School of Molecular and Life Sciences

Characterisation and Genetic Dissection of *Sclerotinia sclerotiorum* Infection in Domesticated and Wild Chickpea

Virginia Wainaina Mwape
ORCID: 0000000274176944

This thesis is presented for the degree of
Doctor of Philosophy
of
Curtin University

October 4, 2021

THESIS DECLARATION

I, Virginia Wainaina Mwape, certify that,

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis does not contain any material, which has been accepted for the award of any other degree or diploma in any university.

No part of this work will, in future, be used in a submission in my name for any other degree or diploma in any university or additional tertiary education without the prior approval of Curtin University.

This thesis does not contain any material previously published or written by another person except where due references have been made in the text and the following Authorship Declaration.

The work(s) described in this thesis was generously funded through a Curtin University RTP scholarship and a top-up scholarship by The Commonwealth Scientific and Industrial Research Organization (CSIRO). Furthermore, it was supported by funds from the bilateral agreement between The Grains Research and Development Corporation (GRDC) and Curtin University, Perth, Australia, under grant CUR00023 and GRDC grant CUR00024.

This thesis contains work submitted for publication and work prepared for publication, including work(s), which have been co-authored. The work(s) are not in any way a violation or infringement of any copyright, trademark, patent or rights whatsoever of any person.

Signature:

Date:

"*Listen to advice and accept discipline, and at the end, you will be counted among the wise"* - Proverbs 19:20

ABSTRACT

The current crop production is not sufficient to feed the projected global population of 9.7 billion in 2050. Therefore, there is a demand for radical changes to food diversification by including plant based protein alternatives such as chickpea. Chickpea has a high protein content that accounts for almost 20% of its weight and potential health benefits such as reducing cardiovascular, diabetic and cancer risks. However, the chickpea industry is challenged by increasing incidences of fungal diseases, including sclerotinia stem rot (SSR) caused by the phytopathogen *Sclerotinia sclerotiorum* in some chickpea cropping areas. *S. sclerotiorum* has a wide host range and the ability to survive in the soil for a long time, making it a challenging disease to manage. Under conducive conditions, *S. sclerotiorum* can cause up to 100% yield loss in chickpea. Currently, there are no registered fungicides to control sclerotinia in chickpea in Australia and no known resistant cultivars. This thesis determines the level of resistance of current Australian chickpea cultivars and a collection of wild chickpea accessions. A recombinant inbred line population was employed, and major quantitative trait loci for stem lesion length were identified. Furthermore, RNA sequencing was employed over time to identify key factors sclerotinia uses in its arsenal to promote disease on a partially resistant and highly susceptible cultivar. Similarly, this data was analysed on the plant side of the interaction to identify differential responses in resistant and susceptible plants. Together, this thesis provides an insight into the chickpea-sclerotinia interaction and resources to improve the resistance to SSR in future chickpea cultivars.

The wild progenitors are much more diverse than domesticated chickpea; the second chapter describes how this relates to *S. sclerotiorum* resistance. Initially, the pathogenicity of nine Australian *S. sclerotiorum* isolates were examined and highly aggressive and moderately aggressive *S. sclerotiorum* isolates were identified. A screening assay was conducted using highly aggressive isolate isolates to evaluate 86 wild *Cicer* accessions (*Cicer reticulatum* and *echinospermum*) and two *C. arietinum* cultivars (PBA HatTrick and Kyabra) for resistance to *S. sclerotiorum*. Wild *Cicer* accessions Karab_084 and Deste_063 demonstrated consistent partial resistance to *S. sclerotiorum* compared to PBA HatTrick and Kyabra. Furthermore, there were significant differences in responses to *S. sclerotiorum* across wild *Cicer* collection sites, highlighting the importance of considering collection sites in the future search for *S. sclerotiorum* resistance. This study demonstrates the first evidence of partial stem resistance

to *S. sclerotiorum* identified in wild *Cicer* germplasm, which can be adopted in chickpea breeding programs to enhance SSR resistance in future chickpea cultivars.

The third chapter assesses the susceptibility levels of Australian chickpea lines, and it demonstrated that PBA HatTrick is moderately resistant and Kyabra is highly susceptible to *S. sclerotiorum*. Two hundred $F_{6:7}$ recombinant inbred lines (RILs) derived from a cross of PBA HatTrick, and Kyabra were phenotyped and genotyped following a stem inoculation method for SSR resistance to identify genomic regions responsible for SSR resistance. Six QTLs on chromosomes 1 (qSSR-1), 4 (qSSR4-1, qSSR4-2, qSSR4-3) and 7 (qSSR7-1, qSSR7-2), which individually accounted for 3.3 - 14.2% of the total estimated phenotypic variation for the response to SSR inoculation, were identified. After proper validation, these QTLs can be used for marker-assisted breeding for SSR resistance in chickpea.

In chapter four, a time-course transcriptional analysis of *S. sclerotiorum* gene expression during chickpea infection was conducted. The results showed 9,491 and 10,487 *S. sclerotiorum* genes were expressed in PBA HatTrick and Kyabra, respectively, relative to *in vitro*. Analysis of the upregulated genes revealed the induction of genes encoding carbohydrate-active enzymes, secondary metabolites, and secreted effectors, which are important during pathogen colonisation of the host. These findings provide the framework for a better understanding of *S. sclerotiorum* interaction with chickpea hosts of varying susceptibility levels. Here, we report for the first time on the *S. sclerotiorum* transcriptome during chickpea infection, which could be important for further studies on the molecular biology of this pathogen.

Chapter five also used the RNA-seq approach to decipher the molecular mechanisms governing partial resistance to *S. sclerotiorum* in chickpea. We observed that phenylpropanoid pathway intermediate enzymes such as alcohol hydrogenase, isoflovanol-2-hydrogenase, chalcone synthase and cinnamoyl-CoA reductase were expressed earlier in PBA HatTrick compared to Kyabra. Similarly, early upregulation of enzymes involved in immunity, cell wall resistance and reactive oxygen species scavengers was observed at the early infection stage in PBA HatTrick compared to Kyabra. Overall, these results suggests that PBA HatTrick resistance to *S. sclerotiorum* in chickpea coincides with an early response to the pathogen and basal expression of resistance genes. In contrast, Kyabra suffered massive infection due to lagging response and repressed signal transduction. This study provides a rich resource for functional characterisation of the genes involved in resistance mechanism and their use in chickpea breeding.

TABLE OF CONTENT

ACKNOWLEDGEMENTS

First, I am very grateful to God, who gave me abundant life and preserved me to see this reality. The undertaking of this PhD has been both challenging and rewarding. This PhD has taught me the power of persistence and the importance of collaboration that underpin all scientific endeavours. I could not have completed this PhD without the support of my collaborators, supervisors, colleagues, friends and loved ones.

My sincere gratitude goes to my lead supervisor Dr Lars Kamphuis, for his endless all-around support, belief in me and professional guidance in all aspects throughout my studies. Apart from his wealth of experience brought to bear, Dr Kamphuis has been of great inspiration to me, and it is heartening that his advice, suggestions, and motivations were helpful. I would also like to thank Dr Fran Ruiz Lopez, who, together with Dr Kamphuis, worked hard to secure a scholarship and extended their kindness by supporting my health insurance to make sure I enrolled for my PhD studies. God bless you.

I want to extend my thanks to my co-supervisors, Dr Toby Newman and Dr Mark Derbyshire, whose support was much appreciated. Your guidance and approach to good science have been invaluable and imparted me with good knowledge and skill to build my career. I would also like to thank Dr Mathew Denton-Giles, who welcomed me and played a significant role at the beginning of my PhD. I have also been very fortunate to work with several wonderful people at the Centre for Crop Disease Management (CCDM), Curtin University; I thank each one of you. I especially enjoyed the moment spent with my other PhD mates Roshan, Evan, and Carolyn. My great appreciation also goes to Yuphin Khentry, who was an excellent technical advisor. Her guidance was invaluable, and working alongside her was thoroughly enjoyable.

Special thanks to Curtin University for the funding received through the Curtin Postgraduate Research Scholarship (CIPRS) and Research Stipend Scholarship and The Commonwealth Scientific and Industrial Research Organization (CSIRO), who provided a top-up scholarship additional operating funds to facilitate my studies. My sincere gratitude also goes to The Grains Research and Development Corporation (GRDC) for the funding under grants CUR00023 and CUR00024, which enabled me to pursue my research to completion. Lastly, I am grateful to the Australasian Plant Pathology Society (APPS) Conference Bursary Award that allowed me to present my research, meet, visit, and learn from other researchers.

My sincere gratitude goes to my wonderful siblings and friends. Your support and belief in me have been so essential to help me keep pushing forward. I especially thank my friends (i) Dr Fredrick Maati and his wife, Dr Margaret Maati; you came into my life when I needed a shoulder in my academic journey. You encouraged, shared, uplifted and cheered me to the end; God bless you; (ii) to my wonderful siblings Thomas, Nancy, Philomena, Chris, Peter and Florence, you all are many miles away in Africa, but the call and texts, the fun you made during the video calls and the pride you carried with the sweet nickname 'Dr Sister' helped me to focus on making you all proud of your little sister; (iii) my amazing mum, Christine. Mum, thank you for believing in the value of education, your continuous spiritual support; you were my strong pillar, especially during the challenging moment. I would also like to thank my stepchildren Violet and Andrew for all their concerns and encouragement.

My deepest appreciation goes to my wonderful husband and special friend, Henry Mwape, his understanding, patience, and support during this period are invaluable. Henry was always there to lift my spirits when things got tough; you helped me to celebrate the high with your light-hearted humour and even occasionally took trips with me over the weekends to water my research plants, so we can spend more time together during your work breaks. To my daughter, Mitchell (17-years-old) took up the many of 'Mum' roles when I was away at school and never disappointed. To my little girl, Maisie (13-year-old), you brought joy and laughter even at my lowest moments that sustained me during the struggle. You two witnessed when I set my first experiment for my PhD at the greenhouse, and you said, "Wow! The sprinklers are beautiful", you had no idea of the tough times ahead of all of us. You sprinkled faith and reassurance whenever the going was tough and made my PhD journey beautiful. These daughters of Zion are two special people that I will forever remain grateful to God for bringing my way.

AUTHORSHIP DECLARATION

This thesis is presented as a thesis-by-publication, containing two papers published in peer-reviewed scientific journals and two additional manuscripts prepared for publication in due course. The bibliography details of these works and declarations of my contributions and those from my co-authors are outlined below.

Details of work:

Mwape VW, Khentry Y, Newman TE, Denton-Giles M, Derbyshire M, Chen K, Berger J, Kamphuis L (2021a) **Identification of sources of *Sclerotinia sclerotiorum* resistance in a collection of wild *Cicer* germplasm**. *Plant Disease*. doi:10.1094/PDIS-02-21-0367-RE.

Location in thesis:

This research paper is included as **CHAPTER 2**

Student contributions:

- Conceived the design of the study with MD, MCD, and LGK
- Designing the experiment with the help of KC
- Conducted all experiment and collecting data with the assistance of KY
- Data analysis with assistance from KC, JDB and TEN
- Lead writer of the manuscript, with comments and suggestions from co-authors.

Co-author signatures:

Signed by LGK, the coordinating, lead supervisor and corresponding author for the research paper on behalf of all co-authors

Details of work:

Manuscript prepared for publication in due course

Mwape VW, Khoo KHP, Kefei C, Khentry Y, Newman TE, Derbyshire MC, Mather DE, Kamphuis LG. (Submitted) **Identification of Sclerotinia stem rot resistance quantitative trait loci in a chickpea recombinant inbred line population.** *Functional Plant Biology***.**

Location in thesis:

This research paper is included as **CHAPTER 3**

Student contributions:

- Conceived the design of the study with TEN, LGK and MCD
- Designed the phenotyping experiment with assistance from KC
- Conducted the phenotyping experiment using materials provided by KK and DEM
- Assisted KC with QTL analysis using genotypic data and a linkage map provided by KK and DEM
- Identified the predicted genes in QTL regions.
- Lead writer of the manuscript, with comments and suggestions from all co-authors

Co-author signatures:

Signed by LGK and DEM, the coordinating project leaders for the research paper on behalf of all co-authors.

Lars Kamphuis: Date:

Diane Mather: Date:

Details of work:

Mwape VW, Mobegi FM, Regmi R, Newman TE, Kamphuis LG, Derbyshire MC (2021b) **Analysis of differentially expressed *Sclerotinia sclerotiorum* genes during the interaction with moderately resistant and highly susceptible chickpea lines**. *BMC Genomics* **22**, 333. doi:10.1186/s12864-021-07655-6"

Location in thesis:

This research paper is included as **CHAPTER 4**

Student contributions:

- Conceived the design of the study with TEN, LGK and MCD,
- Conducted RNAseq experiment and collecting data
- Analysed RNAseq data with assistance from FMM and MCD
- Conducted qPCR experiment and analysed data with assistance from RR
- Lead writer of the manuscript, with comments and suggestions from co-authors.

Co-author signatures:

Signed by LGK, the coordinating, lead supervisor and corresponding author for the research paper on behalf of all co-authors.

Details of work:
Manuscript prepared for publication in due course

Mwape VW, Mobegi FM, Newman TE, Derbyshire MC, Kamphuis LG (2021) **A transcriptome analysis uncovers molecular mechanisms of resistance to *Sclerotinia sclerotiorum* in chickpea.**

Location in thesis:
This research paper is included as **CHAPTER 5**
Student contributions:

- Conceived the design of the study with TEN, LGK and MCD,
- Conducted RNAseq experiment and collecting data
- Analysed RNAseq data with assistance from FMM and MCD
- Conducted qPCR experiment and analysed data
- Lead writer of the manuscript, with comments and suggestions from co-authors.

Co-author signatures:
Signed by LGK, the coordinating lead supervisor and corresponding author for the research paper on behalf of all co-authors

I, **Lars G. Kamphuis**, certify that the student's statements regarding their contribution to each of the work listed above are correct. I hereby authorise the inclusion of the co-authored work in the thesis.

Coordinating/Lead supervisor signature **Student signature**

Date: **Date:**

ABBREVIATIONS

CAZymes: Carbohydrate Active Enzymes
CCDM: Centre For Crop Disease and Management
CWDE: Cell Wall Degrading Enzymes
CYP450: Cytochrome 450
DEG: Differentially expressed genes
DNA: Deoxyribonucleic acid
ETI: Effector triggered immunity
FDR: False discovery rate
GO: Gene ontology
HPI: Hours post-inoculation
ICIM: Inclusive composite interval mapping
LG: Linkage group
LOD: Threshold log-of-odds
LogFC: Log_2 fold change
MAS: Marker-Assisted Selection
NCBI: National Centre for Biotechnology Information
NGS: Next Generation Sequencing
OA: Oxalic acid
PAMP: Pathogen associated molecular pattern
PCA: Principal component analysis
PCD: Programmed cell death
PDA: Potato dextrose agar
PKS: Polyketide synthase
PVE: Phenotypic variance explained
QTL: Quantative Trait Locus
RIL: Recombinant Inbred Line
RNA: Ribonucleic acid
ROS: Reactive oxygen species
SSR: Sclerotinia stem rot

CHAPTER 1

General Introduction

1.1. Background

Approximately 821 million people in the world population suffer from hunger and live without secure food sources due to the effects of global warming, urbanisation and loss of crop diversity (FAO, 2019). Current food production is not adequate to feed the global population, which is predicted to reach over nine billion by 2050. Therefore, radical changes related to food diversification, including other unexploited plant-based foods such as pulses are important (Berners-Lee *et al.* 2018).

Pulses are known for their beneficial nutritional profiles for the human diet and their role in nitrogen fixation; however, they are underexploited in cultivation and consumption and inadequately researched in areas concerning biotic and abiotic stresses (Robinson *et al.* 2019; Foyer *et al.* 2016). Despite their general niche status globally, legume crops remain staple foods along with cereals, especially in Africa and South Asia (Foyer *et al.* 2016). Considering the key roles pulse crops play in the human diet and soil nutrition, advancing studies on these crops will significantly impact food security. Pulse crops favourable for human consumption include butter beans, haricot beans, red kidney beans, black-eyed beans, soyabeans, peas, lentil, lupins and chickpea (Foyer *et al.* 2016).

Chickpea (*Cicer arietinum* L.) is a grain pulse crop popular for the human diet due to its multiple uses (Siddique *et al.* 2000). Chickpea production is third after beans and peas, with over 10 million tonnes produced annually in the world (Merga and Haji 2019). Chickpea is exceptionally high in protein content (20% of its weight) and has potential health advantages such as reduced cancer, diabetic and heart-related ailments (Jukanti *et al.* 2012). Besides human nutrition, chickpea crops fix soil nitrogen through symbiotic nitrogen fixation (Flowers *et al.* 2010). However, limited research efforts compared to cereal grains and increasing disease related challenges affect commercial chickpea production. Therefore, it is critical to consider new disease management strategies, such as developing chickpea genotypes resistant to diseases for future sustainable food security.

Wild crop relatives offer diverse favourable traits that include resistance to disease (Zhang *et al.* 2016). A review by Maxted *et al.* (2007) found that over 60% of previous studies on wild

relatives of pulse crops such as soybeans, faba beans, common beans and chickpeas reported their significance as a source of resistance to abiotic and biotic stresses. The most important biotic stresses that challenge the chickpea industry are Ascochyta blight and Fusarium wilt. Other diseases that cabn cause significant yield losses in the chickpea industry include Phytophthora root rot , root-lesion nematodes, Botrytis grey mould, Powdery mildew, Downy mildew, Pythium root rot and Sclerotinia stem rot (SSR) (Collard *et al*. 2003; Kukreja *et al.* 2018; Pulse Australia, 2020), the latter being the focus of the current research project.

1.2. History and origin of chickpea

Chickpea was domesticated with other crops including wheat, pea, barley, rye, and lentil as part of the evolution of agriculture in the Fertile Crescent 12,000-10,000 years ago (Bar-Yosef 1998). The distribution of old races and wild relatives of chickpea occurred in three main regions: i) Central Asia, Afghanistan and the Himalayan region (ii) the western Mediterranean, Ethiopia, Crete and Greece; and (iii) Asia-minor, Iran and the Caucasus (Singh 1997). The two types of domesticated chickpeas are small angular seeds referred to as Desi and larger and rounder seeds referred to as Kabuli (Thudi *et al.* 2016; Ahmad *et al.* 2005). Domestication of Desi type started in the Mediterranean and spread to southern Europe, northern and eastern Africa (Knights and Hobson 2016). Domestication of the Kabuli type in India started over 4,000 years ago (Abbo *et al.* 2003). However, the commencement of chickpea production in the United States, Canada, and Australia was as recent as the 1950s, 1990s and 1970s, respectively (Thudi *et al.* 2016).

1.3. Global production and importance of chickpea

The global area under chickpea cultivation is approximately 14 million hectares, and chickpea has a mean production of over 10 million tonnes annually (Fig.1) (Merga and Haji 2019; FAO, 2019). India leads the world in chickpea production (65%), followed by Australia, North America, Africa, Europe and Asia, contributing 35% of world production (FAOSTAT, 2019). In Asia, yield increased by over 10%, from 700 kg/ha in 1996 to 800 kg/ha in 2019 (FAOSTAT, 2019).

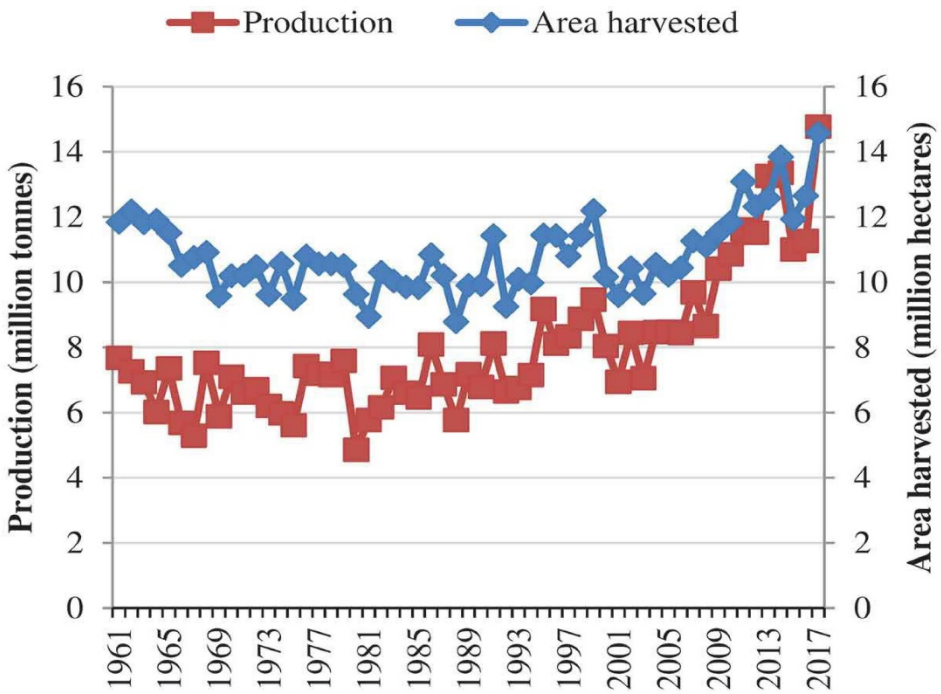


Figure 1.1: Average chickpea production worldwide from 1961 to 2017. The blue line represents the area harvested (million hectares), and the red line represents production (million tons). Illustration adapted from Merga and Haji (2019).

Chickpea is a key player in food security by bridging the gap in the protein level of daily food rations in developing countries (Merga and Haji 2019). Chickpea offers a source of economical and affordable carbohydrates and proteins without compromising nutritional value (Malunga *et al.* 2014). To improve food security opportunities in the major growing areas, both reducing chickpea production risks and increasing production levels are critical. In view of the above, developing chickpea cultivars with improved resistance to devastating diseases such as Ascochyta blight, Fusarium wilt, Root lesion nematodes, and SSR is critical in achieving world food security. Previous research shows that domesticated chickpea genotypes have low genetic diversity, encouraging the search for disease resistance alleles from wild relatives (Abbo *et al.* 2003).

1.4. Wild *Cicer* germplasm gene pool

Wild *Cicer* species are key sources of alleles that may contribute to resistance to diseases (Muehlbauer *et al.* 2006). The wild ancestors of other crops such as lentil, peas, wheat and barley, have a wide distribution geographically, from the eastern Mediterranean to Asia (Abbo

et al. 2003). In a *Cicer* monograph, Van der Maesen (1972) identified 39 *Cicer* species including 31 perennials and eight annuals. Among the *Cicer* species, *C. arietinum* is the sole species under domestication and grown worldwide. The wild *Cicer* had a narrow geographical range, with the *C. reticulatum*, which is the immediate progenitor of chickpea, geographically restricted to the southern province of Turkey (von Wettberg *et al.* 2018). The region had significant differences in elevation and soil types despite the narrow geographical range, which could drive distinct local adaptations (von Wettberg *et al.* 2018). In addition, the wild *Cicer* are not only limited when compared to other crops but the species are endangered due to overgrazing and habitat change in their natural environments driven by climate change (von Wettberg *et al.* 2018). The Grains Research and Development Corporation (GRDC) supported a group of researchers from the USA, Turkey and Australia to collect wild chickpea accessions from the chickpea centre of origin in Turkey (Table 1.1). This collection effort significantly increased the total number of wild *Cicer species* in the world collection which prior to 2013 had 76 wild relatives and now contains 1,209 accessions (Table 1.1). This allowed scientists worldwide to assess this germplasm for desirable characteristics of relevance to their production regions (von Wettberg *et al.* 2018).

Table 1.1: The number of wild chickpea accessions in Australian Grain Genebank before and after collection trips in Turkey between 2013 and 2016 has significantly increased the number of wild accessions.

Species	Prior to 2013	2013 -16 Collection
Cicer bijugum	20	85
Cicer pinnatifidum	28	253
Cicer echinospermum	10	282
Cicer reticulatum	18	589

The current wild *Cicer* species collection provides opportunities to mine potential alleles for introgression into breeding programs. The number of species in the genus *Cicer* was recognized as 45 species with nine annuals and 36 perennials. Importantly, only two of the annual wild *Cicer* species (*C. reticulatum* and *C. echinospermum* P.H Davis) are in the primary and secondary gene pools of cultivated chickpea and are readily inter-fertile with *C. arietinum* (von Wettberg *et al.* 2018). A genetic diversity study reported a successful hybridisation of *C. arietinum* and the wild species *C. reticulatum* and *C. echinospermum* (Croser *et al.* 2003).

Alleles that confer resistance to Phytophthora root rot (Knights *et al.* 2008), Pod borer (Golla *et al.* 2020), Ascochyta blight (Collard *et al.* 2003; Newman *et al.* 2020), Root-lesion nematode (Reen *et al.* 2019), and chilling tolerance (Berger *et al.* 2005) have been identified in the current world *Cicer* collection. Hence, this collection offers the potential for resistance against other chickpea diseases such as SSR to be investigated. Despite potential undesirable agronomic traits (Muñoz *et al.* 2017), genetic diversity and exceptional opportunity to incorporate novel alleles controlling important characteristics has encouraged research on these wild species.

With SSR, which can cause up to 100 % yield losses in chickpea, becoming an emerging disease problem in Australia, the wild chickpea germplasm is an important resource that can be utilised to screen for resistance. (Pulse Australia, 2020). If resistance or partial resistance is found, breeders can introgress the alleles conferring resistance into domesticated chickpea. Moreover, the value of wild species in resistance breeding is important to maintain high productivity, particularly in crops where genetic diversity to the pathogen such as *S. sclerotiorum* is low (Kameswara Rao *et al.* 2003; (Fuhlbohm *et al.* 2003; Njambere *et al.* 2008; Chen *et al.* 2006).

1.5. *Sclerotinia sclerotiorum*

Sclerotinia Sclerotiorum (Lib.), the causal agent of SSR, is a necrotrophic cosmopolitan plant pathogen that infects at least 408 species, including oilseeds, weeds, grasses and pulses (Boland and Hall 1994; Bolton *et al.* 2006). SSR in chickpea is an important disease worldwide and can infect stem, leaves and pods at different developmental stages of the host (Huzar-Novakowiski *et al.* 2017). SSR is an important disease in the USA (Chen *et al.* 2006), Canada (Hilton 2000), India (Haware 1990) and Australia (Bretag and Mebalds 1987). While chemical control has been used to control SSR in canola (Derbyshire and Denton-Giles 2016), there are no registered fungicides to control SSR in Australia (Pulse Australia 2020). Therefore exploring the genetic basis of resistance to *S. sclerotiorum* is novel research that will impact breeding programs focusing on durable, resistant germplasm for environmental and economic benefits.

The source of inoculum for *S. sclerotiorum* is sclerotia, which are hard, black fungal structures that are critical to the long-term survival and sexual reproduction of the pathogen (Lane *et al.* 2019; Brooks *et al.* 2018). Sclerotia can proliferate carpogenically by producing apothecia from

which ascospores are liberated and released to colonise host petals or myceliogenically by the growth of hyphae that infect directly from the base of the plant (Fig. 1.2) (Lane *et al.* 2019). The journey after infection through different plant tissues is important to study since *S. sclerotiorum* virulence and host defence requirements may shift in response to pathogen colonisation and host defence mechanisms.

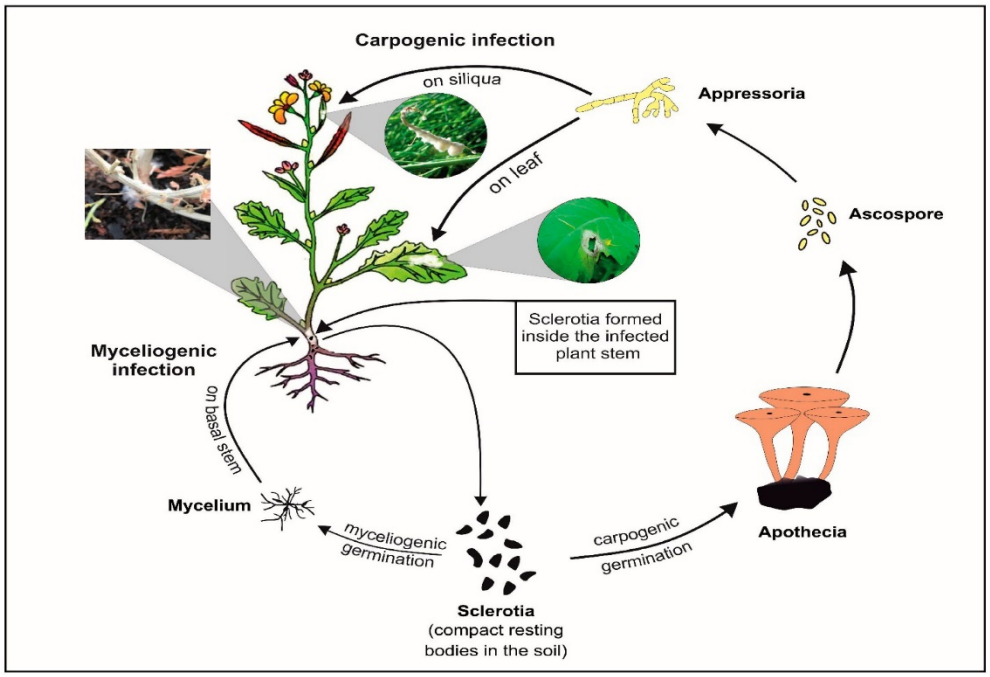


Figure 1.2: A schematic representation of the disease cycle of *Sclerotinia sclerotiorum*. Adapted from Singh *et al.* (2020).

1.5.1. *Sclerotinia sclerotiorum* pathogenesis

Dispersal of inoculum is higher under bushy canopies and during the early growth stage of the host (Bolton *et al.* 2006). For penetration, the host tissue must be moist. Microscopy studies on the mode of penetration of *S. sclerotiorum* showed that the initial attack of the host is by enzymatic degradation of the cuticle and direct penetration by mechanical pressure (Uloth *et al.* 2016). After penetration, the mycelium develops inter and intracellularly and concludes with oxalic acid (OA) production (Cessna *et al.* 2000). OA acidified the host environment, causing chelation of calcium ions and interference with the stomatal closure and starch hydrolysis (Williams *et al.* 2011). Besides OA, the activity of cell wall degrading enzymes, which are released initially, are also important during *S. sclerotiorum* pathogenesis (Williams

et al., 2011). Although mechanical pressure seems to be of major importance in *S. sclerotiorum* infection, colonisation by excretion of key enzymes is reported to play key roles during infection of canola (Oliveira *et al.* 2015; Seifbarghi *et al.* 2017; Chittem *et al.* 2020), soybean (Westrick *et al.* 2019) and chickpea (Mwape *et al.* 2021a). The maceration of the host tissues and the development of watery soft rot after *S. sclerotiorum* infection is caused by the excretion of pectolytic enzymes (Li *et al.* 2004). As the infection progresses, necrosis shows around the infection points and extends within the host tissues. After establishment inside the host, small hyphae appear through the stomata and form a cottony mass on the surface of the host, and small sclerotia are initiated on the mycelium (Willetts and Wong 1980). Research involving *Brasica napus* found that when the host is fully infected, wilting and lodging occur, and necrotic lesions extend on the stem of the host (Derbyshire and Denton-Giles 2016).

1.5.2. Screening for *Sclerotinia sclerotiorum* resistance

Knowledge of the range of aggressiveness within the local *S. sclerotiorum* population offers useful information for breeding and management (Willbur *et al.* 2017). Screening for resistance using weakly aggressive *S. sclerotiorum* isolates can increase the risk of false-positive phenotypes (Denton-Giles *et al.* 2018). Therefore, various isolates representing the overall diversity of *S. sclerotiorum* isolates present in the environment should be used for resistance screening assays. Various non-biological methods are used for diagnosis of *S. sclerotiorum* in the field, such as looking for a white cottony-looking growth that girdles the stem causing the plant wilt and die and small black fruiting bodies (Sclerotia) in the stem and soil. A widespread technique for screening crops for resistance to *S. sclerotiorum* comprises conducting pathogen-host biological assays. To identify host genetic resistance, several assays have been developed to screen for resistance against *S. sclerotiorum*. Cut petiole inoculation (Zhao *et al.* 2004), stem inoculation assays (Denton-Giles *et al.* 2018) and detached leaf inoculation (Li *et al.* 2006) have been employed in assessment for SSR resistance in *B. napus*. Similarly, assays have been developed to screen soybean, dry bean, and sunflower for SSR resistance (Vuong *et al.* 2004; Kim *et al.* 1999). Although previous studies have evaluated screening assays for various hosts, there is no single widely developed and evaluated assay for *S. sclerotiorum* resistance screening in chickpea.

Therefore, the development of a reliable and reproducible phenotyping assay is key in the search for resistance to *S. sclerotiorum* in chickpea germplasm. The current study focuses on

identifying an SSR resistance screening technique in chickpea that will allow dissection of the defence response in resistant and susceptible chickpea lines and identify critical pathways responsible for resistance to *S. sclerotiorum*. The lack of a reliable SSR screening assay and the absence of cultivars with complete or partial resistance to SSR is a limiting factor in breeding resistance to SSR in chickpea.

1.6. Management of *Sclerotinia sclerotiorum*

S. sclerotiorum causes the disease commonly referred to as white mould or sclerotinia collar rot or SSR (Bardin and Huang 2001). Generally, the fungus invades the host tissues, causes watery brown rot and growth of white mycelium over the infected tissues, in which sclerotia eventually form. A large number of sclerotia mount up in the plant stubble or the soil and can survive for over five years (Brooks *et al.* 2018). Germination of sclerotia into infective hyphae or apothecia under favourable conditions and availability of hosts initiates a new infection cycle (Fig.1.2). Management and elimination of SSR, unfortunately, has no single strategy that can be used. Some well-known control techniques include site selection, resistant plants and cultivars, controlling other biotic factors, sanitation, cultural practices, chemical and biological control (Derbyshire and Denton-Giles 2016). Therefore, a combination of management tools provides a long term, consistent management of disease while maintaining crop yield and quality.

1.6.1. Cultural practices

To adhere to sound cultural practices, understanding the basic biology of the pathogen and awareness of favourable conditions that are unconducive to the persistence of the pathogen and the disease are required. For instance, management of SSR in soybean involves clean seed, early planting, soil turnover, and adjustment of plant population to reduce the crop canopy (Mueller *et al.* 2002). This practice aims to reduce underground sclerotia and create non-conducive environs for the pathogen, which spends 90% of its life cycle as sclerotia in the soil (Adam and Ayers, 1979). Pulse growers reduce the amount of sclerotia within their field through crop rotation to disrupt the annual lifecycle of *S. sclerotiorum* and reduce the annual number of sclerotia in the soil. The growth of a host crop accelerates the build-up of the sclerotia in the soils for consecutive years without rotation (Brooks *et al.* 2018). One of the challenges of crop rotation is the ability of *S. sclerotiorum* to infect over 400 hosts, comprising

of common weeds such as shepherd's purse (*Capsella bursa-pastoris*), dandelion (*Taraxacum officinale*) and wild radish (*Raphanus raphanistrum*). Hence, an effective crop rotation strategy to control SSR must include an efficient weed control program to minimise 'green bridge' conditions that would allow *S. sclerotiorum* to persist in the fields (Brooks *et al.* 2018).

The benefit of soil tillage on the persistence of sclerotia in the soil is reducing apothecia production by burying the sclerotia (Kharbanda and Tewari 1996). Sclerotia are viable only in the top 2-3 cm of soil as apothecia cannot grow longer than 3 cm (Brooks *et al.* 2018). Burying sclerotia to a depth of up to 15 cm reduces carpogenic germination and apothecia production (Williams and Stelfox 1980). However, the sclerotia persistence is more remarkable when buried deep in the soil (Merriman *et al*. 1979) because tilling in successive years brings the sclerotia to the topsoil. Hence, this strategy is only successful when the sclerotia remain suppressed underground until they lose their viability.

1.6.2. Chemical and biological control

There are currently no consistent protocols to control SSR through fungicide applications, which is in part due to the variability in canopy coverage and timing of fungicide application following ascospore release (Grau *et al.* 1994). Constant use of concentrated chemicals may cause the pathogen to develop fungicide resistance and negatively impact the environment and non-target species (Mueller *et al.* 2002; Hawthorne and Jarvis 1973). In Brazil, research showed that Procymidone and Fluazinam reduced the production of ascospore/apothecium when sprayed at the onset of flowering in soybean (Sumida *et al.* 2015). Further research is recommended on the testing of unregistered fungicides in Australia to control SSR in chickpea.

The use of microbial agents to control pathogens can harm the environment and economical for integrated management strategies (Mao *et al.* 1997). The mechanisms of biocontrol are classified as antibiosis, parasitism or predation, and competition (Fravel 1988). The strategy is an environmentally acceptable and ecologically viable approach, compatible with many disease management models for integrated disease management (Smolińska and Kowalska 2018). For the past two decades, there has been intensive research on finding biological control agents (BCA) for *S. sclerotiorum*, with only a small number registered to date (Zeng *et al.* 2012). Currently, no registered labels for fungicide treatments or biocontrol options to control SSR in chickpea in Australia exist.

