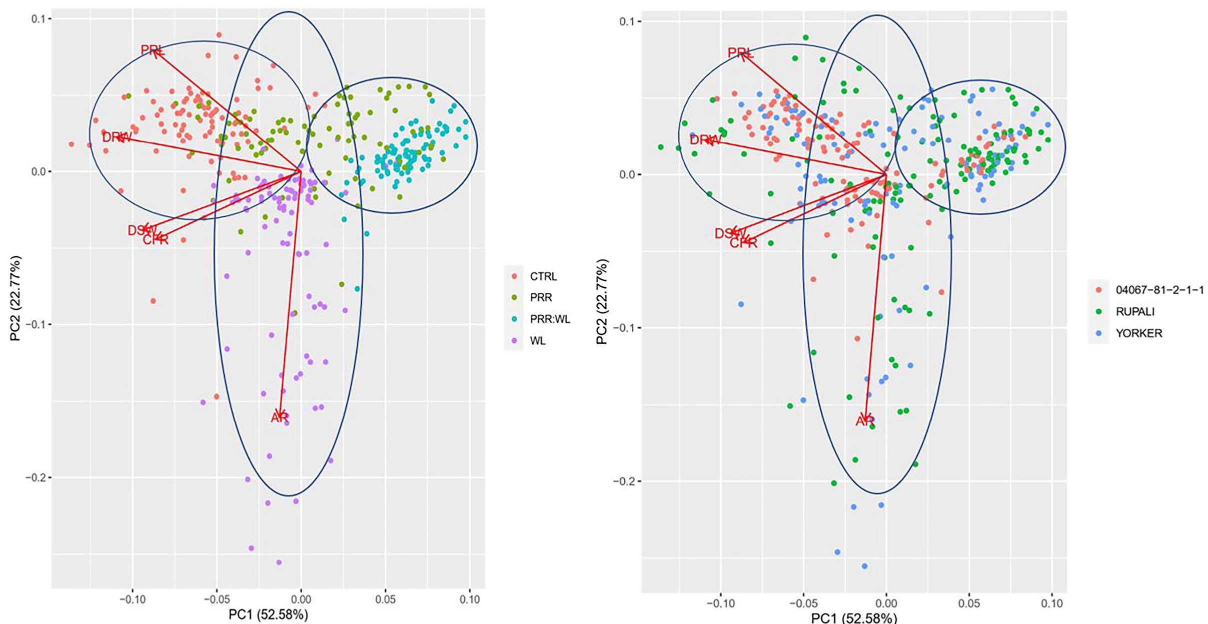

Figure 2. Experiment 1 principal component (PC) analyses indicating the treatment effect (left) and variety effect (right) across chickpea genotypes 04067-81-2-1-1 (resistant), Yorker (moderately susceptible), and Rupali (very susceptible). CTRL = control; PRR = inoculated with *Phytophthora medicaginis*; PRR:WL = inoculated with *P. medicaginis* with waterlogging; and WL = waterlogging treatment. Traits tested include primary root length, dry root weight, dry shoot weight, adventitious root count, and chlorophyll fluorescence ratio.

PRR resistance groups were reflected in DRW, DSW, PRL, and CFR in the resistant *C. echinospermum* BC 04067-81-2-1-1, moderately susceptible Yorker, and very susceptible Rupali in the PRR treatment when compared with the control in experiment 1 (Table 1). The PRR treatment in experiment 2 demonstrated a reduced disease severity; therefore, significant differences in DRW, DSW, PRL, and CFR across lines were not observed.

Table 1: Effects of waterlogging and *Phytophthora medicaginis* inoculation on dry shoot weight (DSW) chlorophyll fluorescence ratio (CFR), dry root weight (DRW), Primary root length (PRL), and adventitious root count (AR) for three chickpea genotypes [a]

Lines/ parameters	Treatment	DSW (mg)		CFR		DRW (mg)		PRL (mm)		AR[b]	
		Ex1	Ex2	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2
04067-81-2-1-1	CTRL	145.6	197.3	1.232	1.02	87.6	183.4	239.2	295.0	0	0
	PRR	116.5	209.5	1.226	1.03	77.9	143.4	196.5	254.3	-1.20(0.3)	0
	WL	150.7	179.6	1.213	1.00	45.5	66.8	92.0	91.8	-0.84(0.4)	0.98(2.7)
	PRR:WL	70.0	159.8	1.066	0.94	14.2	57.2	69.8	86.2	-1.80(0.2)	0.77(2.2)
Yorker	CTRL	136.8	246.3	1.202	1.04	90.1	219.2	230.1	295.3	0.03(1.0)	0.92(2.5)
	PRR	88.5	234.7	1.046	0.98	39.3	197.2	150.5	215.2	-0.31(0.7)	1.30(3.7)
	WL	139.2	186.9	1.235	0.90	45.6	78.2	68.8	84.7	1.19(3.3)	1.85(6.3)
	PRR:WL	76.7	233.0	0.969	0.79	11.6	111.7	51.6	73.0	-2.67(0.1)	1.53(3.2)
Rupali	CTRL	145.7	211.1	1.158	0.98	95.9	203.1	250.4	298.3	-0.47(0.6)	0.00(1.0)
	PRR	80.7	261.5	0.996	0.91	21.0	180.6	99.6	248.8	-1.67(0.2)	0.15(1.2)
	WL	147.1	159.6	1.135	0.68	46.3	74.9	62.7	95.3	1.17(3.2)	1.92(6.8)
	PRR:WL	70.9	150.9	0.956	0.69	9.9	68.9	53.8	65.8	0	1.64(5.2)
LDS[c]		15.6	63.3	0.06	1.17	10.2	60.3	18.2	50.6	1.23	1.05

			P value									
	Df		DSW (mg)		CFR		DRW (mg)		PRL (mm)		AR[b]	
Parameters	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2
Treatment (T)	3	3	<0.001	<0.05	<0.001	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Line (L)	2	15	<0.1	<0.1	<0.001	ns	<0.001	ns	<0.001	ns	<0.001	<0.001
L × T	6	45	ns	ns	ns	ns	<0.001	ns	<0.01	ns	0.01	0.01

[a] Chickpea genotypes are wild *Cicer* backcross 04067-81-2-1-1 and *Cicer aretinum* Yorker and Rupali, Ex1 = experiment 1; Ex2 = experiment 2; and ns= not significant. Treatments: CTRL = control; PRR = *Phytophthora medicaginis* inoculated; WL = waterlogging; and PRR:WL = *P. medicaginis* inoculated and waterlogging in combination.

[b] Back-transformed means in parentheses.

[c] LSD (T × L), $P = 0.05$.

Under WL conditions, a two- to threefold reduction in DRW was observed in both experiments, across all three genotypes, even though there were significant differences in PRL and AR counts (Table 1, Fig. 3). In experiment 1 only, *C. echinospermum* BC 04067-81-2-1-1

maintained an average PRL of 92.0 mm, whereas Yorker and Rupali PRL were significantly shorter at 69.0 and 62.7 mm, respectively (Table 1). WL treatment resulted in a greater AR proliferation in Yorker and Rupali compared with 04067-81-2-1-1 in both experiments (Table 1, Fig. 3). DSW and CFR in the WL treatment did not differ significantly from the control in experiment 1. Reductions were observed in experiment 2, perhaps due to greater duration of treatment application (Table 1).

Figure 3. Root systems of representative seedlings not subjected (no-WL) or subjected to waterlogging (WL) for A, *Phytophthora medicaginis* (PRR)-resistant *Cicer echinospermum* backcross line 04067-81-2-1-1, and B, PRR-susceptible Rupali (*C. arietinum*)

In both experiments, the PRR:WL treatment resulted in severe root disease development and reductions in all measured traits across the three genotypes (Table 1). Plant death occurred in a small number of susceptible Rupali seedlings in both experiments. As observed in the WL treatment, 04067-81-2-1-1 (69.8 mm) maintained a greater average PRL over Yorker (51.6 mm) and Rupali (53.8 mm) in experiment 1 (Table 1). The PRR:WL treatment resulted in a significant reduction of AR in Yorker and Rupali lines when compared with the WL treatment, in experiment 1 (Table 1). Under the higher disease pressure in experiment 1, the combination of PRR and WL resulted in severe rotting of AR roots. Similar trends were observed in experiment 2, in which Yorker and Rupali demonstrated a reduction in AR when compared with the WL treatment.

4.6 Discussion

Waterlogging had a significant effect on chickpea root morphology, which exacerbated PRR severity. Root plasticity of the three chickpea genotypes that differed in PRR resistance was examined and demonstrated significant differences in both PRL and AR branching at the seedling stage under waterlogged conditions. This suggests that chickpea PRR resistance and waterlogging tolerance could be linked, as previously reported in soybean. Further investigation is currently underway using a recombinant inbred line population.

Amalraj et al. (2019a) previously assessed levels of PRR resistance of the chickpea genotypes used in this study under field conditions. Findings showed that *C. echinospermum* BC 04067-81-2-1-1 exhibited improved levels of PRR resistance across two varying seasons, including a high-rainfall (i.e., disease-conducive) season, whereas the moderately susceptible Yorker and highly susceptible Rupali showed a greater range in PRR plant survival (Amalraj et al. 2019a). In this study, BC 04067-81-2-1-1 demonstrated lower DRW losses than Yorker and Rupali, respectively, under PRR infection. Furthermore, BC 04067-81-2-1-1 showed improved CFR over Yorker and Rupali in both PRR and PRR:WL treatments, demonstrating that BC 04067-81-2-1-1 also had improved PRR resistance under both phenotyping conditions.

Chlorophyll content and the associated CFR are often used to phenotype stress responses including waterlogging in plants (Maxwell et al. 2000). However, decreases in CFR were not observed in experiment 1 of this study under WL treatments in the absence of PRR. With the level of root damage incurred by all lines in this study within the WL treatment, a subsequent reduction in photosynthetic potential was expected. The early growth stage of assessment and/or the controlled-environment phenotyping conditions used may have contributed toward the inability of chlorophyll fluorescence to capture root damage caused by waterlogging.

Extending waterlogging may increase the likelihood of CFR detecting differences, as seen in experiment 2.

Genetic variation for waterlogging tolerance has historically been considered to be minimal in Australian chickpea material (Cowie et al. 1996, Palta et al. 2010). However, in this study we found root traits associated with waterlogging tolerance differed significantly between the three genotypes evaluated. Under waterlogged conditions, PRL of all three chickpea genotypes was reduced. However, the *C. echinospermum* BC 04067-81-2-1-1 was able to maintain a greater PRL under both WL and combined PRR:WL conditions over the *C. arietinum* chickpea genotypes Yorker and Rupali, which could be used as an indicator of superior PRR resistance. In support, greater PRL correlated to the level of PRR resistance using this rapid phenotyping method.

The ability of the *C. echinospermum* BC 04067-81-2-1-1 to maintain greater root length under waterlogged conditions may be linked to anaerobic respiration processes, higher root porosity, aerenchyma, and apoplastic barriers of the exo- and endo-dermal tissues. Genotypes that maintain roots at greater depth may have features that support anaerobic respiration or reduce radial oxygen loss, allowing distribution of oxygen to the lower root system (Patrick et al. 1972, Malik et al. 2001, Kotula et al. 2017). Based on the crossover of waterlogging tolerance and PRR resistance in *C. echinospermum* BC 04067-81-2-1-1, the apoplastic barrier of the exo- and endo-dermal tissues may be contributing to the higher levels of PRR resistance observed, as previously demonstrated in soybean (Ranathunge et al. 2008, Nguyen et al. 2012).

Yorker and Rupali had an eightfold and threefold increase in early AR in the epicotyl and hypocotyl region compared with *C. echinospermum* BC 04067-81-2-1-1 under waterlogged conditions in experiments 1 and 2, respectively. Promoting secondary lateral and adventitious root growth at the basal region of the root and stem is a mechanism to escape waterlogging

stress, because this region offers a reprieve from severe hypoxia or anoxia as the soil drains or remains free of flood water. This mechanism has been reported for several crops including wheat and rice (Nishiuchi et al. 2012, Steffens et al. 2016, Kotula et al. 2017). Chickpea is a hypogeal germinating species with an epicotyl that extends from the seed to the soil surface (Cubero 1987). Adventitious roots are not predetermined during early growth, instead developing later in response to a physiological requirement or stress (Verstraeten et al. 2014, Steffens et al. 2016). For adventitious roots to emerge unimpeded, the endodermis, cortex, exodermis, and epidermis must undergo remodeling through reactive oxygen species and peroxidase activity, essentially wounding the root. This may result in a temporary increase in permeability, as previously observed in onion (Peterson et al. 1993). This permeability may be a contributing factor to increased PRR susceptibility under waterlogging conditions.

Understanding the implications of root plasticity and the extent of physiological damage identified within chickpea genotypes Yorker and Rupali under waterlogged conditions is an important piece of the puzzle when considering mechanisms of PRR resistance. *Phytophthora* species have a chemotaxic response to root exudates released by new root tissues, so zoospores are often found in high abundance at branch points and root apices (Kuan et al. 1980, Erwin et al. 1983, Tyler 2002, Suo et al. 2016). Hence, the rapid increase in adventitious root development in response to waterlogging in Yorker and Rupali could hasten pathogen entry and increase inoculum load, thereby increasing susceptibility to PRR. This could explain the observation that the PRR resistance rating of some varieties (such as Yorker) is often poorly expressed in seasons when infection occurs in combination with sustained or repeated periods of waterlogging.

However, in addition to branch points and root apices, stomata below the soil surface on the hypocotyl and epicotyl tissues are the preferred entry point for *P. medicaginis* zoospores (Dale

et al. 1991). Under transient waterlogging conditions, stomatal conductance is increased in a bid to maintain leaf water potential in stressed plants (Bradford et al. 1982). This stomatal closure does not appear to improve the survivability of chickpea to PRR under long-term waterlogging conditions. In addition, root tissues under waterlogging conditions suffer anoxic stress in which cell membranes undergo lipid peroxidation, and subsequently functional integrity is lost (Blokhina et al. 1999). This means that plants would have to rely on other biochemical responses for PRR resistance once they become infected. But under waterlogging conditions plants have reduced energy available to maintain secondary metabolic functions for a biochemical defense response (Blokhina et al. 1999). In chickpea, Dale et al. (1991) found that wounds from PRR-infected susceptible chickpea roots showed an increase in hyphal growth of *P. medicaginis* over resistant chickpea lines, further indicating that there are other biochemical or physiological responses reducing the colonization of *P. medicaginis*. Hence, both the mechanical barrier and maintaining normal metabolism are important for improving PRR resistance in chickpea through waterlogging tolerance.

4.7 Conclusion

Screening for waterlogging tolerance traits in chickpea offers an alternative method for improving PRR resistance. The *C. echinospermum* BC 04067-81-2-1-1, which has superior PRR resistance, maintained a greater rooting length and developed fewer adventitious roots under waterlogged conditions than the two other more susceptible genotypes. Increasing PRR resistance by means of reducing adventitious root development, or root vigor, should be considered carefully as it may impact yield potential. Searching for additional sources of waterlogging tolerance in *Cicer* spp. and extending these preliminary findings to examine responses in adult plants and field scenarios appear to be warranted. Further understanding of underlying PRR resistance mechanisms is essential to rapidly incorporate selected traits into adapted high-yielding chickpea cultivars to limit the losses from this disease. As shown in this

study, adoption of an early stage waterlogging phenotypic screen as a proxy for PRR resistance within breeding programs could be beneficial. This would reduce the reliance on field studies, which are subject to rainfall variability or availability of irrigation. Phenotypic screening for waterlogging also enables the distinction of high and moderate levels of PRR resistance at the seedling stage with improved survivability and subsequent seed recuperation from adult plants.

4.8 Acknowledgments

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4.9 Author Contributions

N.D., K.H., and T.S. developed the phenotyping experiments. N.D. drafted the manuscript. All authors reviewed and revised the manuscript. K.H., T.S., and S.S. supervised the project. S.H. assisted with the experimental designs, principal component plots, and analysis of results for this manuscript.

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Chapter 5: QTL mapping of waterlogging tolerance traits in chickpea (*Cicer arietinum* & *C. echinospermum*) and the genetic relationship with QTL conferring Phytophthora root rot (*Phytophthora medicaginis*) resistance

5.1 Statement of Authorship

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Publication Details	Nicole Dron[1], Tim Sutton[2,3], Julian Taylor[3], Beata Sznajder[3], Julie Hayes[3], and Kristy Hobson[1] [1]NSW Department of Primary Industry Industries, 4 Marsden Park rd, Tamworth NSW 2340; [2]South Australian Research and Development Institute, GPO Box 397, Adelaide SA 5001; [3]School of Agriculture, Food and Wine, University of Adelaide, PMB 1, Glen Osmond SA 5064.

Principal Author

Name of Principal Author (Candidate)	Nicole Dron		
Contribution to the Paper	Contributed to the conceptualisation of the phenotyping method, designed the experiment, conducted the experiment, and collected data. Performed phenotype and QTL analysis. Drafted the manuscript.		
Overall percentage (%)	73%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	17/10/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Tim Sutton		
Contribution to the Paper	5% Helped to evaluate and edit the manuscript. Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	22/5/2023

Co-Author Contributions cont.