1.6.3. Genetic resistance

Host resistance is regarded as one of the most successful strategies for plant disease management. Although complete host resistance to *S. sclerotiorum* has not been reported, partial resistance has been identified in canola (Khot *et al.* 2011; Denton-Giles *et al.* 2018; Taylor *et al.* 2015), soybean (Vuong *et al.* 2004; Kim *et al.* 1999; Kull *et al.* 2003) and chickpea (Mwape *et al.* 2021a). Research has demonstrated that *S. sclerotiorum* pathogenesis is very complex and involves many secreted toxins, including oxalic acid, cell wall degrading enzymes and secreted effectors that cause successful host infection (Lyu *et al.* 2016; Wei *et al.* 2016).

To identify host resistance genes, quantitative trait loci (QTL) analysis is conducted to find the positions of alleles correlated to resistance to *S. sclerotiorum*. For instance, in soybean, 103 QTLs for SSR resistance are reported in the soybean databank (www.soybase.org). Kim and Diers (2000) identified three QTLs underlying partial resistance in soybean cultivar S19-90. Further research identified 28 QTLs in four soybean cultivars, some of which were associated with physiological traits such as flowering time, internode length and lodging, which could be disease escape mechanisms and not physiological resistance. Although several authors have identified partial soybean resistance, there are currently no reports on complete SSR resistance. It is considered that physiological resistance and escape mechanism causes resistance differences in the response of hosts such as soybean to *S. sclerotiorum* (Kim *et al.* 1999). However, unlike soybean, there has been no research conducted focusing on identifying the genetic resistance of chickpea against *S. sclerotiorum*.

Considerable efforts to map genetic loci contributing to SSR resistance in *B. napus* have been made. A mapping population derived from crosses between a partially resistant and a susceptible parent identified resistance in seedling and mature plant stages in *B. napus* (Zhao and Meng 2003; Wu *et al.* 2013). Molecular analysis has also been used to find the host molecular mechanisms associated with disease resistance phenotype. Genes with presumed roles in disease resistance, such as two heat shock proteins, glycine-rich protein, a trypsin inhibitor protein, and a thio-methyltransferase, have been identified and associated with *B. napus* resistance against *S. sclerotiorum* (Wen *et al.* 2013). Similarly, proteins with a putative role in key metabolic pathways such as protein folding, ethylene biosynthesis, antioxidant defence, pathogenesis, protein synthesis, and anthocyanin biosynthesis have also been identified (Garg *et al.* 2013; Zhao *et al.* 2015).

The absence of complete genetic resistance to SSR in crop hosts has prompted the search for genetic resistance within wild crop relatives, intending to introduce this resistance across the species. A high level of resistance against *S. sclerotiorum* was reported in introgression lines derived from hybridisation between wild crucifers and *Brassica* species (Garg *et al.* 2010). Screening of the F_2 population derived from hybridisation of *B. napus* and with *B. oleracea* identified two major QTLs in wild *Brassica* species that highly influenced resistance to SSR compared to cultivated genotypes (Taylor *et al.* 2018). Identification of resistance in wild relatives of SSR hosts will provide novel genetic resources that can be used in future breeding programmes to enhance resistance against SSR.

1.7. Research hypotheses and objectives

Considering the present difficulties in predicting SSR outbreaks and lack of complete resistance in previously studied SSR hosts, it is evident that only a more comprehensive application of phenotypic and genotypic strategies can contribute to substantial advancements in developing commercial chickpea cultivars having an adequate level of resistance against *S. sclerotiorum*. This task will be most rapidly and efficiently achieved with the assistance of explicitly expanding our current understanding of the resistance level of the popular domesticated cultivars and screening wild chickpea relatives for resistance alleles. This knowledge will better inform breeders how best to incorporate those alleles and have academic benefit by understanding the molecular pathways involved during the *S. sclerotiorum* - chickpea interaction.

This thesis tested the following hypotheses:

1) Wild chickpea germplasm will show a diverse range of responses to *S. sclerotiorum* infection compared to domesticated cultivars (PBA HatTrick and Kyabra).
2) Genotyping and phenotyping for *S. sclerotiorum* infection responses of a recombinant inbred line population generated from PBA HatTrick and Kyabra will identify loci associated with resistance to *S. sclerotiorum*.
3) Analysis of S. sclerotiorum transcriptomes will show use of different strategies during the infection of resisatn and susceptible cultivars (PBA HatTrick and Kyabra) by the pathogen.
4) Analysis of the chickpea transcriptomes will show that the two cultivars PBA HatTrick and Kyabra differ in their responses to *S. sclerotiorum* infection.

This thesis' overall objectives were to explore and identify resistance to *S. sclerotiorum* in wild chickpea germplasm and establish the level of susceptibility of two popular Australian chickpea cultivars. To meet the objectives, the following aims were set:

1) Develop a rapid, consistent and reliable chickpea screening technique for *S. sclerotiorum* resistance (Chapter 2).
2) Screen a wild chickpea germplasm collection (n= 86) to identify potential sources of *S. sclerotiorum* resistance (Chapter 2).
3) Initiate development of a segregating recombinant inbred line (RIL) population for resistance to *S. sclerotiorum* using a parent with identified resistance from the wild accessions (Chapter 2).
4) Phenotype an F_7 RIL population derived from a cross between the elite cultivars PBA HatTrick and Kyabra for their response to *S. sclerotiorum* infection and identify QTLs associated with *S. sclerotiorum* resistance (Chapter 3). QTL analysis was based on genotyping work conducted by Khoo et al., (submitted).
5) Study global *S. sclerotiorum* transcriptional changes during chickpea infection of a moderately resistant (PBA HatTrick) and a susceptible (Kyabra) chickpea cultivar (Chapter 4).
6) Analyse chickpea differential gene expression of a moderately resistant (PBA HatTrick) and a susceptible (Kyabra) chickpea cultivar following *S. sclerotiorum* infection and identify pathways that can contribute to the observed resistance to *S. sclerotiorum* in the moderately resistant cultivar (Chapter 5).

1.8. Thesis outline and structure

This thesis is prepared following the higher degree by research (HDR) thesis examination procedures of Curtin University, under Section 1.4 of the guidelines, and presented as a series of scientific papers, including two accepted for publication and two in preparation for submission to journals in due course. Each chapter (i.e. chapter 2, 3, 4, and 5) is self-contained, with an abstract, introduction, methods, results, discussion, conclusion, chapter references and supplementary materials. Parts of the methods sections in Chapter 4 and Chapter 5 are relatively similar, considering a single set of RNA sequencing data was generated and used for molecular studies on *S. sclerotiorum* infection mechanisms (Chapter 4) and chickpea mechanisms response to *S. sclerotiorum* (Chapter 5).

Chapter 1 introduces chickpea as a pulse crop within the context of taxonomy and economic relevance. It also describes SSR and the causal pathogen *S. sclerotiorum* lifecycle, host range, disease epidemiology and current management strategies and future opportunities in the management of SSR.

Chapter 2 describes the development of a robust pathology assay to determine the response of *Cicer* species to *S. sclerotiorum* infection. It also identifies *S. sclerotiorum* isolates with distinct levels of aggressiveness following inoculation of *Cicer* germplasm. It identifies wild *Cicer* accessions with higher levels of partial resistance than two current popular chickpea cultivars (PBA HatTrick and Kyabra) (Mwape *et al.* 2021b).

Chapter 3 describes the genetic dissection of SSR resistance in a segregating chickpea population derived from Australian cultivars PBA HatTrick and Kyabra and reports the identified QTLs and genes underlying those QTL regions.

Chapter 4 covers the transcription analysis of *S. sclerotiorum* gene expression during infection of two chickpea cultivars (PBA HatTrick and Kyabra). This chapter details the gene expression patterns, their roles and significance during *S. sclerotiorum*- chickpea interaction (Mwape *et al.* 2021b).

Chapter 5 includes detailed accounts of host defence-related genes identified in moderately resistant and susceptible *C. arietinum* genotypes through RNAseq analysis.

Chapter 6 summarises the conducted research and highlights the importance of the significant findings of each chapter, further research opportunities identified out of these studies, with further discussions of implications and benefits to both the scientific community and industry.

1.9. References

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

Abbo S, Berger J, Turner NC (2003) Viewpoint: Evolution of cultivated chickpea: four bottlenecks limit diversity and constrain adaptation. *Functional Plant Biology* **30**, 1081–1087.

Ahmad F, Gaur PM, Croser J (2005) Chickpea (*Cicer arietinum* L.). *Genetic Resources, Chromosome Engineering, and Crop Improvement* 187–217.

Bardin SD, Huang HC (2001) Research on biology and control of Sclerotinia diseases in Canada1. *Canadian Journal of Plant Pathology* **23**, 88–98.

Bar-Yosef O (1998) The Natufian culture in the Levant, threshold to the origins of agriculture. *Evolutionary Anthropology: Issues, News, and Reviews* **6**, 159–177.

Berger JD, Buck R, Henzell JM, Turner NC (2005) Evolution in the genus *Cicer*—vernalisation response and low temperature pod set in chickpea (*C. arietinum* L.) and its annual wild relatives. *Australian Journal of Agricultural Research* **56**, 1191–1200.

Berners-Lee M, Kennelly C, Watson R, Hewitt CN (2018) Current global food production is sufficient to meet human nutritional needs in 2050 provided there is radical societal adaptation. *Elem Sci Anth* **6**, 52.

Boland GJ, Hall R (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology* **16**, 93–108.

Bolton MD, Thomma BPHJ, Nelson BD (2006) *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. *Molecular Plant Pathology* **7**, 1–16.

Bretag TW, Mebalds MI (1987) Pathogenicity of fungi isolated from *Cicer arietinum* (chickpea) grown in north-western Victoria. *Australian Journal of Experimental Agriculture* **27**, 141–148.

Brooks KD, Bennett SJ, Hodgson LM, Ashworth MB (2018) Narrow windrow burning canola (*Brassica napus* L.) residue for *Sclerotinia sclerotiorum* (Lib.) de Bary sclerotia destruction. *Pest Management Science* **74**, 2594–2600.

Cessna SG, Sears VE, Dickman MB, Low PS (2000) Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *The Plant Cell* **12**, 2191–2200.

Chen W, Schatz B, Henson B, McPhee KE, Muehlbauer FJ (2006) First Report of Sclerotinia Stem Rot of Chickpea Caused by *Sclerotinia sclerotiorum* in North Dakota and Washington. *Plant Disease* **90**, 114–114.

Chittem K, Yajima WR, Goswami RS, del Río Mendoza LE (2020) Transcriptome analysis of the plant pathogen *Sclerotinia sclerotiorum* interaction with resistant and susceptible canola (*Brassica napus*) lines. *PLoS ONE* **15**,e.0229844.

Collard BCY, Pang ECK, Taylor PWJ (2003) Selection of wild *Cicer* accessions for the generation of mapping populations segregating for resistance to ascochyta blight. *Euphytica* **130**, 1–9.

Croser J, Ahmad F, Clarke H, Siddique K (2003) Utilisation of wild *Cicer* in chickpea improvement - progress, constraints, and prospects. *Australian Journal of Agricultural Research* **54**, 429–444.

Denton-Giles M, Derbyshire MC, Khentry Y, Buchwaldt L, Kamphuis LG (2018) Partial stem resistance in *Brassica napus* to highly aggressive and genetically diverse *Sclerotinia sclerotiorum* isolates from Australia. *Canadian Journal of Plant Pathology* **40**, 551–561.

Derbyshire MC, Denton-Giles M (2016) The control of sclerotinia stem rot on oilseed rape (*Brassica napus)*: current practices and future opportunities. *Plant Pathology* **65**, 859–877.

FAOSTAT, F. 2019 Statistics division of Food and Agriculture Organisation of the United 653 Nations. http://www.fao.org/statistics/en/

Flowers TJ, Gaur PM, Gowda CLL, Krishnamurthy L, Samineni S, Siddique KHM, Turner NC, Vadez V, Varshney RK, Colmer TD (2010) Salt sensitivity in chickpea. *Plant, Cell & Environment* **33**, 490–509.

Foyer CH, Lam H-M, Nguyen HT, Siddique KHM, Varshney RK, Colmer TD, Cowling W, Bramley H, Mori TA, Hodgson JM, Cooper JW, Miller AJ, Kunert K, Vorster J, Cullis C, Ozga JA, Wahlqvist ML, Liang Y, Shou H, Shi K, Yu J, Fodor N, Kaiser BN, Wong F-L, Valliyodan B, Considine MJ (2016) Neglecting legumes has compromised human health and sustainable food production. *Nature Plants* **2**, 1–10.

Fravel DR (1988) Role of Antibiosis in the Biocontrol of Plant Diseases*. *Annual Review of Phytopathology* **26**, 75–91.

Fuhlbohm MJ, Tatnell JR, Ryley MJ (2003) First report of stem rot and wilt of chickpea caused by *Sclerotinia minor* in Queensland, Australia. *Australasian Plant Pathology* **2**, 323–324.

Garg H, Atri C, Sandhu PS, Kaur B, Renton M, Banga SK, Singh H, Singh C, Barbetti MJ, Banga SS (2010) High level of resistance to *Sclerotinia sclerotiorum* in introgression lines derived from hybridisation between wild crucifers and the crop Brassica species *B. napus* and *B. juncea*. *Field Crops Research* **117**, 51–58.

Garg H, Li H, Sivasithamparam K, Barbetti MJ (2013) Differentially expressed proteins and associated histological and disease progression changes in cotyledon tissue of a resistant and susceptible genotype of *Brassica napus* infected with *Sclerotinia sclerotiorum*. *PloS One* **8**, e65205.

Golla SK, Sharma HC, Rajasekhar P, Mishra SP, Jaba J (2020) Biochemical components of wild relatives of chickpea confer resistance to pod borer, *Helicoverpa armigera*. *Arthropod-Plant Interactions* **14**, 623–639.

Grau C, Adee E, Oplinger E (1994) An Integrated Approach to Control Sclerotinia Stem Rot (White Mold) in Soybean. *Proceedings of the Integrated Crop Management Conference*. https://lib.dr.iastate.edu/icm/1994/proceedings/25.

Haware MP (1990) Fusarium wilt and other important diseases of chickpea in the Mediterranean area. *Options Mediterr Ser Semin* **9**, 61–64.

Hawthorne BT, Jarvis WR (1973) Differential activity of fungicides on various stages in the life cycle of *Sclerotinia* spp. *New Zealand Journal of Agricultural Research* **16**, 551–557.

Hilton, S. A. 2000. Canadian Plant Disease Survey, Agriculture and Agri-Food Canada 80:1-151. (http://www.cps-scp.ca/download/cpds_v80.pdf) - Google Search Accessed 22 June 2020.

Huzar-Novakowiski J, Paul PA, Dorrance AE (2017) Host Resistance and Chemical Control for Management of Sclerotinia Stem Rot of Soybean in Ohio. *Phytopathology* **107**, 937–949.

Jukanti AK, Gaur PM, Gowda CLL, Chibbar RN (2012) Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. *British Journal of Nutrition* **108**, S11–S26.

Kameswara Rao N, Reddy LJ, Bramel PJ (2003) Potential of wild species for genetic enhancement of some semi-arid food crops. *Genetic Resources and Crop Evolution* **50**, 707–721.

Kharbanda PD, Tewari JP (1996) Integrated management of canola diseases using cultural methods. *Canadian Journal of Plant Pathology* **18**, 168–175.

Khot SD, Bilgi VN, del Río LE, Bradley CA (2011) Identification of *Brassica napus* lines with partial resistance to *Sclerotinia sclerotiorum*. *Plant Health Progress* **12**, 15.

Kim HS, Diers BW (2000) Inheritance of partial resistance to Sclerotinia stem rot in soybean. *Crop Science* **40**, 55–61.

Kim HS, Sneller CH, Diers BW (1999) Evaluation of soybean varieties for resistance to Sclerotinia stem rot in field environments. *Crop Science* **39**, c003900010010x.

Knights EJ, Hobson KB (2016) Chickpea: Overview. 'Encycl. Food Grains Second Ed.' (Eds C Wrigley, H Corke, K Seetharaman, J Faubion) pp. 316–323. (Academic Press: Oxford) doi:10.1016/B978-0-12-394437-5.00035-8.

Knights EJ, Southwell RJ, Schwinghamer MW, Harden S (2008) Resistance to *Phytophthora medicaginis* Hansen and Maxwell in wild *Cicer* species and its use in breeding root rot resistant chickpea (Cicer arietinum L.). *Australian Journal of Agricultural Research* **59**, 383–387.

Kukreja S, Salaria N, Thakur K, Goutam U (2018) Fungal Disease Management in Chickpea: Current Status and Future Prospects. 'Fungi Their Role Sustain. Dev. Curr. Perspect.'

(Eds P Gehlot, J Singh) pp. 293–309. (Springer: Singapore) doi:10.1007/978-981-13-0393-7_17.

Kull LS, Vuong TD, Powers KS, Eskridge KM, Steadman JR, Hartman GL (2003) Evaluation of resistance screening methods for Sclerotinia stem rot of soybean and dry bean. *Plant Disease* **87**, 1471–1476.

Lane D, Denton-Giles M, Derbyshire M, Kamphuis LG (2019) Abiotic conditions governing the myceliogenic germination of *Sclerotinia sclerotiorum* allowing the basal infection of *Brassica napus*. *Australasian Plant Pathology* **48**, 85–91.

Li C, Li H, Sivasithamparam K, Fu TD, Li YC, Liu SY, Barbetti M (2006) Expression of field resistance under Western Australian conditions to *Sclerotinia sclerotiorum* in Chinese and Australian *Brassica napus* and *Brassica juncea* germplasm and its relation with stem diameter. *Australian Journal of Agricultural Research* **57**, 1131–1135.

Li R, Rimmer R, Buchwaldt L, Sharpe AG, Séguin-Swartz G, Coutu C, Hegedus DD (2004) Interaction of *Sclerotinia sclerotiorum* with a resistant *Brassica napus* variety: expressed sequence tag analysis identifies genes associated with fungal pathogenesis. *Fungal Genetics and Biology* **41**, 735–753.

Lyu X, Shen C, Fu Y, Xie J, Jiang D, Li G, Cheng J (2016) A small secreted virulence-related protein is essential for the necrotrophic interactions of *Sclerotinia sclerotiorum* with its host plants. *PLOS Pathogens* **12**, e1005435.

Malunga LN, Bar-El SD, Zinal E, Berkovich Z, Abbo S, Reifen R (2014) The potential use of chickpeas in development of infant follow-on formula. *Nutrition Journal* **13**, 8.

Mao W, Lewis JA, Hebbar PK, Lumsden RD (1997) Seed treatment with a fungal or a bacterial antagonist for reducing corn damping-off caused by species of pythium and fusarium. *Plant Disease* **81**, 450–454.

Maxted N, Ford-Lloyd BV, Kell SP, Iriondo JM, Dulloo ME, Turok J (Eds) (2007) 'Crop wild relative conservation and use.' (CABI: Wallingford) doi:10.1079/9781845930998.0000.

Merga B, Haji J (2019a) Economic importance of chickpea: Production, value, and world trade (F Yildiz, Ed.). *Cogent Food & Agriculture* **5**, 1615718.

Merga B, Haji J (2019b) Economic importance of chickpea: Production, value, and world trade (F Yildiz, Ed.). *Cogent Food & Agriculture* **5**, 1615718. d

Merriman PR, Pywell M, Harrison G, Nancarrow J (1979) Survival of sclerotia of S*clerotinia sclerotiorum* and effects of cultivation practices on disease. *Soil Biology and Biochemistry* **11**, 567–570.

Muehlbauer FJ, Cho S, Sarker A, McPhee KE, Coyne CJ, Rajesh PN, Ford R (2006) Application of biotechnology in breeding lentil for resistance to biotic and abiotic stress. *Euphytica* **147**, 149–165.

Mueller DS, Dorrance AE, Derksen RC, Ozkan E, Kurle JE, Grau CR, Gaska JM, Hartman GL, Bradley CA, Pedersen WL (2002) Efficacy of fungicides on *Sclerotinia sclerotiorum* and their potential for control of Sclerotinia stem rot on soybean. *Plant Disease* **86**, 26–31.

Muñoz N, Liu A, Kan L, Li M-W, Lam H-M (2017) Potential uses of wild germplasms of grain legumes for crop improvement. *International Journal of Molecular Sciences* **18**,.18020328.

Mwape VW, Khentry Y, Newman TE, Denton-Giles M, Derbyshire M, Chen K, Berger J, Kamphuis L (2021a) Identification of sources of *Sclerotinia sclerotiorum* resistance in a collection of wild *Cicer* germplasm. *Plant Disease*. doi:10.1094/PDIS-02-21-0367-RE.

Mwape VW, Mobegi FM, Regmi R, Newman TE, Kamphuis LG, Derbyshire MC (2021b) Analysis of differentially expressed *Sclerotinia sclerotiorum* genes during the interaction with moderately resistant and highly susceptible chickpea lines. *BMC Genomics* **22**, 333.

Newman TE, Jacques S, Grime C, Kamphuis FL, Lee RC, Berger J, Kamphuis LG (2020) Identification of novel sources of resistance to ascochyta blight in a collection of wild *Cicer* accessions. *Phytopathology®*. **111**(2),339-379.

Njambere EN, Chen W, Frate C, Wu B-M, Temple SR, Muehlbauer FJ (2008) Stem and crown rot of chickpea in california caused by *Sclerotinia trifoliorum*. *Plant Disease* **92**, 917–922.

Oliveira MB, de Andrade RV, Grossi-de-Sá MF, Petrofeza S (2015) Analysis of genes that are differentially expressed during the *Sclerotinia sclerotiorum-Phaseolus vulgaris* interaction. *Frontiers in Microbiology* **6**, 1162.

Pulse Australia - APB Chickpea IDM strategies http://www.pulseaus.com.au/growing-pulses/bmp/chickpea/idm-strategies. Accessed 10 July 2020.

Reen RA, Mumford MH, Thompson JP (2019) Novel sources of resistance to root-lesion nematode (*Pratylenchus thornei*) in a new collection of wild *Cicer* species (*C. reticulatum* and *C. echinospermum*) to improve resistance in cultivated chickpea (*C. arietinum*). *Phytopathology*® **109**, 1270–1279.

Robinson GHJ, Balk J, Domoney C (2019) Improving pulse crops as a source of protein, starch and micronutrients. *Nutrition Bulletin* **44**, 202–215.

Seifbarghi S, Borhan MH, Wei Y, Coutu C, Robinson SJ, Hegedus DD (2017) Changes in the *Sclerotinia sclerotiorum* transcriptome during infection of *Brassica napus*. *BMC Genomics* **18**, 266.

Siddique KHM, Brinsmead RB, Knight R, Knights EJ, Paull JG, Rose IA (2000) Adaptation of chickpea (*Cicer arietinum* L.) and faba bean (*Vicia faba* L.) to Australia. 'Link. Res. Mark. Oppor. Legumes 21st Century Proc. Third Int. Food Legum. Res. Conf.' (Ed R Knight) Current plant science and biotechnology in agriculture. pp. 289–303. (Springer Netherlands: Dordrecht) doi:10.1007/978-94-011-4385-1_26.

Singh KB (1997) Chickpea (*Cicer arietinum* L.). *Field Crops Research* **53**, 161–170.

Singh M, Avtar R, Pal A, Punia R, Singh VK, Bishnoi M, Singh A, Choudhary RR, Mandhania S (2020) Genotype-specific antioxidant responses and assessment of resistance against *Sclerotinia sclerotiorum* causing Sclerotinia rot in indian mustard. *Pathogens* **9**, 892.

Smolińska U, Kowalska B (2018) Biological control of the soil-borne fungal pathogen *Sclerotinia sclerotiorum* — a review. *Journal of Plant Pathology* **100**, 1–12.

Sumida CH, Canteri MG, Peitl DC, Tibolla F, Orsini IP, Araújo FA, Chagas DF, Calvos NS, Sumida CH, Canteri MG, Peitl DC, Tibolla F, Orsini IP, Araújo FA, Chagas DF, Calvos NS (2015) Chemical and biological control of Sclerotinia stem rot in the soybean crop. *Ciência Rural* **45**, 760–766.

Taylor A, Coventry E, Jones JE, Clarkson JP (2015) Resistance to a highly aggressive isolate of *Sclerotinia sclerotiorum* in a *Brassica napus* diversity set. *Plant Pathology* **64**, 932–940.

Taylor A, Rana K, Handy C, Clarkson JP (2018) Resistance to *Sclerotinia sclerotiorum* in wild Brassica species and the importance of *Sclerotinia subarctica* as a Brassica pathogen. *Plant Pathology* **67**, 433–444.

Thudi M, Chitikineni A, Liu X, He W, Roorkiwal M, Yang W, Jian J, Doddamani D, Gaur PM, Rathore A, Samineni S, Saxena RK, Xu D, Singh NP, Chaturvedi SK, Zhang G, Wang J, Datta SK, Xu X, Varshney RK (2016) Recent breeding programs enhanced genetic diversity in both desi and kabuli varieties of chickpea (*Cicer arietinum* L.). *Scientific Reports* **6**, 38636.

Uloth MB, Clode PL, You MP, Barbetti MJ (2016) Attack modes and defence reactions in pathosystems involving *Sclerotinia sclerotiorum*, *Brassica carinata*, *B. juncea* and *B. napus*. *Annals of Botany* **117**, 79–95.

van der Maesen, L. J. G. (1972). *Cicer* L., A monograph of the Genus with Special Reference to the Chickpea (*Cicer arietinum* L.), its ecology and Cultivation, Vol. 72. Wageningen: Mededelingen. Landbouwhooge school, 9–136.

von Wettberg EJB, Chang PL, Başdemir F, Carrasquila-Garcia N, Korbu LB, Moenga SM, Bedada G, Greenlon A, Moriuchi KS, Singh V, Cordeiro MA, Noujdina NV, Dinegde KN, Shah Sani SGA, Getahun T, Vance L, Bergmann E, Lindsay D, Mamo BE, Warschefsky EJ, Dacosta-Calheiros E, Marques E, Yilmaz MA, Cakmak A, Rose J, Migneault A, Krieg CP, Saylak S, Temel H, Friesen ML, Siler E, Akhmetov Z, Ozcelik H, Kholova J, Can C, Gaur P, Yildirim M, Sharma H, Vadez V, Tesfaye K, Woldemedhin AF, Tar'an B, Aydogan A, Bukun B, Penmetsa RV, Berger J, Kahraman A, Nuzhdin SV, Cook DR (2018) Ecology and genomics of an important crop wild relative as a prelude to agricultural innovation. *Nature Communications* **9**, 649.

Vuong TD, Hoffman DD, Diers BW, Miller JF, Steadman JR, Hartman GL (2004) Evaluation of soybean, dry bean, and sunflower for resistance to *Sclerotinia sclerotiorum*. *Crop Science* **44**, 777–783.

Wei L, Jian H, Lu K, Filardo F, Yin N, Liu L, Qu C, Li W, Du H, Li J (2016) Genome-wide association analysis and differential expression analysis of resistance to Sclerotinia stem rot in *Brassica napus*. *Plant Biotechnology Journal* **14**, 1368–1380.

Wen L, Tan T-L, Shu J-B, Chen Y, Liu Y, Yang Z-F, Zhang Q-P, Yin M-Z, Tao J, Guan C-Y (2013) Using proteomic analysis to find the proteins involved in resistance against *Sclerotinia sclerotiorum* in adult *Brassica napus*. *European Journal of Plant Pathology* **137**, 505–523.

Westrick NM, Ranjan A, Jain S, Grau CR, Smith DL, Kabbage M (2019) Gene regulation of *Sclerotinia sclerotiorum* during infection of *Glycine max*: on the road to pathogenesis. *BMC genomics* **20**, 157–157.

Willbur JF, Ding S, Marks ME, Lucas H, Grau CR, Groves CL, Kabbage M, Smith DL (2017) Comprehensive Sclerotinia Stem Rot screening of soybean germplasm requires multiple isolates of *Sclerotinia sclerotiorum*. *Plant Disease* **101**, 344–353.

Willetts HJ, Wong JAL (1980) The biology of *Sclerotinia sclerotiorum*, *S. trifoliorum*, and *S. minor* with emphasis on specific nomenclature. *The Botanical Review* **46**, 101–165.

Williams B, Kabbage M, Kim H-J, Britt R, Dickman MB (2011) Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. *PLOS Pathogens* **7**, e1002107.

Williams JR, Stelfox D (1980) Influence of farming practices in alberta on germination and apothecium production of Sclerotia of *Sclerotinia Sclerotiorum. Canadian Journal of Plant Pathology* **2**, 169–172.

Wu J, Cai G, Tu J, Li L, Liu S, Luo X, Zhou L, Fan C, Zhou Y (2013) Identification of QTLs for resistance to sclerotinia stem rot and BnaC.IGMT5.a as a candidate gene of the major resistant QTL SRC6 in *Brassica napus*. *PloS One* **8**, e67740.

Zeng W, Wang D, Kirk W, Hao J (2012) Use of Coniothyrium minitans and other microorganisms for reducing *Sclerotinia sclerotiorum*. *Biological Control* **60**, 225–232.

Zhang H, Mittal N, Leamy LJ, Barazani O, Song B (2016) Back into the wild—Apply untapped genetic diversity of wild relatives for crop improvement. *Evolutionary Applications* **10**, 5–24.

Zhao X, Han Y, Li Y, Liu D, Sun M, Zhao Y, Lv C, Li D, Yang Z, Huang L, Teng W, Qiu L, Zheng H, Li W (2015) Loci and candidate gene identification for resistance to *Sclerotinia sclerotiorum* in soybean (*Glycine max* L. Merr.) via association and linkage maps. *The Plant Journal* **82**, 245–255.

Zhao J, Meng J (2003) Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.). *TAG Theoretical and applied genetics* **106**, 759–764.

Zhao J, Peltier AJ, Meng J, Osborn TC, Grau CR (2004) Evaluation of Sclerotinia stem rot resistance in oilseed *Brassica napus* using a petiole inoculation technique under greenhouse conditions. *Plant Disease* **88**, 1033–1039.

CHAPTER 2

Identification of sources of *Sclerotinia sclerotiorum* resistance in a collection of wild *Cicer* germplasm

Virginia W. Mwape[1, 2], Yuphin Khentry[1], Toby. E. Newman[1], Matthew Denton-Giles[1,4], Mark C. Derbyshire[1], Kefei Chen[3], Jens Berger[2] and Lars G. Kamphuis[1, 2,]

[1] Centre for Crop and Disease Management, Curtin University, Bentley WA 6102, Australia

[2] Commonwealth Scientific and Industrial Research Organization. Agriculture and Food, Floreat, WA, Australia

[3] Statistics for the Australian Grains Industry -West, Curtin University, Bentley, WA 6102, Australia

[4] Current address: Corteva Agriscience, New Plymouth, Taranaki, New Zealand

This chapter is reproduced, with permission, from Mwape, V. W. *et al*. 2021a. Identification of sources of *Sclerotinia sclerotiorum* in a collection of wild *Cicer* germplasm. *Plant Dis*. https://doi.org/10.1094/PDIS-02-21-0367-RE.

2.1. Abstract

Sclerotinia sclerotiorum is an important fungal pathogen of chickpea (*Cicer arietinum* L.), and it can cause yield losses up to 100%. The wild progenitors are much more diverse than domesticated chickpea, and this study describes how this relates to *S. sclerotiorum* resistance. Initially, the pathogenicity of nine Australian *S. sclerotiorum* isolates was examined on three *Cicer* lines to develop a robust phenotyping assay, and significant differences in isolate aggressiveness were identified, with six isolates being classed as highly aggressive and three as moderately aggressive. We focussed on two *S. sclerotiorum* isolates, CU8.20 and CU10.12, which were highly aggressive and moderately aggressive, respectively. A subsequent phenotyping assay was conducted using the two isolates to evaluate 86 wild *Cicer* accessions (*Cicer reticulatum* and *Cicer echinospermum*) and two *C. arietinum* cultivars for resistance to *S. sclerotiorum*. A subset of 12 accessions was further evaluated, and subsequently, two wild *Cicer* accessions with consistently high levels of resistance to *S. sclerotiorum* were examined using the initially characterised nine isolates. Wild *Cicer* accessions Karab_084 and Deste_063 demonstrated consistent partial resistance to *S. sclerotiorum*. There were significant differences in responses to *S. sclerotiorum* across wild *Cicer* collection sites. The Cermik, Karabahce and Destek sites' responses to the aggressive isolate CU8.20 ranged from resistant to susceptible, highlighting a collection site interaction between isolate genotype and chickpea collection site for sclerotinia stem rot resistance. This is the first evidence of partial stem resistance identified in wild *Cicer* germplasm, which can be adopted in chickpea breeding programs to enhance *S. sclerotiorum* resistance in future chickpea cultivars.

Keywords: *Cicer reticulatum*, *Cicer echinospermum*, *Sclerotinia sclerotiorum*, wild chickpea, disease resistance screening, sclerotinia stem rot, crop wild relatives.

2.2. Introduction

In Australia, chickpea is particularly important as a disease break crop in cereal production systems and for its ability to form a beneficial relationship with nitrogen-fixing Rhizobia bacteria (Siddique *et al.* 2013). Nutritionally, chickpea is high in protein, dietary fibre and essential minerals, and therefore plays a critical role in the fight to reduce hunger and malnutrition in many developing countries (Xing *et al.* 2020; Jukanti *et al.* 2012).

Global chickpea production is threatened by various pathogens including *Ascochyta rabiei*, *Fusarium oxysporum* f. sp. *ciceri, Phytophthora medicaginis*, *Pratylenchus thornei*, *Botrytis cinerea* and *Sclerotinia sclerotiorum,* causal agents of Ascochyta blight, Fusarium wilt, Phytophthora root rot, root lesion nematode disease, Botrytis grey mould and Sclerotinia stem rot (SSR), respectively (Kukreja *et al.* 2018). Among other factors, the narrow genetic base of cultivated cultivars makes chickpea very susceptible and vulnerable to these pathogens, as novel sources of resistance are difficult to identify in the domesticated chickpea gene pool (Thudi *et al.* 2016). Whilst great efforts have been made to incorporate wild sources of resistance to *A. rabiei* (Collard, Pang, and Taylor 2003), *P. thornei*, (Reen *et al*. 2019), and *P. medicaginis* (Knights *et al.* 2008)) into chickpea cultivars, little research efforts have been employed to improve resistance to *S. sclerotiorum*. In Australia, SSR can cause up to 100% yield loss in susceptible chickpea cultivars under favourable conditions (Fuhlbohm *et al*. 2003; Pulse Australia, 2020).

The phytopathogen *S. sclerotiorum* is an economically important necrotrophic fungus with a remarkably broad host range of over 400 species, including wild plants, cereal, oilseed and pulse crops (Boland and Hall 1994). *S. sclerotiorum* is an important pathogen in Canada (Bardin and Huang 2001), America (Chen *et al.* 2006), India (Haware, 1990), and Australia (Fuhlbohm *et al*. 2003). Previous studies in Australia have demonstrated that the *S. sclerotiorum* population is diverse and unpredictable. Their responses depend on existing environmental conditions (Michael *et al.* 2020). In chickpea, SSR infects directly from sclerotia in the soil through myceliogenic germination or from carpogenic germination, resulting in the release of ascospores in the canopy with both infection strategies causing stem whitening, wilting and stem breakage (Chen *et al*. 2006). In Australia, incidences of the disease in pulses are increasing due to the large area of canola grown, resulting in inoculum (sclerotia) build-up in the soil (Brooks *et al.* 2018). Though previous reports have shown partial resistance to *S. sclerotiorum* in some members of the Leguminosae family (Webster *et al*. 2020; Leite *et al*.

2016; Kull *et al*. 2003), there is no published evidence of *S. sclerotiorum* resistance in chickpea to date.

Previous research has demonstrated *S. sclerotiorum* hosts such as model plants *Arabidopsis thaliana*, *Brassica napus*, and *Glycin max* manifest SSR symptoms ranging from high susceptibility to partial resistance to the pathogen, an indication of quantitative resistance responses (Kim *et al*. 1999; Chen and Wang 2005; Perchepied *et al*. 2010; Denton-Giles *et al*. 2018). Previous studies have reported mapped quantitative trait loci for resistance to SSR in pulses such as peanut (Liang *et al*. 2020), pea (Ashtari *et al*. 2020), common bean (Porto *et al.* 2019) and soybean (Boudhrioua *et al.* 2020).

Research has demonstrated that crops wild relatives (CWRs) have the genetic potential to improve *S. sclerotiorum* resistance in other host crops. CWRs have been shown to harbour sources of resistance in other *S. sclerotiorum* hosts such as canola (Taylor *et al.* 2015) and sunflower (Qi *et al.* 2016). Previous studies have shown wild progenitors were used to improve the breeding of crops such as oat, rice and tomatoes using their wild species *Avena sterilis*, *Oryza rufipogon* and *Lycopersicon pimpinellifolium*, respectively (Shakiba and Eizenga 2014; Barrantes *et al*. 2016; Ociepa 2019). Therefore, understanding the disease resistance potential of CWRs is critical in sustaining global food security. Currently, there is no strong resistance to *S. sclerotiorum* in chickpea. Therefore, to improve the cultivated chickpea cultivars, research aiming to reintroduce genes from the wild relatives is important (Abbo *et al*. 2003).