Name of Co-Author	Julian Taylor		
Contribution to the Paper	10% Contributed to conducting linkage map construction and QTL analysis. Reviewed and approved chapter content.		
Signature		Date	19/06/2022

Name of Co-Author	Beata Sznajder		
Contribution to the Paper	5% Contributed to conducting linkage map construction and QTL analysis. Reviewed and approved chapter content.		
Signature		Date	12/10/2022

Name of Co-Author	Julie Hayes		
Contribution to the Paper	2% Helped to evaluate and edit the manuscript.		
Signature		Date	20/05/23

Name of Co-Author	Kristy Hobson		
Contribution to the Paper	5% Helped to evaluate and edit the manuscript. Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	24/05/2023

Abbreviations

AR	Adventitious root count
BC	Backcross
DRW	Dry root weight
DSW	Dry shoot weight
E	Environment
GS	Genomic selection
G	Genotype
H^2	Heritability
MAS	Marker Assisted Selection
MR	Moderately resistant
MS	Moderately susceptible
PRR	Phytophthora root rot
PH	Plant height
PRL	Primary root length
PBA	Pulse Breeding Australia
QTL	quantitative trait locus/loci
RIL	Recombinant inbred line
SNP	Single nucleotide polymorphisms
S	Susceptible
WGAIM	Whole genome average interval mapping

5.2 Abstract

In chickpea, waterlogging is an important precondition for severe Phytophthora root rot (PRR) development. The pathogen, *Phytophthora medicaginis* (E. M. Hansen and D. P. Maxwell), can sporulate readily in transient flooding conditions, increasing disease pressure. Under long term flooding scenarios, and where soil waterlogging occurs, the plant undergoes structural modification and associated stress, increasing susceptibility to disease even in resistant genotypes. This research explores the relationship between waterlogging tolerance, plant architecture and PRR disease resistance. Using an F_6 biparental population of 148 recombinant inbred lines derived from a wild *Cicer* backcross 04067-81-2-1-1 and the *C. arietinum* variety Yorker, an existing linkage map was updated by incorporating SilicoDArT markers into the original SNP map, increasing unique marker loci from 314 to 607 across eight chickpea chromosomes. Waterlogging and plant architecture QTL were identified in chickpea following 14 days of soil saturation, including dry root weight (DRW), dry shoot weight (DSW), plant height (PH), primary root length (PRL) and adventitious root count (RC) or root recovery. Three previously published QTL for field PRR resistance, *QYBprrsi01*, *QRBprrsi02* and *QRBprrsi03*, physically co-located closely with DRW, PRL and RC waterlogging tolerance QTL, respectively, on the Kabuli v1.0 chickpea reference genome. The physical mapping of PRR resistance and waterlogging tolerance QTL to similar genomic regions in chickpea provides novel evidence that these traits may be controlled by common genetic factors. An additional six QTL for waterlogging traits were also identified. This information can be used to link key morphological characteristics and associated genetic markers, to facilitate breeding for resistance to both waterlogging tolerance and PRR disease in chickpea.

5.3 Introduction

Maintaining and improving yield potential and stability under changing climatic conditions is a priority for crop breeders internationally. Australia is the second largest producer of chickpea (*Cicer arietinum*) globally and the majority (>70%) of the Australian chickpea production occurs in the northern grains region (northern New South Wales and southern Queensland) (Boye et al. 2010, ABARES 2018). Chickpea production in this region is constrained by the disease Phytophthora root rot (PRR), caused by the soil-borne oomycete *Phytophthora medicaginis*. Symptoms of PRR are often exacerbated by waterlogging of the vertosol clay soils in high rainfall years. Waterlogging has a compromising effect on the plant and/or motile zoospore inoculum increases, resulting in multiple sites of infection and greater disease pressure (Erwin et al. 1983, Salam et al. 2011, Dron et al. 2022). Current PRR control measures include choosing least susceptible varieties, and paddock selection to avoid low-lying areas prone to waterlogging or where there is a history of PRR. However, these measures have limited effectiveness. Inoculum survives across seasons on alternative hosts (medic weeds and lucerne) and as oospore and chlamydospore structures in the soil, making the control of *P. medicaginis* inoculum through paddock selection and rotational cropping difficult (Bithell et al. 2021). Hence, improving the resistance of chickpea to PRR remains a breeding priority. The improved selection for waterlogging tolerance traits will increase genetic gain in breeding and may improve the current levels of resistance to PRR in chickpea, as has been observed previously in soybean (*Glycine max*) (Nguyen et al. 2012).

Climate change is increasing the frequency and intensity of extreme weather events, resulting in drought and flooding in cropping regions across the world (Hirabayashi et al. 2013, Harrison et al. 2016). Transient and long-term waterlogging in dryland cropping regions can result in crop and yield losses in most cereals, pulses, and oilseed crops. Waterlogging events and the associated damage are difficult to predict due to the influence of soil type (clay content and

structure), timing and duration of the events (Liu et al. 2020). The Australian northern grains region, a sub-tropical to temperate environment with year-round or winter dominant rainfall, has a predicted flooding frequency of one in every five years (BOM 2010). In plants, hypoxia (low oxygen) and anoxia (no oxygen) caused by soil waterlogging can result in the breakdown of physical barriers and loss of hydraulic conductivity and stomatal and aquaporin function, all inherently reducing the plant's ability to maintain essential metabolism and defence responses (Colmer et al. 2009). A reduction in metabolic function and the physical breakdown of root surface barriers will result in an increase in PRR susceptibility, particularly during the highly sensitive late vegetative and flowering stages of chickpea (Cowie et al. 1996, Dron et al. 2022).

Under waterlogging conditions, root systems rot and die at depth in the profile. Roots can survive or develop favourably in the upper soil profile where gas exchange is maintained. Hence, root depth during waterlogging is an indicator of waterlogging tolerance with reduced radial oxygen loss and/or the ability to maintain metabolism (Kotula et al. 2009, Fan et al. 2017, Liu et al. 2020). In addition, specialised secondary roots known as adventitious roots, can develop in the upper soil profile during and after waterlogging. Adventitious roots have been identified in chickpea during waterlogging, but do not appear to increase survival following severe long-term waterlogging; only during early vegetative and transient waterlogging scenarios (Dron et al. 2021, Dron et al. 2022). High levels of waterlogging tolerance in chickpea have not yet been identified (Chauhan 1987., Cowie et al. 1995, Palta et al. 2010, Dron et al. 2022). However, it is possible that waterlogging tolerance may have been indirectly selected through efforts to increase PRR resistance.

Wild relatives of cultivated chickpea such as *Cicer echinospermum* can offer a high level of PRR resistance and have been of significant interest in breeding (Croser et al. 2003). However, the poor agronomic suitability of these related species can present long-term challenges for introgression, including poor harvestability, low yield stability and poor seed quality

(Brinsmead et al. 1985, Knights et al. 2008). A key question remains whether adventitious root growth or root vigour, whilst critical for both yield potential and stability, results in increased permeability and chemotaxis of *P. medicaginis* at root branch sites under waterlogging conditions. If this is the case, breeding for root recovery or vigour traits and improved PRR resistance in unison may be contradictory; resistant genotypes may be less agronomically sound, with low or unstable yield in dryland cropping.

PRR resistance is predominantly identified through field screening, which can be notoriously variable. The soil-borne *Phytophthora* spp. polycyclic lifecycle is highly dependent on soil moisture for establishment and disease pressure. Soil heterogeneity across field experiments increases the difficulty in linking genotypes to phenotype. Phenotyping in a controlled environment overcame this problem and has been used to successfully identify PRR resistant genotypes (Knights et al. 2008, Amalraj et al. 2019b). However, PRR is very destructive and can be terminal, limiting the capacity to recover seed from genotypes with higher levels of resistance. The use of waterlogging response in controlled conditions as a proxy for PRR resistance may present an alternative, rapid phenotyping method that is favourable for both screening consistency and seed recovery.

A previous study utilised two years of irrigated and dryland field PRR survival index phenotyping data across three inter- and intra-specific RIL populations, to identify quantitative trait loci (QTL) contributing to PRR survival (Amalraj et al. 2019a). The *C. arietinum* and *C. echinospermum* resistance QTL differed in location, but both sources contributed to the quantitative PRR resistance in Australian chickpea (Amalraj et al. 2019a). Marker assisted selection (MAS) or updating genomic selection models relies on the understanding of underlying loci or causative genes, especially when aiming to reduce linkage drag and incorporate favourable waterlogging tolerance and PRR resistance QTL from multiple sources.

Here, we hypothesised that measures of waterlogging tolerance in a controlled environment screen could be used as proxies for PRR field resistance. We used a chickpea RIL population with an established linkage map and QTL previously reported for PRR field survival (Amalraj et al. 2019a). The parental lines have been shown to differ in root traits associated with waterlogging tolerance (Dron et al. 2021). The objective of this study was to identify QTL for waterlogging tolerance traits in the RIL population and investigate whether these regions co-locate with PRR survival QTL. This work will: (1) Determine the suitability of waterlogging tolerance and root architecture as a novel phenotyping proxy in early PRR resistance breeding; (2) identify novel waterlogging tolerance QTL for introgression through crossing and selection; and (3) facilitate genetic marker development for breeding both durable resistance to PRR and tolerance to waterlogging.

5.4 Materials and Methods

Plant material

A recombinant inbred line (RIL) population 9024 (n=148) of PRR moderately resistant (MR) *C. echinospermum* backcross (BC) line 04067-81-2-1-1 (04067) (pedigree: Howzat/ILW 245//99039-1013) and the moderately susceptible (MS) *C. arietinum* Yorker (pedigree: 8507-28H)/946-31) which differ in both PRR resistance and waterlogging tolerance root traits was used (Amalraj et al. 2019a, Dron et al. 2021). Included in experiments were RIL population parents and a further three industry PRR check genotypes: Kyabra (susceptible - S) and Pulse Breeding Australia (PBA) PBA Seamer and (MS) PBA chickpea breeding line CICA1815 (MS).

Evaluation of waterlogging tolerance and root phenotyping

The RIL population, parents and industry checks were cultured in a growth room located at the Tamworth Agricultural Institute, New South Wales, Australia. Minimum and maximum temperatures were set diurnally (12 h) at 20 °C and 30 °C, at relative humidities of 30–50%

(environmental control room, Percival Scientific®, Perry, USA). High temperatures were selected for more rapid onset of waterlogging (Dron et al. 2021). The experiment was established as a randomised complete block design with four replicates of each genotype (n=153); each replicate was split across two 32 L tubs (645 × 413 × 276 mm) (Figure 1).

Figure 1: One experimental replicate of chickpea RIL population 9024 (*C. echinospermum* × *C. arietinum**2) seedlings following 14-day waterlogging.

Ten seeds of each chickpea line were scarified and washed for 2 min in 0.1% (W/V) sodium hypochlorite and triple rinsed in reverse osmosis (RO) water. Following sterilisation seeds were transferred to petri dishes with filter paper; 10 mL of 0.5 mM $CaSO_4$ was added to each and covered to vernalise in darkness at 4 °C for five days and 24 °C a further two days. Following germination, four healthy seedlings with uniform radical lengths were transferred into potting tubes (50 × 50 × 100 mm) with pre-moistened sieved (5 mm) potting mix medium (1:1:1 loam, sand, and Greenlife® potting mix) at a depth of 50 mm. Tubes were held in place in a large 32 L sealed tub and filled to the surface with potting mix. Plants were watered with 30 mL of RO every second day until waterlogging treatments were applied at the two-leaf stage. Tubs were

filled with RO water to a height level with the surface of the potting media and maintained to this level of flooding for 14 days.

A single chlorophyll fluorescence ratio (CFR) reading was taken using a hand-held chlorophyll content meter (CCM-300, Opti-sciences Inc., USA) at 10 days post waterlogging as described by Dron et al. (2021). After 14 days, waterlogged plants were removed from the potting-tubes and roots washed prior to recording plant height (PH) (mm), primary root length (PRL) (mm) and hypocotyl adventitious root count (AR). AR count data were collected from the hypocotyl and basal stem regions of each seedling. Dry shoot (DSW) and root weights (DRW), with the cotyledons detached, were recorded for each plant after drying at 60 °C for 72 h.

Genetic linkage map construction

Two marker data sets were created using the DArTseq genotyping-by-sequencing platform at Diversity Arrays Technology (Bruce, ACT, Australia; www.diversityarrays.com/dart-applicaiton-dartseq accessed 02/02/2022). The first data set comprised DArTseq single nucleotide polymorphism (SNP) with co-dominant markers, and the second used SilicoDArT-based presence or absence markers. Construction of the genetic linkage map used a modified method previously described by Gupta et al. (2018); to incorporate SilicoDArT markers that were not used for the original map construction (Amalraj et al. 2019a). The map construction process involved: (1) Importing the RIL population SNP linkage map previously constructed by Amalraj et al. (2019a); (2) generating a SilicoDArT linkage map using the same source of DArTseq; (3) alignment of the SNP and SilicoDArT maps to identify suitable SilicoDArT markers to improve genomic coverage of the SNP map; and (4) construction of the final map with SNP and selected SilicoDArT markers. Map construction and diagnostics were performed using ASMap (Taylor et al. 2017) using the MSTmap algorithm (Wu et al. 2008) in the R Statistical Computing Environment software (R Core Team 2021).

Genotypes previously excluded from the SNP map construction were also excluded from the SilicoDArT mapping. Genotypes with over 98% similarity in detected alleles were merged to form consensus genotypes. Genotypes with a high proportion (>25%) of missing marker data were removed. Genotypic data were re-coded to parental calls. Markers of unknown phase and markers with extreme segregation distortion (family-wise adjusted p value < 1e-10) were removed. The remaining markers were then clustered into linkage groups and ordered into a skeleton linkage map using MSTmap then "pushed" into the existing SNP-based map using the combineMap and alignCross functions of the ASMap package (Taylor et al. 2017). Inspection of the SNP and silicoDArT maps allowed for the visual comparison and identification of markers that were out of phase and required a phase switch (Figure 2). Markers with crossovers in both flanking intervals were manually removed. A heat map displaying pairwise recombination fractions and LOD scores between markers was then generated to examine the physical map to identify potential weaknesses in the linkages between separate marker clusters and patterns of recombination (Figure 3). Using this final linkage map, imputation of missing alleles (Martinez et al. 1994), reduction of co-locating markers to consensus and midpoint interval markers were calculated using whole genome average interval mapping (wgaim) cross2int function (Verbyla et al. 2007).

Linear mixed model and QTL analysis

The analysis of the waterlogging traits for the RIL population was computationally performed using ASReml-R (Butler et al. 2018) in the R Statistical Computing Environment (R Core Team 2021). The AR count trait required a natural logarithm transformation prior to fitting the model. The following linear mixed model was then fitted for all traits to account for genetic and non-genetic sources of variation:

$$\boldsymbol{y} = \boldsymbol{X\beta} + \boldsymbol{Zu} + \boldsymbol{Z_g g} + \boldsymbol{e}$$

where $\boldsymbol{y}$ represents the waterlogging trait vector (PH, DSW, PRL, DRW, CFR and transformed AR), $\boldsymbol{X\beta}$ represents a fixed term that differentiates the population progeny lines from the parental and check lines in the experiment. The random term $\boldsymbol{Zu}$ consists of multiple non-genetic experimental design effects to account for variation between tubs, replicates, sides of the growth chamber as well as spatial effects potentially arising from variation between rows or column within a tub. The term $\boldsymbol{Z_g g}$ contains a set random genetic effect to model the variation of the trait due to RIL lines where the effects are distributed $\boldsymbol{g} \sim N(\boldsymbol{0}, \sigma_{\mathrm{g}}^2 \boldsymbol{I})$ where $\boldsymbol{\sigma_{\mathrm{g}}^2}$ is the genetic variance and $\boldsymbol{I}$ is the identity matrix. The model residual error $\boldsymbol{e}$ is considered distributed $\boldsymbol{e} \sim N(\boldsymbol{0}, \boldsymbol{R})$ where $\boldsymbol{R}$ contains a parameterization for a separable autoregressive spatial model $\mathrm{AR1} \times \mathrm{AR1}$ $(\mathrm{AR1} = \mathrm{auto-regressive\ process\ of\ order\ 1})$ to model natural variation of neighbouring plots through the correlation of observation across columns and rows. Diagnostic tools were utilised to check model assumptions and the ASExtras R package was used to generate variograms of spatial residuals to visually assess extraneous trends across tub columns and rows (Gilmour et al. 1997). No additional random effects were required for the models of this experiment. The set of effects $(\boldsymbol{u}, \boldsymbol{g}, \boldsymbol{e})$ were considered to be mutually independent. Best linear unbiased predictors (BLUPS) were extracted from each of the models and broad sense heritabilities were calculated using Cullis et al. (2006).

For each of the waterlogging traits, QTL analysis was performed using the WGAIM approach described in (Verbyla et al. 2007), implemented in the R package by Taylor et al. (2011), and used in Amalraj et al. (2019a). The intervals identified as containing QTL were summarised by the left and right flanking markers, the estimated effect size, LOD score of significance and percentage of contribution to the total genetic variance. Physical location was determined by anchoring sequence tags of the flanking markers to the Kabuli reference genome v1.0 (Varshney et al. 2013) in a BLAST (Altschul et al. 1990).

5.5 Results

Genetic linkage map

DArT genotyping-by-sequencing generated a total of 2,311 SNPs and 2,762 SilicoDArTs with a total map length of 1359.9 cM and an average marker spacing of 0.44 cM. Strong linkage was demonstrated across linkage groups and chromosomes in a heatmap (Figure 3). The linkage map demonstrated a slight inflation in genetic length from the original SNP map (Amalraj et al. 2019a) following incorporation of the SilicoDArT markers (Figure 2), but remained within the expected limits for chickpea (Thudi et al. 2011, Verma et al. 2015). The map contained 607 consensus markers with higher marker densities on chromosomes 4 and 6 and some chromosomes with lower density (i.e., chromosome 2), as was previously described Amalraj et al. (2019a) (Figure 2).