Currently, the management of SSR in agricultural crops relies on crop rotation and timely, expensive fungicide applications. Therefore, the development of SSR resistant genotypes is desirable. Von Wettberg *et al*. (2018) used genotyping-by-sequencing (GBS) data to demonstrate that approximately 95% of the genetic diversity in the wild chickpea ancestor *C. reticulatum* has been lost in domesticated chickpea. Previous studies have shown that wild *Cicer* species are a source of novel alleles for resistance to diverse chickpea diseases (Abbo *et al*. 2003; Reen *et al*. 2019; Muñoz *et al*. 2017).

Although crosses of other wild *Cicer* species have shown low efficacy, the annual species, *C. reticulatum* and *C. echinospermum,* are the only CWRs that are readily cross-compatible with domesticated chickpea (von Wettberg *et al*. 2018; Croser *et al*. 2003; Reen *et al*. 2019). A recent collection mission to southeastern Turkey significantly increased the global collection of *C. reticulatum* and *C. echinospermum* accessions. This mission was particularly important, as these wild accessions are currently threatened by increasing urbanisation and climate change

(von Wettberg *et al*. 2018). Further, the authors showed that *C. reticulatum* and *C. echinospermum* each have intrinsic genetic differences and classified them into eight and four genetic population groups, respectively. This genetic diversity was largely related to the location of their collection sites. *C. reticulatum* accessions were located on sandstone in disturbed mixed pastures, while *C. echinospermum* accessions were found in open pastures in basaltic soils at a lower elevation (von Wettberg *et al*. 2018; Coyne *et al*. 2020). This germplasm collection has become of interest in the search for chickpea resistance to biotic and abiotic stresses and is a valuable resource for chickpea cultivar improvement (Coyne *et al*. 2020). Resistance to Ascochyta blight, pod-borer (*Helicoverpa armigera*), and root-lesion nematode (*Pratylenchus thornei*) have already been identified in this collection (Newman *et al*. 2020; Reen *et al*. 2019; von Wettberg *et al*. 2018).

Studies have demonstrated the crossing of chickpea using the wild annual *Cicer spp.* can improve the genetic base of cultivated cultivars without compromising their yield or adaptation (Koseoglu *et al*. 2017; Singh and Ocampo 1997). Various crossing programs to elite cultivars have been successfully employed to introduce wild *Cicer* (*C. reticulatum* and *C. echinospermum*) sources of resistance to Ascochyta blight (Collard *et al.* 2001), Botrytis grey mould (Li *et al*. 2015), and Phytophthora root rot (Knights *et al*. 2008; Miranda 2019). These studies indicate that the wild collection harbour useful genes for diverse traits of benefit to the chickpea industry. An assay previously developed by Akem and Kabbabeh (1999) to screen chickpea cultivars in Pakistan for *S. sclerotiorum* resistance under controlled conditions did not identify any substantial resistance. Most Australian chickpea cultivars are susceptible to *S. sclerotiorum* (Chapter 3; Mwape *et al*. unpublished observations). There is a lack of fungicides registered or under permit in Australia for *S. sclerotiorum* control (Pulse Australia 2020). Consequently, the chickpea industry is seeking solutions to this problem; thus, evaluating the wild collection for resistance to *S. sclerotiorum* is of great interest.

To identify novel sources of resistance to SSR in wild chickpea germplasm, we adopted a stem inoculation strategy previously used to screen *Brassica napus* germplasm (Denton-Giles *et al.* 2018). We used this assay to initially assess differences in the response of a representative sample of each *Cicer* spp. (*C. arietinum, C. echinospermum* and *C. reticulatum*). The protocol was subsequently used to assess differences in aggressiveness of nine *S. sclerotiorum* isolates on the three *Cicer* spp. Subsequently, we used one highly aggressive and moderately aggressive *S. sclerotiorum* isolate to characterise the resistance response of 19 *C. echinospermum* and 68 *C. reticulatum* wild accessions, compared to two popular Australian cultivars (PBA HatTrick

XT and Kyabra) under greenhouse conditions. Correlations between wild chickpea geographical location origin and the level of *S. sclerotiorum* infection was determined.

Additionally, *Cicer* spp. with partial stem resistance were identified and evaluated with multiple *S. sclerotiorum* isolates. Karab_084 and Deste_063 showed consistently high levels of partial stem resistance relative to the most resistant chickpea control cultivar. This study is the first evidence of partial stem resistance in wild *Cicer* germplasm, which can be adopted in chickpea breeding programs to enhance SSR resistance in future chickpea cultivars.

2.3. Material and Methods

2.3.1. Sources of *Sclerotinia sclerotiorum* isolates

This study aimed to examine the pathogenicity of nine *S. sclerotiorum* isolates, which are part of a world collection genotyped by Clarkson *et al*. (2017) and previously used to determine differences in the levels of aggressiveness between the isolates in *B. napus* (Denton-Giles *et al*. 2018). The *S. sclerotiorum* isolates were collected from *B. napus* (six isolates) and *Lupinus albus* (three isolates) from different Western Australia sites between 2013 and 2014 (Table 2.1). *S. sclerotiorum* is a broad host range pathogen, and isolates were selected as they belong to the different intergenic spacer (IGS) and mycelial compatibility groups (MCGs) (Table 2.1).

Mature sclerotia were obtained from the Centre for Crop and Disease Management (CCDM), Curtin University, Western Australia. To produce inoculum from actively growing culture, single sterile sclerotia were dissected and placed in a 9 cm Petri dish containing 39 g/L Potato Dextrose Agar (PDA) (Becton Dickinson, USA) and incubated at 20 $^{\circ}$C for 5-7 days in the dark. The cultures were subcultured by using a sterile cork borer and forceps to transfer 5 mm agar plugs from the original plates onto fresh PDA plates, which were incubated for 2 days at 20 $^{\circ}$C to source actively growing mycelia for plant inoculation. Plants were inoculated with 5 mm mycelial plugs taken from the leading mycelial edge of the growing *S. sclerotiorum* culture.

Table 2.1: Australian *Sclerotinia sclerotiorum* isolates used for pathogenicity tests.

Isolate	MCG[a]	IGS[b]	Genome Sequenced	Original Host	Collection location	Year collected
CU4.2	I	7	Yes	*Brassica napus*	Mount-Barker, WA	2014
CU6.1	II	7	Yes	*Brassica napus*	Mount-Barker, WA	2014
CU8.20	III	5	Yes	*Brassica napus*	South-Stirling, WA	2014
CU8.24	IV	3	Yes	*Brassica napus*	South-Stirling, WA	2014
CU10.12	VI	3	Yes	*Brassica napus*	Geraldton, WA	2014
CU10.17	VII	5	Yes	*Brassica napus*	Geraldton, WA	2014
CU11.4	IX	7	No	*Lupinus albus*	Eneabba, WA	2014
CU11.7	X	5	Yes	*Lupinus albus*	Eneabba, WA	2013
CU11.19	XI	5	Yes	*Lupinus albus*	Geraldton, WA	2014

[a] MCG = Mycelial compatibility group from Denton-Giles *et al.* (2018).

[b] IGS = Intergenic spacer region haplotype from Clarkson *et al.* (2017).

2.3.2. Assessing *Sclerotinia sclerotiorum* pathogenicity

The pathogenicity of nine *S. sclerotiorum* isolates (Table 2.1) was evaluated on *C. echinospermum* and *C. reticulatum* accessions S2Drd_065 and Besev_079, respectively, along with a domestic desi cultivar (PBA HatTrick). The *Cicer* accessions S2Drd_065 and Besev_075 were selected because the Chickpea Innovation Lab (University of California, Davis, CA, USA) is generating high-quality genome sequences for these accessions as representatives for *C. echinospermum* and *C. reticulatum,* respectively. We chose PBA HatTrick as it is a popular current Australian *C. arietinum* cultivar.

In all the experiments, the wild *Cicer* seeds were cut at the endosperm end with a scalpel blade to facilitate imbibition for germination. Seeds were sown into 4 L pots with an all-purpose potting mix (UWA mix, Richgro, Perth, Australia). Plants from each accession/cultivar were mock-inoculated with PDA only agar plugs, which served as the negative control that should not produce a stem lesion. At four weeks, plants were fertilised with 2 g Nitrophoska perfkt™ fertiliser (Incitec Pivot fertilisers, Victoria, Australia). Stem inoculation was conducted on eight-week-old plants following a stem inoculation assay previously described in *B. napus* by Denton-Giles *et al*. (2018). A 5 mm PDA plug with *S. sclerotiorum* mycelium from the leading edge of the culture was cut using a sterile cork borer, and a sterile metal spatula was used to transfer the plug onto Parafilm. The plug was placed upright on a strip of Parafilm® and wrapped around the main stem. Measurements of stem lesion length (mm) were taken at 2, 4, 6, 8, 10, and 12 days post inoculation (dpi) to calculate the area under the disease progress curve (AUDPC) according to Jeger and Viljanen-Rollinson (2001).

2.3.3. Screening wild *Cicer* accessions for *S. sclerotiorum* resistance

A set of 86 wild *Cicer* accessions (67 *C. reticulatum* and 19 *C. echinospermum*) and two domestic desi cultivars (PBA HatTrick and Kyabra) (Table 2.2) were screened for *S. sclerotiorum* resistance using the 'highly aggressive' isolate CU8.20 and the 'moderately aggressive' isolate CU10.12. Inoculation with the moderately aggressive isolate (CU10.12) was included in case all *Cicer* accessions were found to equally susceptible to the highly aggressive (CU8.20) isolate. Seeds for the cultivars PBA HatTrick and Kyabra and seeds of the wild accessions were obtained from the Australian Grains Gene Bank (AGG; Horsham, Victoria). The 86 wild accessions are part of a wild *Cicer* collection described and characterised by von Wettberg *et al*. (2018).

2.3.4. Validation of the partial stem resistance of wild *Cicer* accessions over time to *S. sclerotiorum*

Based on the stem assay results of 86 wild *Cicer* accessions, 11 wild accessions were further evaluated using the same highly aggressive isolate (CU8.20) to demonstrate reproducibility. The selected wild *Cicer* accessions demonstrated a range of stem lesion lengths in response to both isolates used in the previous experiment and included a reference *C. arietinum* cultivar, PBA HatTrick. Inoculation, data collection and AUDPC calculation were carried out following the same procedure as previous experiments

2.3.5. Determination of aggressiveness levels of nine *S. sclerotiorum* isolates on accessions Karab_084 and Deste_063

In a subsequent investigation, the aggressiveness of nine isolates characterised above was re-assessed on the two wild *Cicer* accessions, Karab_084 and Deste_063 identified to have the greatest resistance. Inoculation, data collection and AUDPC calculation were carried out following the same procedure as previous experiments.

Table 2.2: Wild chickpea accessions used in this study, classified according to provinces, collection site, species, genetic group, names and code number based on von Wettberg *et al*. (2018) for material collected in 2013/2014 from southeastern Turkey.

Province	Collection site	Species[a]	*N*[b]	Prefix[c]	Suffix[d] and accession code number
Adiyaman					
	Oyali	*C. ret*	4	Oyali	081, 084, 101, 105
Mardin					
	Baristepe 1	*C. ret*	3	Bari1	064, 068, 092
	Baristepe 2	*C. ret*	5	Bari2	062, 064, 067, 072, 074
	Baristepe 3	*C. ret*	4	Bari3	072, 091, 100, 106D
	Besevler	*C. ret*	8	Besev	061, 062, 065, 066, 074, 075, 079, 083
	Dereici	*C. ret*	8	Deric	062, 065, 066, 069, 070, 072, 074, 075,
	Kayatepe	*C. ret*	7	Kayat	061, 063, 064,, 066, 070, 077, 080
	Sarikaya	*C. ret*	9	Sarik	061, 064, 065, 066, 067, 073, 074, 077, 080
	Savur	*C. ret*	1	Savur	063
Diyarbakir					
	Cermik	*C. ech*	3	Cermi	072, 073, 075
	Kesentas	*C. ret*	3	Kesen	062, 065, 075
	Egil	*C. ret*	3	Egill	065, 066, 073
	Kalkan	*C. ret*	4	Kalka	061, 064, 066, 074
	Gunasan	*C. ech*	2	Guna	062, 100
Urfa					
	Destek	*C. ech*	3	Deste	063, 071, 075
	Karabahce	*C. ech*	4	Karab	067, 084, 085C, 092
	Ortanca	*C. ech*	2	Ortan	061, 066
	Siv-Diyar	*C. ech*	5	S2Drd	062, 065, 100, 101, 102
Sirnak					
	CudiA	*C. ret*	4	CudiA	122, 124, 127, 152
	CudiB	*C. ret*	4	CudiB	009, 016, 018, 022C
	Sirnak	*C. ech*	1	Sirna	60

[a] Abbreviation: *C. ret* = *Cicer reticulatum*; *C. ech*= *C. echinospermum* [b] *N*= number of accessions

2.4. Experimental design and statistical analysis

2.4.1. Experimental design

To assess the susceptibility of chickpea accessions, the aggressiveness of *S. sclerotiorum* isolates and their interactions, randomised complete block designs (RCBD) were applied for the first three glasshouse experiments and split-plot design was applied for the fourth

glasshouse experiment. Negative controls with PDA only agar plugs were included for all experiments. All experimental designs were generated using the experimental design tool DiGGer in R (Coombes, 2018).

The first experiment for assessing *S. sclerotiorum* pathogenicity was designed to be an RCBD with cultivar and isolate as treatments in 12 replicates to compare three chickpea cultivars (Besev_079, PBA Hatrick, S2Drd_065) and nine isolates (CU4.2, CU6.1, CU8.20, CU8.24, CU10.12, CU10.17, CU11.4, CU11.7, CU11.19).

The second experiment for screening wild *Cicer* accessions for *S. sclerotiorum* resistance was designed to be an RCBD with cultivar and isolate as treatments in four replicates to compare 86 wild *Cicer* accessions (for details, see Supplementary Table S2) along with domestic desi cultivars (PBA HatTrick and Kyabra) and two isolates (CU10.12, CU8.20).

The third experiment for validating the partial stem resistance of wild *Cicer* accessions was designed to be an RCBD with cultivar as treatments in 12 replicates to compare the susceptibility of the 11 wild *Cicer* accessions (Deste_063, CudiA_122, Oyali_105, Sarik_065, Oyali_084, Egill_065, Karab_084, Besev_074, Besev_079, Deric_062, S2Drd_065) along with domestic desi cultivar (PBA HatTrick) using the highly aggressive isolate (CU8.20).

The fourth experiment for determining aggressiveness levels of nine *S. sclerotiorum* isolates on accessions Karab_084 and Deste_063 was designed to be a split-plot design with cultivar as main-plot and isolate as sub-plot in 4 replicates to compare two wild *Cicer* accessions (Karab_084, Deste_0630) and nine isolates (CU4.2, CU6.1, CU8.20, CU8.24, CU10.12, CU10.17, CU11.4, CU11.7, CU11.19).

All experiments were conducted between January 2018 and June 2019 in the Curtin University field trial area of the Bentley campus. Experiment one and two were conducted in a hoop house and experiment three and four in a glasshouse under natural light and an average temperature of ~ 24 °C.

2.4.2. Statistical analysis

Linear mixed models (LMM) were fitted using ASReml-R (Bulter *et al*., 2018) to examine the spatial variations, including local autocorrelations, global trends and extraneous variations and produce predicted values. As the main objective of the experiments is to determine the difference between specific pairs of treatments, the cultivar, isolate and the interaction term

between cultivar and isolate were fitted as fixed effects (Smith *et al*. 2005). The blocking structures of the experiments were fitted as random effects. Spatial trends and residual variances with auto-regressive correlations at first-order for rows and columns were examined and fitted when the global trends and autocorrelations are significant. Likelihood ratio tests were used for random effects, and conditional Wald tests (Kenward and Roger 1997) were used for fixed effects. Residual diagnostics were performed to examine the validity of the model assumption of normality and homogeneity of variance. For each of the fitted models, the empirical best unbiased linear estimates (eBLUEs) were produced. The R package asremlPlus (Brien, 2020) was used to compute the least significant difference (LSD with $\alpha = 0.05$) values.

The *Cicer* screening data (Exp 2) were also analysed by factorial nested ANOVA in Genstat (V20) to partition variance within and between species, collection sites and accessions and to identify interaction with isolate type. Orthogonal contrasts were used to define differences between wild and domestic *Cicer* and between the two wild species (*C. echinospermum* and *C. reticulatum*). Block effects were fitted to take advantage of the RCBD, residual plots prepared to identify outliers, and confirm that the ANOVA expectations of random and independent error distribution were met.

2.5. Results

2.5.1. A robust *S. sclerotiorum* phenotyping assay shows significant differences in aggressiveness across nine genetically distinct *S. sclerotiorum* isolates on three different *Cicer* species

A robust phenotyping assay was developed, which measures the lesion length over time following inoculation of a chickpea stem with an agar plug containing *S. sclerotiorum.* This allowed us to calculate the area under the disease progress curve (AUPDC) to investigate the response of *Cicer* germplasm to *S. sclerotiorum* isolates, which showed differences in the aggressiveness of *S. sclerotiorum* isolates on chickpea.

Inoculation of the three representatives of *C. reticulatum* (Besev_079), *C. echinospermum* (S2Drd_065) and *C. arietinum* (PBA HatTrick) with nine *S. sclerotiorum* isolates resulted in a consistent infection in all three genotypes (Fig. 2.1). The genotypes showed significant differences ($P < 0.05$, to $LSD_{0.05} = 803.6$) with Besev_079 having higher resistance (lower AUDPC scores) to all the isolates except CU11.19 and CU10.12 compared to PBA HatTrick and S2Drd_065 (Fig. 2.1A, Supplementary Table S2.1). There were significant differences ($P < 0.05$, $LSD_{0.05} = 464$) between the aggressiveness of the nine *S. sclerotiorum* isolates (Fig.

2.1B, Supplementary Table S2.1). Overall, isolates CU8.20, CU10.17, CU4.2, CU8.24, CU11.7, and CU6.1 caused significantly ($P \leq 0.05$, $LSD_{0.05} = 464$) higher AUDPC values (1382-2875) than isolates CU10.12, CU11.4 and CU11.19 (723-1327) (Fig 2.1B, Supplementary Table S2.1). Therefore, isolates CU10.12, CU11.4 and CU11.19 were placed into the moderately aggressive isolate group and isolates CU8.24, CU4.2, CU10.17, CU 6.1, CU 11.7 and CU8.20 in the highly aggressive isolate group. The genotypes exhibited differences ($P < 0.05$, $LSD_{0.05} = 267.9$) with Besev_079 showing significantly higher resistance than PBA HatTrick and S2Drd_065 (Fig. 2.1C). From this initial test, we selected a highly aggressive isolate (CU8.20) and a moderately aggressive isolate (CU10.12) for subsequent screening for *S. sclerotiorum* resistance in a collection of wild *Cicer* germplasm.

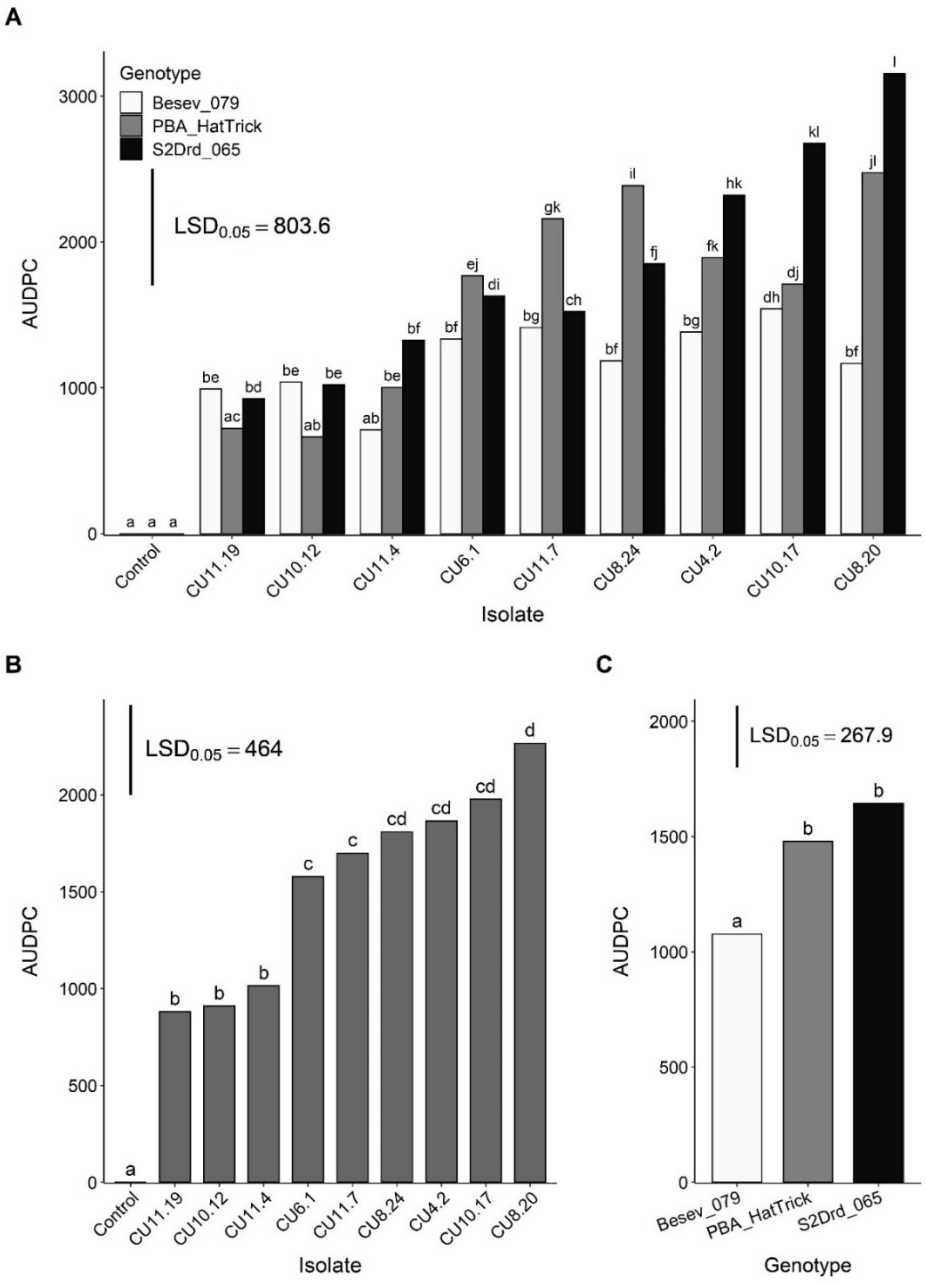


Figure 2.1: Aggressiveness of nine *Sclerotinia sclerotiorum* isolates on *C. reticulatum* (Besev_079), *C. echinospermum* (S2Drd_065) and *C. arietinum* (PBA HatTrick) based on 12 replicates per *Cicer* species for each isolate; (A) Mean area under the disease progress curve (AUDPC) for each isolate by genotype interaction, (B) Mean AUDPC for each isolate on all three *Cicer* species and (C) Mean AUDPC for three chickpea genotypes across all *S. sclerotiorum* isolates. The vertical bars represent the least significant differences (LSD with α = 0.05). The control indicates the lesion length of mock-inoculated plants with a PDA-agar plug without *S. sclerotiorum* mycelium. Different letters above bars represent significant differences between treatments at a significance level of 0.05.

2.5.2. Wild *Cicer* accessions exhibit a wide range of responses to *S. sclerotiorum* inoculation

To investigate resistance levels of 86 wild *Cicer* accessions and two Australian cultivars to *S. sclerotiorum*, the AUDPC value was determined after inoculation with the highly aggressive (CU8.20) or the moderately aggressive (CU10.12) isolate. We used a factorial nested ANOVA approach to partition variance within and between species, collection sites and accessions and to identify interaction with isolate type and orthogonal contrasts to define differences between wild and domestic *Cicer* and between the two wild species (Supplementary Table S2.2).

Orthogonal contrasts revealed large differences between wild and domestic responses and isolate type (Supplementary Table S2.2, $P<0.006$). *C. echinospermum* and *C. reticulatum* were both more resistant to CU10.12 than domestic chickpea ($P<0.001$), whereas there was no wild by domestic differences for the more aggressive CU8.20 ($P_{diff}=0.735$). Within these species, there was a broad range of responses to both isolates which are examined individually (Fig. 2.2A and B, Supplementary Table S2.3) and in terms of interaction (see Fig. 2.3). AUDPC scores of individual accessions ranged from 67.5 to 897.5 for highly aggressive CU8.20 (Fig. 2.2A) and 35.0 to 615.0 for moderately aggressive isolate CU10.12 (Fig. 2.2B). There was a far wider range of responses to the highly aggressive isolate compared to the moderately aggressive isolate. The cultivar PBA HatTrick had lower AUDPC scores to both isolates compared to the cultivar Kyabra, with the difference to isolate CU10.12 being significant (Fig. 2.2). Several wild *Cicer* accessions displayed low mean AUDPC scores to both isolates (*C. echinospermum* accession Cermi_073 and *C. reticulatum* accessions Sarik_073, CudiB_018 and Egill_073). In contrast, accessions Deric_062, Besev_074, Deste_063 and S2Drd_065 showed high mean AUDPC scores and were classed as susceptible to both isolates. The conditional Wald statistic test of the fixed effects from the fitted model provided strong evidence to suggest that the main effects of accession and isolate are statistically significant (P-value < 0.001), and the interactive effect between cultivar and the isolate was statistically significant (P-value = 0.0099).

To visualize this interaction between accessions and isolates directly, we plot mean AUDPC scores of the aggressive (CU8.20) against the moderately aggressive (CU10.12) and include a 1:1 line for reference (Fig. 2.3). (Accessions within 1 LSD of the 1:1 line show no significant isolate interaction, those significantly larger or smaller favour one isolate over the other). Thus, in domestic chickpea, Kyabra shows no interaction, being consistently sensitive to both isolates, while PBA HatTrick was resistant to CU10.12 and moderately resistant to CU8.20.

By plotting species separately and identifying collection site membership, it becomes possible to visualize the variance partitioning identified by the nested ANOVA model (Supplementary Table S2.2).

The greater aggression of CU8.20 over CU10.12 is indicated by higher AUDPC scores (i.e. significantly above the 1:1 line) in most accessions of both wild species (Fig. 2.3A and 2.3B). Most *C. echinospermum* accessions showed resistance to the moderately aggressive isolate and a wide range of responses to the highly aggressive isolate (Fig. 2.3A). Exceptions include Ortanca accessions, which are moderately resistant to both isolates, and Destek 071, which was resistant to the aggressive CU8.20 but sensitive to the less aggressive CU10.12. *C. echinospermum* collection sites were mostly similarly resistant to CU10.12 (except for Ortanca and Destek, mentioned above), but did not discriminate well for responses to the more aggressive CU8.20. Cermik, Karabahce and particularly Destek responses to CU8.20 ranged from susceptible to resistant (Fig. 2.3A), highlighting the significant variance within collection sites. Only the Gunasan, Ortanca and, to a lesser extent, Siv-Diyar accessions responded in a uniform way.

C. reticulatum showed a wider range of responses to both isolates (Fig. 2.3B). Accessions within one LSD of 1:1 line were similarly responsive to both isolates, ranging from consistently resistant (Sarik_073, Deric_075, CudiB_018) to consistently moderately susceptible (Bari3_106D, CudiA_127, Bari2_062). Cudi B was the only collection site returning consistently tolerant accessions to both isolates (but was represented by only two accessions in this study). Most *C. reticulatum* accessions had strong isolate response interactions: typically resistant to CU10.12 but highly or moderately susceptible to CU8.20 (Fig. 2.3B). However, this behaviour was not well defined by the collection site: most *C. reticulatum* collection sites show a wide range of accession responses to CU8.20 (e.g. Kayatepe, Oyali). Only the Kesentas accessions showed consistent isolate responses: resistant to CU10.12, moderately resistant to CU8.20. Other collection sites showed the opposite response: stable for CU8.20 but variable for CU10.12. Thus, Baristepe 1, Cudi A, and Egil accessions were all moderately susceptible to the highly aggressive CU8.20 but varied in response to CU10.12 (Fig. 2.3B). Finally, some collection sites such as Baristepe 2, Sarikaya and Kalkan returned widely different accession responses to both isolates.

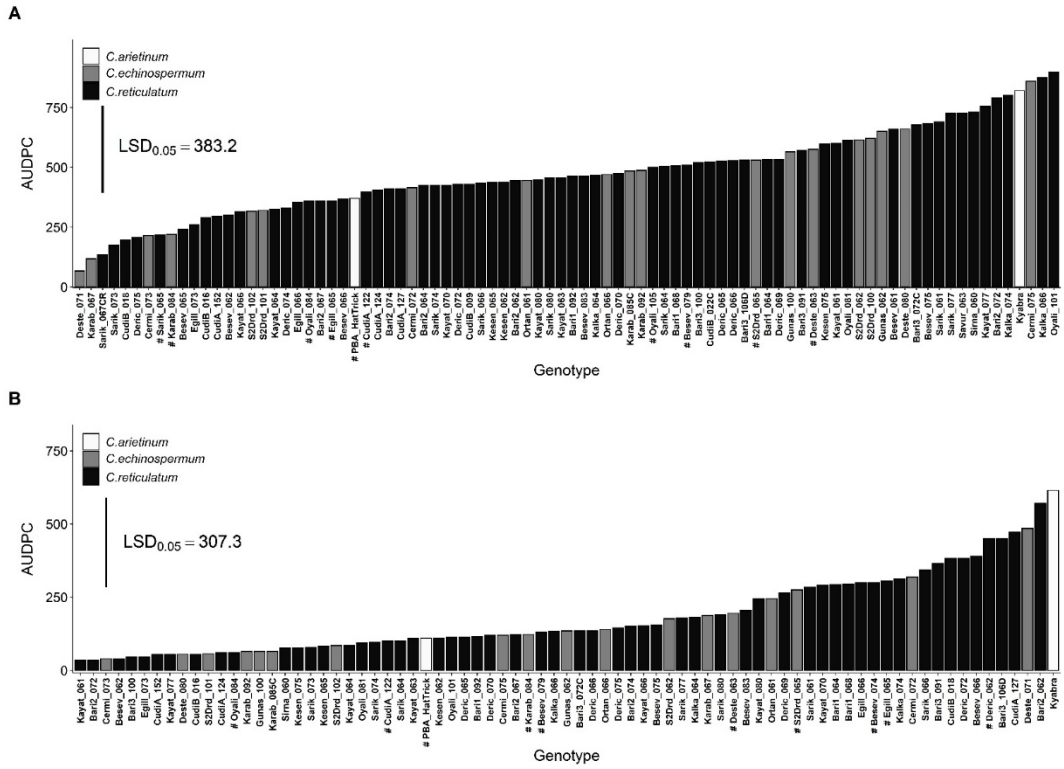


Figure 2.2: The area under the disease progress curve (AUDPC) for 86 wild *Cicer* accessions and two domestic cultivars inoculated with *S. sclerotiorum* isolate (A) CU8.20 representing a highly aggressive isolate ($LSD_{0.05}$ = 383.2). (B) CU10.12 representing moderately aggressive isolate ($LSD_{0.05}$ = 307.3). The mean AUDPC scores are derived from four replicates per accession per isolate, where accessions shaded black represent *C. reticulatum*, grey represent *C. echinospermum* and white represent *C. arietinum* species. The accessions highlighted with a hash (#) for both CU8.20 and CU10.12 indicate the genotypes selected for re-screening.

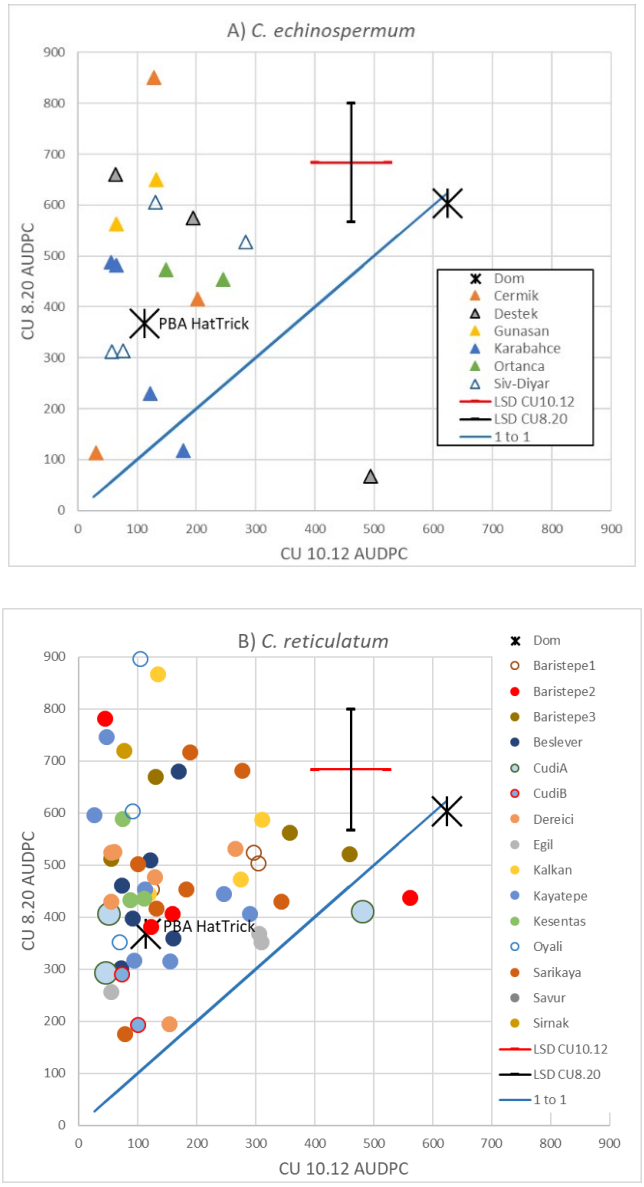


Figure 2.3: Interaction between accessions and isolates represented by plotting AUDPC accession mean values of CU8.20 (highly aggressive) against CU10.12 (moderately aggressive) *S. sclerotiorum* isolates. *C. echinospermum* (A, ▲) and *C. reticulatum* (B, ●) are plotted separately and collection sites identified by unique markers to assist in visualizing the variance partitioning among isolate interactions within/between species, collection sites within species and accessions indicated by nested ANOVA. *C. arietinum* (∗) is included on both A & B as domestic chickpea controls. Vertical and horizontal error bars represent least significant difference (LSD with $\alpha = 0.05$) values ($P < 0.05$) for CU8.20 and CU10.12, respectively. A 1:1 line (—) is included for reference to help identify significant interactions among accessions. (Accessions within 1 LSD of the 1:1 line show no significant isolate interaction, those significantly larger or smaller are favoured by one isolate over the other).

2.5.3. Two *Cicer echinospermum* accessions exhibit robust resistance to *S. sclerotiorum*

To further evaluate the wild material, 11 *Cicer* accessions with diverse responses to the two isolates in the previous experiment were selected, including the moderately resistant *C. arietinum* cultivar PBA HatTrick as a control. Analysis of variance for AUDPC of a subset of the wild *Cicer* accessions showed Karab_084 and Deste_063 resistance responses to *S. sclerotiorum* to be significantly different at 12 dpi ($P < 0.05$) from nine other accessions (Fig. 2.4, Supplementary Table S2.4), while the mean AUDPC scores were significantly lower for Karab_084 and Deste_063 compared to Egill_065 and Deric_062 ($LSD_{0.05} = 226.6$; Fig. 2.4).

Based on AUDPC, *C. echinospermum* accession Karab_084 exhibited strong resistance to *S. sclerotiorum* as compared to accessions Besev_074, Oyali_084, Besev_079, S2Drd_065, Egill_065 and Deric_062 (Fig. 2.4). The two accessions Karab_084 and Deste_063, were screened for resistance to the previously characterised nine *S. sclerotiorum* isolates (Fig. 2.5). The isolate CU8.20 and CU10.12 were found to be significantly ($P < 0.05$) different in aggressiveness to both Karab_084 ($LSD_{0.05} = 537.6$) (Fig. 5A) and Deste_063 ($LSD_{0.05} = 797.9$) (Fig. 2.5B), confirming our previous findings.