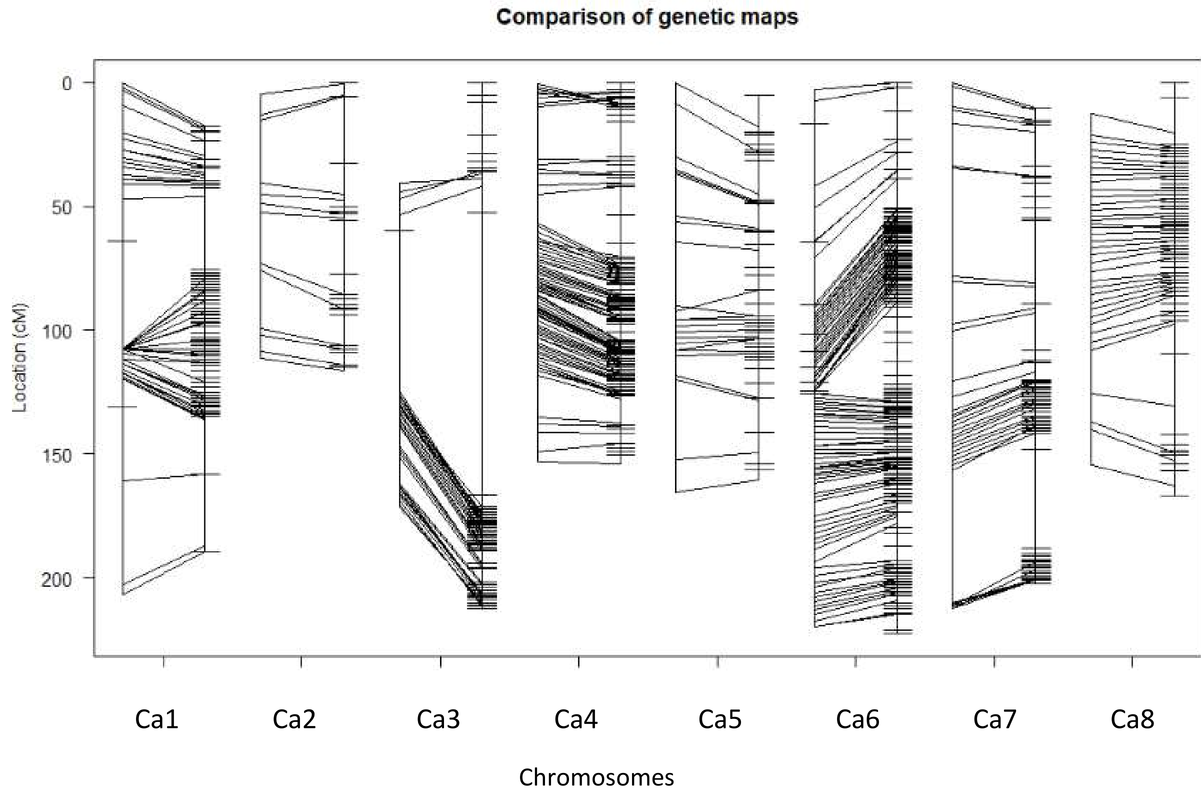

Figure 2. Comparison of the original SNP genetic map (Left for each chromosome) and the genetic map with incorporated SilicoDArT markers (Right for each chromosome) for the chickpea recombinant inbred line population (9024).

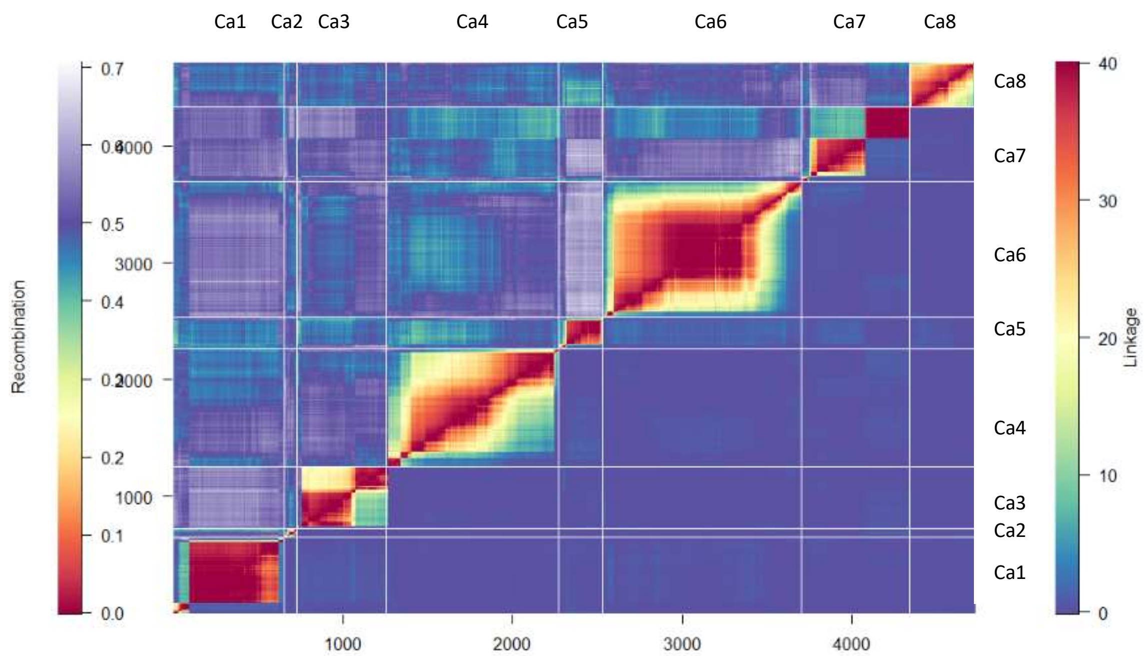

Figure 3: Genetic heat map demonstrating strong linkage for SNP and SilicoDArT markers in a linkage map constructed for the chickpea RIL population 04067 x Yorker.

Waterlogging trait distribution and association

The PRR moderately resistant line 04067 demonstrated greater primary root depth and fewer adventitious roots when compared to the moderately PRR susceptible variety Yorker following waterlogging, as previously observed by Dron et al. (2021) (Table 1). The broad-sense heritability values (H^2) were consistent (0.62-0.68) for all measured traits except for CFR (0.39) that was measured earlier at 10 days and did not capture differences, unlike traits measured at 14 days. This may have been due to the lack of waterlogging pressure at 10 days post treatment, indicating that the level of waterlogging stress must be severe enough to capture variation in waterlogging tolerance using CFR, as described previously by Dron et al. (2021).

Table 1: Chickpea parental and RIL mapping population means and broad sense heritability for measured traits following 14 days waterlogging stress.

	Parental mean		Population		
	04067	Yorker	Mean	Range	Heritability H^2
Dry root weight (g)	0.05	0.06	0.06	0.04 - 0.08	0.64
Dry shoot weight (g)	0.13	0.12	0.13	0.07 - 0.18	0.67
Plant height (mm)	153.2	103.5	136.1	100.8 - 180.0	0.68
Primary root length (mm)	113.8	103.2	102.0	84.4 -119.4	0.62
Adventitious roots count (log(x+1))	0.84	2.45	1.33	0.26-2.65	0.68
Chlorophyll fluorescence ratio (CFR)*	0.70	0.61	0.61	0.61- 0.70	0.39

*Measured at 10 days

QTL analysis and physical mapping

QTL analysis identified genomic regions that associated with waterlogging tolerance traits (Table 2). Major QTL (defined here as having >15% phenotypic variation explained) were identified with phenotypic variance ranging from 16% to 46.7%, and LOD values 1.86 to 5.40 for all traits except CFR. No QTL were identified for CFR, further indicating there was

insufficient phenotypic difference when measured at 10 days post waterlogging. QTL on chromosome 4 for plant height (*QYBwl05*), dry root weight (*QYBwl02*) and dry shoot weight (*QYBwl04*) all fell within a region 74.97 – 76.94 cM, with the favourable alleles derived from the BC line 04067. QTL for dry root weight (*QYBwl03*) and dry shoot weight (*QYBwl06)* on chromosome 6 also mapped to similar regions of the genetic map, with flanking markers sharing a similar physical location in the reference genome. For both QTL, the favourable allele was contributed by Yorker. Unique QTL were identified for root length (*QYBwl07* & *QYBwl08*, both on chromosome 4, with the alleles for long roots contributed by 04067) and adventitious root count (*QYBwl09* & *QYBwl10*, on chromosomes 4 and 8, with the alleles for increased adventitious root growth contributed by Yorker) (Table 2).

Table 2: QTL associated with waterlogging traits from the chickpea RIL population 9024 (n=150)

Trait	Chr.	QTL name	Interval	Genetic map distance (cM)	Physical position (bp)	Size[a]	P Value	% Var.	LOD
Dry Root Weight (g)	Ca3	*QYBwl01*	9024-SNP-121(C): 9024SNP-scf-89(C)	192.97-198.74	36,253,223[b] - 37,664,147[b]	0.005	0.0016	16.0	2.167
	Ca4	*QYBwl02*	9024SNP4-139(C): 9024SNP4-158(C)	76.04-76.94	7,267,692-7,583,978	-0.007	0.0000	31.0	4.014
	Ca6	*QYBwl03*	9024SNP6-358: 9024SNP6-360(C)	147.71-148.67	57,637,102-57,758,667	0.006	0.0003	22.2	2.860
Dry Shoot Weight (g)	Ca4	*QYBwl04*	9024SNP4-146(C): 9024SNP4-139(C)	74.97-76.04	7,413,697-7,267,692	-0.023	0.0001	46.7	3.447
Plant Height (mm)	Ca4	*QYBwl05*	9024SNP4-139(C): 9024SNP4-158(C)	76.04-76.94	7,267,692-7,583,978	-11.162	0.0010	27.3	2.366
	Ca6	*QYBwl06*	9024SD6-81(C): 9024SD6-404	137.66-139.52	53,566,491-53,783,285	10.000	0.0034	21.9	1.863
Primary root length (mm)	Ca4	*QYBwl07*	9024SNP4-90(C): 9024SNP4-89	3.76-4.46	1,066,068-1,065,923	-5.562	0.0027	22.2	1.961
	Ca4	*QYBwl08*	9024-SNP-171(C): 9024SNP4-312(C)	107.95-108.32	14,033,012[b] - 14,122,318	-6.587	0.0008	31.1	2.429
Adventitious Roots count (log(x+1))	Ca4	*QYBwl09*	9024SD4-78: 9024SNP4-67(C)	35.94-37.2	4,369,252-4,476,370	0.551	0.0000	28.8	3.916
	Ca8	*QYBwl10*	9024SD8-167(C): 9024SD8-181(C)	81.18-82.67	7,139,023-7,543,937	0.633	0.0000	38.1	5.398

[a] Positive and negative values indicate that Yorker and 04067 alleles increased the phenotypic values in the 9024 RIL population, respectively. Physical position (bp) in the chickpea Kabuli v1.0 reference genome, where [b] indicates unplaced location of the scaffold marker - estimates were made based on genetic map location.

5.6 Discussion

Waterlogging tolerance has been shown to play an important role in the resistance of plants to soil-borne pathogens (Ranathunge et al. 2008, Nguyen et al. 2012, Li et al. 2016). The RIL population 9024 used in this study is derived from parental lines with the highest levels of PRR resistance currently available: the *C. echinospermum* BC derivative (line 04067) and the *C. arietinum* variety Yorker. Distinct sources of PRR resistance are known to be attributed to each parental genotype (Amalraj et al. 2019a). Similarly, independent QTL identified in this study demonstrated that waterlogging tolerance traits were conferred by both parental genotypes, providing a novel opportunity to further understand the mechanisms of waterlogging tolerance and PRR resistance through genetic mapping and comparative analysis of the QTL regions involved, both at the genetic map and physical DNA sequence level.

The QTL identified in this study indicate that root architecture traits that impact waterlogging tolerance may also be associated with PRR survival. This was evidenced through the co-location or close neighbouring of several QTL for waterlogging traits with those for PRR survival. QTL for dry root weight (*QYBwl01*; physical location on chromosome 3: 36,253,223 bp - 37,664,147 bp) located to a similar physical region of the reference genome as the PRR survival index QTL *QYBprrsi03* (Chromosome 3: 34,523,347 bp – 34,911,684 bp) previously detected in this population (Amalraj et al. 2019a). The QTL for primary root length under waterlogging (*QYBwl08*; physical position on chromosome 4: 14,033,012 bp – 14,122,318 bp) also located to a similar physical region of the reference genome to QTL *QRBprrsi02* (Chromosome 4: 15,542,584-15,752,261 bp) for increased PRR survival identified in a related RIL population (9008), derived from a cross between Rupali and the BC line 04067 (Amalraj et al. 2019a). The proximity of physical regions of the genome associated with both waterlogging and PRR traits described here provides an opportunity for further gene

exploration and potentially in future following validation the ability to phenotype for PRR resistance using waterlogging as a proxy.

The QTL for adventitious root count (*QYBwl10*; physical position on chromosome 8: 7,139,023-7,543,937 bp) co-located with a broad PRR survival QTL (*QRBprrsi03;* physical position 72,201-15,860,254 bp) from the same 9024 RIL population (Amalraj et al. 2019a). The parental genotype, 04067, showed increased survival index under PRR disease pressure, but in this study, Yorker showed greater counts of adventitious roots following waterlogging. A three-fold increase in adventitious root growth was reported in the moderately susceptible Yorker parent when compared to moderately resistant 04067 following waterlogging (Dron et al. 2022). The reduction in adventitious root branching at the epicotyl and hypocotyl region likely reduces the entry and chemotaxis of the *P. medicaginis* pathogen, as previously described (Kuan et al. 1980, Suo et al. 2016, Dron et al. 2021). This supports the theory that breeding programs actively phenotyping and selecting for higher levels of PRR resistance may also be selecting for potentially poor root vigour or smaller root systems, a trait that is generally not desirable in dryland cropping systems in Australia. In contrast, breeders could use waterlogging phenotyping to select root systems with decreased branching and greater root depth to develop varieties targeted for wet seasons, heavy clay soils and/or regions with a high prevalence of PRR.

The shared flanking markers for QTL located on chromosome 4 for plant height (*QYBwl05)*, dry shoot weight (*QYBwl04*), and dry root weight (*QYBwl02*) contributed by alleles from genotype 04067 and QTL on chromosome 6 for dry root weight (*QYBwl03*) and plant height (*QYBwl06*) contributed by alleles from Yorker co-locate in physical position, respectively (Table 2). The detection of similar QTL for different waterlogging tolerance traits supports the potential to phenotype aerial plant characteristics in place of difficult to measure root traits when selecting waterlogging tolerance traits and/ or PRR resistance. This information may be

used in future to assist breeding target phenotypes and pyramiding tolerance to both PRR and waterlogging.

Selection with molecular markers flanking QTL identified from low density genetic maps could result in linkage drag in a crossing program. This is particularly important when QTL might co-locate with other regions that affect yield and agronomic parameters. Adding to this difficulty, resistance to PRR is known to be quantitative in nature, with resistance genes likely to be pleiotropic with traits for agronomic performance and yield. Further research is warranted to understand the nature of the interaction between resistance genes, those that are involved in traits that impart waterlogging tolerance and potential pleiotropic effects on other agronomic traits.

MAS has been a popular plant breeding technology for the rapid introgression of agronomic, abiotic and biotic traits with major-effect genes. Further identification of more closely linked markers and genes for implementation in a MAS strategy is possible but has shown limited success when breeding for quantitative disease resistance. The implementation of genomic selection (GS) in plant breeding can accelerate genetic gain, reduce costs and is better suited to evaluate and select for resistance or tolerance traits in breeding, especially for quantitative traits typically characterised by contribution from many small effect additive genes from diverse sources (Jannink 2010, Olatoye et al. 2019). The QTL identified in the RIL populations described here (9024 and 9001) could be used to guide further fine mapping to identify markers for MAS. Following this, selection based on high-throughput waterlogging phenotyping, genotyping using diagnostic MAS, and a longer-term GS approach with a larger training set established from breeding material would be suitable for application in chickpea breeding (Sudheesh et al., 2021). Incorporating all favourable genes is essential to ensure high levels of PRR resistance and ensure that waterlogging tolerance is achieved without selecting for traits that limit yield or agronomic performance.

Waterlogging tolerance can be attributed to a number of mechanisms including: tolerance of nutritional constraints and toxic element accumulation in the soil (Setter et al. 2009, Yaduvanshi et al. 2012); ability to maintain hydraulic conductance (Bramley et al. 2010); ability to maintain photosynthesis and metabolism with regards to nutrient, oxygen and water deficiencies (Colmer et al. 2009); and the ability to reduce oxidative stress damage (Gill et al. 2019). More detailed phenotyping of waterlogging tolerance mechanisms across diverse genetic material would facilitate the discovery of traits contributing to improving both waterlogging tolerance and PRR resistance in chickpea. RNA sequencing analysis identified a high number of responsive genes associated with the upregulation of oxidative burst during PRR infection in the susceptible variety Rupali (Amalraj 2019), suggesting that the hypersensitive response may only be partially effective as a response to infection from phytophthora. Oxidative stress response could potentially be measured by phenotyping under waterlogging conditions and may offer an ability to explore this pathway further in chickpea. RNA sequencing analysis identified that aquaporins were also upregulated following PRR infection and mapped to a similar QTL region for PRR resistance in chickpea (Amalraj 2019). Stomatal conductance and aquaporin function in the plasma membrane are critical for waterlogging tolerance, and genotypic variability is known in soybean (Jitsuyama 2017). Further pre-breeding and breeding targeting these two mechanisms is warranted to fully explore the currently available PRR resistance and waterlogging tolerance of Australian chickpea.

5.7 Conclusion

Several QTL for plant architecture traits associated with waterlogging tolerance in a chickpea RIL population co-locate with PRR survival index QTL previously identified by Amalraj et al. (2019), suggesting that waterlogging tolerance may be a suitable proxy for PRR resistance in chickpea. The use of waterlogging as a proxy for PRR resistance may be useful as a high-

throughput selection technique in early breeding where large numbers of lines are phenotyped. Further fine mapping of QTL and validation of linked markers in breeding populations could identify priority regions for MAS, facilitating introgression of both *Cicer echinospermum* and *C. arietinum*-derived resistance loci. Alternatively, this proxy method and/or RIL training set populations could be integrated into a GS approach. Specifically, generating mechanistic information around PRR resistance and root phenotyping will improve the efficiency of breeding resistance to PRR and tolerance to waterlogging whilst limiting the penalties to agronomic fit and yield of chickpea that currently exist.

5.8 Acknowledgments

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5.9 Author Contributions

ND and KH developed the phenotyping experiments. ND conducted the experimentation and drafted the manuscript. JT and BS assisted with the updating of the linkage map and QTL analyses. All authors reviewed and revised the chapter. KH, JH, and TS supervised the project.

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Chapter 6: Flavonoid accumulation and QTL mapping in chickpea *(Cicer arietinum & C. echinospermum)* infected with Phytophthora root rot *(Phytophthora medicaginis)*

6.1 Statement of Authorship

Title of Paper	Flavonoid accumulation and QTL mapping in chickpea (*C. arietinum* L. & *C. echinospermum*) infected with Phytophthora root rot (*P. medicaginis*)
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Nicole Dron[1], Tim Sutton[2,3], Julian Taylor[3], Beata Sznajder[3], Julie Hayes[3], and Kristy Hobson[1] [1]NSW Department of Primary Industry Industries, 4 Marsden Park rd, Tamworth NSW 2340; [2]South Australian Research and Development Institute, GPO Box 397, Adelaide SA 5001; [3]School of Agriculture, Food and Wine, University of Adelaide, PMB 1, Glen Osmond SA 5064.

Principal Author

Name of Principal Author (Candidate)	Nicole Dron		
Contribution to the Paper	Contributed to the conceptualisation of the phenotyping method, designed the experiment, conducted the experiment, and collected data. Performed phenotype and QTL analysis. Drafted the manuscript.		
Overall percentage (%)	65%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	17/10/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Tim Sutton		
Contribution to the Paper	5% Helped to evaluate and edit the manuscript. Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	22/5/2023

Co-Author Contributions cont.

Name of Co-Author	Jason Ng		
Contribution to the Paper	5% Contribution to LC-MS/MS protocol adaptation, troubleshoot and data analysis		
Signature		Date	17/07/2022

Name of Co-Author	Ulrike Mathesius		
Contribution to the Paper	5% Contribution to the planning and help with mass spectrometry.		
Signature		Date	02/07/2022

Name of Co-Author	Julian Taylor		
Contribution to the Paper	5% Contributed to conducting linkage map construction and QTL analysis. Reviewed and approved chapter content.		
Signature		Date	19/06/2022

Name of Co-Author	Beata Sznajder		
Contribution to the Paper	5% Contributed to conducting linkage map construction and QTL analysis. Reviewed and approved chapter content.		
Signature		Date	12/10/2022

Co-Author Contributions cont.

Name of Co-Author	Julie Hayes		
Contribution to the Paper	5% Helped to evaluate and edit the manuscript.		
Signature		Date	20/05/23

Name of Co-Author	Kristy Hobson		
Contribution to the Paper	5% Helped to evaluate and edit the manuscript. Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	24/05/2023

Abbreviations

BC	Backcross
GS	Genomic selection
H^2	Broad-sense Heritability
MAPK	mitogen-activated protein kinase
MR	Moderately resistant
MS	Moderately susceptible
MS	Marker Assisted Selection
NLR	nucleotide-binding leucine-rich repeats
PAMPs	Pathogen-associated molecular patterns
PBA	Pulse Breeding Australia
PR	Pathogenesis-Related
PRR	Phytophthora root rot
PRRs	pattern recognition receptors
QTL	quantitative trait locus/loci
R genes	resistance genes
RIL	Recombinant inbred line
S	Susceptible
SA	salicylic acid
SNP	Single nucleotide polymorphisms
WAKs	cell wall associated kinase
WGAIM	Whole genome average interval mapping

6.2 Abstract

Metabolites exuded by plant roots play an important role in plant-microbe interactions within the rhizosphere, especially in leguminous species such as chickpea (*Cicer arietinum*). Phenolic compounds modulate nodulation and are also linked to improved levels of plant disease and pest resistance. Phytophthora root rot (PRR), caused by the oomycete *Phytophthora medicaginis* (E. M. Hansen and D. P. Maxwell), results in significant losses to the Australian chickpea industry annually. This study explored the influence of PRR infection on the specific exudation of 47 target flavonoid compounds from chickpea roots, eight days post infection using *P. medicaginis* mycelial-oospore suspension in a hydroponic system. Twelve flavonoids were measured and accumulated variably with up to 8-fold differences between the PRR moderately resistant wild *Cicer* back cross line 04067-81-2-1-1, *C. arietinum* moderately susceptible variety Yorker and the susceptible variety Rupali. Using an F_6 bi-parental population of 148 recombinant inbred lines derived from a cross between 04067-81-2-1-1 and Yorker, flavonoid QTL were identified in chickpea associated with formononetin, maackiain, biochanin A, genistin (genistein-7-O-glucoside) and morin. Two previously published QTL for field PRR survival, *QYBprrsi01* and *QYBprrsi02*, were physically near to QTL associated with the flavonoid morin, *QYBmoprr02* on chromosome 3 and *QYBmoprr04* on chromosome 6. The co-location of loci controlling PRR resistance and flavonoid exudation QTL with similar genomic regions in chickpea provides novel evidence that these traits may be controlled by the same genetic factors. This information can be used to link key metabolites and PRR resistance, identifying associated genetic markers that could be used to facilitate PRR resistance breeding and novel forms of biocontrol for PRR in chickpea.

6.3 Introduction

The largest chickpea growing region of Australia is in New South Wales and Queensland, where production is constrained by Phytophthora root rot (PRR) caused by the hemibiotrophic oomycete *Phytophthora medicaginis*. The commonly grown varieties in this region, moderately susceptible PBA Seamer and PBA HatTrick, can suffer up to 70% yield loss due to PRR infection, as there is no cost-effective, in-crop control for the disease (Bithell et al. 2021). The narrow genetic base of cultivated chickpea dictates that interspecific crossing is essential for increasing diversity and introducing novel sources of disease resistance (Croser et al. 2003). High levels of resistance were previously identified in relatively small collections of uncultivated *Cicer* spp. (*C. echinospermum* P.H. Davis and *C. reticulatum* Ladizinsky) (Brinsmead et al. 1985, Knights et al. 2008, Amalraj et al. 2019). However, wild *Cicer* germplasm is un-adapted, often prostrate in growth habit, has poor yield stability and low seed quality, collectively making breeding and selection for introgression of resistance from these sources difficult (Knights et al. 2008). The accessions ILW 245 and ILW 246 were successfully backcrossed into domestic *C. arietinum* to partially recover adaptation traits, yield, and quality attributes essential for agronomic relevance to growers (Brinsmead et al. 1985, Knights et al. 2008). It remains unclear whether genes for PRR resistance can be fully uncoupled from traits associated with a negative impact on adaptation.

Plant disease resistance can be established through molecular and physiological mechanisms, or avoidance and escape mechanisms. The identification and incorporation of resistance genes (R genes) is important in breeding to build durable host resistance that can: (1) prevent immune suppression by pathogen virulence genes, (2) inhibit the release of pathogen effector molecules elicited by pathogen virulence genes, or (3) create a hostile environment for the pathogen by eliciting host defence responses. In some cases, R genes are incompatible or overcome by the pathogen, failing to result in resistance and in some cases even increasing susceptibility of the

host (Balint-Kurti 2019). The environment, plant and pathogen genetics, and the timing of infection all influence the host's ability to detect a pathogen and to scale a resistance response. A number of complex detection receptors (i.e., nucleotide-binding leucine-rich repeats (NLR), pattern recognition receptors (PRRs), cell wall associated kinases (WAKs), and pathogen-associated molecular patterns (PAMPs)) have been shown to be involved in pathogen detection and initiating a cascade of signalling pathways that can trigger immunity (PAMP-triggered immunity and effector-triggered immunity) (Andersen et al. 2018, Liu et al. 2019). Defence responses include NAPDH oxidase-dependent reactive oxygen species (ROS) production as a component of the hypersensitive response, activation of Ca^{2+}-induced stomatal closure, mitogen-activated protein kinase (MAPK) and trimeric G-protein signalling pathways, induction of salicylic acid (SA) biosynthesis, pathogenesis-related (PR) genes, callose deposition, and biosynthesis of phytoalexins, in addition to transcriptome reprogramming to prevent further pathogen colonisation (Liu et al. 2019). Morphological mechanisms of PRR resistance in chickpea are associated with mechanisms of escape (adventitious root growth) and avoidance (small root systems without branching) (Dron et al. 2021), but further exploration of systemic resistance including possible molecular and biochemical components is required.

In plants, flavonoids have important roles in the transport of auxin, root and shoot development, pollination, regulation of ROS, and signalling with symbiotic bacteria in the legume-rhizobium symbiosis (Hassan et al. 2012, Weston et al. 2013). Some flavonoids have phytoalexin properties that are antibacterial, antifungal and antiviral. Within the plant, flavonoids are transported both inter- and intra-cellularly and are specifically exuded or released following degradation of root cells in the rhizosphere, influencing plant-microbe interactions (Aoki et al. 2000, Hassan et al. 2012, Weston et al. 2013). In chickpea, there are several examples of flavonoids having a role in plant pathogen defence. The pathogen *Streptomyces rolfsii* caused

significant increases in ROS regulatory enzymes (i.e., superoxide dismutase (SOD) and peroxidase (PO)), reducing oxidative stress and lipid peroxidation, and reducing cell damage in young infected seedlings (Singh et al. 2017). An increase in the enzyme phenylalanine ammonia lyase (PAL) that initiates the flavonoid pathway was also observed in this study, resulting in the biosynthesis of phytoalexins and enhanced resistance to *S. rolfsii* (Singh et al. 2017). Increased accumulation of phytoalexin flavonoids, maackiain and medicarpin, have also been associated with increased resistance of chickpea to fusarium root rot (*Fusarium oxysporum* f. sp. *ciceri*) (Cachinero et al. 2002, Kumar et al. 2015). The involvement of flavonoids in *P. medicaginis* resistance of chickpea is yet to be explored. Large-scale metabolomic studies are now possible, and understanding the function of flavonoids in chickpea PRR resistance may forge the way for new breeding approaches (i.e., biomarkers for selection) or novel methods of control (i.e., plant priming or bio-fungicides).

The utilisation of novel sources of resistance in chickpea through introgression have resulted in a reduction in PRR disease severity but not provided complete resistance, indicative of the presence of minor quantitative resistance genes (Amalraj 2019). To develop durable quantitative PRR resistance, the identification and pyramiding of minor-effect resistance QTL is necessary but requires ongoing exploratory pre-breeding research. PRR resistance QTL have been mapped from both *C. arietinum* intraspecific populations, and interspecific *C. arietinum* x *C. echinospermum* backcross populations, identifying multiple chromosomal regions associated with resistance traits that were distinctly derived from wild or domestic sources (Amalraj et al. 2019). Transcriptomic studies examining chickpea responses to PRR in the parental genotypes found several metabolic pathways differentially up- and down-regulated following PRR infection (Amalraj 2019, Plett et al. 2021, Coles et al. 2022). Identified pathways included those related to the synthesis of auxin, abscisic acid, ethylene, salicylic acid, jasmonic acid and jasmonate. Each represent synthesis and signalling pathways which

influence the accumulation of flavonoids, providing signals for metabolic cascades within plant cells (Stevenson et al. 1997, Wasson et al. 2006, Weston et al. 2013, Plett et al. 2016, Plett et al. 2021, Coles et al. 2022). The important effects that flavonoids have on plant-microbe interactions in legumes, involving both pathogens and beneficial nitrogen fixing rhizobium (*Mesorhizobium ciceri*), warrants further investigation in the context of PRR in chickpea.

In this study, an interspecific chickpea RIL population was phenotyped using a controlled-environment hydroponic system, quantifying the exudation of target flavonoids from roots following *P. medicaginis* infection. Genetic analysis identified regions of the genome that were associated with accumulation of specific flavonoids. *In vitro* testing of the effect of these flavonoids on *P. medicaginis* pathogen growth was also conducted. The objective of this study was to identify QTL for flavonoid accumulation in root exudates and investigate whether these QTL co-locate with PRR field resistance QTL identified by Amalraj et al. (2019a). This work will (1) identify specific target flavonoids in root exudates following infection with *P. medicaginis*, (2) identify novel flavonoid QTL that can be targeted for introgression for improved PRR resistance, and (3) measure the effects of identified flavonoids on the *in vitro* growth of *P. medicaginis*. This will facilitate the development of genetic markers or metabolite screening methodologies that could be applied in PRR resistance breeding in chickpea. Outcomes of this research may also facilitate the development of novel methods for control of the disease.

6.4 Materials and Methods

Plant and oomycete material

A recombinant inbred line (RIL) population, 9024 (n=148; F_6 generation), was derived from PRR moderately resistant (MR), uncultivated *C. echinospermum* backcross (BC) line 04067-81-2-1-1 (pedigree: Howzat/ILW245//99039-1013) and the moderately susceptible (MS) *C. arietinum* variety Yorker (pedigree: 8507-28H)/946-31). The population was used for

screening along with the varieties Kyabra (susceptible - S), Rupali (S), Pulse Breeding Australia (PBA) HatTrick (MS), PBA Seamer (MS), PBA chickpea breeding line CICA1815 (MS) and ICRISAT accessions ICC02629 and ICC12607. A subset of twelve genotypes, including 04067-81-2-1-1 (04067), Yorker, Rupali, CICA1815, PBA HatTrick, Kyabra, ICC02629 and ICC12607, along with four highest PRR resistance 9024 RIL lines (D09024B>F6RIL>030, D09024B>F6RIL>040, D09024C>F6RIL>010 and D09024D>F6RIL>028) and four least PRR resistance RIL lines (D09024B>F6RIL>001, D09024C>F6RIL>005, D09024C>F6RIL>016 and D09024A>F6RIL>034), as determined by PRR field phenotyping (Amalraj et al. 2019), were included in an uninfected experimental control. The *P. medicaginis* isolate 4046, recovered from a PRR-infected chickpea plant in Moree, New South Wales (Bithell et al. 2022), was prepared as per Dron et al. (2021) for PRR infection.

Experimental design and conditions

The study was undertaken in a growth room located at the NSW Department of Primary Industries, Tamworth Agricultural Institute, New South Wales. Diurnal temperatures were set to 25/ 15 °C with 12 h day light and 12 h night. The experiment was a randomised complete block design with six replicates, with each replicate split into two 12 L tubs. The control was conducted at the same time in two tubs randomly placed amongst the PRR treatments, with six replicates of each genotype.

In planta infection and exudate collection

Chickpea seeds were washed in 0.1% (W/V) sodium hypochlorite and triple-rinsed in reverse osmosis (RO) water. Aerated 12 L tubs of 0.25 strength modified Hoagland's nutrient solution was used to germinate the seedlings on mesh (Figure 1a). The composition of the nutrient solution in RO water at full-strength was (mM): 5.0 Ca^{2+}, 5.0 K^{+}, 0.625 NH_4^{+}, 0.4 Mg^{2+}, 0.2 Na^{+}, 5.4 SO_4^{2-}, 4.4 NO_3^{-}, 0.2 $H_2PO_4^{-}$, 0.1 SiO_3^{2-}, 0.1 Fe-sequestrene, 0.05 Cl^{-}, 0.025 BO_3^{3-},

0.002 Mn^{2+}, 0.002 Zn^{2+}, 0.0005 Cu^{2+}, 0.0005 MoO_4^{2-} and 0.001 Ni^{2+}. The solution was buffered with 1.0 mM MES (2-[N-morpholino] ethane sulfonic acid) to a pH of 6.5 (Samineni et al. 2011). Germination occurred at 20 °C for seven days in darkness, after which seedlings of the same size were transferred into fresh 12 L tubs filled with full-strength aerated nutrient solution in a growth chamber under experimental conditions (Figure 1b). Light was introduced three days prior to inoculation. Inoculations were made at a concentration of 30 oospores mL^{-1} in mycelial-oospore slurry as per Dron et al. (2021). The control set remained uninfected.