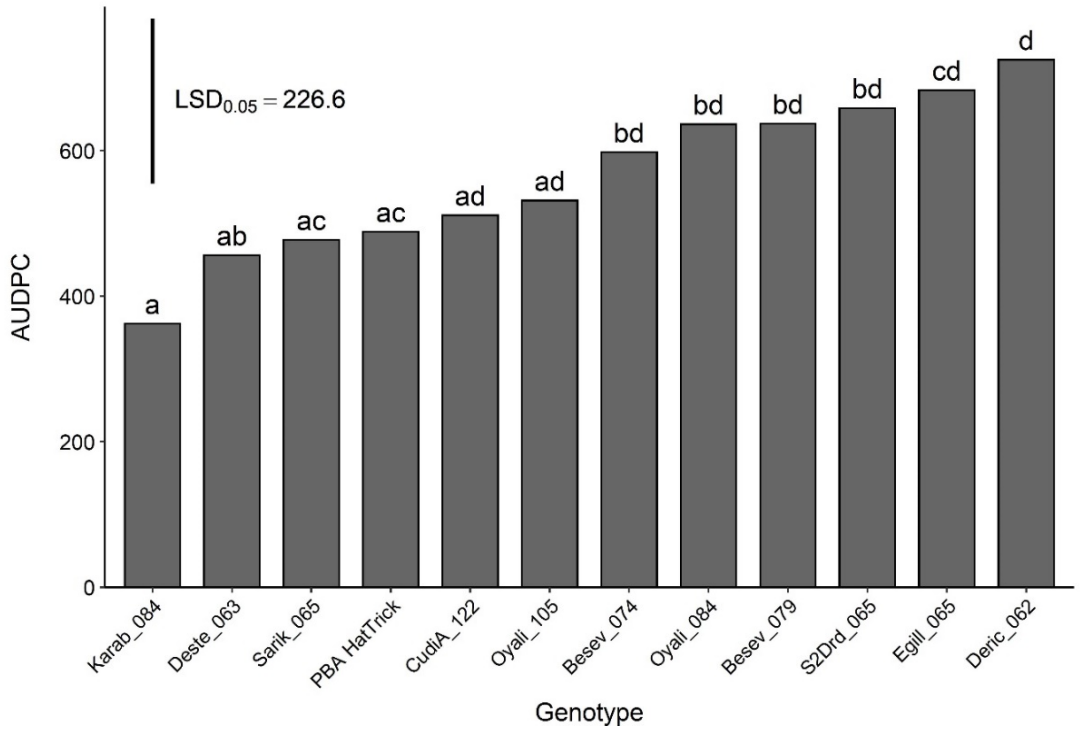


Figure 2.4: Area under disease progress curve (AUDPC) for 11 selected wild *Cicer* accessions and one *C. arietinum* cultivar (PBA HatTrick) inoculated with the highly aggressive *S. sclerotiorum* isolate CU8.20. The mean AUDPC values are derived from 12 replicates per accession/cultivar. The vertical bar represents the average least significant difference (LSD with $\alpha = 0.05$) value across all accessions. Different letters above bars of each genotype time point represent significant differences at a significance level of 0.05.

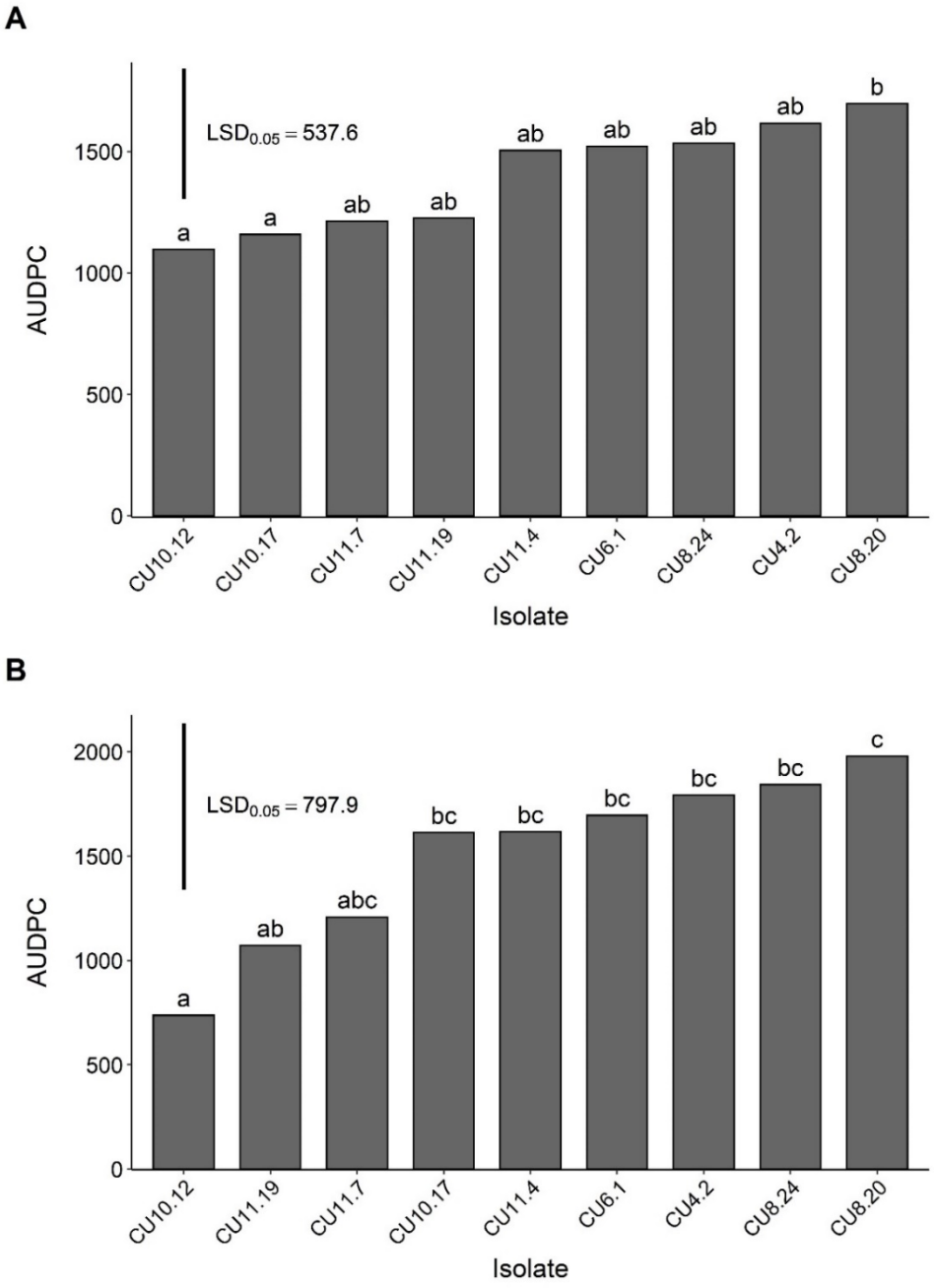


Figure 2.5: The mean area under the disease progression curve (AUDPC) scores for wild *Cicer* accessions (A) Karab_084 and (B) Deste_063 following inoculation with nine *S. sclerotiorum* isolates. The mean AUDPC values are derived from 12 replicates per accession for each isolate. The vertical bar represents the average least significant difference (LSD with $\alpha = 0.05$) value across all isolates. Different letters above bars of each isolate represent significant differences at a significance level of 0.05.

2.6. Discussion

This is the first study utilising the recent collection of wild *Cicer* species (von Wettberg *et al*. 2018) for the evaluation of Sclerotinia stem rot resistance. In the collection, we identified accessions with higher levels of partial resistance to *S. sclerotiorum*, including *C. reticulatum* and *C. echinospermum,* when compared to the cultivated *C. arietinum* cultivars PBA HatTrick and Kyabra. Two findings in our present study have important implications for future investigation of SSR on chickpea. First, a robust stem inoculation assay showed the diverse pathogenicity levels of Western Australian *S. sclerotiorum* isolates on chickpea. Second, wild *Cicer* accessions with improved resistance compared to moderately resistant domesticated cultivars were identified.

The status of resistance to SSR has been reported after screening various legume host crops. A study by Vuong *et al*. (2004) reported significant differences in *S. sclerotiorum* resistance in soybean (*Glycine max*) and dry bean (*Phaseolus vulgaris*) cultivars, which were screened following a cut stem inoculation method. To improve *S. sclerotiorum* management in pea (*Pisum sativum*), screening of a core collection for resistance to SSR under different controlled conditions identified two partially resistant accessions that could be used in pea breeding programs (Porter 2012). These reports indicate that partial resistance is an important attribute in breeding for pulses with SSR resistance and can be a vital resource in optimising breeding strategies to increase production.

Researchers have developed several methods of screening for SSR resistance on different hosts (Denton-Giles *et al*. 2018; Hoffman *et al*. 2002; Porter 2012). *S. sclerotiorum* inoculation methods can use either whole plant or different parts of the plant and inoculation with the fungus; however, the stem inoculation method has been more reliable (Li *et al*. 2009; Bennett *et al*. 2016; Denton-Giles *et al*. 2018). *S. sclerotiorum* naturally infects its host and limits the flow of water and nutrients through the xylem (Seifbarghi *et al.* 2017). Therefore, the stem inoculation method used in this study simulated the infection strategy naturally employed by *S. sclerotiorum* in the field (Denton-Giles *et al*. 2018; Garg *et al*. 2010; Uloth *et al*. 2013). In the current study, inoculation was conducted on the main stem just before chickpea flowering (Fuhlbohm *et al*. 2003). For these reasons, introgression of the identified resistance into chickpea cultivars would likely reduce natural *S. sclerotiorum* infection in the field.

The aggressiveness of a pathogen is its relative ability to attack, colonise and cause damage, and partial resistance of a host to a pathogen can result in a significant reduction of disease

rather than a total absence of the disease (Denton-Giles *et al*. 2018; Deacon 1997). Previous research has reported partial resistance and characterisation of *S. sclerotiorum* aggressiveness levels in different hosts, including canola (*B. napus*) (Denton-Giles *et al*. 2018), sunflower (*Helianthus annus* L.) (Seiler *et al.* 2017); Vuong *et al*. 2004), soybean (*G. max*) (Hoffman *et al.* 2002), pea (*P. sativum* L.) (Porter 2012) and dry beans (*P. vulgaris*) (Miorini *et al.* 2019). A recent study by Miorini *et al*. (2019) showed that *S. sclerotiorum* pathotypes could influence the outcome of host resistance assays. Therefore, knowledge of the aggressiveness of *S. sclerotiorum* isolates on chickpea is an important resource for any future chickpea - *S. sclerotiorum* infection study. The distinction in the aggressiveness of *S. sclerotiorum* isolates in different plant species was previously shown to be due to the physiological specialisation of the pathogen (Garg *et al*. 2010). For instance, two separate studies on *B. napus* (Taylor *et al*. 2015; Denton-Giles *et al*. 2018) and one study on dry beans (Viteri *et al.* 2015) found *S. sclerotiorum* isolates to have different levels of pathogenicity on these host crops.

The nine Australian isolates used in this study showed different levels of aggressiveness in chickpea, similar to previous isolate aggressiveness classifications found by Denton-Giles *et al*. (2018). However, *S. sclerotiorum* isolate CU11.19 was found to be moderately aggressive in our study and highly aggressive in *B. napus* Denton-Giles *et al*. (2018). This suggests some degree of isolate-host specialisation, which is reported as a possible *S. sclerotiorum* characteristic (Liang and Rollins 2018); however, further work is needed with a greater range of chickpea genotypes to test this hypothesis. Aggressiveness variations of *S. sclerotiorum* when infecting different hosts have been reported in other regions of the world. For instance, isolates collected from different plant species in the UK exhibited a different range of aggressiveness when inoculated onto *B. napus* (Taylor *et al*. 2015). In the current study, differential levels of pathogenicity of nine Australian isolates on chickpea were demonstrated, and the results enabled the selection of two isolates, moderately and highly aggressive, for screening a collection of wild *Cicer* germplasm.

In a range of crops, adoption of partial resistance to SSR is an alternative to improve susceptible cultivars, especially in crop species with low genetic diversity and high disease pressure (Hoffman *et al*. 2002; Denton-Giles *et al*. 2018; Porter 2012). Wild *Cicer* spp. offer desirable resistance to multiple chickpea diseases compared to *C. arietinum* (Croser *et al*. 2003). Improved resistance to ascochyta blight and root-lesion nematodes has been found in *C. echinospermum* (Newman *et al*. 2020; Reen *et al*. 2019; Collard *et al*. 2003). Despite not finding total resistance to SSR, our findings demonstrate that wild *Cicer* accessions also

possess promising sources of *S. sclerotiorum* resistance, with *C. echinospermum* accession Karab_084 showing a greater level of resistance among the 88 genotypes assessed. Plant breeders look for resistance to specific diseases by screening available germplasm for resistance, but more often than not, complete resistance is not identified (Li *et al*. 2015). In the current study, a quantitative continuum of responses to SSR was observed among wild *Cicer* accessions. However, it must be recognized that this study represents only the beginning of the characterization of wild *Cicer* as a source of resistance. While the numbers of accessions screened in this study are large in a historical context, far exceeding the world's germplasm resource prior to this collection (Berger *et al.* 2003) (Berger *et al*., 2003), there is much more material that remains to be evaluated in both *C. reticulatum* and *C. echinospermum*. Our wild chickpea germplasm resistance assessment strategy has been employed in previous studies aiming to identify improved resistance to chickpea diseases from wild *Cicer* accessions (Newman *et al*. 2020; Reen *et al*. 2019; Croser *et al*. 2003; Knights *et al*. 2008). To the authors' knowledge, this is the first report of resistance to SSR in wild *Cicer* accessions.

The wild *Cicer* accessions used in the current study were collected from 21 sites with a narrow geographical area and a wide range of physical and environmental properties such as location, altitude, slope, climate and soil type (von Wettberg *et al.* 2018). Our data demonstrate that it is very important to consider the collection site when searching for SSR resistance because there has clearly been selection for different disease responses across sites. However, these interactions are complex and resist classification along climatic or even geographic lines. For instance, accessions from the three Baristepe collection sites showed very different responses to *S. sclerotiorum* despite being collected in an apparently homogenous environment within 5 km of each other. *S. sclerotiorum* is a pathogen with a wide range of hosts (Boland and Hall 1994); hence the disease will vary around the collection sites depending on the presence/absence of other hosts. While collection sites can have big effects, the present study indicates they are very hard to understand and probably reflect a complex interaction between geography, climate and biological history. This suggests that future sampling strategies should try to balance accessions and collection sites to maximise diversity in respect to SSR. Therefore, future collection of wild *Cicer* accessions may need to take passport data into consideration before embarking on screening studies to avoid unbalanced comparisons where collection sites are both under or over represented.

A range of tests for SSR resistance has been recommended for screening germplasm for diseases resistance (Taylor *et al*. 2015). The results in our study highlight the consistency of

found resistance in wild *Cicer* accession, Karab_084, after re-screening and analysing the rate of stem lesion development (calculated as AUDPC). Furthermore, the levels of aggressiveness of nine isolates initially used to develop the assay were also consistent when re-evaluating the two most resistant accessions (Karab_084 and Deste_063) with these isolates. This assessment revealed that there was a lack of genotype by isolate interaction. The analysis of resistance based on *S. sclerotiorum* responses in multiple experiments increased the confidence of identifying the most resistant genotype. Thus it can be concluded that an agar plug stem inoculation method employed herein can be used to robustly screen chickpea germplasm for partial stem resistance to *S. sclerotiorum* infection and that this aligns with studies in canola and other pulses (Denton-Giles *et al*. 2018; Webster *et al*. 2020)

Screening for SSR resistance in the field has been reported to be challenging due as it is highly dependent upon favourable weather conditions (Denton-Giles *et al*. 2018). We, therefore, recommend further studies on developing reproducible assays for field *S. sclerotiorum* resistance testing on chickpea. These assays will facilitate validation of the identified wild *Cicer* partial stem resistance for durability in the field. It is vital to identify resistance to local aggressive *S. sclerotiorum* isolates; however, it will also be interesting to test the resistance of the identified wild *Cicer* accessions using other national and international isolates. The development of recombinant inbred lines by crossing the partially resistant wild accession with a susceptible domestic cultivar will also enable genetic dissection of the underlying loci conferring resistance to *S. sclerotiorum* in wild *Cicer* species.

In conclusion, we have developed a robust phenotyping assay to screen domestic and wild *Cicer* species for their response to *S. sclerotiorum* infection. Using this approach, wild *Cicer* accessions have been identified that show partial levels of resistance to *S. sclerotiorum*. Introgression of the resistance into modern chickpea cultivars has the potential to improve resistance to this economically damaging disease. Furthermore, isolates with distinct levels of aggressiveness on *Cicer* species were identified, and these results provide further insight into the *S. sclerotiorum* – chickpea interaction and sources of partial resistance to improve future chickpea cultivars.

2.7. References

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

Abbo S, Berger J, Turner NC (2003) Viewpoint: Evolution of cultivated chickpea: four bottlenecks limit diversity and constrain adaptation. *Functional Plant Biology* **30**, 1081–1087.

Akem C, Kabbabeh S (1999) Screening for resistance to sclerotinia stem rot in chickpea: A simple technique.

Ashtari Mahini R, Kumar A, Elias EM, Fiedler JD, Porter LD, McPhee KE (2020) Analysis and Identification of QTL for Resistance to *Sclerotinia sclerotiorum* in Pea (Pisum sativum L.). *Frontiers in Genetics* **11**,587968.

Bardin SD, Huang HC (2001) Research on biology and control of Sclerotinia diseases in Canada1. *Canadian Journal of Plant Pathology* **23**, 88–98.

Barrantes W, López-Casado G, García-Martínez S, Alonso A, Rubio F, Ruiz JJ, Fernández-Muñoz R, Granell A, Monforte AJ (2016) Exploring New Alleles Involved in Tomato Fruit Quality in an Introgression Line Library of *Solanum pimpinellifolium*. *Frontiers in Plant Science* **7**,01172.

Berger J, Abbo S, Turner NC (2003) Ecogeography of Annual Wild *Cicer* Species. *Crop Science* **43**, 1076–1090.

Boland GJ, Hall R (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology* **16**, 93–108.

Boudhrioua C, Bastien M, Torkamaneh D, Belzile F (2020) Genome-wide association mapping of *Sclerotinia sclerotiorum* resistance in soybean using whole-genome resequencing data. *BMC Plant Biology* **20**,02401-8.

Brien C (2020) Employing asremlPlus, in conjunction with asreml, to calculate and use information criteria.

Brooks KD, Bennett SJ, Hodgson LM, Ashworth MB (2018) Narrow windrow burning canola (*Brassica napus* L.) residue for *Sclerotinia sclerotiorum* (Lib.) de Bary sclerotia destruction. *Pest Management Science* **74**, 2594–2600.

Chen W, Schatz B, Henson B, McPhee KE, Muehlbauer FJ (2006) First report of sclerotinia stem rot of chickpea caused by *Sclerotinia sclerotiorum* in North Dakota and Washington. *Plant Disease* **90**, 114–114.

Chen Y, Wang D (2005) Two Convenient Methods to Evaluate Soybean for Resistance to *Sclerotinia sclerotiorum*. *Plant Disease* **89**, 1268-1272.

Clarkson JP, Warmington RJ, Walley PG, Denton-Giles M, Barbetti MJ, Brodal G, Nordskog B (2017) Population Structure of *Sclerotinia subarctica* and *Sclerotinia sclerotiorum* in England, Scotland and Norway. *Frontiers in Microbiology* **8**,00490.

Collard BCY, Ades PK, Pang ECK, Brouwer JB, Taylor PWJ (2001) Prospecting for sources of resistance to ascochyta blight in wild *Cicer* species. *Australasian Plant Pathology* **30**, 271–276.

Collard BCY, Pang ECK, Ades PK, Taylor PWJ (2003) Preliminary investigation of QTLs associated with seedling resistance to ascochyta blight from *Cicer echinospermum*, a wild relative of chickpea. *Theoretical and Applied Genetics* **107**, 719–729.

Collard BCY, Pang ECK, Taylor PWJ (2003) Selection of wild *Cicer* accessions for the generation of mapping populations segregating for resistance to ascochyta blight. *Euphytica* **130**, 1–9.

Coyne CJ, Kumar S, Wettberg EJB von, Marques E, Berger JD, Redden RJ, Ellis THN, Brus J, Zablatzká L, Smýkal P (2020) Potential and limits of exploitation of crop wild relatives for pea, lentil, and chickpea improvement. *Legume Science* **2**, e36.

Croser J, Ahmad F, Clarke H, Siddique K (2003) Utilisation of wild *Cicer* in chickpea improvement - progress, constraints, and prospects. *Australian Journal of Agricultural Research* **54**, 429–444.

Deacon JW (1997) Glossary of Plant-Pathological Terms. By M. C. Shurtleff and C. W. Averre III. 24×16 cm. Pp. iv+361. St Paul: APS Press, 1997. Price h/b: $US72, ISBN 0 89054 176 0. *New Phytologist* **137**, 371–372.

Denton-Giles M, Derbyshire MC, Khentry Y, Buchwaldt L, Kamphuis LG (2018) Partial stem resistance in *Brassica napus* to highly aggressive and genetically diverse *Sclerotinia sclerotiorum* isolates from Australia. *Canadian Journal of Plant Pathology* **40**, 551–561.

Fuhlbohm MJ, Tatnell JR, Ryley MJ (2003) First report of stem rot and wilt of chickpea caused by *Sclerotinia minor* in Queensland, Australia. *Australasian Plant Pathology* **2**, 323–324.

Garg H, Li H, Sivasithamparam K, Kuo J, Barbetti MJ (2010) The infection processes of *Sclerotinia sclerotiorum* in cotyledon tissue of a resistant and a susceptible genotype of *Brassica napus*. *Annals of Botany* **106**, 897–908.

Haware MP (1990) Fusarium wilt and other important diseases of chickpea in the Mediterranean area. Conference Proceedings, FAO-UN : 61–64.

Hoffman DD, Diers BW, Hartman GL, Nickell CD, Nelson RL, Pedersen WL, Cober ER, Graef GL, Steadman JR, Grau CR, Nelson BD, del Rio LE, Helms T, Anderson T, Poysa V, Rajcan I, Stienstra WC (2002) Selected soybean plant introductions with partial resistance to *Sclerotinia sclerotiorum*. *Plant Disease* **86**, 971–980.

Jeger MJ, Viljanen-Rollinson SLH (2001) The use of the area under the disease-progress curve (AUDPC) to assess quantitative disease resistance in crop varieties. *Theoretical and Applied Genetics* **102**, 32–40.

Jukanti AK, Gaur PM, Gowda CLL, Chibbar RN (2012) Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. *British Journal of Nutrition* **108**, S11–S26.

Kenward MG, Roger JH (1997) Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* **53**, 983–997.

Kim HS, Sneller CH, Diers BW (1999) Evaluation of soybean varieties for resistance to sclerotinia stem rot in field environments. *Crop Science* **39**, 3900010010x.

Knights EJ, Southwell RJ, Schwinghamer MW, Harden S (2008) Resistance to *Phytophthora medicaginis* Hansen and Maxwell in wild *Cicer* species and its use in breeding root rot resistant chickpea (*Cicer arietinum* L.). *Australian Journal of Agricultural Research* **59**, 383–387.

Koseoglu K, Adak A, Sari D, Sari H, Oncu Ceylan F, Toker C (2017) Transgressive segregations for yield criteria in reciprocal interspecific crosses between *Cicer arietinum* L. and *C. reticulatum* Ladiz. *Euphytica* **213**, 116.

Kukreja S, Salaria N, Thakur K, Goutam U (2018) Fungal disease management in chickpea: current status and future prospects. 'Fungi and their role in sustainable development: current perspectives'. (Eds P Gehlot, J Singh) pp. 293–309. (Springer: Singapore)

Kull LS, Vuong TD, Powers KS, Eskridge KM, Steadman JR, Hartman GL (2003) Evaluation of resistance screening methods for sclerotinia stem rot of soybean and dry bean. *Plant Disease* **87**, 1471–1476.

Leite ME, Dias JA, Souza DA de, Alves FC, Pinheiro LR, Santos JB dos, Leite ME, Dias JA, Souza DA de, Alves FC, Pinheiro LR, Santos JB dos (2016) Increasing the resistance of common bean to white mold through recurrent selection. *Scientia Agricola* **73**, 71–78.

Li CX, Liu SY, Sivasithamparam K, Barbetti MJ (2009) New sources of resistance to Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* in Chinese and Australian *Brassica napus* and *B. juncea* germplasm screened under Western Australian conditions. *Australasian Plant Pathology* **38**, 149–152.

Li H, Rodda M, Gnanasambandam A, Aftab M, Redden R, Hobson K, Rosewarne G, Materne M, Kaur S, Slater AT (2015) Breeding for biotic stress resistance in chickpea: progress and prospects. *Euphytica* **204**, 257–288.

Liang Y, Cason JM, Baring MR, Septiningsih EM (2020) Identification of QTLs associated with Sclerotinia blight resistance in peanut (*Arachis hypogaea* L.). *Genetic Resources and Crop Evolution*.

Liang X, Rollins JA (2018) Mechanisms of broad host range necrotrophic pathogenesis in *Sclerotinia sclerotiorum*. *Phytopathology*® **108**, 1128–1140.

Michael PJ, Lui KY, Thomson LL, Stefanova K, Bennett SJ (2020) Carpogenic germinability of diverse *Sclerotinia sclerotiorum* populations within the Southwestern Australian grain belt. *Plant Disease* **104**, 2891–2897.

Miorini TJJ, Kamvar ZN, Higgins RS, Raetano CG, Steadman JR, Everhart SE (2019) Differential aggressiveness of *Sclerotinia sclerotiorum* isolates from North and South America and partial host resistance in Brazilian soybean and dry bean varieties. *Tropical Plant Pathology* **44**, 73–81.

Miranda JH (2019) Single plant selection for improving root rot disease (*Phytophthora medicaginis*) resistance in Chickpeas (*Cicer arietinum* L.). *Euphytica* **215**, 91.

Muñoz N, Liu A, Kan L, Li M-W, Lam H-M (2017) Potential uses of wild germplasms of grain legumes for crop improvement. *International Journal of Molecular Sciences* **18**,18020328.

Newman TE, Jacques S, Grime C, Kamphuis FL, Lee RC, Berger J, Kamphuis LG (2020) Identification of novel sources of resistance to ascochyta blight in a collection of wild *Cicer* accessions. *Phytopathology*®. **111**, 369-379.

Ociepa T (2019) The oat gene pools – review about the use of wild species in improving cultivated oat. *Journal of Central European Agriculture* **20**, 251–261.

Perchepied L, Balagué C, Riou C, Claudel-Renard C, Rivière N, Grezes-Besset B, Roby D (2010) Nitric oxide participates in the complex interplay of defense-related signalling pathways controlling disease resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions*® **23**, 846–860.

Porter LD (2012) Pea Germplasm with partial resistance to *Sclerotinia sclerotiorum* Extends the time required by the pathogen to infect host tissue. *Crop Science* **52**, 1044–1050.

Porto ACM, Cardon CH, Vasconcellos RCC, Novaes E, Leite ME, Chalfun-Junior A, Pereira WA, Santos JB (2019) Expression of candidate genes related to white mold resistance in common beans. *Tropical Plant Pathology* **44**, 483–493.

Qi L, Long Y, Talukder ZI, Seiler GJ, Block CC, Gulya TJ (2016) Genotyping-by-sequencing uncovers the introgression alien segments associated with Sclerotinia basal stalk rot resistance from wild species—I. *Helianthus argophyllus* and *H. petiolaris*. *Frontiers in Genetics* **7**,00219.

Reen RA, Mumford MH, Thompson JP (2019) Novel sources of resistance to root-lesion nematode (Pratylenchus thornei) in a new collection of wild *Cicer* species (*C. reticulatum* and *C. echinospermum*) to improve resistance in cultivated chickpea (*C. arietinum*). *Phytopathology*® **109**, 1270–1279.

Seifbarghi S, Borhan MH, Wei Y, Coutu C, Robinson SJ, Hegedus DD (2017) Changes in the *Sclerotinia sclerotiorum* transcriptome during infection of *Brassica napus*. *BMC Genomics* **18**, 266.

Seiler GJ, Misar CG, Gulya TJ, Underwood WR, Flett BC, Gilley MA, Markell SG (2017) Identification of novel sources of resistance to sclerotinia basal stalk rot in south african sunflower germplasm. *Plant Health Progress* **18**, 87–90.

Shakiba E, Eizenga GC (2014) Unraveling the secrets of rice wild species. *Rice - Germplasm, Genetics and Improvement*. doi:10.5772/58393.

Siddique KHM, Erskine W, Hobson K, Knights EJ, Leonforte A, Khan TN, Paull JG, Redden R, Materne M (2013) Cool-season grain legume improvement in Australia—use of genetic resources. *Crop and Pasture Science* **64**, 347–360.

Singh KB, Ocampo B (1997) Exploitation of wild *Cicer* species for yield improvement in chickpea. *Theoretical and Applied Genetics* **95**, 418–423.

Smith AB, Cullis BR, Thompson R (2005) The analysis of crop variety breeding and evaluation trials: an overview of current mixed model approaches. *The Journal of Agricultural Science* **143**, 449–462.

Taylor A, Coventry E, Jones JE, Clarkson JP (2015) Resistance to a highly aggressive isolate of *Sclerotinia sclerotiorum* in a *Brassica napus* diversity set. *Plant Pathology* **64**, 932–940.

Thudi M, Chitikineni A, Liu X, He W, Roorkiwal M, Yang W, Jian J, Doddamani D, Gaur PM, Rathore A, Samineni S, Saxena RK, Xu D, Singh NP, Chaturvedi SK, Zhang G, Wang J, Datta SK, Xu X, Varshney RK (2016) Recent breeding programs enhanced genetic diversity in both desi and kabuli varieties of chickpea (*Cicer arietinum* L.). *Scientific Reports* **6**, 38636.

Uloth MB, You MP, Finnegan PM, Banga SS, Banga SK, Sandhu PS, Yi H, Salisbury PA, Barbetti MJ (2013) New sources of resistance to *Sclerotinia sclerotiorum* for crucifer crops. *Field Crops Research* **154**, 40–52.

Viteri DM, Otto K, Terán H, Schwartz HF, Singh SP (2015) Use of four *Sclerotinia sclerotiorum* isolates of different aggressiveness, three inoculations per plant, and delayed multiple evaluations to select common beans with high levels of white mold resistance. *Euphytica* **204**, 457–472.

Vuong TD, Hoffman DD, Diers BW, Miller JF, Steadman JR, Hartman GL (2004) Evaluation of soybean, dry bean, and sunflower for resistance to *Sclerotinia sclerotiorum*. *Crop Science* **44**, 777–783.

Webster RW, Roth M, Reed H, Mueller B, Groves C, McCaghey M, Chilvers MI, Mueller DS, Kabbage M, Smith DL (2020) Identification of soybean (*Glycine max*) check lines for evaluating genetic resistance to sclerotinia stem rot. *Plant Disease*. doi:10.1094/PDIS-10-20-2193-RE.

von Wettberg EJB, Chang PL, Başdemir F, Carrasquila-Garcia N, Korbu LB, Moenga SM, Bedada G, Greenlon A, Moriuchi KS, Singh V, Cordeiro MA, Noujdina NV, Dinegde KN, Shah Sani SGA, Getahun T, Vance L, Bergmann E, Lindsay D, Mamo BE, Warschefsky EJ, Dacosta-Calheiros E, Marques E, Yilmaz MA, Cakmak A, Rose J, Migneault A, Krieg CP, Saylak S, Temel H, Friesen ML, Siler E, Akhmetov Z, Ozcelik H, Kholova J, Can C, Gaur P, Yildirim M, Sharma H, Vadez V, Tesfaye K, Woldemedhin AF, Tar'an B, Aydogan A, Bukun B, Penmetsa RV, Berger J, Kahraman

A, Nuzhdin SV, Cook DR (2018) Ecology and genomics of an important crop wild relative as a prelude to agricultural innovation. *Nature Communications* **9**, 649.

Xing Q, Dekker S, Kyriakopoulou K, Boom RM, Smid EJ, Schutyser MAI (2020) Enhanced nutritional value of chickpea protein concentrate by dry separation and solid state fermentation. *Innovative Food Science & Emerging Technologies* **59**, 102269.

CHAPTER 3

Identification of Sclerotinia stem rot resistance quantitative trait loci in a chickpea recombinant inbred line population

Virginia W. Mwape[1, 2], Kelvin H. P. Khoo [3], Kefei Chen[4], Yuphin Khentry[1], Toby. E. Newman[1], Mark C. Derbyshire[1], Diane E. Mather[4], and Lars G. Kamphuis[1, 2]

[1] Centre for Crop Disease Management, Curtin University, Bentley WA 6102, Australia.

[2] Commonwealth Scientific and Industrial Research Organization. Agriculture and Food, Floreat, WA, 6913, Australia.

[3] School of Agriculture, Food and Wine, Waite Research Institute, University of Adelaide, Urrbrae SA 5064, Australia.

[4] Statistics for the Australian Grains Industry -West, Curtin University, Bentley, WA 6102, Australia.

This chapter consist of work that has been submitted to *Functional Plant Biology* journal

3.1 Abstract

Sclerotinia stem rot (SSR), caused by *Sclerotinia sclerotiorum*, is an economically important diseases in chickpea (*Cicer arietinum* L). No complete resistance is available in chickpea to this disease, and the inheritance of partial resistance is not understood. Two hundred F_7 recombinant inbred lines (RILs) derived from a cross between a partially resistant cultivar PBA HatTrick, and a highly susceptible cultivar Kyabra were characterised for one year for their responses to SSR inoculation. Quantitative trait locus (QTL) analysis was conducted for the area under the disease progress curve (AUDPC) after RIL infection with *S. sclerotiorum*. Four QTLs on chromosomes, Ca4 (qSSR4-1, qSSR4-2), Ca6 (qSSR6-1) and Ca7 (qSSR7-1), individually accounted for between 4.2 and 15.8 % of the total estimated phenotypic variation for the response to SSR inoculation. Candidate genes located in these QTL regions are predicted to be involved in a wide range of processes, including phenylpropanoid biosynthesis, plant-pathogen interaction, and plant hormone signal transduction. This is the first study investigating the inheritance of resistance to *S. sclerotiorum* in chickpea. Markers associated with the identified QTLs could be employed for marker-assisted selection in chickpea breeding.

Keywords: Chickpea, disease resistance, quantitative trait locus analysis, Sclerotinia stem rot, polygenic disease resistance.

3.2 Background

Chickpea (*Cicer arietinum* L.) is a self-pollinated diploid (2n = 2x = 16) pulse crop with a genome size of approximately 738 Mb (Varshney *et al.* 2013). Chickpea is produced in over 50 countries, including India, Australia, USA, Canada, Turkey and Ethiopia, and is third in the world among pulse crops in production, behind dry bean (*Phaseolus vulgaris* L.) and field pea (*Pisum sativum* L.) (Merga and Haji 2019). Together with other pulse crops such as soybean (*Glycine max* L.), lupin (*Lupinus* spp.), and lentil (*Lens culinaris* L.), chickpea contributes a significant amount of protein to a plant-based diet, particularly in low-income countries (Bohra *et al.* 2014). Chickpea is also a valuable source of micronutrients such as phosphorous, calcium, magnesium, iron and zinc (Jukanti *et al.* 2012). Additionally, through symbiosis with rhizobacteria, chickpea plants are able to fix up to 80% of their nitrogen requirement (Gaur *et al.* 2012).

Chickpea is produced under diverse agro-ecological conditions, and achieving stable yields is constrained by various abiotic and biotic stresses (Jha *et al.* 2014; Abbo *et al.* 2003). Currently, chickpea is produced on approximately 14 Mha, with an average production of 760 kg per ha globally (FAOSTAT, 2019). Research has shown that a chickpea crop that is free from biotic and abiotic stresses can produce up to 3,500 kg per ha (Merga and Haji 2019). Among the biotic constraints affecting chickpea are diseases such as Fusarium wilt, Ascochyta blight, Botrytis grey mould and Sclerotinia stem rot (SSR), caused by *Fusarium oxysporum* f. sp. *ciceris*, *Ascochyta rabiei, Botrytis cinerea*, and *Sclerotinia sclerotiorum,* respectively (Knights and Hobson 2016). Increasing resistance to diseases would both enhance and stabilise yields in chickpea production throughout the world. In addition, the identification of molecular markers associated with resistance may help to speed up the breeding process.

In Australia, SSR is a destructive chickpea disease that can cause up to 100% yield loss under conducive conditions (Pulse Australia, 2020). Fuhlbohm *et al.* (2003) reported the first incidence of SSR in chickpea in eastern Australia. Since then, the pathogen *S. sclerotiorum* has emerged as a threat in all Australian chickpea growing regions due to its remarkably diverse hosts that include cultivated crops such as canola and weeds such as wild radish (Boland and Hall 1994). The cultivation of chickpea as a break crop in rotation with canola (*Brassica napus*), an important *S. sclerotiorum* host, increases the incidence of disease outbreaks in chickpea crops. In Australia, SSR can be controlled in canola with timely fungicide applications, but there are no fungicides registered for controlling SSR in chickpea (Pulse Australia, 2020). Further, the *S. sclerotiorum* resting structures (sclerotia) can survive in the

soil for over seven years (Lane *et al.* 2019; Brooks *et al.* 2018). Management of SSR in chickpea requires observing cultural practices such as crop rotation, which can sometimes be ineffective due to *S. sclerotiorum*'s broad host range (Boland and Hall 1994). Therefore, exploring the feasibility of breeding for disease resistance to *S. sclerotiorum* infection should be explored.

No sources of resistance to *S. sclerotiorum* have been identified in chickpea. However, lines with partial resistance to various chickpea diseases have been identified and were successfully used in chickpea breeding to develop new resistant cultivars. QTL mapping of an intra-specific recombinant inbred line (RIL) population identified major and minor QTLs responsible for both Fusarium wilt and Ascochyta blight resistance (Garg *et al.* 2018). Single dominant and recessive genes controlling Ascochyta blight have been reported in chickpea (Li *et al.* 2017; Dey and Singh 1993; Tewari and Pandey 1986). Another study involving intraspecific crosses of desi and kabuli chickpea cultivars identified three QTLs responsible for Botrytis grey mould (Anuradha *et al.* 2011) caused by *Botrytis cinerea*, a closely related pathogen of *S. sclerotiorum*.