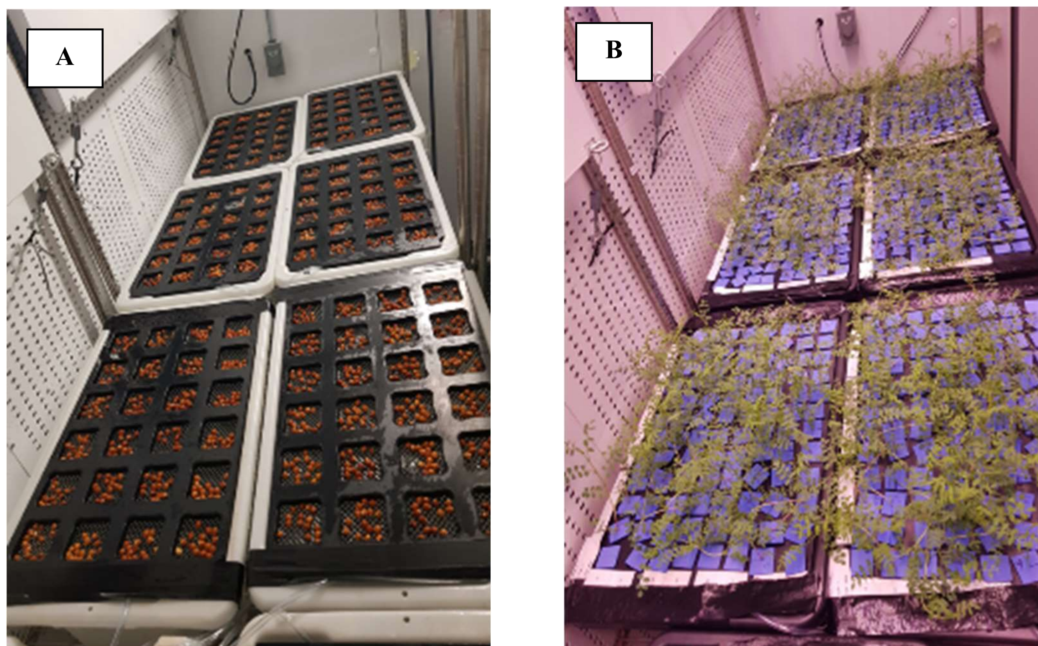

Figure 1: a) Germination of chickpea on mesh over modified aerated modified Hoagland's solution b) Three experimental replicates of chickpea seedlings in hydroponics, infected with Phytophthora root rot for root exudate collection.

Six days post-inoculation, shoot and cotyledons were cut away from the root system and shoot and root fresh weight and lengths were measured for each seedling. Infected plant roots of susceptible varieties had a water-soaked appearance with minor browning. Root exudates were collected by submerging root tissues in 15 mL RO water for 24 h at 21 °C in a plastic zip-lock

bag, agitating frequently. Exudates were then transferred to 15 mL conical Falcon® tubes. An internal standard (20 ng umbelliferone; Sigma-Aldrich) was added to each sample prior to freezing at -20 °C for 24 h. Samples were then stored at -80 °C before freeze drying in a SP VirTis general purpose freeze dryer (SP Industries, Warminster, Pennsylvania) with sample temperature control set to -20 °C. Dried exudate samples were resuspended in 1 mL 80% (v/v) LC-MS grade methanol (Merck) and vortexed for 20 s, followed by 5 min centrifugation at 16,000 g. The supernatant was transferred to 1 mL tubes and concentrated to dryness in a speedvac centrifuge. Samples were resuspended in 200 µL 80% (v/v) LC-MS grade methanol, vortexed for 10 s and filtered through 0.2 µm regenerated cellulose micro-spin filters (CIRO, USA), and resuspended in 50 µL 80% (v/v) LC-MS grade methanol in brown glass vials.

Liquid chromatography electrospray-ionisation tandem mass spectrometry

Prepared samples were subjected to targeted analysis of flavonoids in a Thermo QE Plus UPLC-Orbitrap at the Joint Mass Spectrometry Facility, Australian National University (ACT, Australia), following a modified method described by Ng et al. (2016). Flavonoid standards were sourced as per supplementary material (product list, Appendix 1 – Table 1). Samples and standards were separated in an Agilent Zorbax Eclipse 1.8 mm XDB-C18 2.1 x 50 mm column at 40 °C on a linear gradient from 5-90 % of 0.1 % aqueous formic acid to 99.9% methanol containing 0.1% formic acid at a flow rate of 200 µL min^{-1}. Data were collected using the positive ion mode and collision energies were optimised for each flavonoid. The heated electrospray ionisation (HESI-II) probe was operated with the following settings: ultra-high purity nitrogen gas was used as the sheath gas (45 L min^{-1}), auxiliary gas (10 L min^{-1}) and sweep gas (2 L min^{-1}); the spray voltage was 3.5 kV and capillary temperature 250 °C; the S-lens RF level was 50 V; the auxiliary gas heater temperature was 300 °C. Tandem mass spectrometry was performed using the parallel reaction monitoring mode with a mass resolution of 17,500 at 1.0 microscan. The Automatic Gain Control target value was set at

1.0e05 counts, maximum accumulation time was 50 ms and the isolation window was set at m/z 4.0. Standards for each of the target flavonoids were dissolved in 80% methanol at 1 ppm and analysed alongside the test samples. Thermo Scientific Xcalibur 4.0 software was used to extract and analyse sample and standard data.

Linear mixed model and QTL analysis

Phenotypic analysis of flavonoid accumulation in root exudates for the RIL population was performed using ASReml-R (Butler et al. 2018) in the R Statistical Computing Environment (R Core Team 2021). The equation below describes the calculation for ng per g fresh root tissues based on the sample umbelliferone internal standard (std.) and the separate LC-MS-MS run target standard describe previously by Ng et al. (2016). A response factor was included to correct for variation across the extended sample run time and align measured signals to actual concentrations of target compounds for accurate quantitative analysis. All flavonoid exudate accumulation data required a transformation prior to fitting the model. The transformation was of the following form:

$$log\left(\underbrace{\left(\left(\frac{1}{1.6}\right)\times\left(\frac{Internal\ std.area}{Target\ std.\ area}\right)\right)}_{\text{Response factor}}\times\left(\frac{Target\ area}{Internal\ std.\ area}\right)\times\left(\frac{20\ ng\ internal\ std.}{Root\ weight\ (g)}\right)\right)$$

(Ng et al. 2016)

The following linear mixed model was then fitted for all transformed traits to account for genetic and non-genetic sources of variation:

$$\boldsymbol{y} = \boldsymbol{X\beta} + \boldsymbol{Zu} + \boldsymbol{Z_g g} + \boldsymbol{e}$$

where $\boldsymbol{y}$ represents the transformed flavonoid exudate accumulation (liquiritigenin, isoliquiritigenin, formononetin, maackiain, medicarpin, afromosin, naringenin-7-O-glucoside (prunin), morin, apigenin, genistein, genistin (genistein-7-O-glucoside) and biochanin A), $\boldsymbol{X\beta}$ represents a fixed term differentiating the progeny lines, parental and check lines in the experiment. The random term $\boldsymbol{Zu}$ potentially consists of multiple random non-genetic experimental design effects to account for variation between tubs, replicates, sides of the growth chamber as well as spatial effects potentially arising from variation between rows or column within a tub. The term $\boldsymbol{Z_g g}$ contains a set of random genetic effects to model the variation of the trait due to RIL lines where the effects are distributed $\boldsymbol{g} \sim N(\boldsymbol{0}, \sigma_{\mathrm{g}}^2 \boldsymbol{I})$ where $\boldsymbol{\sigma_{\mathrm{g}}^2}$ is the genetic variance and $\boldsymbol{I}$ is the identity matrix. The model residual error $\boldsymbol{e}$ was considered distributed $\boldsymbol{e} \sim N(\boldsymbol{0}, \boldsymbol{R})$ where $\boldsymbol{R}$ contained a parameterization for a separable autoregressive spatial model $\mathrm{AR1} \times \mathrm{AR1}$ $(\mathrm{AR1} = \mathrm{auto} - \mathrm{regressive\ process\ of\ order\ 1})$ to model natural variation of neighbouring plots through the correlation of observation across columns and rows. Diagnostic tools were utilised to check model assumptions and the ASExtras R package was used to generate variograms of spatial residuals to visually assess extraneous trends across tub column and rows (Gilmour et al. 1997). No additional random effects were required for the models of this experiment. The set of effects $(\boldsymbol{u}, \boldsymbol{g}, \boldsymbol{e})$ were considered to be mutually independent. Best linear unbiased predictors (BLUPS) were extracted from each of the models and broad sense heritabilities were calculated using Cullis et al. (2006).

Target flavonoids with high levels of detection across the data set (maackiain, formononetin, biochanin A, genistin (genistein-7-O-glucoside) and morin) underwent QTL analysis, using the

WGAIM approach derived in (Verbyla et al. 2007, Taylor et al. 2011) using the updated QTL linkage map described in Chapter 5. Putative QTL were then summarised with the left and right interval markers, the estimated effect size, LOD score of significance and percentage of contribution to the total genetic variance. Physical location was determined by anchoring sequence tags of the flanking markers to the Kabuli reference genome v1.0 (Varshney et al. 2013) in a BLAST (Altschul et al. 1990). QTL regions with common flanking markers underwent a further BLAST to identify target genes for flavonoid biosynthesis enzymes.

Phytophthora medicaginis DNA quantification

Additional seedlings of 04067, Yorker and Rupali were included in each of the experimental tubs. Root systems were harvested as described for infected seedlings of the population at the termination of the experiment and dried at 40 °C for 24 h. qPCR quantification of *P. medicaginis* in the dried roots was assayed using the commercial Predicta®B test by the South Australian Research and Development Institute (SARDI, SA, Australia). Mean kDNA copies g^{-1} dry root weight and standard deviation are reported for each line. Predicated means and standard deviations were analysed in R using a one-way ANOVA and Tukey honestly significant difference (HSD) post hoc test with 95 % confidence interval.

Determining the effect of detected flavonoids on P. medicaginis using an agar disc-diffusion method

A paper disc-diffusion method was used to test *P. medicaginis* activity against detected flavonoids including biochanin A, formononetin, genistin (genistein-7-O-glucoside), maackiain, and morin. Filter paper discs (5mm in diameter) were washed, sterilised and placed on fresh V8 agar plates with 2.5% calcium carbonate and saturated with 20 µL of test flavonoids at three concentrations (250 µg mL^{-1}, 500 µg mL^{-1} and 1000 µg mL^{-1}) along with a dimethyl sulphoxide (DMSO) control. Mother plates of isolate *P. medicaginis* 4046 were prepared, as previously described by Dron et al. (2022). After five days of culture, 5 mm plugs

from the growing edge were taken from the mother plates and placed in the centre of each test plate. Each test flavonoid was replicated four times. Plates were incubated at 25 °C and diameters of the mycelial growth were measured from the plug to the growing edge every 6 h, from 48 - 84 h of incubation. Data was analysed using a linear mixed model that combined linear regression with non-linear splines across hours for individual concentration and compound treatment combinations. From this model, combined linear and non-linear predictions and their standard errors were extracted for each treatment combination across hours, and half-LSD bars were calculated. The resulting predictions and half-LSD coverage areas were then plotted using the ggplot2 R package (Wickham et al. 2007). Linear mixed models were fitted using the analysis package ASReml-R (Butler et al. 2018). Analyses were performed in the R Statistical Computing Environment software (R Core Team 2021).

6.5 Results

Flavonoid trait distribution and association

Flavonoid accumulation was successfully detected in root exudates for twelve of 47 targeted flavonoids (Table 1) with LC-MS-MS peaks aligning with retention times, qualifier and quant ions compound standards (Appendix 1 – Table 2). The estimated broad sense heritability values (H^2) were low, and variable, ranging from 7.1×10^{-8} to 0.396 (Table 1). The low heritabilities may be explained by the population structure with similar allelic frequencies, a skewed population towards a phenotype, or the flavonoid accumulation being heavily influenced by the environment and the associated variability in the phenotyping method including *P. medicaginis* pathogen infection and exudate collection.

Table 1: Back-transformed mean values (ng g^{-1} root tissue), range and broad sense heritability for twelve flavonoids detected in root exudates of chickpea varieties and breeding lines (04067 (PRR - MR), Yorker (MS) and Rupali (S)) and a RIL mapping population (04067/Yorker), following six days *P. medicaginis* infection in hydroponics. Back-transformed ANOVA means for uninfected parent lines are also presented.

	Uninfected			Infected					
	Genotype			Genotype			Population (04067 / Yorker)		
	04067	Yorker	Rupali	04067	Yorker	Rupali	Mean	Range	Heritability H^2
Liquiritigenin	3.78	5.37	3.53	1.54	3.64	1.87	1.21	1.21-1.21	-
Isoliquiritigenin	2.75	0.29	0.76	2.53	3.9	1.98	1.26	0.53-2.02	-
Formononetin	334	194	318	92	233	224	139	44.9-284	0.396
Maackiain	31.2	14.4	33.1	9.61	47.8	25.1	22.5	13.6-36.2	0.308
Medicarpin	8.58	7.54	10.0	10.2	26.8	33.0	21.0	17.2-24.4	0.121
Afromosin	3.9	5.21	2.25	43.1	5.03	10.8	10.6	4.1-19.7	0.244
Naringenin-7-O-glucoside (prunin)	4.46	3.78	6.34	4.06	1.02	0.8	2.05	2.05-2.05	-
Morin	37.7	47.9	27.1	332	219	111	177	59.90-406.25	0.380
Apigenin	2.53	0.61	1.92	6.9	1.36	2.79	4.27	3.61-5.37	0.104
Genistein	4.3	4.01	2.66	6.6	4.76	2.64	4.97	2.78-6.78	0.272
Genistin (genistein-7-O-glucoside)	1.58	0.16	0.86	1.79	0.48	0.29	0.5	0.28-0.69	0.168
Biochanin A	639	692	392	65.9	24.1	106	89.8	58.6-137	0.236

n.d. – not detected

Moderately susceptible Yorker had a 1.2-fold increase in accumulation of formononetin in root exudates, whilst moderately resistant genotype 04067 and susceptible Rupali showed 3.6-fold and 1.4-fold decreases following PRR infection, compared to the uninfected control, respectively (Table 1 & Figure 2). Yorker showed a 3.3-fold greater accumulation in maackiain whilst genotypes 04067 (-3.2-fold) and Rupali (-1.3-fold) demonstrated reductions following infection compared to the uninfected control (Table 1 & Figure 2). All three genotypes demonstrated a reduction in biochanin A accumulation following PRR infection when compared to the uninfected treatment in Yorker (-28.7-fold), 04067 (-9.7-fold) and Rupali (-3.7-fold) (Table 1 & Figure 2). Genistin (genistein-7-O-glucoside) accumulated 3.0-fold in Yorker exudates but was reduced in 04067 (-1.1-fold) and Rupali (-3.0-fold) following PRR infection when compared to the uninfected control treatment. Morin exudation increased following infection in all genotypes with increases of 8.8-fold in 04067, 4.6-fold in Yorker and 4.1-fold in Rupali (Table 1 & Figure 2).

Comparing flavonoid levels in exudates between the three parental genotypes when infected with PRR, Yorker had 2.5-fold more formononetin and 5.0-fold more maackiain compared to 04067 (Table 1 & Figure 2). 04067 exudates had 8.6-fold higher levels of afromosin and 5-fold higher levels of apigenin compared with Yorker. Genistin (genistein-7-O-glucoside) was 3.7-fold higher in 04067 exudates compared to Yorker (Table 1 & Figure 2). 04067 exudates had 8.6-fold higher levels of afromosin compared to Yorker in the infected treatment. Morin levels were 1.5-fold higher in exudates of 04067 when compared to Yorker (Table 1 & Figure 2).

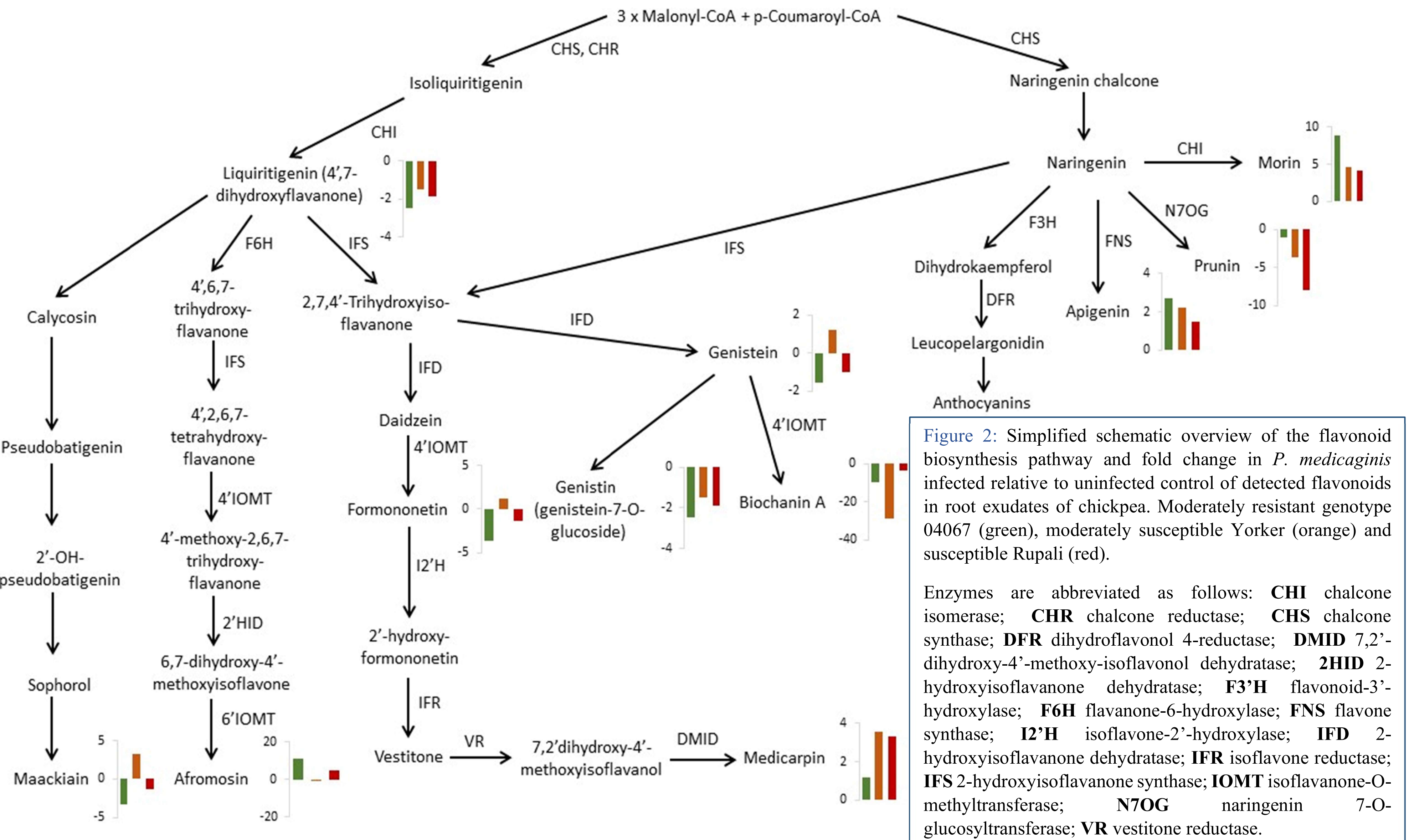

Figure 2: Simplified schematic overview of the flavonoid biosynthesis pathway and fold change in *P. medicaginis* infected relative to uninfected control of detected flavonoids in root exudates of chickpea. Moderately resistant genotype 04067 (green), moderately susceptible Yorker (orange) and susceptible Rupali (red).