Cultivated chickpea has a high morphological diversity but narrow genetic variation for trait improvement (Udupa *et al.* 1993; Abbo *et al.* 2003); therefore, research on whether SSR resistance alleles exist in this narrow gene pool is needed. Recently, a RIL population derived from Australian cultivars PBA HatTrick and Kyabra, (developed within the plant growth facilities at University of Western Australia in 2016) was employed to map QTLs associated with resistance to the root-lesion nematode *Pratylenchus thornei* (Khoo et al. submitted). The parents of this population differ in their responses to *S. sclerotiorum* infection, with PBA HatTrick having partial stem resistance and Kyabra showing high susceptibility (Mwape *et al.* 2021a; Mwape *et al.* 2021b). In the research reported here, the F_7 generation of that population was used to investigate the response of 200 F_7 RILs to *S. sclerotiorum* inoculation and to map QTLs associated with SSR resistance.

3.3 Materials and Methods

3.3.1 Plant material and growth conditions

The Australian desi chickpea cultivars PBA HatTrick and Kyabra were obtained from the Australian Grains Genebank (AGG, Horsham, Victoria, Australia). An additional 18 Australian chickpea cultivars and 29 breeding lines were obtained from Dr. Kristy Hobson of the Chickpea Breeding Australia (CBA) program at the New South Wales Department of Primary Industries

(NSW-DPI, Tamworth, NSW, Australia). An F_7 recombinant inbred line (RIL) population (n=200) derived from cultivars PBA HatTrick and Kyabra (Khoo et al., submitted) was used. One seed per pot were sown in 4 litres (L) pots filled with all-purpose potting mix (UWA plant biology mix, Richgro, Perth, WA, Australia). Plants were watered regularly for optimal growth, and at four weeks, they were fertilised with Nitrophoska Perfect^{TM} fertiliser (Incitec Pivot fertilisers, Victoria, Australia). The experiment to evaluate the 49 chickpea lines was conducted in a hoop house environment under natural light and an average temperature of 24 °C day/ 18 °C night at the field trial area, Curtin University, Bentley, WA, Australia (32° 0' 19.272" S, 115° 53' 38.144" E) between June 2019 and August 2019. The F_7 RIL population, along with their parental lines PBA HatTrick and Kyabra, were screened for SSR response under a controlled greenhouse environment, under natural light and an average temperature of 24 °C day/ 18 °C night, at the Shenton Park field station of the University of Western Australia (31° 57' 2.2" S, 115° 47' 52.3" E) from September to November 2019.

3.3.2 *Sclerotinia sclerotiorum* inoculum production

The isolate CU8.20 is a highly aggressive isolate of *S. sclerotiorum* collected from *B. napus* fields in Western Australia and has been tested and found to be pathogenic to *B. napus* and chickpea (Denton-Giles *et al.* 2018; Mwape *et al.* 2021a). Single sterile sclerotia were dissected and germinated on a 9 cm Petri dish containing 39g/L potato dextrose agar (PDA) (Becton Dickinson, NJ, USA) and incubated at 20 °C for 5-7 days in the dark to produce inoculum from actively growing culture. The cultures were further sub-cultured by using a sterile cork borer and forceps to transfer 5 mm agar plugs from the original plates onto fresh media for two days at 20 °C to source actively growing mycelia for plant inoculation.

3.3.3. Evaluation of the response to *Sclerotinia sclerotiorum* infection

Stem inoculation was conducted on eight-week-old plants following a stem inoculation assay previously described in *B. napus* by Denton-Giles *et al.* (2018) and adapted for chickpeas as described by Mwape *et al.* (2021a). Briefly, a 5 mm PDA plug with *S. sclerotiorum* mycelium from the leading edge of the culture was cut using a sterile cork borer, and a sterile metal spatula was used to transfer the plug onto Parafilm. The plug was placed upright on a strip of Parafilm® and wrapped around the middle of the main stem of an individual plant, with the mycelium making direct contact with the chickpea stem. Plants were phenotyped by measuring stem lesion length development over time. The stem lesion length was measured at 3, 7, 10, 14, 17, and 21 days after inoculation (dai) using a ruler. At the end of the assessments, the stem

lesion length data obtained were used to calculate the area under the disease progress curve (AUDPC) (Jeger and Viljanen-Rollinson 2001) for each line evaluated.

3.3.4 Experimental design

Randomised complete block designs (RCBD) were applied for both the hoop house evaluation of the 49 chickpea lines and the glasshouse evaluation of the RIL population to assess the susceptibility of all the chickpea genotypes to *S. sclerotiorum*. Negative controls with PDA-only agar plugs were included for all experiments. The experiment assessing 49 chickpea lines for *S. sclerotiorum* resistance was designed with three replicates per line, and the experiment evaluating the RIL population with four replicates per line.

3.3.5 Statistical analysis

For both the evaluation of the 49 chickpea lines and the RIL population, linear mixed models (LMM) were fitted using ASReml-R (Butler *et al.* 2018) to examine spatial variations, including local autocorrelations, global trends and extraneous variations. The cultivar, isolate, and the interaction term between cultivar and isolate were fitted as fixed effects (Smith *et al.* 2005). The blocking structures of the experiments were fitted as random effects. Spatial trends and residual variances with auto-regressive correlations at first-order for rows and columns were examined and fitted when the global trends and autocorrelations were significant. Likelihood ratio tests were used for random effects, and conditional Wald tests (Kenward and Roger 1997) were used for fixed effects. Residual diagnostics were performed to examine the validity of the model assumptions of normality and homogeneity of variance. For each of the fitted models, the empirical best unbiased linear estimates (eBLUEs) were produced. The R package AsremlPlus (Brien 2020) was used to compute the least significant difference (LSD with $\alpha = 0.05$) values.

3.3.6 Genotyping and linkage mapping

Genotypic data of the RIL population were as described by Khoo *et al.* (submitted). Briefly, leaf tissue was sampled from the parents and individual F_6 plants. DNA was isolated and subjected to genotyping-by-sequencing analysis by Diversity Arrays Technology Pty. Ltd. (Bruce, ACT, Australia) using its chickpea DArTseq (1.0) GBS platform. A linkage map was constructed using the R package ASMap (Taylor and Butler 2017). Linkage groups were assigned to chromosomes and oriented based on BLASTn analysis (Altschul *et al.* 1990) of GBS sequence tags against the kabuli chickpea reference genome (Version 2.6.3) (Edwards, 2016).

3.3.7 QTL Mapping

QTL analysis was conducted using the inclusive composite interval mapping (ICIM) method, which is implemented in the integrated software for QTL mapping (QTL IciMapping v4.1) available at http://www.isbreeding.net/ (Meng *et al.* 2015). The QTL mapping was conducted using the functionality of inclusive composite interval mapping of additive and dominant QTL (ICIM-ADD) (Li *et al.* 2007, Zhang *et al.* 2008). The stepwise regression was performed to identify the most significant markers and marker-pairs at a significance level of 0.001 and a scanning step of 1 cM. The threshold LOD (logarithm of the odds) score to declare significant QTL at a chromosome-wise type I error rate of 0.05 (Churchill and Doerge 1994) were determined by performing 1,000 permutations.

3.3.8 Candidate gene identification

The CDC Frontier reference genome v2.6.3; http://www.cicer.info/databases.php/downloads/kabuli2.6.3rawdata.zip (Edwards 2016) was interrogated by filtering out the genes between the flanking markers of each QTL to identify candidate genes within the same intervals as the estimated QTL positions. Subsequently, the coding sequences (CDSs) were translated into amino acid sequences, and the Pfam database (http://pfam.xfam.org/) queried for putative domains to infer function (Finn *et al.* 2014; Bateman *et al.* 2002).

3.3.9 Expression of candidate resistance gene

Gene expression analysis was conducted to assess the candidate genes in the QTL regions during the infection of the two RIL parents, the moderately resistant (MR: PBA HatTrick) and susceptible (S: Kyabra) parents, using RNAseq data collected in a previous study (Mwape *et al.* 2021b). Briefly, the data was generated by stem inoculation of six-week-old plants with *S. sclerotiorum* isolate CU8.20 at 0 (control), 6, 12, 24, 48, and 72 hours post-inoculation with three biological replicates for each of the treatments. RNA extracted from stem segments was resequenced by Novogene (Beijing, China) on an Illumina HiSeq 2500 platform. The sequenced reads were aligned to the reference chickpea genome (Edwards, 2016) and gene expression analysis was conducted using the Limma package in R v4.0.2. A false discovery rate (FDR) cut-off of 0.05 was applied, and a $\log_2$ fold change cut-off of ≥ 1 to indicate upregulation and ≤ -1 to indicate down-regulation. The RNAseq data has been deposited as an NCBI sequence read archive under BioProject ID: PRJNA687280.

3.4 Results

3.4.1 Responses of Australian chickpea cultivars and breeding lines to Sclerotinia stem rot

A set of 20 cultivars and 29 breeding lines were evaluated for their responses to inoculation with *S. sclerotiorum* isolate CU8.20 to determine if there is any resistance to *S. sclerotiorum* in the Australian chickpea breeding program. Lesion length measurements over time were used to calculate the area under the disease progress curve (AUDPC) for each cultivar/breeding line. The 49 lines showed significant differences ($P < 0.05$, $LSD_{0.05} = 445.3$) in disease responses, with PBA HatTrick showing the lowest susceptibility with a mean AUDPC of 2,169 and Moti showing the highest susceptibility with a mean AUDPC of 5,221 (Fig.,1, Supplementary Table S3.1).

3.4.2 Responses of recombinant inbred line population derived from PBA HatTrick and Kyabra to Sclerotinia stem rot

The cultivars PBA HatTrick and Kyabra differed in their response to *S. sclerotiorum* isolate CU8.20, with mean AUDPCs of 2,169 and 4,281, respectively (Fig. 1). Therefore, a RIL population derived from these parents was inoculated with the same isolate to investigate the underlying genetic control of the partial resistance observed in PBA HatTrick. The Shapiro-Wilk test of normality ((Shapiro and Wilk 1965; Royston 1982) for the AUDPC of the RIL population indicated that the data were approximately normally distributed with the Shapiro-Wilk statistic of 0.9875 (P-value > 0.05)) (Fig. 2). The results showed that the AUDPC values were different for PBA HatTrick (3329) and Kyabra (3724) and a broader range of AUDPC variation among the RILs: AUDPC range of 2489 – 4609 (Fig. 2, Supplementary Table S3.1). The mean AUDPC scores across each individual RIL and the parents showed a continuous trait distribution with significant differences ($LSD_{0.05} = 885.3$) between the parents and among the RILs (Supplementary Table S3.2).

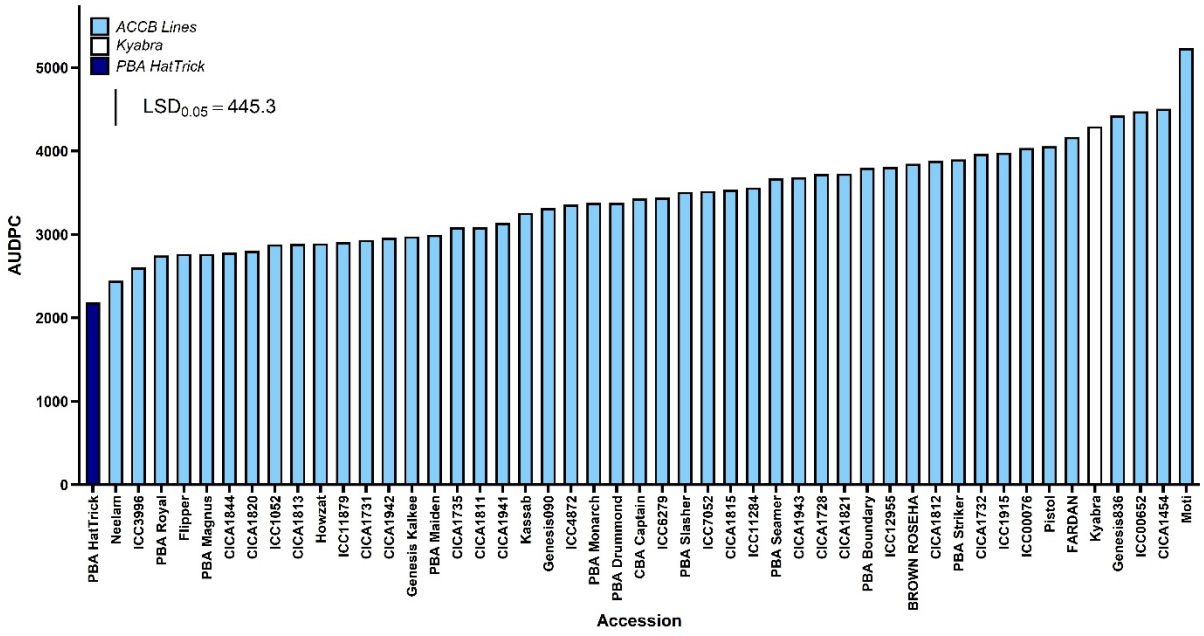


Figure 3.1: The mean area under the disease progress curve (AUDPC) scores for Australian chickpea cultivars and breeding lines following inoculation with an aggressive *S. sclerotiorum* isolate. The vertical bar represents the least significant difference (LSD with $\alpha = 0.05$) value across all genotypes (y-axis). Indicated in dark blue is the partially resistant cultivar PBA HatTrick and in white, the susceptible cultivar Kyabra that are the parents of the recombinant inbred line population used in this study.

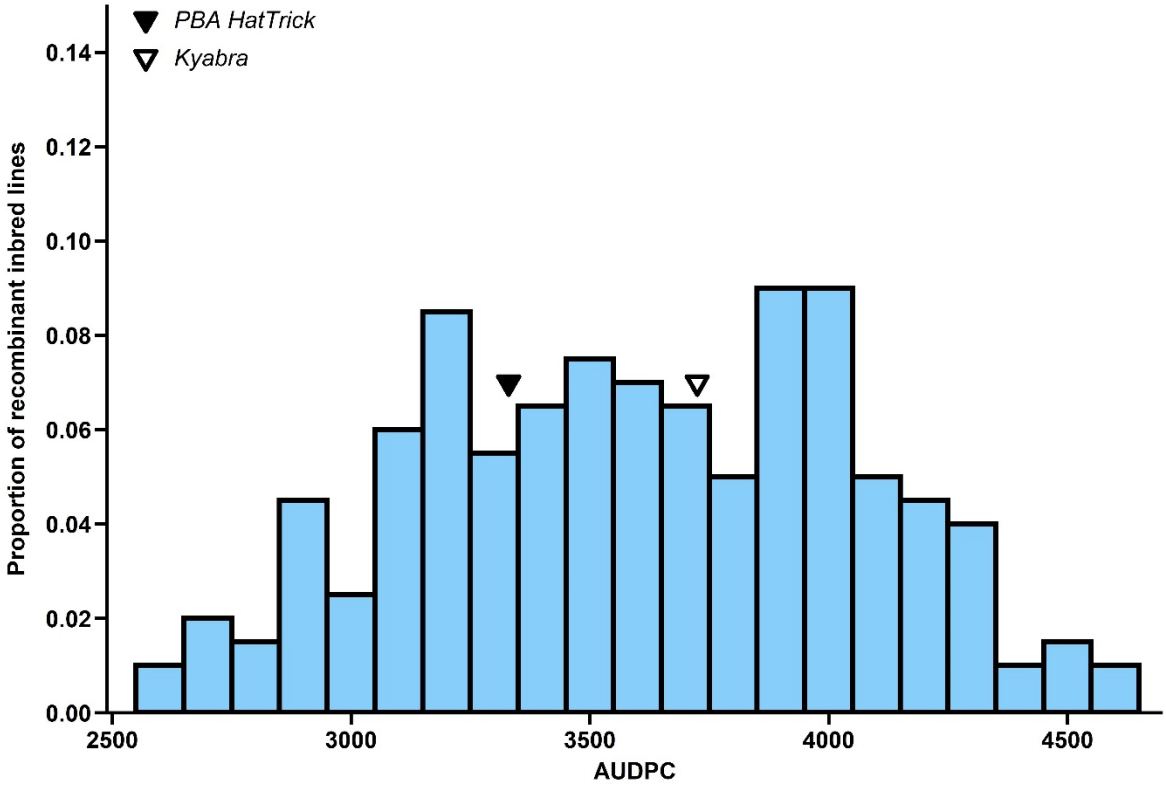


Figure 3.2: Phenotypic distribution of the area under the disease progress curve (AUDPC) after *S. sclerotiorum* inoculation of 200 RIL population and the parents. The x-axis shows the mean AUDPC, and the y-axis shows the RIL population and their parents. The white and black arrowheads indicate AUDPC scores for the highly susceptible parent (Kyabra) and moderately resistant parent (PBA HatTrick), respectively.

3.4.3 QTLs for resistance to Sclerotinia stem rot

QTL analysis to detect loci contributing to the observed variation in SSR resistance in the RIL population was conducted using the mean AUDPC values. In the present study, four QTLs, with phenotypic variation explained (PVE) ranging from 4.2 - 15.8 %, were detected and mapped on three chromosomes (Table 1 and Fig. 3): Ca4 (qSSR4-1, qSSR4-2), Ca6 (qSSR6-1) and Ca7 (qSSR7-1). The most significant of these was qSSR4.1 (PVE = 15.8 %, LOD = 10.6) between markers 11062500|F|0-36:G>A-36:G>A and 8822765|F|0-8:C>T-8:C>T (Fig. 3).

Table 3.1: List of quantitative trait loci (QTL) associated with resistance response to a highly aggressive *S. sclerotiorum* isolate in PBA HatTrick x Kyabra chickpea recombinant inbred line population (n = 200).

QTL name	Chromosome	Position (cM)	Left Marker	Right Marker	LOD^{+}	PVE^{b} (%)	Additive effect#
qSSR4-1	4	0	11062500	8822765	10.6188	15.8219	-193.286
qSSR4-2	4	74	5825802	5826178	3.0732	4.238	100.0106
qSSR6-1	6	2	5825910	13144413	3.2097	4.6532	104.7948
qSSR7-1	7	46	29967333	29967370	4.7374	6.7445	-126.295

LOD^{+} = Logarithm of the odds PVE^{b} =Phenotypic variance explained LG = Linkage group
#: The negative effects indicate that the favourable allele was derived from PBA HatTrick, and the positive additive effects indicate that the favourable allele was derived from Kyabra.

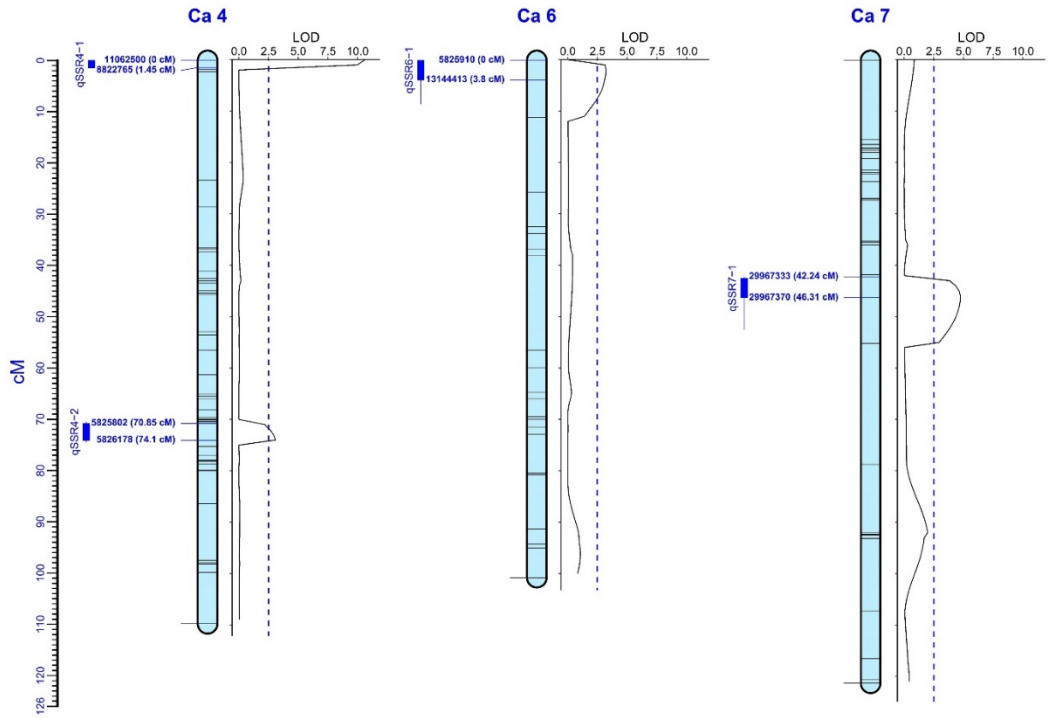


Figure 3.3: Genetic positions of the QTLs associated with Sclerotinia stem rot resistance on chromosomes Ca4, Ca6 and Ca7. Resistance is expressed using the area under the disease progress curve (AUDPC) derived from the stem lesion length measurements for 200 F_7 individuals of the PBA HatTrick x Kyabra population. On the right side of the chromosome are the markers and their LOD, while on the left side are their corresponding positions in

centimorgans (cM). The red dots indicate the marker positions in cM. The names of the major QTLs, their flanking markers and confidence intervals are depicted in blue. The blue dashed line indicates the significance threshold with a logarithm of the odds (LOD) score of 2.5.

3.4.4 Candidate genes in QTL regions

To determine which genes underlying the QTLs might be involved in the resistance response to *S. sclerotiorum* infection, the physical regions in the chickpea cultivar CDC Frontier reference genome were assessed. A total of 52, 102, 622 and, 351 genes in the intervals between the flanking markers for QTLs qSSR4-1, qSSR4-2, qSSR6-1, and qSSR7-1 were identified, respectively (Supplementary Table S3.3).

Genes involved in the plant hormone signal transduction pathway, including auxin-induced protein (LOC101514996), three ethylene transcription factors (LOC101502435, LOC101502737 and LOC101504146), and signal recognition particle protein (LOC101512174), were identified in the region for qSSR4-1 (Supplementary Table S3.3 and S4). Other genes located in this region related to disease resistance were a WAT1-related protein (LOC101504468), an F-box/LRR-repeat protein 17 (LOC101514669), a calmodulin-like protein (LOC101491221), which is involved in plant-pathogen interactions, a glutathione reductase (LOC101514119) involved in glutathione metabolism, and a beta-carotene isomerase (LOC101489176) involved in carotenoid biosynthesis (Supplementary Table S3.3).

In the region of qSSR4-2, genes involved in the biosynthesis of secondary metabolites, purine metabolism, metabolic pathways, biosynthesis of antibiotics, phenylpropanoid biosynthesis, oxidoreductase and plant-pathogen interaction were identified (Supplementary Table S3.4). Genes related to disease-resistance pathways identified in this region included ethylene-responsive transcription factor (LOC101505675), calcium-dependent protein kinase 4-like (LOC101502238), cellulose synthase-like protein (LOC101514030, LOC101514359, and LOC101506417) peroxidase 5-like (LOC101492647), aminoacylase-1 (LOC101489624), uricase-2 isozyme 1-like (LOC101500535), WAT1-related protein (LOC101497530), and serine/threonine-protein kinase (LOC101499994) (Supplementary Table S3.3)

The highest number of genes was identified in the qSSR6-1 region. These genes are involved in pathways including plant hormone signal transduction, biosynthesis of antibiotics, biosynthesis of secondary metabolites, phenylalanine metabolism, ABC transporters, and plant-pathogen interaction pathways (Supplementary Table S3.4). Genes involved in plant hormone signal transduction included serine/threonine-protein kinase (LOC101489210, LOC101489210, LOC101489533, and LOC101494601), signal recognition particle subunit

(LOC101498515, and LOC101504832), two-component response regulator (LOC101509325 and LOC101497765), putative ETHYLENE INSENSITIVE 3-like 4 protein (LOC101500668) and histidine kinase 3-like (LOC101506212). Genes involved in plant-pathogen interaction included calcium-dependent protein kinase (LOC101493107) and respiratory burst oxidase (LOC101491892) (Supplementary Table S3.3).

Genes involved in plant resistance, including plant hormone signal transduction, biosynthesis of antibiotics, plant-pathogen interaction, phenylpropanoid biosynthesis and metabolism and oxidative phosphorylation, were identified in the region of qSSR7-1 (Supplementary Table S3.4). Five genes that play a role in plant hormone transduction included two-component response regulators ARR2 (LOC101510188) and protein TIFY 3B (LOC101502388), which were located in the qSSR7-1 region. Plant-pathogen interaction pathway-related genes including *pto*-interacting protein (LOC101511806) and squidulin (LOC101501000), defence response pathway (ABA-responsive protein ABR18-like: LOC101511589 and LOC101511270) and those involved in the biosynthesis of antibiotics (dihydrolipoyl dehydrogenase, alpha-aminoadipic semi-aldehyde synthase, ATP-citrate synthase beta chain protein 2-like and 1-deoxy-D-xylulose-5-phosphate synthase) were identified in the region of qSSR7-1.

3.4.5 Differential expression of candidate genes between moderately resistant and susceptible parents

The list of candidate genes was narrowed to assess the expression of the genes underlying the QTLs. A time course from 0 to 72 hours post-inoculation with *S. sclerotiorum* isolate CU8.20 was generated for both the moderately resistant (MR) cultivar PBA HatTrick and the susceptible (S) cultivar Kyabra. Of the 1,127 genes across the four QTLs, 120 genes showed differential expression patterns between non-inoculated (time 0) and inoculated samples (time points 6-72 hpi) for both parents (Fig. 4, 5 and 6). There were 10, 8, 47 and 55 differentially expressed genes in the qSSR4-1, qSSR4-2, qSSR6-1 and qSSR7-1 regions, respectively.

The ethylene-responsive transcription factor (LOC101502737) was identified in the qSSR4-1 region and was expressed earlier in the MR parent than in the S parent (Fig. 4). The peroxidase 5-like (LOC101492647), Armadillo repeat-containing protein 6 (LOC101513382) and WAT1-related protein (LOC101497530) were upregulated while glycerol-3-phosphate dehydrogenase (LOC101495780) downregulated in the MR line only (Fig. 4)

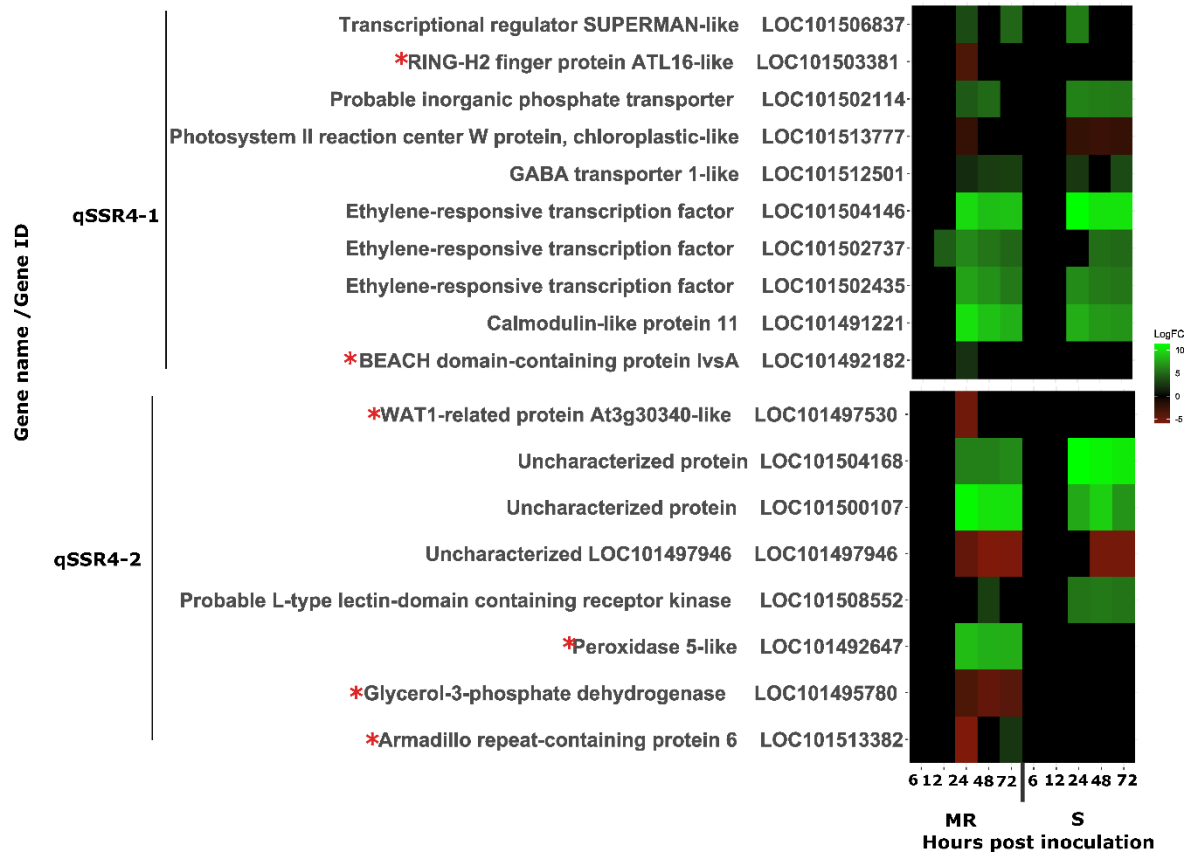


Figure 3.4: Heat maps showing the patterns of expression of all the differentially expressed genes (relative to 0 hpi) identified in the regions of QTLs, qSSSR4-1 and qSSR4-2. Positive LogFC values (shown in green) represent upregulation relative to expression at the time of inoculation, LogFC values of 0 (shown in black) represent no significant change in expression and negative LogFC values (shown in red) represent down-regulation of expression at 6, 12, 24, 48 and 72 hpi for moderately resistant (MR: PBA HatTrick) and susceptible (S: Kyabra) chickpea parent lines. The genes with different patterns of expression between parents are marked with asterisks. The vertical axis represents the genes, and the horizontal axis represents the chickpea line and time points.

Of the genes located in qSSR6-1, sucrose synthase (LOC101494314), serine/threonine-protein kinase (LOC101511372 and LOC101503660), monothiol glutaredoxin-S2-like (LOC101503006 and LOC101503330), mitochondrial thiamine pyrophosphate carrier-like (LOC101495703), F-box protein (LOC101497457), chitotriosidase-1-like (LOC101501002), basic 7S globulin-like (LOC101500045), alpha-aminoadipic semialdehyde synthase (LOC101500267), alkaline/neutral invertase A, mitochondrial (LOC101491074) and ABC transporter (LOC101511590) were differentially expressed in the MR line only. A pathogenicity related protein (LOC101512575) located in qSSR6-1 was upregulated earlier (24 hpi) in the MR line compared to the S line (72 hpi) (Fig. 5).

Among the genes differentially expressed in qSSR7-1 were a leucine-rich repeat receptor-like kinase (LOC101503887), probable WRKY transcription factor 11 (LOC101513839), pathogen-associated molecular pattern-induced protein A70 (LOC101492689), alpha-trehalose-phosphate synthase (LOC101507748), calcium-binding protein CML45 (LOC101505709), homeobox-leucine zipper protein (LOC101489128), probable serine/threonine-protein kinase (LOC101514185), a probable WRKY transcription factor 11 (LOC101513839) and a caffeoyl shikimate esterase (LOC101510635) (Fig. 6). A probable F-box protein (LOC101501001) located in the qSSR7-1 region was differentially expressed at 24-72 hpi in the MR line and 24 hpi in the S line (Fig. 6).

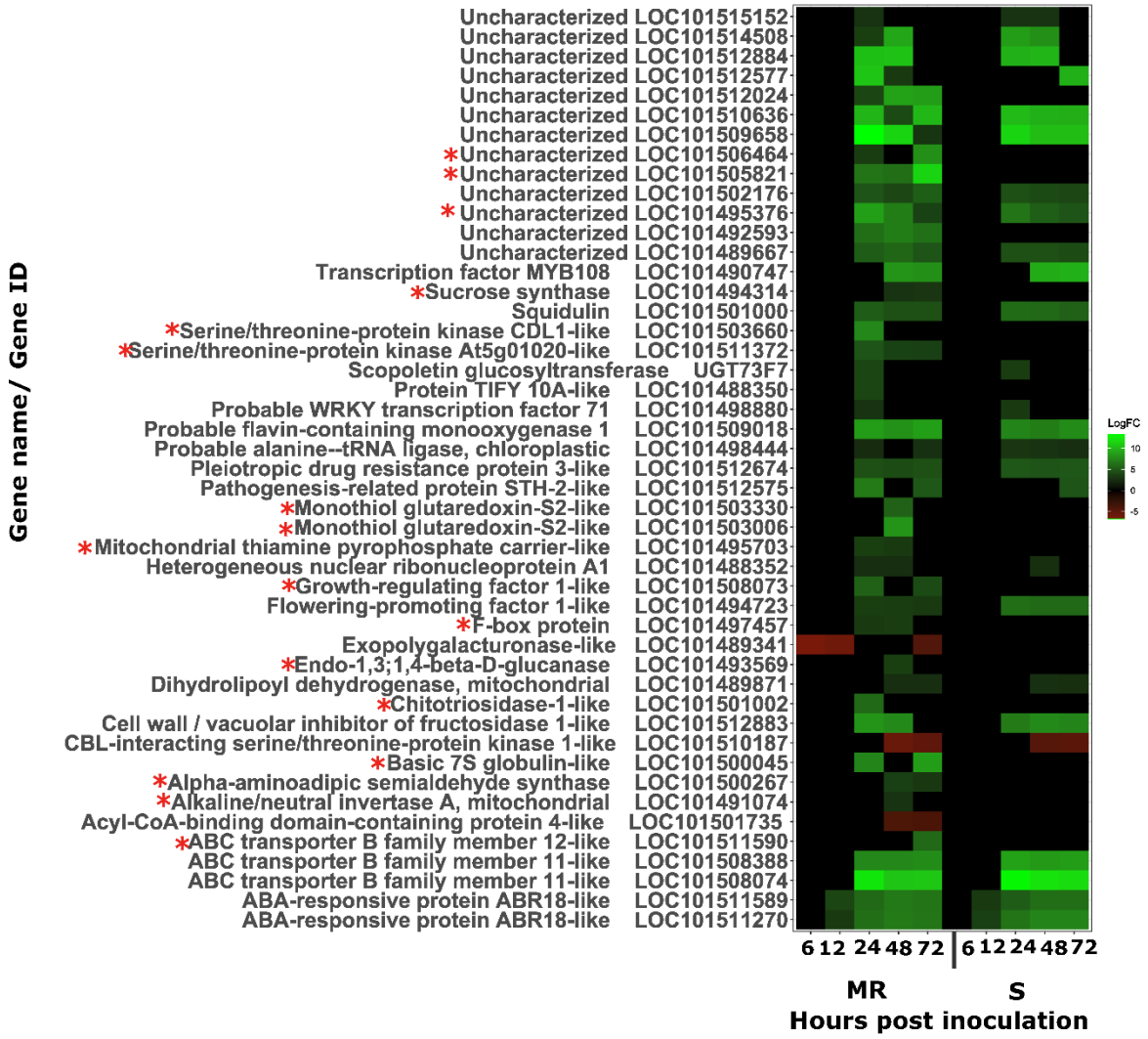


Figure 3.5: Heat maps showing the patterns of expression of all the differentially expressed genes identified in the regions of QTLs qSSR6-1. The green colour represents the upregulated, black no significant change in expression and red colour down-regulated genes at 6, 12, 24, 48 and 72 hpi for moderately resistant (MR: PBA HatTrick) and susceptible (S: Kyabra) chickpea lines. Marked with the red asterisks are the genes with different patterns of expression between parents. The vertical axis represents the genes, and the horizontal axis represents the chickpea line and time points.

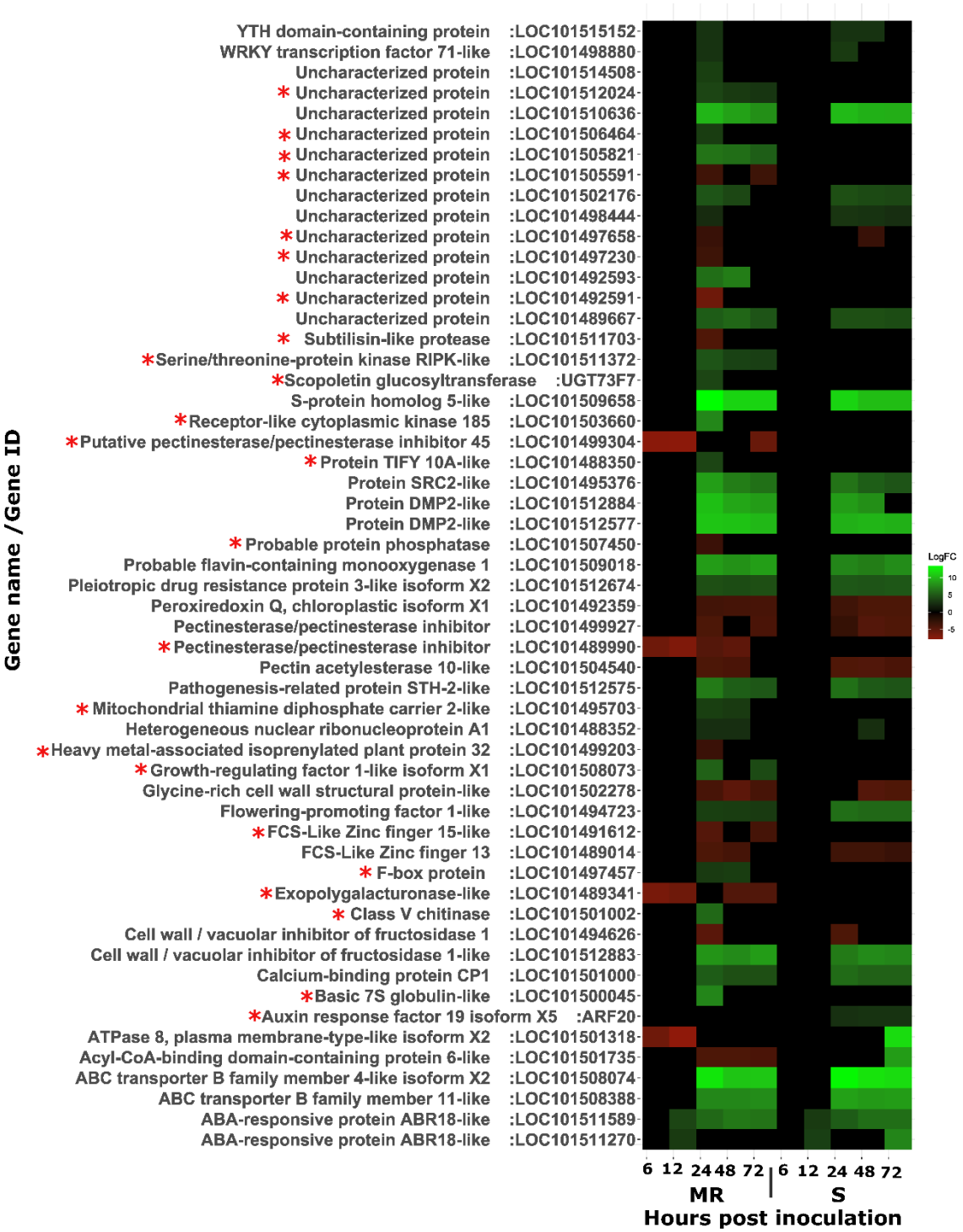


Figure 3.6: Heat maps showing the patterns of expression of all the differentially expressed genes identified in the regions of QTLs qSSR7-1. The green colour represents the upregulated, black no expression and red colour down-regulated genes at 6, 12, 24, 48 and 72 hpi for moderately resistant (MR: PBA HatTrick) and susceptible (S: Kyabra) chickpea lines. Marked with the red asterisks are the genes with different patterns of expression between parents. The vertical axis represents the genes, and the horizontal axis represents the chickpea line and time points.

3.5 Discussion

This study investigated the inheritance of resistance to *S. sclerotiorum* in a bi-parental mapping population derived from Australian cultivars PBA HatTrick and Kyabra, following the evaluation of 49 cultivars and 200 breeding lines. Evaluation of the 49 chickpea lines identified cultivar PBA HatTrick as having the lowest mean AUDPC scores, whereas cultivar Kyabra was one of the more susceptible cultivars and had a significantly different AUDPC score from PBA HatTrick. As such, the bi-parental RIL population was employed to dissect the genetic inheritance of the partial resistance identified in PBA HatTrick. Our findings indicate that resistance to *S. sclerotiorum* in chickpea is a complex quantitative trait and is affected by several genes with small effects, which is consistent with previous studies in *B. napus* (Yin *et al.* 2010), soybean (Arahana *et al.* 2001), common bean (Ender and Kelly 2005), and sunflower (Yue *et al.* 2008). Breeding cultivars that are resistant to SSR is important for crops such as chickpea, which is a highly valued crop for human consumption (Kottapalli *et al.* 2009). Thus, it is important to identify SSR resistance-related genes through QTL mapping from the current chickpea germplasm. This finding provides direct genetic resources for genetic improvement of SSR resistance and the knowledge required for developing effective strategies for SSR resistance breeding.

To date, complete resistance to *S. sclerotiorum* has not been identified in crop hosts. However, partial resistance has been reported in *B. napus* (Denton-Giles *et al.* 2018), soybean (Kim *et al.* 1999), sunflower, dry bean (Vuong *et al.* 2004) and chickpea (Mwape *et al.* 2021). Partial resistance to *S. sclerotiorum* was found in chickpea line PBA HatTrick, while the Kyabra line showed high susceptibility to SSR compared to other Australian cultivars and breeding lines (Fig. 1). Similar findings of partial resistance to *S. sclerotiorum* in the PBA HatTrick line and high susceptibility in the Kyabra line compared to a subset of wild chickpea germplasm were reported previously by Mwape *et al.* (2021). The ability of PBA HatTrick to maintain durable partial resistance in the field remains to be tested.

A stem inoculation assay measures the host-pathogen interaction under a consistent favourable environment but does not measure escape mechanisms such as early flowering, early maturity, and canopy size. Here we used a highly reproducible stem inoculation technique that has previously been used for SSR resistance screening of canola and chickpea under controlled environment conditions (Denton-Giles *et al.* 2018; Mwape *et al.* 2021). The frequency distribution of RILs for SSR on length (calculated as AUDPC) depicted a distribution for a

continuous trait indicating that genetic control of resistance to *S. sclerotiorum* may be complex. There were significant differences in AUDPC among RILs (LSD $_{0.05}$ = 885.3), with 32% of phenotyped lines showing higher resistance to *S. sclerotiorum* than the moderately resistant parent PBA HatTrick (Fig. 2; Supplementary Fig. S2).

In recent years, extensive efforts have been made using identified partial resistance to detect the loci controlling resistance to *S. sclerotiorum* in soybean (McCaghey *et al.* 2017; Arahana *et al.* 2001) canola (Qasim *et al.* 2020), pea (Ashtari Mahini *et al.* 2020) peanut (Liang *et al.* 2021) and dry bean (Miklas 2007), an indication of the importance of understanding the genetic basis of SSR resistance. In the present study, we mapped four QTLs: two on chromosome Ca4 (qSSR4-1 and qSSR4-2), one on chromosome Ca6 (qSSR6-1) and one on chromosome Ca7 (qSSR7-1). Each of these explained between 3.5 and 14.2 % of the phenotypic variation, with the favourable alleles contributed by both parents (Fig. 3; Table 1). The detection of some QTLs with favourable alleles from PBA HatTrick and some with favourable alleles from Kyabra is consistent with the observation that some lines had more extreme phenotypes than the parents (Fig. 2). There were 18 RILs that had the favourable alleles from both parents and showed overall higher levels of resistance compared to PBA HatTrick. This is the first QTL report for resistance to *S. sclerotiorum* in chickpea to the best of our knowledge.

The earlier mapped resistance loci in chickpea included those for resistance to Ascochyta blight, Fusarium wilt and Botrytis grey mould (Deokar *et al.* 2019) (Sabbavarapu *et al.* 2013; Anuradha *et al.* 2011; Garg *et al.* 2018). A chickpea genomic region on Ca4 has consistently been reported to contain QTLs for Ascochyta blight resistance (Sharma and Ghosh 2016) and Fusarium wilt (Garg *et al.* 2018). In the present study, QTLs with major effects (qSSR4-1 and qSSR4-2), explaining the phenotypic variance of 8.8 - 14.2%, were located on chromosome Ca4. Genes involved in known pathways involved in plant disease responses such as plant-pathogen interaction, phenylpropanoid pathways and plant hormone signalling were located in these regions. Our findings and previous studies on chickpea fungal diseases show that the Ca4 genomic region may be highly associated with disease resistance. The chickpea chromosomes Ca4 and Ca7 have also been reported to harbour QTLs responsible for Ascochyta blight resistance (Deokar *et al.* 2019).

The identified SNP markers associated with the *S. sclerotiorum* resistance loci could be adopted for marker-assisted selection by chickpea breeding programs, which will allow them to retain the partial resistance that exists in the breeding program whilst also incorporating novel sources of partial stem resistance such as those identified in a collection of wild *Cicer* germplasm (Mwape *et al.* 2021a). Investigation into the genomic regions of QTLs identified

several candidate genes that were differentially expressed in response to *S. sclerotiorum*. With the help of the RNA sequencing data, we were able to identify some genes that may play important roles in resistance to *S. sclerotiorum*. For instance, a gene involved in the thiamine biosynthesis pathway (LOC101495703) was identified in the region of qSSR6-1. Thiamine is known to play a key role in enhancing anti-oxidative capacity in the plant; thus, an increase in plant thiamine increases resistance to biotic stresses (Zhao *et al.* 2011; Zhou *et al.* 2013). Thiamine metabolism was associated with modulation of the redox environment, reducing the disease progress during *S. sclerotiorum* infection in *Arabidopsis* (Zhou *et al.* 2013).

A putative ethylene-responsive factor (ERF) gene present in the qSSR4-1 region showed an early upregulation in the MR compared to the S parent. ERF transcription factors play important roles in plant development and response to biotic and abiotic stresses (Licausi *et al.* 2013). Overexpression of ERF genes enhanced resistance to *S. sclerotiorum* in broccoli (Jiang *et al.* 2019). Plant calcium-dependent protein kinases (CDPKs) are important in downstream calcium signalling and regulating diverse plant immune responses, including production of ROS, transcription reprogramming of genes and hypersensitive response (Gao *et al.* 2014). Previous research reported CDPK positively regulates resistance to *S. sclerotiorum* and *Pseudomonas syringae* through promoting H_2O_2 accumulation and phosphorylation in tomatoes (Wang *et al.* 2015).

Members of the plant WRKY transcription factor family are implicated in regulating defence-related genes in response to fungal pathogens (Yang *et al.* 2009). A WRKY transcription factor identified in the QTL regions qSSR6-1 showed upregulation during infection at 24 hpi (Fig. 5). Other candidate genes identified in qSSR7-1 regions encode auxin response factors (ARFs) known for their signalling role during plant growth and development and have been linked to disease resistance (Li *et al.* 2016; Yamada 1993). In *Arabidopsis*, auxin signalling mutants that had defects in response to auxin showed increased susceptibility to *B. cinerea* and *Plectosphaerella cucumerina,* indicating that auxin signalling is important resistance to these necrotrophs (Llorente *et al.* 2008).

Plants have receptors that harbour a C-terminal LRR domain and can directly or indirectly perceive pathogen effectors to activate multiple defence signal transduction pathways that may result in a hypersensitive response to limit pathogen growth (Sagi *et al.* 2017). Genes with LRR domains were identified in the QTL regions of Ca4 and Ca7. Another domain is known to play a role in pathogen recognition and downstream signalling is the zinc finger protein (Carpita

and Gibeaut 1993). Zinc finger protein-encoding genes were identified in the region of QTL qSSR7-1.

Peroxidases are known for their role in the resistance-related oxidative burst response in plants (Dmochowska-Boguta *et al.* 2013). Four peroxidase proteins were identified in the qSSR7-1 region; however, they did not show differential expression during infection at 6-72 hpi. The plant hormone abscisic acid (ABA) is known to promote resistance in some plant-pathogen interactions and susceptibility in others and is linked with salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signalling to affect pathogen resistance (Wang *et al.* 2012). Two ABA response proteins were identified in the regions of qSSR7-1 (Supplementary Table S3.3 and were shown to be upregulated in both lines at 12-72 hpi. Three *Arabidopsis* mutants with defects in ABA signalling showed a complete loss of resistance to *S. sclerotiorum* (Perchepied *et al.* 2010). This suggests that ABA signalling may be involved in partial chickpea defence against *S. sclerotiorum*.

Putative candidate genes with defence functional categories related to pathogenesis-related (PR) genes were identified at qSSR7-1. Pathogen-associated molecular pattern-induced proteins A70 and pathogenesis-related proteins (PR1 and PR2) were identified in the qSSR7-1 region (Supplementary Table S3.2). PR proteins produce glycosidic fragments, which weaken and decompose fungal cell walls containing glucans, chitin and proteins (Ali *et al.* 2018). PR-1 genes involved in signalling and plant defences were identified within regions of QTLs qSSR6.1 (Supplementary Table S3.2).

The markers closely linked with SSR resistance QTLs identified in this study could facilitate identifying the genes that contribute to the partial resistance phenotype and may be used to retain these through pyramiding in one genotype. The QTLs identified in this study can facilitate marker-assisted breeding for SSR resistance.

3.6 Acknowledgements

The Australian Grains Research and Development Corporation (GRDC) supported the development of the population and linkage map under grant UA00157 and the research on Sclerotinia stem rot under grants CUR00023 and CUR00024. A Curtin University RTP Scholarship and CSIRO Research Plus Top-up scholarship were awarded to VWM. We thank Dr Kristy Hobson of Chickpea Breeding Australia for kindly providing the chickpea cultivars and breeding lines.

3.6 Funding

The Australian Grains Research and Development Corporation (GRDC) supported the development of the population and linkage map under grant UA00157 and the research on Sclerotinia stem rot under grants CUR00023 and CUR00024. A Curtin University RTP Scholarship and CSIRO Research Plus Top-up scholarship were awarded to VWM.

3.7 Availability of data and materials

The raw data used in this study for RNA sequence analysis was obtained from NCBI sequence read archive deposit under BioProject ID: PRJNA687280.

3.8 References

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

Abbo S, Berger J, Turner NC (2003) Viewpoint: Evolution of cultivated chickpea: four bottlenecks limit diversity and constrain adaptation. *Functional Plant Biology* **30**, 1081–1087.

Ali S, Ganai BA, Kamili AN, Bhat AA, Mir ZA, Bhat JA, Tyagi A, Islam ST, Mushtaq M, Yadav P, Rawat S, Grover A (2018) Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. *Microbiological Research* **212–213**, 29–37.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410.

Anuradha C, Gaur PM, Pande S, Gali KK, Ganesh M, Kumar J, Varshney RK (2011) Mapping QTL for resistance to botrytis grey mould in chickpea. *Euphytica* **182**, 1–9.

Arahana VS, Graef GL, Specht JE, Steadman JR, Eskridge KM (2001) Identification of QTLs for Resistance to *Sclerotinia sclerotiorum* in Soybean. *Crop Science* **41**, 180–188.

Ashtari Mahini R, Kumar A, Elias EM, Fiedler JD, Porter LD, McPhee KE (2020) Analysis and 1dentification of QTL for resistance to *Sclerotinia sclerotiorum* in pea (*Pisum sativum* L.). *Frontiers in Genetics* **11**,587968.

Bateman A, Birney E, Cerruti L, Durbin R, Etwiller L, Eddy SR, Griffiths-Jones S, Howe KL, Marshall M, Sonnhammer ELL (2002) The Pfam protein families database. *Nucleic Acids Research* **30**, 276–280.

Bohra A, Pandey MK, Jha UC, Singh B, Singh IP, Datta D, Chaturvedi SK, Nadarajan N, Varshney RK (2014) Genomics-assisted breeding in four major pulse crops of developing countries: present status and prospects. *Theoretical and applied genetics* **127**, 1263–1291.

Boland GJ, Hall R (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology* **16**, 93–108.

Brien C (2020) Employing AsremlPlus, in conjunction with Asreml, to calculate and use information criteria. https://cran.r-project.org/web/packages/asremlPlus/vignettes.

Brooks KD, Bennett SJ, Hodgson LM, Ashworth MB (2018) Narrow windrow burning canola (*Brassica napus* L.) residue for *Sclerotinia sclerotiorum* (Lib.) de Bary sclerotia destruction. *Pest Management Science* **74**, 2594–2600.

Butler, D.G., Cullis, B. R., Gilmour, A. R., Gogel, B. J. and Thompson, R. (2018). ASReml-R reference manual version 4, VSN International Ltd, Hemel Hempstead, HPI 1ES, UK.

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal* **3**, 1–30.

Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. *Genetics* **138**, 963–971.

Denton-Giles M, Derbyshire MC, Khentry Y, Buchwaldt L, Kamphuis LG (2018) Partial stem resistance in *Brassica napus* to highly aggressive and genetically diverse *Sclerotinia sclerotiorum* isolates from Australia. *Canadian Journal of Plant Pathology* **40**, 551–561.

Deokar A, Sagi M, Daba K, Tar'an B (2019) QTL sequencing strategy to map genomic regions associated with resistance to ascochyta blight in chickpea. *Plant Biotechnology Journal* **17**, 275–288.

Dey SK, Singh G (1993) Resistance to ascochyta blight in chickpea - Genetic basis. *Euphytica* **68**, 147–153.

Dmochowska-Boguta M, Nadolska-Orczyk A, Orczyk W (2013) Roles of peroxidases and NADPH oxidases in the oxidative response of wheat (*Triticum aestivum*) to brown rust (*Puccinia triticina*) infection. *Plant Pathology* **62**, 993–1002.

Edwards David (2016) Improved kabuli reference genome. CyVerse Data Commons. http://doi.org/10.7946/P2G596.

Ender M, Kelly JD (2005) Identification of QTL associated with white mold resistance in common Bean. *Crop Science* **45**, 2482–2490.

FAOSTAT http://www.fao.org.dbgw.lis.curtin.edu.au/faostat/en/. Accessed 29 May 2020.

Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J, Sonnhammer ELL, Tate J, Punta M (2014) Pfam: the protein families database. *Nucleic Acids Research* **42**, D222–D230.

Fuhlbohm MJ, Tatnell JR, Ryley MJ (2003) First report of stem rot and wilt of chickpea caused by *Sclerotinia minor* in Queensland, Australia. *Australasian Plant Pathology* **2**, 323–324.

Gao X, Cox KL, He P (2014) Functions of calcium-dependent protein kinases in plant innate immunity. *Plants* **3**, 160–176.

Garg T, Mallikarjuna BP, Thudi M, Samineni S, Singh S, Sandhu JS, Kaur L, Singh I, Sirari A, Basandrai AK, Basandrai D, Varshney RK, Gaur PM (2018) Identification of QTLs for resistance to Fusarium wilt and Ascochyta blight in a recombinant inbred population of chickpea (*Cicer arietinum* L.). *Euphytica* **214**, 45.

Gaur PM, Jukanti AK, Varshney RK (2012) Impact of genomic technologies on chickpea breeding strategies. *Agronomy* **2**, 199–221.

Jeger MJ, Viljanen-Rollinson SLH (2001) The use of the area under the disease-progress curve (AUDPC) to assess quantitative disease resistance in crop varieties. *Theoretical and Applied Genetics* **102**, 32–40.

Jha UC, Chaturvedi SK, Bohra A, Basu PS, Khan MS, Barh D, Varshney R (2014) Abiotic stresses, constraints and improvement strategies in chickpea. *Plant breeding*. https://agris.fao.org/agris-search/search.do?recordID=US201400065632.

Jiang M, Ye Z, Zhang H, Miao L (2019) Broccoli Plants Over-expressing an ERF Transcription factor gene BoERF1 facilitates both salt stress and Sclerotinia stem rot resistance. *Journal of Plant Growth Regulation* **38**, 1–13.

Jukanti AK, Gaur PM, Gowda CLL, Chibbar RN (2012) Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. *British Journal of Nutrition* **108**, S11–S26.

Kenward MG, Roger JH (1997) Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* **53**, 983–997.

Kim HS, Sneller CH, Diers BW (1999) Evaluation of soybean varieties for resistance to Sclerotinia stem rot in Field Environments. *Crop Science* **39**, 003900010010x.

Knights EJ, Hobson KB (2016) Chickpea: Overview. 'Encycl. Food Grains Second Ed.' (Eds C Wrigley, H Corke, K Seetharaman, J Faubion) pp. 316–323. (Academic Press: Oxford) doi:10.1016/B978-0-12-394437-5.00035-8.

Kottapalli P, Gaur PM, Katiyar SK, Crouch JH, Buhariwalla HK, Pande S, Gali KK (2009) Mapping and validation of QTLs for resistance to an Indian isolate of Ascochyta blight pathogen in chickpea. *Euphytica* **165**, 79–88.

Lane D, Denton-Giles M, Derbyshire M, Kamphuis LG (2019) Abiotic conditions governing the myceliogenic germination of *Sclerotinia sclerotiorum* allowing the basal infection of *Brassica napus*. *Australasian Plant Pathology* **48**, 85–91.

Li Y, Ruperao P, Batley J, Edwards D, Davidson J, Hobson K, Sutton T (2017) Genome analysis identified novel candidate genes for Ascochyta blight resistance in chickpea using whole genome re-sequencing data. *Frontiers in Plant Science* **8**,00359

Li S-B, Xie Z-Z, Hu C-G, Zhang J-Z (2016) A review of auxin response factors (ARFs) in plants. *Frontiers in Plant Science* **7**,00047.

Li H, Ye G, Wang J (2007) A modified algorithm for the improvement of composite interval mapping. *Genetics* **175**, 361–374.

Liang Y, Cason JM, Baring MR, Septiningsih EM (2021) Identification of QTLs associated with Sclerotinia blight resistance in peanut (*Arachis hypogaea* L.). *Genetic Resources and Crop Evolution* **68**, 629–637.

Licausi F, Ohme-Takagi M, Perata P (2013) APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. *New Phytologist* **199**, 639–649.

Llorente F, Muskett P, Sánchez-Vallet A, López G, Ramos B, Sánchez-Rodríguez C, Jordá L, Parker J, Molina A (2008) Repression of the auxin response pathway increases arabidopsis susceptibility to necrotrophic fungi. *Molecular Plant* **1**, 496–509.

McCaghey M, Willbur J, Ranjan A, Grau CR, Chapman S, Diers B, Groves C, Kabbage M, Smith DL (2017) Development and evaluation of *Glycine max* germplasm lines with quantitative resistance to *Sclerotinia sclerotiorum*. *Frontiers in Plant Science* **8**, 01495.

Meng L, Li H, Zhang L, Wang J (2015) QTL IciMapping: Integrated software for genetic linkage map construction and quantitative trait locus mapping in biparental populations. *The Crop Journal* **3**, 269–283.

Merga B, Haji J (2019) Economic importance of chickpea: Production, value, and world trade (F Yildiz, Ed.). *Cogent Food & Agriculture* **5**, 1615718.

Miklas PN (2007) Marker-assisted backcrossing QTL for partial resistance to Sclerotinia white mold in dry bean. *Crop Science* **47**, 935–942.

Mwape VW, Khentry Y, Newman TE, Denton-Giles M, Derbyshire M, Chen K, Berger J, Kamphuis L (2021a) Identification of sources of *Sclerotinia sclerotiorum* resistance in a collection of wild *Cicer* germplasm. *Plant Disease*. doi:10.1094/PDIS-02-21-0367-RE.

Mwape VW, Mobegi FM, Regmi R, Newman TE, Kamphuis LG, Derbyshire MC (2021b) Analysis of differentially expressed *Sclerotinia sclerotiorum* genes during the interaction with moderately resistant and highly susceptible chickpea lines. *BMC Genomics* **22**, 333.

Perchepied L, Balagué C, Riou C, Claudel-Renard C, Rivière N, Grezes-Besset B, Roby D (2010) Nitric oxide participates in the complex interplay of defense-related signalling pathways controlling disease resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions®* **23**, 846–860.

Pulse Australia - APB Chickpea IDM strategies http://www.pulseaus.com.au/growing-pulses/bmp/chickpea/idm-strategies. Accessed 10 July 2020.

Qasim MU, Zhao Q, Shahid M, Samad RA, Ahmar S, Wu J, Fan C, Zhou Y (2020) Identification of QTLs containing resistance genes for Sclerotinia stem rot in *Brassica napus* using comparative transcriptomic studies. *Frontiers in Plant Science* **11**, .00776.

Royston JP (1982) An extension of Shapiro and Wilk's W test for normality to large samples. *Journal of the Royal Statistical Society Series C (Applied Statistics)* **31**, 115–124.

Sabbavarapu MM, Sharma M, Chamarthi SK, Swapna N, Rathore A, Thudi M, Gaur PM, Pande S, Singh S, Kaur L, Varshney RK (2013) Molecular mapping of QTLs for resistance to Fusarium wilt (race 1) and Ascochyta blight in chickpea (*Cicer arietinum* L.). *Euphytica* **193**, 121–133.

Sagi MS, Deokar AA, Tar'an B (2017) Genetic analysis of NBS-LRR gene family in chickpea and their expression profiles in response to ascochyta blight infection. *Frontiers in Plant Science* **8**, 00838.

Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). *Biometrika* **52**, 591–611.

Sharma M, Ghosh R (2016) An update on genetic resistance of chickpea to ascochyta blight. *Agronomy* **6**, 18.

Smith AB, Cullis BR, Thompson R (2005) The analysis of crop varieties breeding and evaluation trials: an overview of current mixed model approaches. *The Journal of Agricultural Science* **143**, 449–462.

Taylor J, Butler D (2017) R Package ASMap: Efficient genetic linkage map construction and diagnosis. *Journal of Statistical Software* **79**,06.

Tewari SK, Pandey MP (1986) Genetics of resistance to ascochyta blight in chickpea (*Cicer arietinum* L.). *Euphytica* **35**, 211–215.

Udupa SM, Sharma A, Sharma RP, Pai RA (1993) Narrow genetic variability in *Cicer arietinum* L. as revealed by RFLP analysis. *Journal of Plant Biochemistry and Biotechnology* **2**, 83–86.

Varshney RK, Song C, Saxena RK, Azam S, Yu S, Sharpe AG, Cannon S, Baek J, Rosen BD, Tar'an B, Millan T, Zhang X, Ramsay LD, Iwata A, Wang Y, Nelson W, Farmer AD, Gaur PM, Soderlund C, Penmetsa RV, Xu C, Bharti AK, He W, Winter P, Zhao S, Hane JK, Carrasquilla-Garcia N, Condie JA, Upadhyaya HD, Luo M-C, Thudi M, Gowda CLL, Singh NP, Lichtenzveig J, Gali KK, Rubio J, Nadarajan N, Dolezel J, Bansal KC, Xu X, Edwards D, Zhang G, Kahl G, Gil J, Singh KB, Datta SK, Jackson SA, Wang J, Cook DR (2013) Draft genome sequence of chickpea (*Cicer arietinum*) provides a resource for trait improvement. *Nature Biotechnology* **31**, 240–246.

Vuong TD, Hoffman DD, Diers BW, Miller JF, Steadman JR, Hartman GL (2004) Evaluation of soybean, dry bean, and sunflower for resistance to *Sclerotinia sclerotiorum*. *Crop Science* **44**, 777–783.

Wang J-P, Munyampundu J-P, Xu Y-P, Cai X-Z (2015) Phylogeny of plant calcium and calmodulin-dependent protein kinases (CCaMKs) and functional analyses of tomato CCaMK in disease resistance. *Frontiers in Plant Science* **6**, 01075.

Wang Z, Tan X, Zhang Z, Gu S, Li G, Shi H (2012) Defense to *Sclerotinia sclerotiorum* in oilseed rape is associated with the sequential activations of salicylic acid signaling and jasmonic acid signaling. *Plant Science* **184**, 75–82.

Yamada T (1993) The role of auxin in plant-disease development. *Annual Review of Phytopathology* **31**, 253–273.

Yang B, Jiang Y, Rahman MH, Deyholos MK, Kav NN (2009) Identification and expression analysis of WRKY transcription factor genes in canola (*Brassica napus* L.) in response to fungal pathogens and hormone treatments. *BMC Plant Biology* **9**, 68.

Yin X, Yi B, Chen W, Zhang W, Tu J, Fernando WGD, Fu T (2010) Mapping of QTLs detected in a *Brassica napus* DH population for resistance to *Sclerotinia sclerotiorum* in multiple environments. *Euphytica* **173**, 25–35.

Yue B, Radi SA, Vick BA, Cai X, Tang S, Knapp SJ, Gulya TJ, Miller JF, Hu J (2008) Identifying quantitative trait loci for resistance to Sclerotinia head rot in two USDA sunflower germplasms. *Phytopathology* **98**, 926–931.

Zhang L, Li H, Li Z, Wang J (2008) Interactions between markers can be caused by the dominance effect of quantitative trait loci. *Genetics* **180**, 1177–1190.

Zhao W, Cheng X, Huang Z, Fan H, Wu H, Ling H-Q (2011) Tomato LeTHIC is an Fe-requiring HMP-P synthase involved in thiamine synthesis and regulated by multiple factors. *Plant & Cell Physiology* **52**, 967–982.

Zhou J, Sun A, Xing D (2013) Modulation of cellular redox status by thiamine-activated NADPH oxidase confers Arabidopsis resistance to *Sclerotinia sclerotiorum*. *Journal of Experimental Botany* **64**, 3261–3272.

Zhou J, Sun A, Xing D (2013) Modulation of cellular redox status by thiamine-activated NADPH oxidase confers Arabidopsis resistance to *Sclerotinia sclerotiorum*. *Journal of Experimental Botany* **64**, 3261–3272.

CHAPTER 4

Analysis of differentially expressed *Sclerotinia sclerotiorum* genes during the interaction with moderately resistant and highly susceptible chickpea lines

Virginia W. Mwape[1, 2], Fredrick M. Mobegi[1], Roshan Regmi[1, 2], Toby. E. Newman[1], Lars G. Kamphuis[1, 2*], Mark C. Derbyshire[1]

[1] Centre for Crop and Disease Management, Curtin University, Bentley WA 6102, Australia

[2] Commonwealth Scientific and Industrial Research Organization. Agriculture and Food, Floreat, WA, Australia

Published: (2021) *BMC Genomics*. **22**, 333 https://doi.org/10.1186/s12864-021-07655-6

This chapter is reproduced, by permission, from Mwape. *et al*. (2021b) Analysis of differentially expressed *Sclerotinia sclerotiorum* genes during the interaction with moderately resistant and highly susceptible chickpea lines. *BMC Genomics* **22**, 333. doi:10.1186/s12864-021-07655-6.

4.1. Abstract

Sclerotinia sclerotiorum, the cause of Sclerotinia stem rot (SSR), is a host generalist necrotrophic fungus that can cause major yield losses in chickpea (*Cicer arietinum*) production. This study used RNA sequencing to conduct a time course transcriptional analysis of *S. sclerotiorum* gene expression during chickpea infection. It explores pathogenicity and developmental factors employed by *S. sclerotiorum* during interaction with chickpea. During infection of moderately resistant (PBA HatTrick) and highly susceptible chickpea (Kyabra) lines, 9,491 and 10,487 *S. sclerotiorum* genes, respectively, were significantly differentially expressed relative to *in vitro*. Analysis of the upregulated genes revealed enrichment of Gene Ontology biological processes, such as oxidation-reduction process, metabolic process, carbohydrate metabolic process, response to stimulus, and signal transduction. Several gene functional categories were upregulated *in planta*, including carbohydrate-active enzymes, secondary metabolite biosynthesis clusters, transcription factors and candidate secreted effectors. Differences in expression of four *S. Sclerotiorum* genes on cultivars with different levels of susceptibility were also observed. These findings provide a framework for a better understanding of *S. sclerotiorum* interactions with hosts of varying susceptibility levels. Here, we report for the first time on the *S. sclerotiorum* transcriptome during chickpea infection, which could be important for further studies on this pathogen's molecular biology.

Keywords: *Sclerotinia sclerotiorum*, *Cicer arietinum*, CAZymes, secondary metabolites, secreted effectors, transcription factors, infection.

4.2. Background

Sclerotinia sclerotiorum is a necrotrophic fungal pathogen with a remarkably broad host range of over 600 plant species (Boland and Hall 1994; Liang and Rollins 2018). The hosts of *S. sclerotiorum* include economically important crops such as *Brassica napus* (canola), *Glycine max* (soybean), *Phaseolus vulgaris* (common beans), *Pisum sativum* (field pea), *Helianthus annuus* (sunflower) and *Cicer arietinum* (chickpea) (Boland and Hall 1994). Research on genetic and molecular management of various fungal pathogens in chickpeas, such as *Aschochyta rabiei* and *Fusarium oxysporum* f. sp. *ciceris*, has led to the identification of genetic and pathological variabilities leading to shifting from cultural practices to the development of new genetic and molecular management approaches (Kukreja *et al.* 2018). However, limited information is available on the molecular biology of *S. sclerotiorum* during chickpea infection, despite the fact that, in a conducive environment, a disease caused by *Sclerotinia* species can cause up to 100% chickpea yield loss (Fuhlbohm *et al.* 2003).

S. sclerotiorum is generally described as a necrotroph. As such, it derives its energy from dead plants to complete its lifecycle; this contrasts with biotrophs, which feed on living plant cells. However, recent studies indicate that *S. sclerotiorum* undergoes a brief biotrophic phase soon after penetration (Kabbage *et al.* 2015). Expression of biotrophy-related genes, including those with Lysin Motif (LysM) domains, within the first 24 hours post-inoculation (hpi) during the *S. sclerotiorum* - *B. napus* interaction has been reported (Seifbarghi *et al.* 2017). Furthermore, previous studies have shown that *S. sclerotiorum* integrin-like protein (*SSITL*) and chorismate mutase (*SsCm1*) may suppress host defence signalling during the biotrophic phase (Kabbage *et al.* 2015; Seifbarghi *et al.* 2017; Tang *et al.* 2020). The pathogenesis journey through the two phases requires regulation of metabolic, virulence and defence enzymes in response to challenges associated with the type of host tissue, nature of energy source, acidity, and oxidative stress (Zhu *et al.* 2013; Kabbage *et al.* 2013).

The *S. sclerotiorum* reference genome has revealed several potential pathogenicity and virulence factors, including cell wall degrading enzymes (CWDES), metabolites, detoxification enzymes and candidate secreted effectors (Li *et al.* 2011; Xiao *et al.* 2013; Derbyshire *et al.* 2017). We refer to pathogenicity factors as genes that are essential for causing disease and virulence factors as genes that contribute in a quantitative manner to pathogen aggressiveness; any genes that have an impact on growth away from the plant host are referred to in this article as ‘developmental factors’, and these may also be pathogenicity or virulence

factors at the same time (Bolton *et al.* 2006; Amselem *et al.* 2011; Casadevall and Pirofski 1999). Amselem *et al.* (2011) compared the genomes of *S. sclerotiorum* and its relative *B. cinerea* and found a variety of putative secreted enzymes, including carbohydrate-active enzymes (CAZymes) such as xylanases, pectinases, polygalacturonases (PGs), hemicellulases, and cellulases. CAZymes play a crucial role in host cell wall degradation to simpler monomers that serve as a carbon source (Kubicek *et al.* 2014). Disruption of the *S. sclerotiorum* CAZymes arabinofuranosidase/β-xylosidase and an endo-β-1, 4-xylanase showed reduced or lost virulence (Yajima *et al.* 2009), an indication of their importance in the growth and virulence of the pathogen.

Secreted effector candidates have also been found in *S. sclerotiorum*. These are proteins that manipulate host cell functions and suppress plant defence to promote infection (Derbyshire *et al.* 2017). Some of these candidates have been functionally characterised. For example, secreted protein *SsSSVP1* manipulates plant energy metabolism for full virulence (Lyu *et al.* 2016). Disruption of *SsSSVP1* in *S. sclerotiorum* significantly reduces virulence in *B. napus* and *Arabidopsis thaliana*, compared to the wild type (Lyu *et al.* 2016). *S. sclerotiorum* strains lacking *SSITL* cause rapid induction of plant defence genes associated with the salicylic acid and jasmonic acid/ethylene signalling pathway, suggesting *SSITL* as a possible effector that plays a key role in suppressing host immunity at an early stage of infection (Kabbage *et al.* 2015; Casadevall 2007).

Transcription factors (TFs) act as pivotal regulators of gene expression by binding to gene promoters to activate or repress expression (Amselem *et al.* 2011). Several *S. sclerotiorum* transcription factors have been characterised. For example, in response to reduced acidity, the *S. sclerotiorum* gene encoding a zinc finger transcription factor (*Pac1*) triggers oxalic acid (OA) biosynthesis, causing an increase in expression of exo-polygalacturonase (*Sspg1*), which is involved in pectin degradation, a significant constituent of the plant cell wall (Poussereau *et al.* 2001). Although not directly involved in pathogenicity, *Pac1* plays a role in OA and *Sspg1* accumulation.

Recent studies of *S. sclerotiorum* gene expression on different hosts found that a gene encoding oxaloacetate acetylhydrolase (*Ssoah1*), known to be vital for OA production, was expressed in a similar pattern during infection of *B. napus* (Fuhlbohm *et al.* 2003; Kubicek *et al.* 2014) and *P. vulgaris* (Oliveira *et al.* 2015). However, *Ssoah1* expression was not observed during *G. max* infection (Westrick *et al.* 2019). Intrinsic host immunity may also affect the pattern of *S.*

sclerotiorum gene expression as demonstrated in *B. napus,* where a gene encoding a polygalacturonase, *Sspg1*, was upregulated in a resistant cultivar, with no upregulation in a susceptible cultivar relative to *in vitro* (Chittem *et al.* 2020). These discrepancies indicate that *S. sclerotiorum* gene expression may depend on the host species and intraspecific differences in levels of resistance.