Enzymes are abbreviated as follows: **CHI** chalcone isomerase; **CHR** chalcone reductase; **CHS** chalcone synthase; **DFR** dihydroflavonol 4-reductase; **DMID** 7,2'-dihydroxy-4'-methoxy-isoflavonol dehydratase; **2HID** 2-hydroxyisoflavanone dehydratase; **F3'H** flavonoid-3'-hydroxylase; **F6H** flavanone-6-hydroxylase; **FNS** flavone synthase; **I2'H** isoflavone-2'-hydroxylase; **IFD** 2-hydroxyisoflavanone dehydratase; **IFR** isoflavone reductase; **IFS** 2-hydroxyisoflavanone synthase; **IOMT** isoflavanone-O-methyltransferase; **N7OG** naringenin 7-O-glucosyltransferase; **VR** vestitone reductase.

QTL analysis of flavonoid accumulation

Following genetic map construction and refinement for the RIL population 9024, QTL analysis detected loci associated with accumulation of five flavonoids including maackiain, formononetin, biochanin A, genistin (genistein-7-O-glucoside) and morin. The remaining flavonoids (Table 1 & Appendix 1 - Table 1) measured in this experiment either showed little variation between parental genotypes or the RIL population, or had a high percentage of lines that were below detectible limits. Sixteen major QTL (defined here as >15% phenotypic variation explained) and four minor QTL (<15% phenotypic variation explained) were identified, with phenotypic variance ranging from 3.7% to 50.3%, and LOD values 1.3 to 7.2 (Table 2). Commonality between flanking markers of QTL *QYBfprr01*, *QYBbprr02* and *QYBmoprr01* for formononetin, biochanin A and morin, as well as *QYBfprr06* and *QYBmaprr03* for formononetin and maackiain, suggest the presence of a common regulatory locus, or biosynthetic pathway enzyme (Table 2 & Figure 2). The morin QTL *QYBmoprr02* on chromosome 3 and *QYBmoprr04* on chromosome 6 (Table 2) co-locate in physical position with previously identified QTL for PRR field tested survival index, *QYBprrsi01* and *QYBprrsi02* (Amalraj et al. 2019), for the same population.

Table 2: QTL associated with flavonoid accumulation in root exudates of chickpea RIL population 9024 infected with *P. medicaginis*.

Flavonoid	Chr.	QTL name	Interval	Genetic map distance (cM)	Physical position (bp)	Effect size[a]	P Value	% Var.	LOD
Maackiain	Ca3	*QYBmaprr01*	9024SNP2-28: 9024SNP-scf-215	35.58-39.21	1,198,062 – 316,009[b]	-0.3075	0.0006	19.5	2.5815
	Ca4	*QYBmaprr02*	9024SNP4-393: 9024SNP-scf-78	127.8-137.54	74,103[b]– 19,899,247	0.4940	0.0000	50.3	5.8523
	Ca5	*QYBmaprr03*	9024SNP5-124(C): 9024SD5-189(C)	123.21-135.79	38,110,075 – 34,403,372	0.3577	0.0002	26.4	2.9950
Formononetin	Ca2	*QYBfprr01*	9024SD2-10: 9024SNP2-7	115.1-116.29	35,396,628-35,515,963	-0.6433	0.0000	18.6	7.1661
	Ca4	*QYBfprr02*	9024SNP4-91(C): 9024SD4-104(C)	5.47-6.2	1,207,282-1,474,681	0.2881	0.0131	3.7	1.3360
	Ca4	*QYBfprr03*	9024SD4-248: 9024SNP4-247(C)	95.16-95.48	11,336,083-11,346,591	-0.6178	0.0000	17.1	4.0577
	Ca4	*QYBfprr04*	9024SD4-355: 9024SD4-349	122.85-124.05	17,399,034-17,701,880	0.7656	0.0000	26.3	6.2244
	Ca5	*QYBfprr05*	9024SD-445(C): 9024SNP5-137	43.43-44.07	39,868,795[b]-28,638,623	0.5713	0.0000	14.6	5.5174
	Ca5	*QYBfprr06*	9024SNP5-124(C): 9024SD5-189(C)	123.21-135.79	38,110,075-34,403,372	0.4234	0.0007	8.0	2.5121
	Ca6	*QYBfprr07*	9024-SNP-156(C): 9024SNP6-383	186.29-187.85	59,252,181[b]-59,252,208	0.3737	0.0013	6.3	2.2557
	Ca7	*QYBfprr08*	9024SD-286: 9024SD-scf-210	190.91-192.22	5,016,178[b]-315,068	0.3280	0.0033	4.8	1.8761
Biochanin A	Ca1	*QYBbprr01*	9024SD-scf-28(C): 9024SD-scf-49(C)	75.95-76.44	824,683-309,621	0.3944	0.0000	49.8	3.5869
	Ca2	*QYBbprr02*	9024SD2-10: 9024SNP2-7	115.1-116.29	35,396,628-35,515,963	-0.3695	0.0001	43.7	3.4194
Genistin (genistein-7-O-glucoside)	Ca1	*QYBgprr01*	9024SD-580(C): 9024SD-5	96.39-98.87	-	0.5883	0.0000	26.4	5.2328
	Ca2	*QYBgprr02*	9024SNP2-25: 9024SNP2-26(C)	5.4-5.89	5,358,564-5,387,533	0.4837	0.0000	17.9	4.2146
	Ca4	*QYBgprr03*	9024SNP4-71(C): 9024-SNP-165(C)	8.34-8.99	676,271[b] - 402,539	0.3725	0.0007	10.6	2.4735
	Ca7	*QYBgprr04*	9024SNP7-26: 9024SNP-scf-216	70.82-72.07	10,088,392-56,666	0.5329	0.0000	21.7	5.5935
	Ca8	*QYBgprr05*	9024SD8-105: 9024SD8-101	55.06-56.2	5,314,413-5,246,264	-0.5430	0.0000	22.5	4.2754
Morin	Ca2	*QYBmoprr01*	9024SD2-10: 9024SNP2-7	115.1-116.29	35,396,628-35,515,963	-0.4790	0.0000	20.1	3.6640
	Ca3	*QYBmoprr02*	9024SNP3-19(C): 9024SNP3-44(C)	206.43-207.03	39,711,310-39,585,946	-0.4764	0.0001	19.9	3.3711
	Ca4	*QYBmoprr03*	9024SD4-390(C): 9024SNP4-407(C)	138.22-138.87	26,411,645-28,045,178	0.5840	0.0000	29.9	5.0334
	Ca6	*QYBmoprr04*	9024SD-596: 9024SNP6-12	78.46-78.96	18,331,227[b]-50,535,991	-0.5295	0.0000	24.5	3.6000

[a] Positive and negative regression values indicate that Yorker and 04067 alleles increased the phenotypic values in the 9024 RIL population, respectively. Physical position mapped to the chickpea Kabuli v1.0 reference genome; [b] indicates unplaced location of the scaffold marker - estimates were made based on genetic map location.
n.d. – not detected.

Predicted biosynthesis enzyme genes for root exudate flavonoid accumulation

Candidate enzymes in shared flavonoid biosynthesis pathways include chalcone synthase and chalcone isomerase (Figure 2). Annotated genes (UniRef90) encoding these enzymes were identified in the chickpea reference Kabuli v1 genome (Varshney et al. 2013), to determine if any are located within the identified QTL. No genes encoding chalcone synthase or chalcone isomerase were identified within the region on Ca2 that was associated with multiple flavonoids. A gene for chalcone synthase at ~42 Mb is located at ~4 Mb away from the Ca5 QTL interval. Annotations for chalcone isomerase include a predicted gene at ~7 Mb from the Ca5 interval, and four predicted genes at ~18 Mb on Ca4, which may fall inside a region associated with the accumulation of both maackiain (*QYBmaprr02*; 0.7 – 19.9 Mb) and formononetin (*QYBfprr04*; 17.4 – 17.7 Mb).

Phytophthora medicaginis DNA quantification

As this experiment was harvested prior to the onset of visual symptoms, PRR disease establishment within the experiment was confirmed by qPCR (Figure 4) and the reduction in root fresh weight and primary root length compared to a control set of plants (Appendix 1 – Table 3 & 4). Moderately resistant 04067, moderately susceptible Yorker and susceptible Rupali roots were collected from each experimental tub for quantification of *P. medicaginis* DNA using qPCR. *P. medicaginis* was not detected in the uninfected control tubs. 04067 roots contained the least amount of *P. medicaginis* DNA, followed by Yorker and Rupali, correlating with the known PRR resistance ratings for each genotype. There was high variability across the experiment, but significant differences between moderately resistant phenotype, 04067, and susceptible, Rupali were demonstrated (Figure 4).

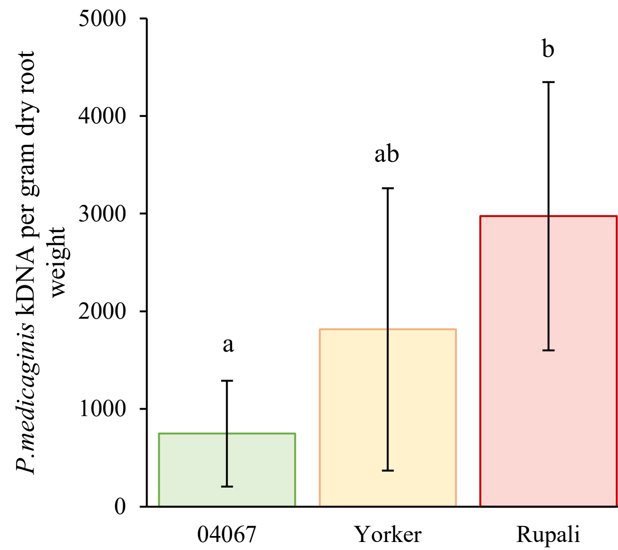

Figure 4: Mean kDNA copies of *P. medicaginis* per gram of root tissue at six days post-infection in hydroponics, following qPCR analysis of roots of moderately resistant 04067, moderately susceptible Yorker and susceptible Rupali. Samples (n=12) of each variety were taken from across twelve experimental tubs and data are shown mean ±sd. ANOVA, F = 10.5, df = 2, P < 0.0001. Means not sharing the same letter are significantly different (Tukey HSD, P < 0.05).

The effect of selected flavonoids on P. medicaginis growth using an agar disc-diffusion method

Mycelial growth of *P. medicaginis* isolate (4046) was tested against 1 mL of five flavonoid compounds at three concentrations (250 μg mL^{-1}, 500 μg mL^{-1} and 1000 μg mL^{-1}) applied to each disc. Compounds were serially diluted in DMSO, which was also used as a control, as depicted in Figure 5. Both the flavonoid compound and flavonoid concentration had significant effects on mycelial growth ($P < 0.001$). The interaction between compound, concentration and hours was however not significant with $P < 0.1$ (Figure 6). Maackiain and morin treatments at the 500 μg mL^{-1} and 1000 μg mL^{-1} concentrations demonstrated inhibition of *P. medicaginis* mycelial growth when compared to biochanin A, formononetin and genistin (genistein-7-O-glucoside) (Figure 5). However, similar inhibition trends were observed in the control (0 μg mL^{-1}) treatments. This may indicate that the control treatment was affected by the test flavonoid compound through the diffusion into other quadrants of the agar plate, and by the interconnected nature of mycelial growth of oomycyetes. It is reccomended that this experiment be repeated and conducted with different compounds and concentrations in individual, separate plates, and by testing the compounds at higher concentrations.

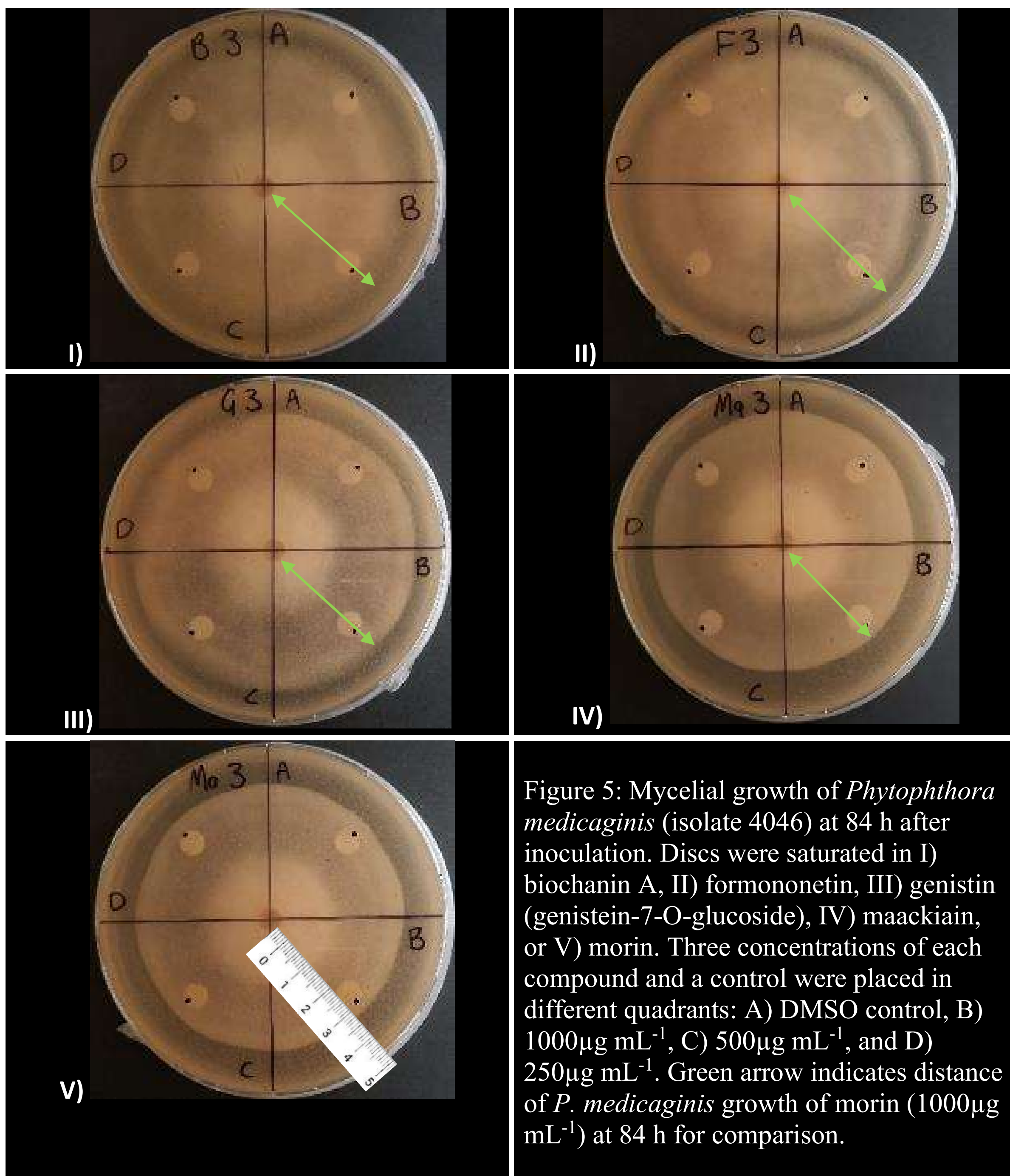

Figure 5: Mycelial growth of *Phytophthora medicaginis* (isolate 4046) at 84 h after inoculation. Discs were saturated in I) biochanin A, II) formononetin, III) genistin (genistein-7-O-glucoside), IV) maackiain, or V) morin. Three concentrations of each compound and a control were placed in different quadrants: A) DMSO control, B) 1000µg mL^{-1}, C) 500µg mL^{-1}, and D) 250µg mL^{-1}. Green arrow indicates distance of *P. medicaginis* growth of morin (1000µg mL^{-1}) at 84 h for comparison.

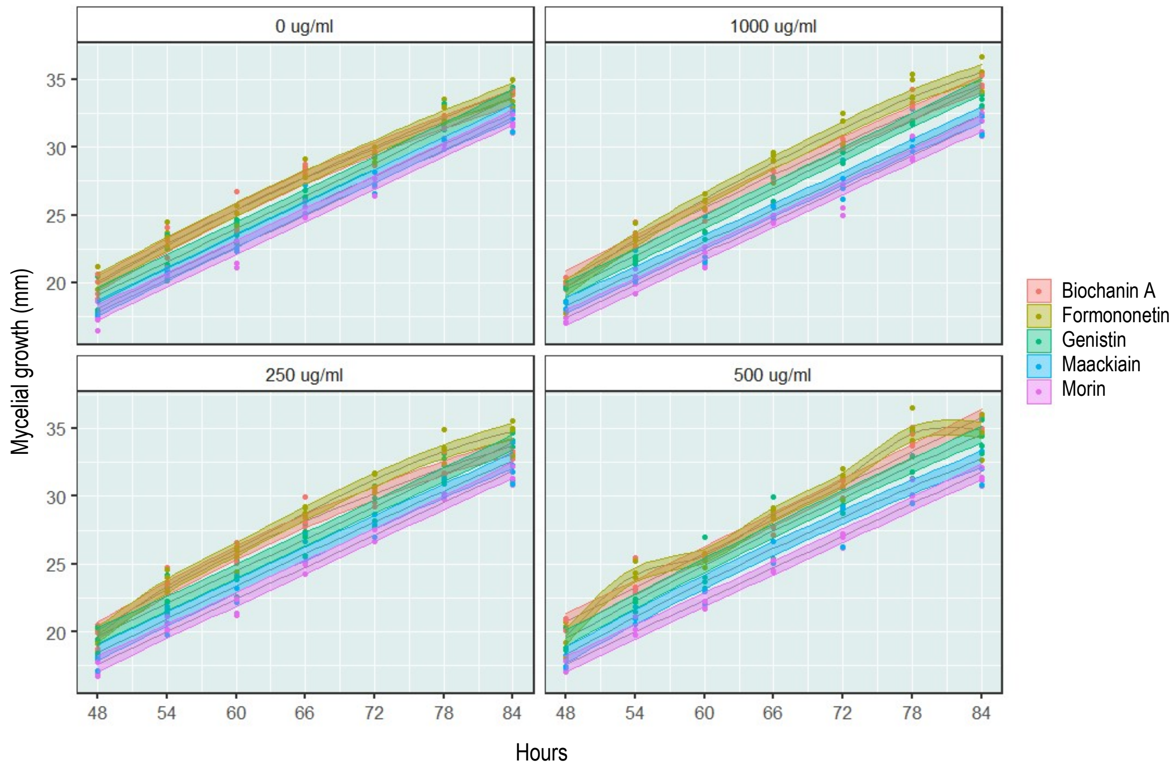

Figure 6: *Phytophthora medicaginis* mycelial growth predictions with half-LSD bars for flavonoids (biochanin A, formononetin, genistin (genistein-7-O-glucoside), maackiain and morin) on agar at three concentrations (250 ug mL^{-1}, 500 ug mL^{-1} and 1000 ug mL^{-1}) and a control (0 ug mL^{-1}). Non-overlapping ribbons are considered to be significantly different from each other.

6.6 Discussion

Up- and down-regulation of target flavonoids was demonstrated in parental genotypes 04067 (*C. echinospermum* BC) and Yorker (*Cicer arietinum*), and between the infected and non-infected control treatments (Table 1 & Figure 2). Liquiritigenin, genistin (genistein-7-O-glucoside) and biochanin A were down-regulated in all parental genotypes following PRR infection, whilst medicarpin was increased in all genotypes. Morin increased in all parental genotypes following infection compared to the control, but accumulated to 34% higher concentrations in exudates from infected 04067 compared to infected Yorker. Maackiain was down-regulated in 04067 and Rupali in response to PRR infection, but was up-regulated in Yorker. QTL for maackiain and morin in PRR-infected root exudates were detected in the RIL population derived from 04067 and Yorker (Table 2). The Yorker allele contributed positively

to the accumulation of maackiain, whilst the 04067 allele contributed positively to that of morin.

Defence signalling pathways are interconnected, with numerous transcription factors, biochemical processes and phytohormones involved. The detection of the same QTL region(s) for multiple flavonoids is likely attributed to a common link in biosynthesis pathways. In this work, the co-location of QTL for formononetin, biochanin A and morin, as well as for formononetin and maackiain (Table 1 and Figure 2), suggests that the underlying gene(s) or regulatory factors are targeting early enzymatic steps common to biosynthesis of each of the two groups of flavonoids. This is supported by the detection of genes encoding chalcone synthase and chalcone isomerase within common QTL regions on Ca2, Ca4 and Ca5. Additional genes, annotated with UniRef90, on the chickpea Kabuli v1 genome (Varshney et al. 2013) that were physically located between QTL flanking markers and with known function in flavonoid regulation were also identified. Two genes involved in flavonoid regulation localized within the formononetin (*QYBfprr01*), biochanin A (*QYBbprr02*) and morin (*QYBmoprr01*) QTL. These were: (1) an ABC transporter C family member 3-like genes (Ca_09705) that is involved in the cellular transport of various compounds, including phytochemicals and associated signaling molecules (Kang et al. 2011); (2) a premnaspirodiene oxygenase-like genes (Ca_09706); and (3) cytochrome P450 genes (Ca_ 07770) that have been shown to regulate the biosynthetic pathways of phenylpropanoid compounds (i.e., flavonoids) (Takahashi et al. 2007, Baba et al. 2020). Examination of the maackiain QTL, *QYBmaprr03*, revealed a candidate gene coding for a dual specificity protein phosphatase (Ca_01398), an enzyme that is involved in cellular removal of phosphate groups from proteins in response to external stimuli, including biotic stress (Jiang et al. 2018). The morin QTL *QYBmoprr02* physical region contained a gene coding for 3-ketoacyl-CoA synthase (Ca_01346), a protein putatively involved in the regulation of ion transporters, the accumulation of osmolytes, and

the maintenance of membrane integrity in *Arabidopsis thaliana* (Yang et al. 2020). These candidates could be investigated further to determine a possible association between expression level or sequence polymorphism across lines contrasting for flavonoid accumulation and/or PRR resistance.

The QTL identified for the exudation of morin, *QYBmoprr02* (chromosome 3: 39,711,310-39,585,946 bp), located in a similar physical region to both the PRR survival QTL *QYBprrsi01* (chromosome 3: 34,523,347 – 34,911,684 bp), and the single environment PRR survival QTL identified by Amalraj et al. 2019 (chromosome 3: 36,361,648 – 37,759,375 bp). A second QTL identified in this study for morin, *QYBmoprr04* (chromosome 6: 18,331,227 – 50,535,991 bp), co-located with the PRR survival QTL *QYBprrsi02* (chromosome 6: 30,089,649 - 31,011,058 bp), also identified by Amalraj et al. (2019). Morin has antioxidant properties that protect cells from damage through anti-lipid peroxidation, free radical scavenging and cytotoxic effects, that have been predominantly studied in human pharmacology (Kok et al. 2000, Subash et al. 2009). In a single study relevant to plants, Hussain et al. (2014) demonstrated that morin on PDA agar at three concentrations (i.e., 50, 200 and 500 ppm) has the capacity to reduce growth of plant pathogenic fungi, including *Aspergillus niger*, *Fusarium oxysporum*, *Chaetomium globosum*, and *Alternaria alternata*. Morin is a promising antioxidant agent, scavenging superoxide and neutralising damaging ROS produced during stress (Hussain et al. 2014). This ROS-regulating response has also been linked to an increase in resistance in avocado to *P. cinnamomi* (Teresa et al. 2014). These findings suggests that ROS-regulating enzymes and antioxidants may be key to improving PRR resistance in chickpea.

RNAseq transcript profiling studies of the same chickpea genotypes investigated here have shown a down-regulation in enzyme-coding genes involved in complex redox state signalling and hormone signalling pathways that regulate auxin, abscisic acid and ethylene in the moderately resistant genotype 04067 when infected with PRR, compared to uninfected control

plants (Amalraj 2019). However, protein kinases, such as MAPK cascades, that are controlled by flavonoids were only activated in 04067 (Amalraj 2019). Upregulation of flavonoid signalling pathways could be the necessary trigger to launch an oxidative stress defence response, reducing the rate of apoptosis, maintaining cellular structure and consequently disease resistance (Brunetti et al. 2013). In further support of this idea, recent research in cotton (*Gossypium hirsutum*) revealed that low levels of ROS act as a signal that induces tolerance to environmental extremes by altering the expression of defence genes (Qamer et al. 2021). These findings are in agreement with observations in this study which showed that the more resistant line 04067 had greater levels of morin than more susceptible lines and a greater fold change between the control and the infected treatment for flavonoid accumulation.

In contrast, in Yorker the upregulation of genes associated with brassinosteroid and jasmonic acid hormone signalling pathways was observed following *Phytophthora* infection (Amalraj 2019). Plant hormones, for example jasmonic acid, have been shown to be crucial elicitors for secondary metabolites, including flavonoids (Nabi et al. 2021). This could explain the higher levels of formononetin, medicarpin and maackiain in Yorker root exudates following infection. In disc-diffusion plate experiments, the flavonoids medicarpin and maackiain also showed antifungal activity against *F. oxysporum* of chickpea, and roots of fusarium wilt-resistant chickpea varieties were shown to accumulate significantly greater concentrations of medicarpin and maackiain than susceptible varieties (Stevenson et al. 1997, Cachinero et al. 2002). The differences in flavonoid accumulation profiles between Yorker and 04067 following PRR infection may relate to a difference in the defence responses of these lines. It is important to consider the suitability of these R-gene responses, as some resistance traits are only effective under specific infection scenarios. As observed in previous studies, oxidative burst and R-gene signalling following pathogen infection in Rupali could relate to its high level of susceptibility due to the incompatibility of the defence response (Liu et al. 2017, Amalraj

2019). Employing gene editing to silence, reduce or overexpress candidate genes related to flavonoid biosynthesis and secretion would help to define the role of each candidate in PRR resistance, and also help quantify effects on the soil and rhizosphere micro-biome, particularly nitrogen fixing bacteria.

An important consideration for this experiment is that the accumulation of exudates could occur as a result of damage to root tissues, causing electrolyte leakage (Oburger et al. 2018). The level of electrolyte leakage has been reported to correlate to level of PRR resistance and disease progression in chickpea (Coles et al. 2022). Experimental variability could also arise from the spatial or temporal nature of infection. Root exudates were harvested at a single time point prior to development of severe root disease in order to limit the extent of associated electrolyte leakage. Also, exudates measures were normalised to fresh root tissue weight to account for differences in root size. Future experimental work aiming to examine the role of flavonoids in PRR resistance could involve a more detailed experimental design, to include additional sampling over a time series of harvest, post-infection. Amalraj et al. (2019) showed that all genotypes reached a plateau of root *P. medicaginis* DNA content approximately eight hours post-inoculation with zoospore culture. Here and in Amalraj et al. (2019), PRR susceptible Rupali contained a significantly greater quantity of *P. medicaginis* DNA compared to the moderately resistant 04067, an observation that correlates with the relative levels of resistance and ability to reduce pathogen invasion and growth. The mycelial-oospore hydroponics method developed and applied in this research could be implemented as a more rapid and high throughput phenotyping tool in chickpea breeding to screen for PRR resistance in large numbers of breeding lines, without the difficulty of generating zoospores.

To hasten the improvement of PRR resistance in chickpea, rapid metabolite phenotyping methods could be developed to efficiently select lines in a breeding program with higher morin and maackiain levels. This work has identified a number of candidate genes that may contribute

to durable PRR resistance in chickpea. Agronomic opportunities also exist to manipulate the metabolic profile by priming the plant or seed prior to infection. For example, priming chickpea seed with the endophyte *Streptomyces* induces defence-related enzymes with antioxidant properties, including phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) superoxide dismutase (SOD), peroxidase (PO), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX), as well as an increase in phenolics in the growing plants (Singh et al. 2017). The primed plants also showed improved resistance to *Sclerotium rolfsii* (Singh et al. 2017). In another study, the application of calcium products ($Ca(NO_3)_2$ and $CaCl_2$) reduced the level of disease caused by *P. sojae* in soybean, but only in some varieties, suggesting the involvement of specific and inherited physiological pathways including for flavonoid biosynthesis (Sugimoto et al. 2005). Added calcium has been shown to reverse the effects of oxidative damage caused by heat damage in *Arabidopsis thaliana* (Larkindale et al. 2002). Novel bio-stimulants and calcium treatments may function to prime antioxidant status for improved PRR resistance of chickpea and are worthy of future investigation.

6.7 Conclusion

This study has identified QTL in chickpea that are associated with flavonoid exudation following infection with *P. medicaginis*. Some of these flavonoid compounds have reported phytoalexin and antioxidant properties (Aoki et al. 2000, Brunetti et al. 2013). Two major QTL for morin in PRR-infected root exudates, with the high-value allele coming from moderately PRR resistant 04067, aligned with major QTL for PRR field resistance and may play a part in reducing oxidative damage caused by *P. medicaginis* infection. The upregulation of maackiain in root exudates in response to PRR infection was highest in the moderately susceptible Yorker. There was indication that both morin and maackiain inhibited the mycelial growth of *P. medicaginis* in an *in vitro* assay, but further studies are required to confirm this initial finding. Additional research is required to validate linked markers with resistance in larger populations

of breeding material, and fine mapping of QTL regions could identify key resistance genes or develop closely linked markers for marker-assisted selection. The development of a high-throughput and cost-effective metabolite screening methodology to advance breeding for improved PRR resistance could also be advantageous. Alternatively, this information could be used to develop novel biocontrol approaches to better manage the agronomic impact of this disease in chickpea.

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6.9 Author Contributions

ND, JN, UM, KH, and TS developed the phenotyping experiments. ND conducted the experimentation and drafted the manuscript. JN and UM developed the LC-MS-MS protocol and assisted with the sample processing. JT and BS assisted with the updating of the linkage map as well as QTL and data analyses. All authors reviewed and revised the chapter. KH, JH, and TS supervised the project.

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Chapter 7 : General conclusion and future directions

7.1 General Discussion

7.1.1 Overview

This research was initiated to improve PRR resistance in chickpea through the improvement of waterlogging tolerance. Severe PRR disease and associated yield losses occur in chickpea in high rainfall seasons, due to soil flooding and waterlogging (Erwin et al. 1996, Bithell et al. 2021). The inherent link between flooding, pathogen inoculum proliferation and increased disease severity is well documented (Erwin et al. 1983, Li et al. 2016). Further to this, waterlogging tolerance has been shown to improve host resistance to soil-borne disease in several crops, as described in soybean (*Glycine max*) and *P. sojae*, and common bean (*Phaseolus vulgaris*) and *Pythium* spp. (Nguyen et al. 2012, Li et al. 2016). However, the effects of waterlogging on chickpea in the presence of *P. medicaginis* had not been reported, and it was unknown if waterlogging tolerance could also increase PRR resistance in chickpea. As part of this study, an initial priority was to understand the effects of waterlogging on both the *P. medicaginis* pathogen and the chickpea host.

Waterlogging creates a hostile environment for plants and aerobic pathogens, each with specific oxygen requirements (Moore 1975, Jackson 2004). This study found that long term soil waterlogging, and the associated hypoxia, reduced *P. medicaginis* zoospore concentration in nutrient solution and DNA levels (Predicta®B) in soil and root tissues (Chapter 3). Under severe waterlogging conditions, *P. medicaginis* pathogen sporulation is therefore likely to be compromised. In addition, waterlogging itself had a significant effect on the ability of the plant to maintain disease resistance under waterlogging stress. This work highlighted that waterlogging is a major contributor to the severe incidence of PRR in chickpea and searching for improved waterlogging tolerance warranted further investigation.

Chickpea waterlogging experiments have demonstrated that waterlogging tolerance in chickpea is inherently low when compared to wheat, rice and faba bean (Setter et al. 2009,

Nishiuchi et al. 2012, Munir 2016). However, in this research, morphological and physiological differences in waterlogging response between genotypes were identified (Chapter 4), with some chickpea genotypes having significantly deeper root growth under waterlogging (04067) and others showing increased adventitious root growth (Yorker). *Phytophthora* spp. preferentially infect branch points and new root tissues, and have also demonstrated hastened hyphal infection through compromised root barriers (Erwin et al. 1983, Peterson et al. 1993). Adventitious root growth may therefore compromise chickpea disease resistance in waterlogging conditions. The PRR moderately resistant 04067 genotype in this study demonstrated a reduction in adventitious root growth following waterlogging and generally had greater root depth, which is indicative of reduced radial oxygen loss and maintenance of intact root barriers, commonly associated with waterlogging tolerance (Kotula et al. 2009, Kotula et al. 2017).

Exploring the co-incidences of previously identified field PRR resistance QTL and new waterlogging tolerance QTL (Chapter 5), provided a means to further understand the mechanisms of waterlogging tolerance and PRR resistance, and where they may overlap. It also offered the opportunity to identify novel genetic loci associated with waterlogging tolerance that could be incorporated to improve PRR resistance in chickpea. This work has shown the potential to further improve PRR resistance through waterlogging tolerance. It is, however, ultimately limited by the low inherent waterlogging tolerance of chickpea. Modern breeding technologies such as genetic transformation or gene editing may allow the incorporation of novel waterlogging tolerance mechanisms from other species such as rice or wheat (Setter et al. 2009, Nishiuchi et al. 2012).