Our study aimed to (1) understand further how the *S. sclerotiorum* transcriptome is deployed *in planta* relative to *in vitro* conditions; (2) catalogue upregulated and downregulated genes in the *S. sclerotiorum* - chickpea pathosystem; and (3) evaluate the differences in gene regulation during *S. sclerotiorum* infection of a moderately resistant and a susceptible chickpea line. The current study hypothesised that (i) *S. sclerotiorum* would deploy an array of factors to facilitate chickpea infection, and (ii) *S. sclerotiorum* will express genes that are specific to moderately resistant and susceptible cultivars. This study reveals the activation of primary *S. sclerotiorum* pathogenesis factors, including CAZymes and affiliated proteins, putative secreted effector proteins, secondary metabolites and genes involved in regulating the production of and tolerance to reactive oxygen species (ROS) such as catalases and peroxidases.

4.3. Material and Methods

4.3.1. Plant material

Two desi chickpea cultivars with different levels of susceptibility to *S. sclerotiorum* were used as hosts in this study (Mwape *et al.* 2021a). One seed per pot of moderately resistant (PBA HatTrick) and highly susceptible (Kyabra) chickpea cultivars were planted in 5 cm pots with an all-purpose potting mix (UWA mix, Richgro, Perth, Australia) and grown for eight weeks in a plant growth chamber with a 16-hour photoperiod, a 22/16ºC day/night temperature, and 60% relative humidity. Once germinated, seedlings were watered as necessary and fertilised at four weeks with Nitrophoska perfekt™ fertiliser (Incitec Pivot fertilisers, Victoria, Australia). These two cultivars are herein referred to as the MR line (moderately resistant PBA HatTrick) and S line (susceptible Kyabra).

4.3.2. Fungal material preparation and inoculation

An aggressive *S. sclerotiorum* isolate CU8.20 was previously found to be aggressive in *B. napus* (Denton-Giles *et al.* 2018) and chickpea (Mwape *et al.* 2021a). The isolate CU8.20 culture was prepared from dry sclerotia, which were cut in half and placed mycelium-side down on potato dextrose agar (PDA) (Becton Dickinson, USA) and incubated at 20°C for 5-7 days in the dark plates. Subsequently, a mycelial plug was cut from actively growing edges of the

PDA culture and sub-cultured in a fresh PDA plate at 20°C for two days. Only plates with consistent mycelial growth were used for inoculation. Eight-week-old plants were infected following a stem inoculation assay that involved cutting a 5 mm plug from the actively growing mycelium and placing it in the middle of the plant stem. The plug was secured using Parafilm® to maintain moisture. The *in vitro* control samples were generated by inoculating potato dextrose broth (PDB) with 5 mm minimal media agar plugs and incubated at 26 ºC with shaking at 160 rpm for 96 h.

4.3.3. Sample collection

S. sclerotiorum mycelium (*in vitro* samples) was collected from PDB and flash-frozen in liquid nitrogen. Inoculated plant stem sections were collected by cutting the stem 1 cm above and below the point of inoculation or the lesion at 6, 12, 24, 48, and 72 hours post-inoculation (hpi), immediately put in an Eppendorf tube and flash-frozen in liquid nitrogen within 10 s of collection and stored at -80 °C until RNA extraction. Each treatment (time point /cultivar) consisted of three biological replicates. Six stem sections were collected from four individual plants and pooled for one biological replicate.

4.3.4. RNA extraction and sequencing

The fungal mats and the infected stem samples were ground into a fine powder with liquid nitrogen pre-cooled in an RNAse-free mortar and pestle. Total RNA was extracted from inoculated chickpea stem tissues and *S. sclerotiorum* mycelium following the Trizol™ Reagent protocol (Invitrogen Corp., Carlsbad, CA, USA). RNA quantity and quality were assessed using the Qubit fluorometry assay (Invitrogen Corp., Carlsbad, CA, USA). Novogene performed library preparation (150 bp paired-end) and sequencing on an Illumina HISEq 2500 platform.

4.3.5. RNA sequencing data quality control

Quality assessment on raw fastq reads and cleaned reads were conducted using the FastQC tool (V. 0.11.8) (www.bioinformatics.babraham.ac.uk/projects/fastqc). Trimmomatic v.0.38 was used to trim low-quality base calls and filter adapters and low quality reads (Bolger *et al.* 2014). The following trimmomatic parameters were used, ILLUMINACLIP: TruSeq3-SE: 2:30:10 MINLEN: 36 LEADING: 3 TRAILING: 3 SLIDINGWINDOW: 4:15.

4.3.6. Read alignment

The trimmed reads were split between the pathogen (*S. sclerotiorum* strain 1980 genome, Bioproject PRJNA348385, assembly ASM185786v1) (Derbyshire *et al.* 2017) and the host (*Cicer arietinum,* Bioproject PRJNA190909, assembly ASM33114v1) (Varshney *et al.* 2013) using BBSplit tool v.38.12 (https://sourceforge.net/projects/bbmap/). Quality filtered *S. sclerotiorum in planta* and *in vitro* reads were aligned to the reference genomes using HISat2 v2.1.0 (Kim *et al.* 2015). The resulting alignments in SAM format were then converted to BAM format, sorted and indexed using SAMtools v.0.1.19 (Li *et al.* 2009). The number of reads that mapped to each gene in the reference (read counts/gene counts) were generated using HTseq package v 0.12.4 (Anders *et al.* 2015).

4.3.7. Differential gene expression analysis

The *S. sclerotiorum in planta* differential gene expression analysis was conducted using edgeR and limma Bioconductor packages in R v4.0.2 (Robinson *et al.* 2010; Ritchie *et al.* 2015). The raw count data were normalised using the Trimmed Mean of M-values (TMM) method. Principle coordinate analysis (PCoA) plots were generated using the *plot-MDS ()* function from limma and the *heatmap.2 ()* function from gplots to determine the relatedness of the biological replicates. Pairwise contrasts were performed using quasi-likelihood F tests (Lun *et al.* 2016). A false discovery rate (FDR) cut-off of 0.05 was applied, and a $\log_2$ fold change cut-off of ≥ 2 to indicate upregulation and ≤ -2 to indicate down-regulation. Differentially expressed genes (DEGs) were considered at each time point for each host in relation to the *in vitro*/vegetative growth culture (hypothetical time-point 0).

4.3.8. Gene Ontology enrichment analysis of DEGs

S. sclerotiorum gene ontology (GO) terms were derived from a previous InterPro annotation (Derbyshire *et al.* 2017). To test for significantly enriched (GO) categories, we used the R Bioconductor package TopGO (2007) to implement the classical method and Fisher's exact test with a P-value threshold of ≤ 0.05 (Alexa and Rahnenführer,2006). GO terms for the full *S. sclerotiorum* total gene list were used as the background list for enrichment analysis.

4.3.9. CAZymes and secreted protein effector analysis

CAZymes were predicted using DBCan2 web server v8.0 (Zhang *et al.* 2018). The *S. sclerotiorum* strain 1980 protein sequences were used as the input for CAZymes prediction (GCA_001857865) (Derbyshire *et al.* 2017). The CAZymes prediction was made using three databases, HMMER, DIAMOND and Hotpep (Zhang *et al.* 2018). Only those CAzymes

identified by at least two databases and with positive SignalP scores were considered for analysis. This study considered previously predicted effectors by Derbyshire *et al.* (2017) and Guyon *et al.* (2014).

4.3.10. Validation of RNA-seq data using reverse transcription-quantitative PCR

RNA-Seq data was validated by performing reverse transcription quantitative polymerase chain reactions (RT-qPCRs) on five upregulated genes and one downregulated gene. Two-time points were chosen to represent the early stage (12 hpi) and late stage (48 hpi) of MR and S line infection. RNA samples used for qPCR validation were the same samples used for Illumina sequencing. The RNA samples were reverse transcribed using the First-Strand cDNA Synthesis Kit for RT-PCR (AMV) (NEB Inc. Ipswitch, MA) according to the manufacturer's instruction. The cDNA samples were then diluted at 1:20 before qPCR. Real-time quantification for MR line was performed with iTaq Universal SYBR Green Supermix and S line with PowerUp SYBR Green master mix with the following cycling conditions: 95 ºC for 2 min, then 95 ºC for 15 sec, 60 ºC for 30 sec and 72 ºC for 15 sec, repeated 40 cycles, followed by 72 ºC for 2 min. The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with the fungal β-tubulin gene (sscle_02g015170) used as an endogenous control. Three biological replicates were used, and three technical replicates per biological replicate to determine the expression levels for the six genes relative to fungal β-tubulin. The qPCR experiment consisted of three biological replicates per sample and three technical replicates per biological replicate.

4.4. Results and discussion

4.4.1. Processing and filtering of transcriptome data

RNA-seq was used to compare *S. sclerotiorum* gene expression between samples taken during infection of two *C. arietinum* lines and during growth *in vitro*. Between 1.8 to 61.8 % of sequence reads derived from the infected moderately resistant (MR) line samples collected between 6 – 72 hpi mapped to the reference genome of *S. sclerotiorum*. On the other hand, between 0.7% to 68.1% of sequence reads derived from infected susceptible line samples collected between 6- 72 hpi mapped back to the *S. sclerotiorum* genome (Table 4.1). At 72 hpi, the average percentage of reads mapping to the fungal genome in the S line was higher (68.1%) than in the MR line (61.8%), suggesting that the S line tissues may be more heavily colonised than those of the MR line (Table 4.1). The larger lesions found on the S line at the later stage of infection during the current study (results not shown) and greater abundance of fungal RNA

in the S line samples together suggest that it exhibited greater levels of fungal colonisation than the MR line. Such differences have been reported in previous *S. sclerotiorum* transcriptome studies (Seifbarghi *et al.* 2017; Oliveira *et al.* 2015; Westrick *et al.* 2019; Chittem *et al.* 2020).

Table 4.1: Summary of the Illumina sequence reads generated by RNA – seq obtained from inoculation of a moderately resistant (MR) chickpea line PBA HatTrick and a susceptible (S) chickpea line Kyabra. The values for each time point are the averages of the three biological replicates.

Host	**Hours post inoculation (hpi)**	**Total raw read pairs**	**Trimmomatic reads retention (%)**	**BBSplit reads separation**	
				S. sclerotiorum	***C. arietinum***
MR	6	67,354,385	98.7	1,207,201 (1.8%)	66,147,184 (98.2%)
	12	68,680,857	98.76	5,929,006 (8.6%)	62,751,851 (91.4%)
	24	61,114,985	98.91	28,078,637 (45.9%)	33,036,348 (54.1%)
	48	56,616,306	98.85	40,566,767 (71.7%)	16,049,538 (28.3%)
	72	63,109,260	98.92	39,012,318 (61.8%)	24,096,941 (38.2%)
S	6	58,025,893	98.4	414,371 (0.7%)	57,611,521 (99.2%)
	12	72,896,961	98.4	1,851 043 (2.5%)	71,045,918 (97.5)
	24	54,049,381	98.4	18,414,027 (31.%)	35,635,354 (65.9%)
	48	60,727,165	98.5	36,714,863 (60.4%)	24,012,301 (39.5%)
	72	57,636,636	98.5	39,273,084 (68.1%)	18,363,551 (31.9%)
				**20, 961,027	**40,875,050
In vitro	0	56,566, 082	96.8	53,907,476 (95.3%)	NA

**averages number of reads

The similarity of the three biological replicates and the accuracy of the RNA-seq analysis was demonstrated using classic multidimensional scaling (MDS), which shows the MDS plot of distances between gene expression profiles (Fig. 4.1). The MDS showed a distinct grouping of samples grown *in vitro* and *in planta* at the early (6-12 hpi), the mid (24 hpi) and late (48-72 hpi) stage of infection (Fig. 4.1). There was a clear distinction between the *S. sclerotiorum* transcriptomes at 24 and 48-72 hpi, an indication of the significant differences in the types of genes expressed at these time points.

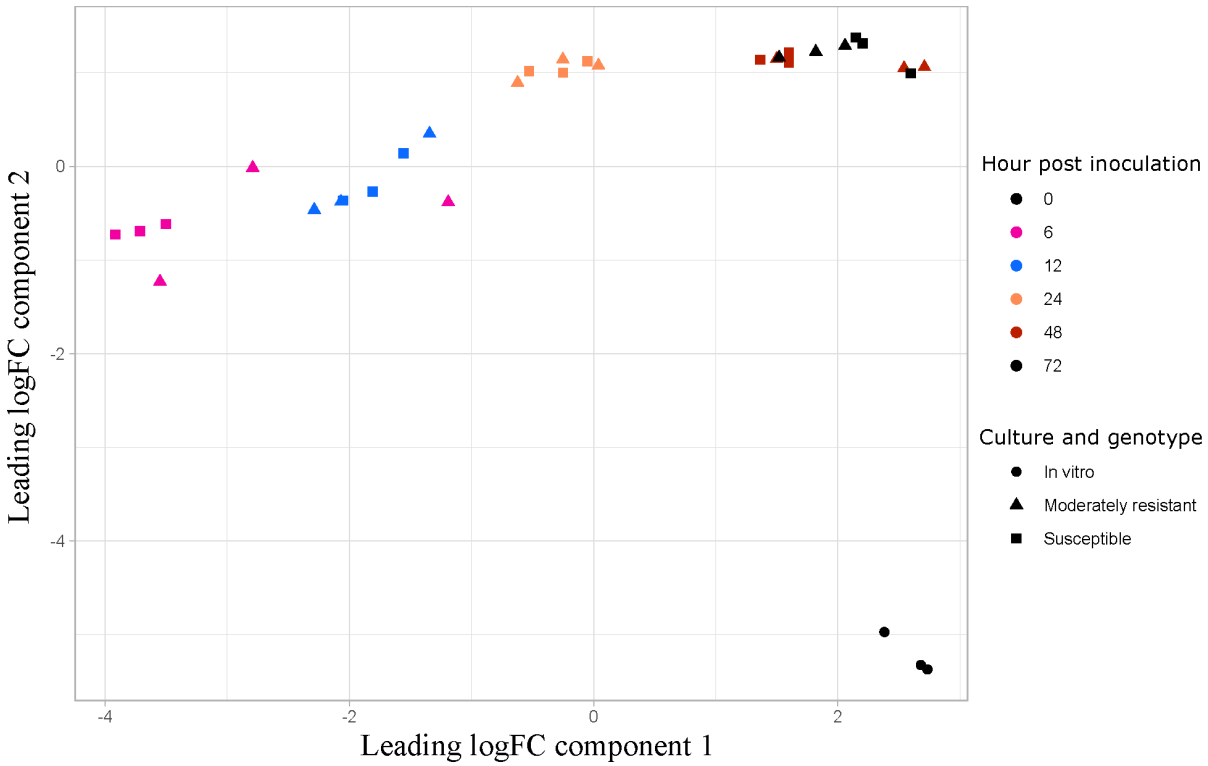


Figure 4.1: A multidimensional scaling (MDS) plot showing the relatedness of *Sclerotinia sclerotiorum* samples used for RNA-Seq analysis. Samples were collected from moderately resistant (MR) and susceptible (S) chickpea lines at 6, 12, 24, 48 and 72 hours post inoculation (hpi), as well as samples from an *in vitro* culture. The symbol ▲represents the MR, ■ the S and ● the *in vitro* samples. The x and y-axis represent Euclidean dimensions, distinct colours represent each treatment, and individual dots represent each sample.

4.4.2. Validation of RNA-seq data using reverse transcription-quantitative PCR

To validate the accuracy of the RNA-seq data, five upregulated genes and one downregulated gene in both chickpea lines at 12 hpi (early infection stage) and 48 hpi (late infection stage) were quantified using reverse transcription quantitative polymerase chain reaction (RT-qPCR) (Fig. 4.2). Six genes that according to RNA-seq analysis, five were significantly upregulated (sscle_05g041810, sscle_11g084430, sscle_08g067130, sscle_04g033880 and sscle_01g003110) and one was significantly downregulated (sscle_16g108230) , were randomly selected for validation. These genes, their putative functions and the primer sequences are listed in Supplementary Table S4.1. The expression patterns for each gene in our qPCR assay (Fig. 4.2A) were similar to the expression observed in the RNA-seq data (Fig. 4.2B). These results thus show a correlation between our qPCR and RNA-seq data.

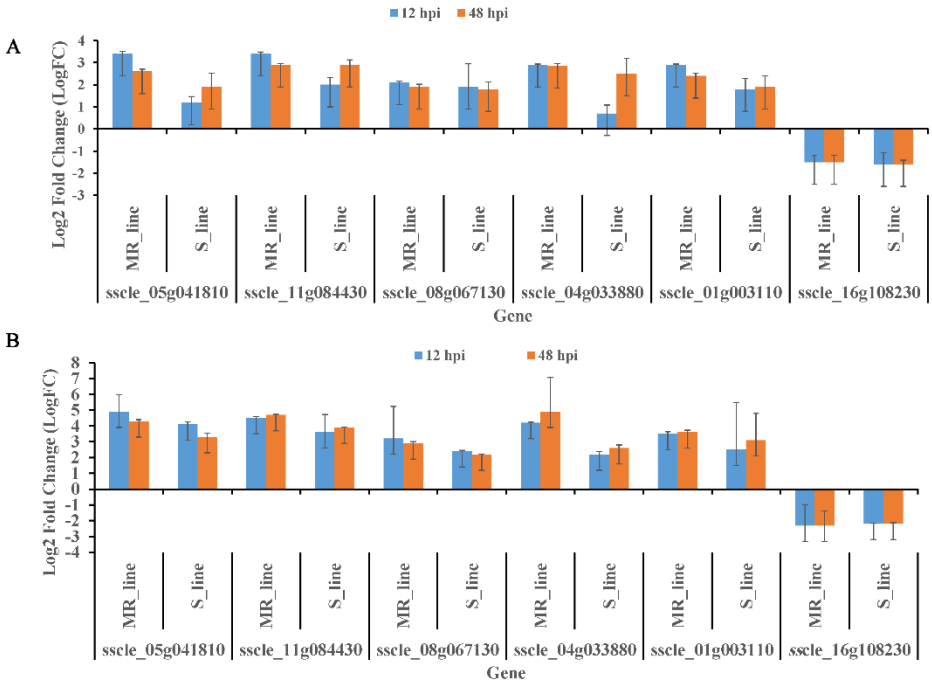


Figure 4.2: Reverse transcription-quantitative PCR (RT-qPCR) validation of RNA sequencing (RNA-Seq) data in the moderately resistant (MR) and susceptible (S) chickpea lines following infection with *Sclerotinia sclerotiorum*. Log_2(fold change) (LogFC) values were generated for qPCR samples by comparing the expression of genes at each time point of infection vs the *in vitro* control sample using the $2^{-\Delta\Delta Ct}$ method (Fig. 4.2A). LogFC values were generated for RNA-Seq samples by comparing the average raw read counts at each time point of infection vs *in vitro*/vegetative growth culture (Fig. 4.2B). Pairwise contrasts were performed using quasi-likelihood F tests. The data are presented as means ± standard error (SE) from three biological replicates for 12 hpi (early stage of infection) and 48 hpi (late stage of infection).

4.4.3. Genotype-specific and genotype non-specific differential gene expression during *Sclerotinia sclerotiorum* infection of chickpea

Based on the distinct differences between the *in planta* and *in vitro* samples demonstrated in the MDS plot (Fig. 4.1), we expected that many *S. sclerotiorum* genes would be differentially expressed *in planta* relative to *in vitro*, irrespective of the susceptibility level of the host line. Therefore, we first assessed whether there were significant differences in read counts for each of the infection time points for each host relative to *in vitro*. We identified upregulation of 2,150 and 3,593 and downregulation of 7,341 and 6,894 *S. sclerotiorum* genes during MR and S line infection, respectively (Fig. 4.3 A and B, Supplementary Table S4.2, Fig. S4.1). There were 171 common genes upregulated in the MR line (Fig. 4.3A) and 230 common genes

upregulated in the S line (Fig. 4.3B). A comparative analysis of the upregulated genes between the MR and S genotypes during the early stage (6-12 hpi) and late stage (48-72 hpi) of infection revealed that 511 genes were differentially expressed relative to *in vitro* at the same time points on both the MR and S lines (Fig. 4.3C, Supplementary Table S4.3). A gene encoding an alcohol oxidase (*SsAOX*; sscle_03g024060) was the most upregulated gene common to the two chickpea genotypes (Fig. 4.3D). An alcohol oxidase in *Cladosporium fulvum* has been suggested to be a key component in the detoxification of antifungal compounds released from the plant cell wall during infection (Segers *et al.* 2001). Similarly, two putative hydrophobic cell surface proteins (sscle_12g091650 (logFC = 9.6-12.5) and sscle_09g070510 (LogFC = 7.3-8.6) were the most highly upregulated at an early stage of infection relative to *in vitro* across both cultivars. The gene sscle_12g091650 contains a hydrophobic surface binding protein A (HsbA) domain (PF12296) which was originally identified in *Aspergillus oryzae* as a surface protein that plays a key role in both the adhesion to and degradation of hydrophobic surfaces (Ohtaki *et al.* 2006). Similarly, sscle_09g070510 contains a repeated fasciclin domain (PF02469) which has been reported in *Magnaporthe oryzae* to be important in adhesion and binding to hydrophobic surfaces (Liu *et al.* 2009). Our findings suggest these two genes might have a role during the *S. sclerotiorum* biotrophic phase during chickpea infection.

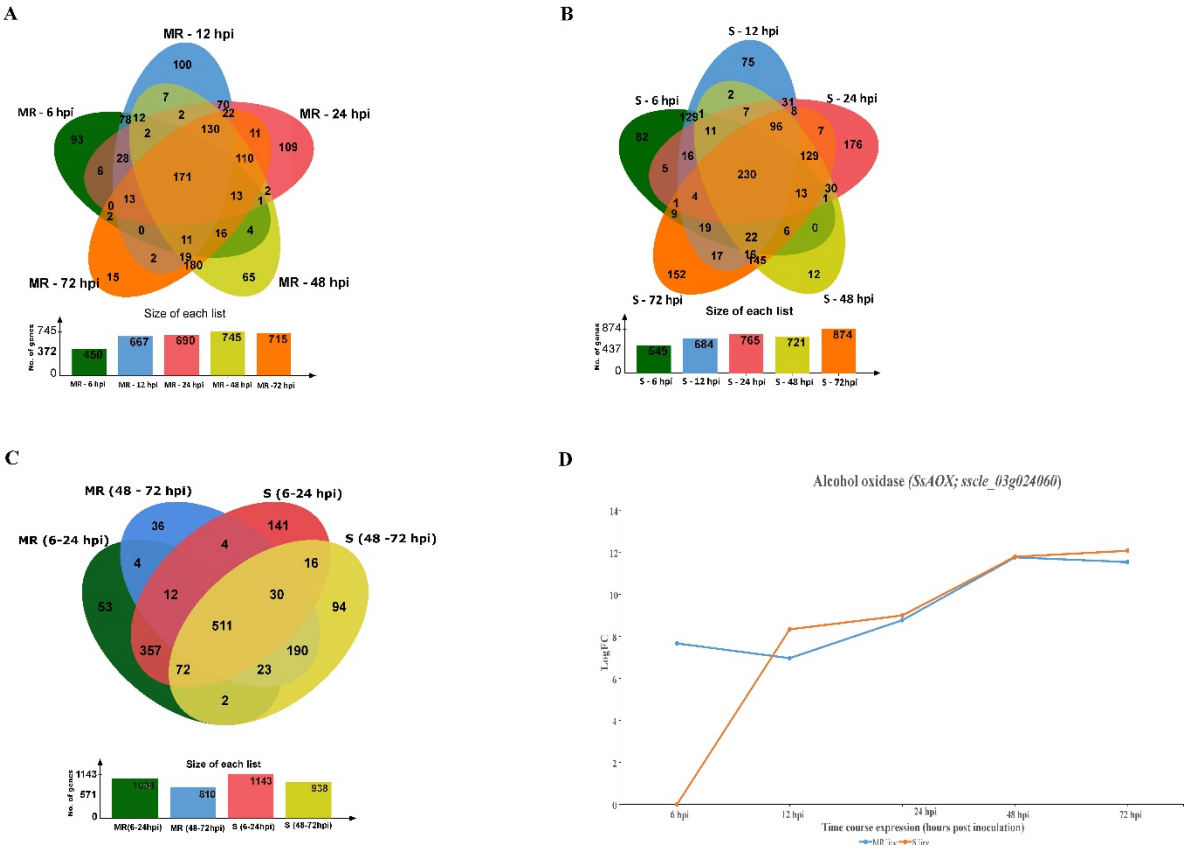


Figure 4.3: Venn diagram and graph showing upregulated *Sclerotinia sclerotiorum* genes during interaction with chickpea. Venn diagram shows the number of common and unique genes at time points 6, 12, 24, 48, and 72 hpi in (A) moderately resistant (MR), and (B) susceptible (S) lines (C) Comparison of MR and S genes (D).A graph showing expression pattern during the time course of infection of the most highly expressed common gene between MR and S line.

The current study demonstrates common genes between MR and S line when compared to *in vitro* (Supplementary Table S4.3). Further, comparing the transcription changes in MR and S lines, we found 82 and 251 genes upregulate in MR only and S only, respectively (Additional files: Fig. S4.2, Supplementary Table S4.4). There were 42 genes with known functions expressed either in MR or S line only and are involved in cell wall degradation, secondary metabolites, transportation, detoxification, and signalling (Supplementary Fig. S4.2). The common gene and these exclusively upregulate genes are discussed in various sections below. To note are two genes upregulated in the MR only which are involved in sugar glucose and carboxylate catabolism, metabolism and anabolism (sscle_01g005580 and sscle_05g040510) (Supplementary Fig. S4.2), indicating the importance of hydrolytic activities during infection of chickpea. Previous research has found pentose phosphate is critical in fungal pathogens for supplying cells with NADPH for detoxification of ROS and virulence (Liu *et al.* 2009; Stincone *et al.* 2015). A gene involved in the pentose-phosphate pathway (sscle_01g005580) was upregulated in the MR line only. The full virulence of *S. sclerotiorum* requires detoxification of ROS, an important component of the host defence response (Stotz *et al.* 2011), suggesting that *S. sclerotiorum* upregulation of sscle_01g005580 may be a managing strategy of host resistance responses.

Expression analysis of the MR versus S line at each time point showed that there were only four genes that were differentially expressed between genotypes at any given time point (Supplementary Table S4.2). This included two genes downregulated in the MR relative to the S line (upregulated in the S line) at 6 hpi and the other two upregulated in the MR relative to the S line (downregulated in S line) at 48 hpi. The genes sscle_09g073140 (logFC = 5.1, p_{adj} = 0.02) and sscle_04g033530 (log FC = 4.2, p_{adj}=0.04) were differentially expressed at 6 hpi and sscle_16g111070 (logFC=5.3, p_{adj} = 0.004) and sscle_05g047520 (logFC = 5.3) were differentially expressed at 48 hpi. These four genes are predicted in the *S. sclerotiorum* genome, but they have no known functional domains. Therefore, it is not possible to speculate much on their role during specific interactions between MR and S chickpea genotypes.

We also performed an analysis where we included the genotype x time point interaction. The final design as a factor and found that this interaction was not significant for any genes (P_{adj} = 0.05), suggesting that all genes had temporally similar expression patterns between the two lines. We did not include hosts (*C. arietinum*) differentially expressed genes in the current manuscript, as this will form a discrete study along with other data in future. However, the limited differences in expression of *S. sclerotiorum* genes between the two hosts would suggest that they present a qualitatively similar environment to the pathogen despite one of them, the MR line, reducing the extent of pathogen growth.

4.4.4. Gene Ontology term enrichment analysis of upregulated genes identifies multiple biological and molecular functions associated with infection

Gene Ontology (GO) enrichment analysis is a powerful technique for analysing differential gene expression data to gain insight into the broader biological processes (BP), molecular functions (MF) and cellular components (CC) of genes. *S. sclerotiorum* expressed genes in the current study were significantly enriched with a wide range of upregulated (Supplementary Table S4.5, Supplementary Fig. S4.3) and downregulated (Supplementary Table S4.5, Supplementary Fig. S4.4) GO categories.

The BPs highly enriched during the early stage of infection included oxidation-reduction process (GO:0055114), protein metabolic process (GO:0019538), proteolysis (GO:0006508), cellular response to stimulus (GO:0051716) signal transduction (GO:0007165), carbohydrate metabolic process (GO:0005975) and metabolic processes (GO:0008152) (Supplementary Table S4.5). Early defence of *Aschochyta rabiei* in chickpea has been associated with a strong accumulation of reactive oxygen species (ROS) in resistant chickpea cultivars compared to susceptible chickpea cultivars (Sambasivam *et al.* 2020). Similarly, previous research found *A. thaliana* enhanced host ROS increased resistance to *S. sclerotiorum*, and coordinately *S. sclerotiorum* genes involve in response to oxidative stress were overexpressed (Ding *et al.* 2020). The BP category oxidation-reduction process (GO:0055114) was highly enriched exclusively in genes upregulated in the MR line at 6 hpi and 48 hpi, suggesting that *S. sclerotiorum* may focus on regulating the environment redox status during MR line infection to counter host resistance responses.

GO term enrichment analysis also provided an insight into the temporal aspects of the *S. sclerotiorum*-chickpea interaction. Genes involved in cellular communication (GO:0007154), signalling (GO;0023052), response to stimulus (GO:0050896), and signal transduction (GO:0007165) were enriched in genes upregulated in both lines at the early stage of infection

(6-24 hpi; Fig. 4.3C), indicating the importance of rapid adaptation to *in planta* growth. Among genes upregulated in both lines at the late stage of infection (48-72 hpi; Fig. 4.3 C), the enriched GO categories included carbohydrate metabolic process (GO:0005975) and metabolic process (GO:0008152), among others, an indication of the importance of utilisation of energy sources during the necrotic phase of *S. sclerotiorum* infection. The most significantly enriched GO categories in the current study grouped into carbohydrate-active enZYmes (CAZymes), proteases, transporters, transcription factors and other secondary metabolites. Genes were categorised based on their functions and predicted roles to simplify the study, as discussed below.

4.4.5. Genes involved in the degradation of the host cuticle

The plant cuticle is the first physical barrier to pathogen invasion and is composed of lipid-derived polyester and cuticular waxes (Heredia and Dominguez 2009). In the current study, *S. sclerotiorum* genes encoding cutinases and lipases were upregulated throughout infection. Interestingly, four *S. sclerotiorum* genes encoding lysophospholipase (sscle_02g020060), carboxylesterase (sscle_03g027590), GDSL-lipase-acylhydrolase (sscle_01g004820), and triacylglycerol lipase (sscle_01g008640) were significantly upregulated at the late stage of infection, specifically in the S line (Supplementary Table S4.6). This suggests the induction of lipolytic enzymatic activity in *S. sclerotiorum* may depend on the immunity of the host. Lipases were also reported to act as virulence factors in the fungal phytopathogen *B. cinerea* (Reis *et al.* 2005), suggesting *S. sclerotiorum* lipases may play a role in virulence.

Table 4.2: The number of *in planta* upregulated *S. sclerotiorum* genes involved in the cell wall and cuticle degradation.

Substrate	CWDE category	Number of upregulated genes in the category
Lipid/cutin	Cutin	14
Polysaccharides	Cellulose	19
	Arabinogalactan	6
	Hemicellulose	16
	Mannan	7
	Pectin	16
	Starch	3
Proteins/peptides	Protein	17

4.4.6. Genes involved in the degradation of the host cell wall

As a necrotroph, degradation of the host cell wall is important during *S. sclerotiorum* infection to achieve the required plant cell death for growth and development (Lehtinen 1993). A portion of the numerous cell wall degrading enzymes (CWDEs) identified in the *S. sclerotiorum* genome (Amselem *et al.* 2011), including those involved in the degradation of lipids, cellulose, arabinogalactan, hemicellulose, mannan, pectin, starch and proteins, were upregulated during infection of chickpea (Table 4.2, Supplementary Table S4.6). After breaching the cuticle, polygalacturonases (PGs) are often the first lytic enzymes produced by a pathogen (Alghisi and Favaron 1995; ten Have *et al.* 1998). A putative exo-PG (sscle_05g046840, LogFC=3.2-8.2) was the most upregulated relative to *in vitro* in the current study in both chickpea cultivars relative to *in vitro* throughout the infection (Supplementary Table S4.6). Four previously characterised PGs: endo-PGs *Sspg1* (sscle_16g108170) and *Sspg3* (sscle_09g070580), and exo-PGs *Ssxpg1* (sscle_02g018610) and *Ssxpg2* (sscle_04g035440) were also upregulated in the current study, relative to *in vitro* (Supplementary Table S4.5). Infiltration of purified endo-PG into plant leaf tissues causes rapid loss of cell wall integrity followed by cell death (Bashi *et al.* 2012; Kars *et al.* 2005), suggesting the importance of *Sspg1* and *Sspg3* in tissue maceration during *S. sclerotiorum* infection. Orthologs of *Ssxpg1* and *Ssxpg2* in *B. cinerea* (*BcPG1* and *BcPG2*) showed necrosis inducing activities and disruption of either of the genes reduced virulence(Stincone *et al.* 2015; Wilson *et al.* 2007), an indication of the significant role exo-PGs play in lesion development and host colonisation.

Proteases are hydrolytic enzymes that act as important virulence factors in many fungal plant pathogens by degrading host proteins that are involved in the immune response (Franceschetti *et al.* 2017). The *in planta* upregulation relative to *in vitro* of non-aspartyl acid protease (*acp1*; sscle_11g082980) was observed at all time points, peaking in expression at 24 hpi in both lines (LogFC= 7.2-7.9) (Supplementary Table S4.5). Several factors control *acp1* induction, including glucose levels, nitrogen starvation and acidification (Poussereau *et al.* 2001). Previous studies found upregulation of *acp1* at a later stage of *S. sclerotiorum* infection in *H. annuus* cotyledons (Poussereau *et al.* 2001), *G. max* petioles (Westrick *et al.* 2019), and *B. napus* leave (Seifbarghi *et al.* 2017), suggesting that *acp1* has a possible role in virulence on multiple plant species and that it responds to cues present at different infection stages in different hosts. Another gene encoding an aspartyl protease, sscle_07g058540, was upregulated at all stages of infection in the current study, with a peak expression relative to *in vitro* at 24 hpi (Supplementary Table S4.5). The gene sscle_07g058540 is a homologue of several aspergillopepsin-like proteins (cd06097) in aspergillosis of humans, which act as a cofactor for

the persistence of colonisation (Rementeria *et al.* 2005). Putting this all together, sscle_07g058540 may be a catalyst that assists *S. sclerotiorum* growth and development during infection.

4.4.7. *S. sclerotiorum* secondary metabolite synthesis and detoxification enzymes

Secondary metabolite (SM) polyketide synthases (PKSs) and non-ribosomal peptide synthases (NRPSs) were the major enzymes associated with SM synthesis in *S. sclerotiorum* and make up to 47.2% of the upregulated SM biosynthesis clusters in the current study (Supplementary Table S4.6). The SM biosynthesis gene expressed at the highest level (LogFC = 7.6-9.2) was a gene encoding the PKS responsible for dihydroxy naphthalene (DHN) melanin biosynthesis (*PKS13*; sscle_03g031520) at 6-12 hpi as compared to the *in vitro* control, indicating a possible role in penetration during chickpea infection (Supplementary Table S4.6). In a previous study, disruption of *S. sclerotiorum* genes involved in melanin biosynthesis showed no change in pathogenicity; however, slower development of mycelial and hyphal branching was observed (Liang *et al.* 2018). The current results indicate the importance of melanin to aid appressoria mediated penetration of *S. sclerotiorum*.

Table 4.3: *Sclerotinia sclerotiorum* detoxification enzymes upregulated (LogFC) *in planta* relative to *in vitro.*

		MR[a] line hpi*					S[b] line hpi*				
Gene ID	**Description**	**6**	**12**	**24**	**48**	**72**	**6**	**12**	**24**	**48**	**72**
sscle_01g003110	Glutathione S-transferase	3.6	3.5	4.9	3.6	4.1	-	2.5	5.2	3.1	3.8
sscle_01g005000	Glutathione S-transferase	-	-	3.7	3.1	2.9	-	-	3.2	-	-
sscle_08g067590	Glutathione S-transferase	-	-	-	2.8	2.9	-	-	-	-	-
sscle_02g021570	Laccase	4.3	-	-	-	-	4.6	4.5	-	-	-
sscle_01g005590	Cytochrome P450	-	-	-	3.3	2.9	-	-	-	2.9	3.1
sscle_04g033880	Cytochrome P450	4.1	4.2	5.6	4.9	5.9	-	2.2	2.3	2.6	5.7

[a] moderately resistant line; [b] susceptible line; * hpi = hours post-inoculation.

Glutathione S-transferases (GSTs) play critical roles in the detoxification of xenobiotic chemicals in fungi by reducing them to glutathione (Gullner *et al.* 2018). The *S. sclerotiorum* GST most upregulated relative to *in vitro* in this study was a UDP-glucosyltransferase (*Ssbgt1*; sscle_01g003110, LogFC = 3.6-5.2) (Table 4.3). *Ssbgt1* plays a role in the degradation of the antimicrobial compound brassinin through glycosylation and is induced in response to the presence of a cultivar of plant phytoalexins (Sexton *et al.* 2009).

Other GSTs induced *in planta* in the current study were sscle_01g005000 (logFC = 2.9-3.7) in both lines and sscle_08g067590 (logFC = 2.8-2.9) in the MR line, at 24-72 hpi (Table 4.3). Disruption of GST genes *AbGSOT1* and *AbUre2pB1* in the host generalist brassica pathogen *Alternaria brassicicola* led to reduced virulence (Calmes *et al.* 2015). This indicates the importance of xenobiotic compound detoxification during fungal infection. The greatest upregulation of GSTs was observed in the MR line, possibly a reflection of host resistance exerted by the release of host defence-related antifungal compounds during infection.