Flavonoids are important modulators of plant disease resistance, but the links between flavonoid synthesis and PRR resistance had not been researched in chickpea. Prior to undertaking the experimental work for this thesis, the biochemical response of chickpea to *P.*

medicaginis had not been reported, making an investigation of the role of flavonoids in PRR response novel. After six days of PRR infection, variability in the accumulation of flavonoids in root exudates was observed both in response to infection and between genotypes (Chapter 6). Flavonoid species measured were targeted for their known phytotoxic effects on microbial and/or fungal activity and activation of ROS regulatory pathways. An *in vitro* experiment also showed that two flavonoids (maackiain and morin) potentially reduced *P. medicaginis* mycelial growth. These preliminary observations require further validation (Chapter 6, Figure 5). Improved understanding of the biochemical pathways involved in PRR resistance unlocks opportunities to improve breeding strategies through MAS with either genetic markers or metabolite screening, as well as develop novel in-crop agronomic solutions such as bio-fungicides, that could reduce the impact of PRR.

A recent mapping study of chickpea in the 9024 (04067/ Yorker) RIL population identified QTL associated with PRR survival (Amalraj et al. 2019). Here, the same RIL population was used to identify chromosomal regions linked to PRR-induced flavonoid exudation and waterlogging tolerance traits in chickpea (Chapters 5 and 6). Through comparative analyses of genomic locations of the identified QTL regions, it could be demonstrated that waterlogging tolerance and flavonoid exudation are likely to have been indirectly selected for during breeding for PRR resistance. Additional QTL that were identified offer further opportunity to enhance PRR resistance, through fine mapping and the identification of linked molecular markers. Pyramiding QTL may also offer a successful approach for increasing PRR resistance in new varieties.

7.2 Key Findings and Future Research Perspectives

7.2.1 Development of rapid phenotyping systems for PRR and waterlogging in chickpea

Three novel, controlled environment phenotyping systems were developed to screen chickpea responses to waterlogging and PRR, both separately and in combination. Application of the systems across multiple experiments enabled: (1) the identification of germplasm with improved PRR resistance in the presence of waterlogging, (2) an investigation of the impact of waterlogging on both chickpea and that of the pathogen *P. medicaginis*, and (3) measurement of flavonoid accumulation in chickpea root exudates following *P. medicaginis* infection. Chapter 3 describes the first phenotyping method, a large-scale long-term pot study that measured the effects of early- and late-vegetative waterlogging and PRR treatments on eight chickpea genotypes. The second method (applied in Chapters 3, 4 and 5) is a rapid, small-pot system using potting media to measure the effects of both PRR and waterlogging on chickpea seedlings. The final method is a hydroponic system used to measure the effect of hypoxia on chickpea response to *P. medicaginis* and was applied in Chapters 3 and 5. This method also facilitated the harvest of root exudates to investigate flavonoid exudate accumulation from an infected RIL population and a set of control lines.

The hydroponic screening system used easy to propagate mycelial-oospore PRR inoculum as an alternative to the zoospore inoculum method employed by Amalraj et al. (2019). The use of mycelial-oospore suspension alleviates the need for the otherwise difficult production of PRR zoospores. Using this method, susceptible and resistant seedlings could be distinguished at 10 days post inoculation. The method has now been upscaled and adopted by Chickpea Breeding Australia (CBA), to enable rapid screening of approximately 700 breeding lines within four weeks. Previously, the breeding program inoculated field trials over the winter cropping season (approximately 6 months) where results were variable due to soil moisture and climate.

7.2.2 Effects of waterlogging on *P. medicaginis* and chickpea

This study measured the effects of waterlogging on chickpea PRR resistance, and on the PRR causing pathogen *P. medicaginis* (Chapter 3). A large-scale factorial experiment investigated the effect of early- and late-vegetative waterlogging and PRR on chickpea root disease in three reference genotypes. Late-season waterlogging alone resulted in an average 88% reduction in biomass in the absence of PRR, and 94% reduction when in combination with PRR infection. Waterlogging caused significant root damage in chickpea, particularly when imposed late in the season. However, *P. medicaginis* inoculum load under periods of long-term waterlogging in potting media and oxygen-reduced conditions in hydroponics did not significantly increase. In fact, zoospore counts were reduced when exposed to hypoxic conditions that reflect soil waterlogging conditions. This suggests that chickpea suffers physiological damage and constraints from exposure to waterlogging alone, which in turn results in reduced PRR resistance, severe disease and yield loss (Dron et al. 2022). These findings support the need for ongoing research to identify additional sources of waterlogging tolerance in *Cicer* spp. or genetic modification to increase waterlogging tolerance to reduce both waterlogging damage and PRR disease in chickpea.

7.2.3 Screening for waterlogging tolerance to enhanced PRR resistance in chickpea

Controlled environment seedling experiments in hydroponic and potting media showed that a relatively low level of waterlogging tolerance exists in the screened chickpea materials (Chapter 3, 4 and 5). However, the PRR moderately resistant wild *Cicer* backcross line 04067 showed a 3-fold reduction in adventitious root growth, whilst maintaining a significantly greater rooting depth when compared to moderately susceptible Yorker under waterlogging conditions. *P. medicaginis* preferentially infects branch points and new root tissues (Erwin et al. 1983), which may be associated with the superior PRR resistance of 04067. However,

increasing resistance by reducing adventitious root formation or potentially root vigor, should be considered carefully as it may negatively impact adaptation and yield potential: The reduced vigor phenotype of line 04067 (Chapter 3) (Dron et al. 2022) is illustrative of this. It is also likely that reduced adventitious root formation and reduced vigor will be difficult to genetically un-couple. Selection for primary root depth under waterlogging or the early establishment of roots with aerenchyma, may be more suitable traits to use in selecting for PRR resistance. Overall, this study verified that component root traits under waterlogging are useful proxy phenotypes for targeting PRR resistance in chickpea pre-breeding and breeding.

7.2.4 Flavonoid accumulation in root exudates of chickpea following PRR infection

Measures of chickpea flavonoid accumulation in root exudates and the effect on *P. medicaginis* growth of specific flavonoids *in vitro* had not previously been undertaken. Following six days of PRR infection using a hydroponic screening method, seedling root exudates were harvested (Chapter 6). Root exudates were processed and analysed using targeted LC-MS-MS. Variability in flavonoid accumulation was observed between the 9024 RIL population parental genotypes 04067 and Yorker, for twelve of the 45 target flavonoids that have a reported role in soil-root microbial activity including pathogen resistance (Chapter 6 – Table 1). Specifically, Yorker exudates collected from PRR-infected roots had 5.0-fold more maackiain compared to 04067, whilst 04067 exudates had 1.5-fold more morin when compared to Yorker (Chapter 6 - Table 1). Maackiain and morin demonstrated a reduction in *P. medicaginis* mycelial growth when compared to other detected flavonoids in an *in vitro* agar experiment, but this finding requires further validation (Chapter 6 - Figure 6). This preliminary understanding of the potential role of flavonoid exudation in PRR resistance may provide a unique opportunity for future research. Additional research to measure the impact of variation in flavonoid exudation

on other biological process such as rhizobia infection, nodulation and nitrogen fixation, will also be required.

7.2.5 Co-location of waterlogging tolerance and flavonoid accumulation QTL with PRR survival QTL

The RIL population 9024 (04067/Yorker) was phenotyped for both waterlogging root traits and PRR induced flavonoid accumulation (n=148). Genetic analyses identified ten QTL associated with traits including plant dry matter, adventitious root growth and root depth following waterlogging. In addition, 21 QTL for flavonoid accumulation in root exudates were identified for maackiain, formonetin, biochanin A, genistin (genistein-7-O-glucoside) and morin following *P. medicaginis* infection. QTL were mapped to physical regions of the kabuli reference genome v1.0 to enable comparative analysis (Varshney et al. 2013). Some of the detected waterlogging and flavonoid accumulation QTL physically co-located with those previously identified for PRR survival by Amalraj et al. (2019). This was an encouraging finding, supporting the hypothesis that waterlogging tolerance and flavonoid exudation contributes to PRR resistance in chickpea.

The morin QTL, *QYBmoprr02* on chromosome 3 and *QYBmoprr04* on chromosome 6 (Table 2), co-locate in physical position with previously identified QTL for PRR field-tested survival index (*QYBprrsi01* and *QYBprrsi02*; Amalraj et al. 2019). QTL for waterlogging traits that co-located with previously identified PRR survival regions included loci for root dry weight (*QYBwl01*), primary root length (*QYBwl08*), and adventitious root count (*QYBwl10*) (Chapter 5). QTL analysis showed that alleles for adventitious root count were contributed by Yorker following waterlogging treatment. Interestingly though, Amalraj et al. (2019) demonstrated that 04067 contributed the alleles for PRR survival. This supports the hypothesis that selection for improved PRR resistance may be indirectly selecting for fewer adventitious roots or poor root vigour. As *Phytophthora* spp. are attracted to branch points and new root tissue, having

fewer adventitious roots reduces the amount of tissue at high risk of PRR infection. Again, the co-location of QTL associated with both waterlogging and PRR survival suggests a link between waterlogging and PRR resistance, offering potential to phenotype for PRR resistance using waterlogging as a proxy.

Findings from QTL analysis provide a basis for the future development of genetic markers that can be utilised in breeding selection. The quantitative nature of PRR resistance and waterlogging tolerance also implies that genomic selection strategies in chickpea breeding may enable significant progress to be made. Methods to screen metabolite profiles and implement selection based on specific flavonoid accumulation profiles may also provide an effective path for increasing resistance in new varieties.

7.3 Conclusion

Waterlogging is a common environmental stress that can result in significant yield and crop losses. It also exacerbates PRR disease in chickpea. Key findings from this research included that in some scenarios, such as during long-term soil waterlogging and associated hypoxic soil conditions, the *P. medicaginis* inoculum load does not increase. Therefore, symptoms of severe PRR disease during waterlogging are largely associated with the physiological and morphological changes imposed on plant roots by waterlogging. There is minimal waterlogging tolerance in chickpea, particularly during severe late season exposure. However, improving or selecting for PRR resistance can be achieved through selection of specific, component waterlogging tolerance root traits such as root depth. Greater root depth in waterlogging conditions infers greater levels of aerenchyma or root porosity, lower surface area to volume, and reduced radial oxygen loss. Similarly, maintaining plant metabolism under waterlogging conditions would ensure that plants can respond to *P. medicaginis* infection through, for example, root exudates. Flavonoids are differentially accumulated in root exudates between chickpea genotypes when infected with *P. medicaginis* and may play an important

role in PRR resistance. These mechanisms and the linked QTL collectively provide opportunity for targeted selection in breeding that will assist plant breeders to develop new adapted varieties with high levels of PRR resistance and waterlogging tolerance.

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Appendix 1 – Supplementary Materials

Table 1. List of targeted flavonoids and standard providers.

Flavonoid	Source
2'-hydroxyflavone, 3'-hydroxyflavone, 6,7,4'-trihydroxyisoflavone, 7,3',4'-trihydroxyflavone, 7,4'-dihydroxyflavone, Afromosin, 5,7-dihydroxyflavone (Chrysin), Daidzein -7-O-glucoside (Daidzin), Eriodyctiol, Esculetin, Genistin, Glycitein, Isoliquiritigenin, Luteolin, Madecassoside, Naringenin-7-O-glucoside (Prunin), Formononetin-7-O-glucoside (Ononin), Prunetin, Puerarin, Resveratrol, Rutin, Taxifolin	Indofine Chemical Company, Hillsborough NJ, USA
Genistein, Hesperitin, Kaempferol-7-O-glucoside, Kaempferol-3-O-glucoside (Astragalin); Liquiritigenin, Morin	Extrasynthese, Genay Cedex, France
Coumestrol, Daidzein, Kaempferol	Cayman Chemical Company
Medicarpin, 2'-O-methylliquiritigenin	Carbosynth, Compton, UK
Apigenin, Apigenin-7-neohesperidoside, Naringenin-7-O-rhamnoglucoside (Naringin), 3',5,7-trihydroxy-4'-methoxyflavone 7-rutinoside, Biochanin A, Formononetin, Naringenin, Quercetin, Quercetin-3-glucoside (Isoquercetin)	Sigma-Aldrich, Castle Hill Australia

Table 2. Detected flavonoid LC-MS-MS retention times (RT) and parent, quant, and qualifier ions peak time.

Flavonoid	CE	RT	Parent ion	Quant ion	Qualifier ions				
Umbeliferone	25	5.46	163.0388	107.0493	91.0545	119.0492	55.9350	95.0494	72.9376
Morin	40	8.06	303.0499	123.0442	153.0180	149.0232	121.0648	213.0544	185.0597
Genistin (genistein-7-O-glucoside)	12	6.19	433.1128	271.0599	272.0631				
Naringenin-7-O-glucoside (prunin)	10	6.47	435.1275	273.0754	274.0787				
Medicarpin	15	8.55	271.0961	137.0596	138.0629	161.0595	123.0440	147.0439	243.1012
Liquiritigenin	23	7.14	257.0804	137.0233	147.0440	119.0492	138.0267		
Daidzein	38	7.40	255.0659	91.0545	137.0232	181.0645	199.0751	153.0698	128.0621
Genistein	38	7.72	271.0604	91.0545	153.0180	215.0700	169.0646	197.0595	68.9976
Isoliquiritigenin	23	8.23	257.0804	139.0703	147.0439	119.0491	138.0266		
Apigenin	47	7.73	271.0601	91.0545	153.0180	68.9976	119.0492	67.0546	146.0362
Formononetin	38	8.52	269.0793	197.0594	253.0491	226.0617	118.0413	237.0542	213.0907
Biochanin A	40	9.11	285.0755	213.0542	242.0567	269.0439	124.0154	118.0414	153.0181
Afromosin	38	8.60	299.0913	256.0726	227.0696	255.0647	138.0310	283.0600	184.0366
Maackian	38	8.44	285.0747	151.0388					

Table 3: Chickpea parental and RIL mapping population means for measured root traits following six days *P. medicaginis* inoculation.

	Control		Infected				
	Parental		Parental		Population		
	04067	Yorker	04067	Yorker	Mean	Range	Heritability H^2
Root weight (g)	1.16	1.83	1.05	1.29	1.15	0.76-1.48	0.644
Root length (mm)	250.2	286.3	195.5	236.9	207.7	157.9-257.6	0.564

Table 4: QTL associated with PRR root traits from chickpea RIL wild *Cicer echinospermum* BC and *C. arietinum* following six days *P. medicaginis* inoculation.

Trait	Chr.	Interval	Genetic map distance (cM)	Physical position (bp)	Size	P Value	% Var.	LOD
Root weight	C6	9024SNP6-434(C): 9024SNP6-45(C)	35.42-35.91	10,500,428-10,259,500	0.077	0.013	22.9	1.3383
Root length	C7	9024SNP7-72(C): 9024SD7-120	110.98- 111.32	377,293-3,653,166	-21.98	0.0000	53.4	4.0675

[a] Positive and negative values indicate that Yorker and 04067 alleles increased the phenotypic values in the 9024 RIL population, respectively. Physical position mapped to the chickpea Kabuli v1.0 reference genome; [b] indicates unplaced location of the scaffold marker estimates based on genetic map location.
n.d. – not detected.

Appendix 2 - Candidature Milestones

June 2017	Introduction presentation at the Annual ARC Industrial Transformation Research Hub - Legumes for Sustainable Agriculture (LSA; IH140100013) meeting
July 2017	Admitted into PhD program at The University of Adelaide
January 2018	Offered and Accepted a GRDC and NSW DPI Grains Industry Research Scholarship through the Grains Agronomy and Pathology Partnership (GAPP)
January 2018	Started PhD
March 2018	Annual GAPP report
April 2018	Completed University of Adelaide "Minor review"
April 2018	Contribute to LSA annual report
June 2018	Presentation at the LSA Hub meeting
October 2018	Completed "Core Component of the Structured Program"
October 2018	Poster presentation at the International Plant Phenotyping Symposium. Adelaide, Australia
February 2019	GRDC ground cover article
March 2019	Annual GAPP report
April 2019	Completed University of Adelaide "Minor review"
April 2019	Contribute to LSA annual report
June 2019	Presentation at the LSA Hub meeting
June 2019	Australian National University Lab presentation
July 2019	Change mode of study from part time to full time
July 2019	GAPP project update presentation
July 2019	NSW DPI Northern Grower Report
November 2019	Poster presentation at the Australasian Plant Pathology Society (APPS) conference. Melbourne, Australia
August 2019	Completed the "Major review of Progress for Doctoral Programs"
March 2020	Presentation at GRDC updates
March 2020	Annual GAPP report
April 2020	Contribute to LSA annual report
April 2020	Completed University of Adelaide "Minor review"
July 2020	NSW DPI Northern Grower Report
October 2020	Awarded best pathology presentation by APPS at the Adelaide university post-graduate symposium
April 2021	Completed "Pre-Submission Review" #1
April 2021	Completed University of Adelaide "Minor review"
April 2021	Contribute to LSA annual report
June 2021	GAPP project update presentation
October 2021	Published in Plant Health Progress
December 2021	Published in Agronomy Journal
January 2022	Adelaide University Biometry Hub Internship exit seminar.
March 2022	Presentation at GRDC updates
March 2022	Draft GAPP final technical report
April 2022	Completed University of Adelaide "Minor review"
May 2022	Completed "Pre-Submission Review" #2
May 2022	Submitted "Notification to Submit"
June 2022	GAPP final technical report submitted
July 2022	NSW DPI Northern Grower Report
August 2022	Oral and poster presentation Australasian soil-borne disease symposium
May 2023	Submitted thesis to Adelaide graduate centre for review
November 2023	Submitted final thesis