Benzoic acid derivatives are aromatic compounds arising from the plant defence β- ketoadipate pathway (Harwood and Parales 1996). The CYP enzyme, benzoate 4-hydroxylase, from *Aspergillus niger,* was reported to play a role in the hydroxylation of benzoic acid to 4-hydroxybenzoate (Faber *et al.* 2001). An *S. sclerotiorum* CYP gene, sscle_01g005590, encoding benzoate 4-hydroxylase, was upregulated at 48-72 hpi in both chickpea lines (Table 4.3), which may suggest higher pressure from host defence toxins at the late stage during *S. sclerotiorum*-chickpea interaction.

4.4.8. *Sclerotinia sclerotiorum* signalling pathways are vital during chickpea infection

A total of 24 *S. sclerotiorum* transcription factors were upregulated in the current study (Supplementary Table S4.7). Two functionally characterised *S. sclerotiorum* TFs *Pac1* (sscle_06g049830) (Rollins 2003), and *Ssfkh1* (sscle_06g049780) (Fan *et al.* 2017) were upregulated *in planta*. *Pac1* was upregulated at 12 hpi in the S line only (LogFC = 2.8) during chickpea infection (Supplementary Table S4.6). *Pac1* triggers oxalic acid (OA) biosynthesis in response to increased pH and reduces the ambient pH, which in turn causes an increase in *Sspg1* and *acp1* expression and promotes sclerotial development (Rollins 2003). The upregulation of *Pac1* on the S line may suggest that S line tissues were more alkaline than those of the MR line.

Fungal histidine kinases play a vital role in controlling signalling pathways that regulate osmotic and oxidative stress responses, cell cycle control and virulence (Amselem *et al.* 2011). We found that the two-component sensor histidine kinase *Shk1* (sscle_16g107650) was upregulated at 12-72 hpi relative to inoculum in both lines (Supplementary Table S4.7). A previous study showed disruption of *Shk1* led to reduced and altered hyphal growth and failed sclerotia formation (Duan *et al.* 2013). Although *Shk1* mutants exhibited normal virulence, they showed sensitivity to osmotic stress and increased resistance to fungicides, which suggest that *Shk1* likely works upstream of the MAPK cascade to control these processes.

4.4.9. A substantial portion of putative effectors are upregulated on both host cultivars during infection

We compared the *S. sclerotiorum* expressed genes with the 523 secreted proteins identified in the *S. sclerotiorum* genome (Derbyshire *et al.* 2017) to determine specific temporal changes in their regulation during chickpea infection. Of these, 173 were upregulated in both cultivars, and 148 downregulated, with nine of the upregulated genes observed in the MR cultivar only and 27 in the S cultivar only (Supplementary Table S4.8). Of the identified *S. sclerotiorum* secreted proteins, 78 were predicted to be candidate effectors by Guyon *et al*. (Guyon *et al.* 2014) and 70 by Derbyshire *et al*. (Derbyshire *et al.* 2017). Of these candidate effectors, 32 were upregulated, and 40 downregulated on both hosts during the current study (Supplementary Table S4.9).

In addition to putative candidate effectors, we also considered the expression of experimentally characterised *S. sclerotiorum* effectors. Previous studies showed that *S. sclerotiorum* small cysteine-rich secreted protein *SsSSVP1* (sscle_01g003850) plays an essential role during infection by interfering with host respiration and inducing localised tissue necrosis (Lyu *et al.* 2016). In the current study, *SsSSVP1* was upregulated at 48 hpi in the MR line and 72 hpi in the S line (logFC = 5.1 and 5.7), respectively (Supplementary Table S4.9). *S. sclerotiorum SsSSVP1* mutants showed reduced virulence in *B. napus* and *A. thaliana* (Lyu *et al.* 2016). Similarly, *SsSSVP1* upregulation was previously reported during the late stage of infection in *B. napus* (Fuhlbohm *et al.* 2003; Lehtinen 1993) and at all-time points in *G. max* (Westrick *et al.* 2019). The earlier induction *SsSSVP1* of in the MR line (48 hpi) compared to the S line (72 hpi) may suggest that temporal regulation of expression of *SsSSVP1* may depend on host susceptibility level.

Two *S. sclerotiorum* necrosis and ethylene-inducing (NEP) proteins (*SsNEP1* and *SsNEP2*) were characterised in a previous study on *Nicotiana benthamiana* and were reported to function as necrotrophic effectors (Dallal Bashi *et al.* 2010). The previous study showed upregulation of both genes at mid to later stages of infection with SsNEP2 expressed at a higher level than SsNEP1. In the current study, *SsNEP1* (sscle_04g039420) was not differentially expressed, and *SsNEP2* (sscle_12g090490) was upregulated at the later stages of infection (48 hpi in MR and at 48-72 hpi In S lines) relative to *in vitro* (Supplementary Table S4.9). Orthologs of these two genes in *B. cinerea* (*BcNEP1* and *BcNEP2*) are both proteins capable of inducing necrosis in dicotyledonous but not monocotyledons host (Schouten *et al.* 2008).

4.4.10. Expression of genes related to oxalic acid production and reactive oxygen species regulation

Oxalic acid (OA) has roles in weakening the host cell wall, activating hydrolytic enzymes, suppressing the oxidative burst and intensifying programmed cell death (PCD) leading to full colonisation (Kim *et al.* 2008). A gene encoding oxalate decarboxylase (*Ss-odc2*: sscle_09g069850) was highly upregulated at the very early stage (6 - 24 hpi) of infection (LogFC = 6.5-8.4) and showed lower expression at the later stage (LogFC = 3.8-4.2) of infection relative to *in vitro*, in both chickpea lines (Table 4.4). *Ss-odc2* protects the pathogen cells by preventing excess accumulation of OA (Heard *et al.* 2015). A previous study suggested that an alternative route of OA biosynthesis may be utilised during *S. sclerotiorum* early stages of infection (Li *et al.* 2004). Expression of *Ss-odc2* without the induction of *Ssoah1* in the current study may support previous findings that OA is not the only source of acidification or determinant of *S. sclerotiorum* virulence expression (Xu *et al.* 2015), or alternatively, the host tissue was already acidic enough for growth.

An *S. sclerotiorum* gene, sscle_09g069850, with a bicupin domain, was previously reported to be a possible oxalate oxidase enzyme (Kim *et al.* 2011). This gene was highly upregulated at 6-12 hpi (logFC= 7.6-8.7) and expression decreased at 48-72 hpi (logFC = 3.8-4.2) with no expression at 24 hpi, in both chickpea lines, relative to *in vitro* (Table 4.4). A previous study suggested that oxalate oxidases play a role in inducing programmed cell death (Kim *et al.* 2008). The pattern of sscle_09g069850 expression in the current study may suggest involvement in both the biotrophic and necrotic stages during chickpea interaction.

Catalases and peroxidases are also important *S. sclerotiorum* ROS scavengers (Schaffer and Bronnikova 2012). Three catalases, sscle_03g026200 (*Sscat1*), sscle_04g037170, and sscle_15g104430, were upregulated during the late stage of infection (48 -72 hpi) in the MR and 72 hpi in the S line (Table 4.4). The most upregulated catalase during the current study was the previously characterised *Scat1* (sscle_04g037170). *Scat1* mutants show slower radial growth, a higher number of small sclerotia and hypovirulence (Yarden *et al.* 2014). The upregulation of catalases and peroxidases was observed at an early stage in the MR line and later stage in the S line during infection, suggesting *S. sclerotiorum* induces ROS scavengers depending on the host speed of employing defence responses.

Table 4.4: *Sclerotinia sclerotiorum* reactive oxygen scavenging (ROS) enzymes upregulated (LogFC) during chickpea infection relative to *in vitro*.

		MR[a] line hpi*					S[b] line hpi*				
Gene ID	**Description**	**6**	**12**	**24**	**48**	**72**	**6**	**12**	**24**	**48**	**72**
sscle_15g104430	Catalase	-	-	-	5.1	5.7	-	-	-	-	4.5
sscle_04g037170	Catalase	-	-	-	5.6	5.8	-	-	-	-	4.7
sscle_03g026200	Catalase	-	-	-	-	-	-	-	-	-	2.9
sscle_09g069850	Oxalate decarboxylate	8.4	6.5	-	4.1	4.2	8.2	8.1	-	-	3.8
sscle_04g035020	Peroxidase	-	3.5	3.5	4.3	5.1	-	-	5.3	5.4	4.7
sscle_15g102360	Peroxidase	3.6	-	-	-	-	4.1	3.8	-	-	-
sscle_09g070530	Peroxidase	4.1	-	-	-	-	-	3.6	-	-	3.6
sscle_08g065740	Peroxidase heme-thiolate	4.5	-	-	-	-	4.6	-	-	-	-

[a] moderately resistant line; [b] susceptible line; * hpi = hours post-inoculation.

4.5. Conclusion

The current study demonstrates there were differences in *S. sclerotiorum* genes expression in MR and S line when compared to *in vitro* with 82 and 251 genes upregulated in MR only and S only, respectively (Supplementary Fig. S4.2 and Supplementary Table S4.3). Our study demonstrates a continuum of activities that occurs during infection and colonisation of *C. arietinum* by *S. sclerotiorum*. In support of our study hypothesis, we observed significant upregulation of *S. sclerotiorum* genes *in planta* irrespective of the host's susceptibility level. To our knowledge, this is the first RNA-seq study to investigate *in planta* gene expression in *S. sclerotiorum* during *C. arietinum* infection. The current findings showed that *S. sclerotiorum* induced numerous virulence factors, including CAZymes, transportation enzymes, detoxification enzymes, metabolites and putative secreted effector proteins during penetration and subsequent proliferation through the host.

In conclusion, the present study provides an insight into global transcriptional changes in *S. sclerotiorum* during infection of chickpea cultivars differing in their susceptibility to the pathogen. Our findings further emphasise the role of CAZymes and proteases, in addition to secreted effectors, transporters and detoxifying enzymes during the growth and development of *S. sclerotiorum* within chickpea plants. Temporal changes in expression have demonstrated that *S. sclerotiorum* specific gene expression may depend on host susceptibility level. Detailed investigation of the identified genes could elucidate their precise roles and determine if they represent viable targets for disease management.

4.6. Acknowledgements

The authors would like to acknowledge funding from the Australian Grains Research and Development (GRDC), Curtin University, Commonwealth Scientific and Industrial Research Organization (CSIRO) and support from the Pawsey Supercomputing Centre. We thank Mathew Denton-Giles for his collaboration at the early stage of project conception.

4.7. Availability of data and materials

The raw data used in this study has been deposited to the sequence read archive under BioProject ID: PRJNA687280. The gene identifiers prefixed with 'sscle_' used throughout this manuscript are locus tags of *S. sclerotiorum* proteins that are available in the NCBI protein database.

4.8. References

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

Alexa A, Rahnenführer J, Lengauer T. Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. Bioinform Oxf Engl. 2006;22(13):1600–7.

Alghisi P, Favaron F (1995) Pectin-degrading enzymes and plant-parasite interactions. *European Journal of Plant Pathology* **101**, 365–375.

Amselem J, Cuomo CA, Kan JAL van, Viaud M, Benito EP, Couloux A, Coutinho PM, Vries RP de, Dyer PS, Fillinger S, Fournier E, Gout L, Hahn M, Kohn L, Lapalu N, Plummer KM, Pradier J-M, Quévillon E, Sharon A, Simon A, Have A ten, Tudzynski B, Tudzynski P, Wincker P, Andrew M, Anthouard V, Beever RE, Beffa R, Benoit I, Bouzid O, Brault B, Chen Z, Choquer M, Collémare J, Cotton P, Danchin EG, Silva CD, Gautier A, Giraud C, Giraud T, Gonzalez C, Grossetete S, Güldener U, Henrissat B, Howlett BJ, Kodira C, Kretschmer M, Lappartient A, Leroch M, Levis C, Mauceli E, Neuvéglise C, Oeser B, Pearson M, Poulain J, Poussereau N, Quesneville H, Rascle C, Schumacher J, Ségurens B, Sexton A, Silva E, Sirven C, Soanes DM, Talbot NJ, Templeton M, Yandava C, Yarden O, Zeng Q, Rollins JA, Lebrun M-H, Dickman M

(2011) Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *PLoS Genetics* **7**, (8):e. 1002230.

Anders S, Pyl PT, Huber W (2015) HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169.

Bashi ZD, Rimmer SR, Khachatourians GG, Hegedus DD (2012) *Brassica napus* polygalacturonase inhibitor proteins inhibit *Sclerotinia sclerotiorum* polygalacturonase enzymatic and necrotizing activities and delay symptoms in transgenic plants. *Canadian Journal of Microbiology* **59**, 79–86.

Boland GJ, Hall R (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology* **16**, 93–108.

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120.

Bolton MD, Thomma BPHJ, Nelson BD (2006) *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. *Molecular Plant Pathology* **7**, 1–16.

Calmes B, Morel-Rouhier M, Bataillé-Simoneau N, Gelhaye E, Guillemette T, Simoneau P (2015) Characterization of glutathione transferases involved in the pathogenicity of *Alternaria brassicicola*. *BMC Microbiology* **15**, 123.

Casadevall A (2007) Determinants of virulence in the pathogenic fungi. *Fungal Biology Reviews* **21**, 130–132.

Casadevall A, Pirofski L (1999) Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infection and Immunity* **67**, 3703–3713.

Chittem K, Yajima WR, Goswami RS, del Río Mendoza LE (2020) Transcriptome analysis of the plant pathogen *Sclerotinia sclerotiorum* interaction with resistant and susceptible canola (*Brassica napus*) lines. *PLoS ONE* **15**(3): e0229844.

Dallal Bashi Z, Hegedus DD, Buchwaldt L, Rimmer SR, Borhan MH (2010) Expression and regulation of *Sclerotinia sclerotiorum* necrosis and ethylene-inducing peptides (NEPs). *Molecular Plant Pathology* **11**, 43–53.

Denton-Giles M, Derbyshire MC, Khentry Y, Buchwaldt L, Kamphuis LG (2018) Partial stem resistance in *Brassica napus* to highly aggressive and genetically diverse *Sclerotinia sclerotiorum* isolates from Australia. *Canadian Journal of Plant Pathology* **40**, 551–561.

Derbyshire M, Denton-Giles M, Hegedus D, Seifbarghy S, Rollins J, van Kan J, Seidl MF, Faino L, Mbengue M, Navaud O, Raffaele S, Hammond-Kosack K, Heard S, Oliver R (2017) The complete genome sequence of the phytopathogenic fungus *Sclerotinia sclerotiorum* reveals insights into the genome architecture of broad host range pathogens. *Genome Biology and Evolution* **9**, 593–618.

Ding Y, Mei J, Chai Y, Yang W, Mao Y, Yan B, Yu Y, Disi JO, Rana K, Li J, Qian W (2020) *Sclerotinia sclerotiorum* utilizes host-derived copper for ROS detoxification and infection. *PLOS Pathogens* **16**, e1008919.

Duan Y, Ge C, Liu S, Wang J, Zhou M (2013) A two-component histidine kinase Shk1 controls stress response, sclerotial formation and fungicide resistance in *Sclerotinia sclerotiorum*. *Molecular Plant Pathology* **14**, 708–718.

Faber BW, van Gorcom RFM, Duine JA (2001) Purification and characterization of benzoate-para-hydroxylase, a Cytochrome P450 (CYP53A1), from *Aspergillus niger*. *Archives of Biochemistry and Biophysics* **394**, 245–254.

Fan H, Yu G, Liu Y, Zhang X, Liu J, Zhang Y, Rollins JA, Sun F, Pan H (2017) An atypical forkhead-containing transcription factor SsFKH1 is involved in sclerotial formation and is essential for pathogenicity in *Sclerotinia sclerotiorum*. *Molecular Plant Pathology* **18**, 963–975.

Franceschetti M, Maqbool A, Jiménez-Dalmaroni MJ, Pennington HG, Kamoun S, Banfield MJ (2017) Effectors of filamentous plant pathogens: commonalities amid diversity. *Microbiology and molecular biology reviews: MMBR* **81**,e00066-16.

Fuhlbohm MJ, Tatnell JR, Ryley MJ (2003) First report of stem rot and wilt of chickpea caused by *Sclerotinia minor* in Queensland, Australia. *Australasian Plant Pathology* **2**, 323–324.

Gullner G, Komives T, Király L, Schröder P (2018) Glutathione S-Transferase Enzymes in Plant-Pathogen Interactions. *Frontiers in Plant Science* **9**, e01836.

Guyon K, Balagué C, Roby D, Raffaele S (2014) Secretome analysis reveals effector candidates associated with broad host range necrotrophy in the fungal plant pathogen *Sclerotinia sclerotiorum*. *BMC Genomics* **15**, 1–19.

Harwood CS, Parales RE (1996) The β-ketoadipate pathway and the biology of self-identity. *Annual Review of Microbiology* **50**, 553–590.

ten Have A, Mulder W, Visser J, van Kan JA (1998) The endopolygalacturonase gene Bcpg1 is required for full virulence of *Botrytis cinerea*. *Molecular plant-microbe interactions: MPMI* **11**, 1009–1016.

Heard S, Brown NA, Hammond-Kosack K (2015) An interspecies comparative analysis of the predicted secretomes of the necrotrophic plant pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *PLOS ONE* **10**, e0130534.

Heredia A, Dominguez E (2009) The Plant Cuticle: A complex lipid barrier between the plant and the environment. An overview. InDishovsky C, Pivovarov A (eds) 'Counteraction Chem. Biol. Terror. East Eur. Ctries.', Dordrecht. NATO Science for peace and security series A: Chemistry and Biology. 109–116. (Springer Netherlands: Dordrecht) doi:10.1007/978-90-481-2342-1_14.

Kabbage M, Williams B, Dickman MB (2013) Cell death control: The Interplay of apoptosis and autophagy in the pathogenicity of *Sclerotinia sclerotiorum*. *PLoS Pathogens* **9**,e1003287.

Kabbage M, Yarden O, Dickman MB (2015) Pathogenic attributes of *Sclerotinia sclerotiorum*: Switching from a biotrophic to necrotrophic lifestyle. *Plant Science* **233**, 53–60.

Kars I, Krooshof GH, Wagemakers L, Joosten R, Benen JAE, Kan JALV (2005) Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *The Plant Journal* **43**, 213–225.

Kim H, Chen C, Kabbage M, Dickman MB (2011) Identification and characterization of *Sclerotinia sclerotiorum* NADPH Oxidases. *Applied and Environmental Microbiology* **77**, 7721–7729.

Kim D, Langmead B, Salzberg SL (2015) HISAT: a fast spliced aligner with low memory requirements. *Nature Methods* **12**, 357–360.

Kim KS, Min J-Y, Dickman MB (2008) Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Molecular Plant-Microbe Interactions*® **21**, 605–612.

Kubicek CP, Starr TL, Glass NL (2014) Plant cell wall–degrading enzymes and their secretion in plant-pathogenic fungi. *Annual Review of Phytopathology* **52**, 427–451. d

Kukreja S, Salaria N, Thakur K, Goutam U (2018) Fungal disease management in chickpea: Current status and future prospects. 'Fungi their role sustain. Dev. Curr. Perspect.' (Eds P Gehlot, J Singh) pp. 293–309. (Springer: Singapore)

Lehtinen U (1993) Plant cell wall degrading enzymes of *Septoria nodorum*. *Physiological and Molecular Plant Pathology* **43**, 121–134.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R,(2009) 1000 Genome Project Data Processing Subgroup. The Sequence Alignment/Map format and SAMtools. *Bioinformatics (Oxford, England)* **25**, 2078–2079.

Li M, Liang X, Rollins JA (2011) *Sclerotinia sclerotiorum* γ-Glutamyl transpeptidase (Ss-Ggt1) is required for regulating glutathione accumulation and development of sclerotia and compound appressoria. *Molecular Plant-Microbe Interactions*® **25**, 412–420.

Li R, Rimmer R, Buchwaldt L, Sharpe AG, Séguin-Swartz G, Hegedus DD (2004) Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: cloning and characterization of endo- and exo-polygalacturonases expressed during saprophytic and parasitic modes. *Fungal genetics and biology: FG & B* **41**, 754–765.

Liang X, Rollins JA (2018) mechanisms of broad host range necrotrophic pathogenesis in *Sclerotinia sclerotiorum*. *Phytopathology*® **108**, 1128–1140.

Liang Y, Xiong W, Steinkellner S, Feng J (2018) Deficiency of the melanin biosynthesis genes SCD1 and THR1 affects sclerotial development and vegetative growth, but not pathogenicity, in *Sclerotinia sclerotiorum*. *Molecular Plant Pathology* **19**, 1444–1453.

Liu T, Chen G, Min H, Lin F (2009) MoFLP1, encoding a novel fungal fasciclin-like protein, is involved in conidiation and pathogenicity in *Magnaporthe oryzae*. *Journal of Zhejiang University SCIENCE B* **10**, 434–444. doi:10.1631/jzus.B0920017.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. *Methods* **25**, 402–408.

Lun ATL, Chen Y, Smyth GK (2016) It's DE-licious: A recipe for differential expression analyses of rna-seq experiments using quasi-likelihood methods in edgeR. *Methods in Molecular Biology (Clifton, NJ)* **1418**, 391–416.

Lyu X, Shen C, Fu Y, Xie J, Jiang D, Li G, Cheng J (2016) A small secreted virulence-related protein is essential for the necrotrophic interactions of *Sclerotinia sclerotiorum* with its host plants. *PLOS Pathogens* **12**, e1005435.

Mwape VW, Yuphin K, Newman, TE, Denton-Giles M, Derbyshire MC, Chen K, Kamphuis LG (2021a) Identification of sources of Sclerotinia sclerotiorum resistance in a collection of wild Cicer germplasm. *Plant Disease*. https://doi.org/10.1094/PDIS-02-21-0367-RE.

Ohtaki S, Maeda H, Takahashi T, Yamagata Y, Hasegawa F, Gomi K, Nakajima T, Abe K (2006) Novel hydrophobic surface binding protein, HsbA, produced by *Aspergillus oryzae*. *Applied and Environmental Microbiology* **72**, 2407–2413.

Oliveira MB, de Andrade RV, Grossi-de-Sá MF, Petrofeza S (2015) Analysis of genes that are differentially expressed during the *Sclerotinia sclerotiorum-Phaseolus vulgaris* interaction. *Frontiers in Microbiology* **6**, 1162.

Poussereau N, Creton S, Billon-Grand G, Rascle C, Fevre M (2001) Regulation of acp1, encoding a non-aspartyl acid protease expressed during the pathogenesis of *Sclerotinia sclerotiorum*. *Microbiology,* **147**, 717–726.

Reis H, Pfiffi S, Hahn M (2005) Molecular and functional characterization of a secreted lipase from *Botrytis cinerea*. *Molecular Plant Pathology* **6**, 257–267.

Rementeria A, López-Molina N, Ludwig A, Vivanco AB, Bikandi J, Pontón J, Garaizar J (2005) Genes and molecules involved in *Aspergillus fumigatus* virulence. *Revista Iberoamericana De Micologia* **22**, 1–23.

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research* **43**, e47–e47.

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)* **26**, 139–140.

Rollins JA (2003) The *Sclerotinia sclerotiorum* pac1 gene is required for sclerotial development and virulence. *Molecular Plant-Microbe Interactions®* **16**, 785–795.

Sambasivam P, Mehmood Y, Bar I, Davidson J, Hobson K, Moore K, Ford R (2020) Evidence of recently increased pathogenicity within the Australian Ascochyta rabiei population. *bioRxiv* 2020.06.28.175653.

Schaffer WM, Bronnikova TV (2012) Peroxidase-ROS interactions. *Nonlinear Dynamics* **68**, 413–430. d

Schouten A, van Baarlen P, van Kan JAL (2008) Phytotoxic Nep1-like proteins from the necrotrophic fungus *Botrytis cinerea* associate with membranes and the nucleus of plant cells. *The New Phytologist* **177**, 493–505.

Segers G, Bradshaw N, Archer D, Blissett K, Oliver RP (2001) Alcohol oxidase is a novel pathogenicity factor for *Cladosporium fulvum*, but aldehyde dehydrogenase is dispensable. *Molecular Plant-Microbe Interactions®* **14**, 367–377.

Seifbarghi S, Borhan MH, Wei Y, Coutu C, Robinson SJ, Hegedus DD (2017) Changes in the *Sclerotinia sclerotiorum* transcriptome during infection of *Brassica napus*. *BMC Genomics* **18**, 266.

Sexton AC, Minic Z, Cozijnsen AJ, Pedras MSC, Howlett BJ (2009) Cloning, purification and characterisation of brassinin glucosyltransferase, a phytoalexin-detoxifying enzyme from the plant pathogen *Sclerotinia sclerotiorum*. *Fungal Genetics and Biology* **46**, 201–209.

Stincone A, Prigione A, Cramer T, Wamelink MMC, Campbell K, Cheung E, Olin-Sandoval V, Grüning N-M, Krüger A, Alam MT, Keller MA, Breitenbach M, Brindle KM, Rabinowitz JD, Ralser M (2015) The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. *Biological Reviews* **90**, 927–963.

Stotz HU, Sawada Y, Shimada Y, Hirai MY, Sasaki E, Krischke M, Brown PD, Saito K, Kamiya Y (2011) Role of camalexin, indole glucosinolates, and side chain modification of glucosinolate-derived isothiocyanates in defense of Arabidopsis against *Sclerotinia sclerotiorum*. *The Plant Journal* **67**, 81–93.

Tang L, Yang G, Ma M, Liu X, Li B, Xie J, Fu Y, Chen T, Yu Y, Chen W, Jiang D, Cheng J (2020) An effector of a necrotrophic fungal pathogen targets the calcium-sensing receptor in chloroplasts to inhibit host resistance. *Molecular Plant Pathology* **21**, 686–701.

Varshney RK, Song C, Saxena RK, Azam S, Yu S, Sharpe AG, Cannon S, Baek J, Rosen BD, Tar'an B, Millan T, Zhang X, Ramsay LD, Iwata A, Wang Y, Nelson W, Farmer AD, Gaur PM, Soderlund C, Penmetsa RV, Xu C, Bharti AK, He W, Winter P, Zhao S, Hane JK, Carrasquilla-Garcia N, Condie JA, Upadhyaya HD, Luo M-C, Thudi M, Gowda CLL, Singh NP, Lichtenzveig J, Gali KK, Rubio J, Nadarajan N, Dolezel J, Bansal KC, Xu X, Edwards D, Zhang G, Kahl G, Gil J, Singh KB, Datta SK, Jackson SA, Wang J, Cook DR (2013) Draft genome sequence of chickpea (*Cicer arietinum*) provides a resource for trait improvement. *Nature Biotechnology* **31**, 240–246.

Westrick NM, Ranjan A, Jain S, Grau CR, Smith DL, Kabbage M (2019) Gene regulation of *Sclerotinia sclerotiorum* during infection of *Glycine max*: on the road to pathogenesis. *BMC genomics* **20**, 157–157.

Wilson RA, Jenkinson JM, Gibson RP, Littlechild JA, Wang Z-Y, Talbot NJ (2007) Tps1 regulates the pentose phosphate pathway, nitrogen metabolism and fungal virulence. *The EMBO Journal* **26**, 3673–3685.

Xiao X, Xie J, Cheng J, Li G, Yi X, Jiang D, Fu Y (2013) Novel secretory protein Ss-Caf1 of the plant-pathogenic fungus sclerotinia sclerotiorum is required for host penetration and normal sclerotial development. *Molecular Plant-Microbe Interactions®* **27**, 40–55.

Xu L, Xiang M, White D, Chen W (2015) pH dependency of sclerotial development and pathogenicity revealed by using genetically defined oxalate-minus mutants of *Sclerotinia sclerotiorum*. *Environmental Microbiology* **17**, 2896–2909.

Yajima W, Liang Y, Kav NNV (2009) Gene disruption of an arabinofuranosidase/beta-xylosidase precursor decreases *Sclerotinia sclerotiorum* virulence on canola tissue. *Molecular plant-microbe interactions: MPMI* **22**, 783–789.

Yarden O, Veluchamy S, Dickman MB, Kabbage M (2014) *Sclerotinia sclerotiorum* catalase SCAT1 affects oxidative stress tolerance, regulates ergosterol levels and controls pathogenic development. *Physiological and Molecular Plant Pathology* **85**, 34–41.

Zhang H, Yohe T, Huang L, Entwistle S, Wu P, Yang Z, Busk PK, Xu Y, Yin Y (2018) dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. *Nucleic Acids Research* **46**, W95–W101.

Zhu W, Wei W, Fu Y, Cheng J, Xie J, Li G, Yi X, Kang Z, Dickman MB, Jiang D (2013) A secretory protein of necrotrophic fungus *Sclerotinia sclerotiorum* that suppresses host resistance. *PLoS ONE* **8**,e0053901.

CHAPTER 5

A transcriptome analysis uncovers molecular mechanisms of resistance to *Sclerotinia sclerotiorum* in chickpea

Virginia W. Mwape[1,2], Fredrick M. Mobegi[1], Toby. E. Newman[1], Mark C. Derbyshire[1] and Lars G. Kamphuis[1,2]

[1] Centre for Crop and Disease Management, Curtin University, Bentley WA 6102, Australia

[2] Commonwealth Scientific and Industrial Research Organization. Agriculture and Food, Floreat, WA, Australia

This chapter is prepared for publication in due course.

5.1. Abstract

Sclerotinia sclerotiorum is a necrotroph pathogen with a remarkable host range and causes Sclerotinia stem rot (SSR) in chickpea (*Cicer arietinum* L.). Molecular mechanisms governing resistance against this pathogen in chickpea are poorly researched, therefore limiting molecular breeding. We used an RNA sequencing approach to study the molecular processes leading to *S. sclerotiorum* resistance in chickpea. The transcriptome of a moderately resistant (PBA HatTrick) and a susceptible (Kyabra) chickpea line after *Sclerotinia sclerotiorum* infection was analysed across a time course of 6-72 hours post inoculation. Our analysis shows that chickpea resistance to *S. sclerotiorum* is related to timely activation of pathways, including pathways linked to plant-pathogen interaction, phenylpropanoid pathway, plant hormone signal transduction, and scavenging reactive oxygen species. Early induction of key enzymes in these pathways appeared to be an important defence mechanism of chickpea plants against *S. sclerotiorum*. Generally, our findings support a model where plant resistance to pathogens depends on the speed of responses to the pathogen invasion. The extent and time of expression of defence related genes against *S. sclerotiorum* may be vital in discriminating moderately resistant from susceptible chickpea genotypes. Here, we report for the first time a transcriptome study of chickpea genotypes with varying susceptibility levels in response to *S. sclerotiorum* infection. This study provides a rich resource and a framework for better understanding the mechanisms of interaction between *C. arietinum* and *S. sclerotiorum* that will be useful to the design of molecular breeding strategies in chickpea.

Key words: *Cicer arietinum*, *S. sclerotiorum*, gene expression, genotype, defence machanisms.

5.2. Introduction

Chickpea (*Cicer arietinum* L.) is a self-pollinated pulse crop with immense value as a source of protein, carbohydrates, phosphorous, calcium, magnesium, iron and zinc (Jukanti *et al.* 2012). However, chickpea production is constrained by various biotic stresses, including sclerotinia stem rot (SSR), caused by the fungus *Sclerotinia sclerotiorum*, which can cause up to 100% yield losses under favourable conditions (Pulse Australia, 2020). *S. sclerotiorum* is a necrotroph with a diverse host range and prolonged survival of resting sclerotia (Boland and Hall 1994, Brooks *et al.* 2018, Lane *et al.* 2019). Although there is no complete resistance to *S. sclerotiorum*, partial resistance has been reported in chickpea (CHAPTER 2; Mwape *et al.* 2021a). *S. sclerotiorum* control in most crop hosts depends heavily on fungicide application. However, there are no fungicides registered for controlling *S. sclerotiorum* on chickpea in Australia (Pulse Australia, 2020). Another way of combating diseases like *S. sclerotiorum* is through breeding. Advancing our understanding of the fundamental molecular mechanisms responsible for SSR resistance may facilitate the improvement of genetic resistance in chickpea.

S. sclerotiorum is known to secrete an array of molecules during infection, such as oxalic acid and cell wall degrading enzymes (CWDE's) that facilitate the killing of the host tissues to derive nutrients (Kim *et al*. 2008; Yu *et al*. 2017). *S. sclerotiorum* exhibits a biphasic feeding strategy with a short biotrophic phase during the early stages followed by a transition to the necrotrophic stage (Kabbage *et al.* 2015). In return, hosts use multifaceted strategies to defend themselves against the invasion of this pathogen, which consist of pathogen recognition plant intracellular resistance (R) genes, signal transduction by mitogen-associated protein kinase (MAPK) and defence responses including transcription factors (Piffanelli *et al.* 1999; Uloth *et al.* 2013; Wang *et al.* 2019). Overexpression of MAPK gene was found to significantly enhance resistance to *Botrytis cinerea* and *S. sclerotiorum* in *Brassica napus* and by possibly regulating jasmonic acid mediated defence response (Wang *et al.* 2009).

Pathogen perception in the host is coordinated in part through pathogen-associated molecular pattern (PAMP) recognition by plant cell surface receptors termed pattern recognition receptors (PRRs) or recognition of plant virulence molecules (Zipfel 2014). The host R gene products are responsible for recognition of the pathogen virulence factors, which results in effector-triggered immunity (ETI), and recognition of PAMPs by PRRs results in PTI (Jones and Dangl

2006). The interaction between pathogen effectors and in plant resistant genes leads to activation of ETI, a model referred to "gene for gene interaction" (Newman and Derbyshire 2020). On the contrary, the necrotrophic pathogen causes elicitation of host defence by inducing necrotic effectors that result to host programmed cell death for their own advantage, an interaction known as 'inverse gene-for-gene interaction' (Tan *et al.* 2010).

Host damage-associated molecular patterns (DAMPs) triggers host immunity (Andersen *et al.* 2018). Wall-associated kinases (WAKs) detect DAMPs that result from cellular damage associated with pathogen infection. Plant receptors with leucine-rich repeats detect pathogen effectors that play a role in facilitating infection (Dangl *et al.* 2013). The polygalacturonase inhibitor proteins (PGIPs) are leucine-rich proteins that inhibit fungal polygalacturonases, therefore reducing the potential of pathogen destruction; they may also trigger plant defence responses induced by oligogalaturonides (Ferrari *et al.* 2008, Ferrari *et al.* 2013, Dallal *et al.* 2012). A previous study on the interaction between *S. sclerotiorum* and *Phaseolus vulgaris* showed that the pathogen's endo-polygalacturonases contribute to the infection process by promoting the release of plant oligogalaturonides, which are signalling molecules that may cause host cell death, and may also activate plant defences such as PGIPs (Oliveira *et al.* 2010).

Current research indicates that plant defence to *S. sclerotiorum* involves various biological processes, including defence-related proteins, redox homeostasis, lipid and calcium signalling (Cao *et al.* 2016). Initiating PTI and ETI is connected to the signalling pathway that results in the upregulation of host defence-associated genes (Lewis *et al.* 2015; Zipfel 2014). The hormones such as jasmonic acid (JA), salicylic acid (SA), ethylene (ET), abscisic acid (ABA) and auxin role as immune signalling molecules (Wang *et al.* 2012). Previous research has shown that SA, JA, and ET signalling pathways are involved in defence against *S. sclerotiorum* (Guo and Stotz 2007). An investigation of *S. sclerotiorum* infection of *B. napus* found that proteins related to ET and JA signalling were induced; however, limited SA responsive proteins were differentially regulated following infection (Zhao *et al.* 2009). Previous studies have also shown that SA can antagonise the JA signalling pathway, a phenomenon that has been identified during *S. sclerotiorum* infection in oilseed rape (Wang *et al.* 2012). In general, JA and ET act synergistically to cause defence, leading to improved host resistance to pathogens (Glazebrook 2005).

Previous research showed that resistance against *S. sclerotiorum* in soybean is linked to increased antifungal activities associated with the phenylpropanoid pathway (Ranjan *et al.* 2019). The study showed the downregulation of genes involved in phenylpropanoid pathways, including phenylalanine ammonia-lyase (PAL), chalcones synthase and flavonol synthase (FLS), in resistant genotype compared to the susceptible genotype (Ranjan *et al.* 2019). On the other hand, genes involved in anthocyanin and phytoalexin biosynthesis, including isoflavone synthase (IFR), were upregulated in resistant line (Ranjan *et al.* 2019). Accumulation of metabolites cinnamic, caffeic acid, ferulic, and benzoic in the resistance line compared to the S line was observed (Ranjan *et al.* 2019)

Previous studies have shown that *S. sclerotiorum* produces CWDE's that enhances its pathogenicity (Amselem *et al.* 2011). Studies have shown that CWDE's *S. sclerotiorum* pathogenicity relies on oxalic acid (OA) production and other virulence factors that target the host responses (Kim *et al.* 2008; Cessna *et al.* 2000). Reactive oxygen species (ROS), hormones and nitric oxide (NO) and are vital signalling molecules that the host rapidly generates after pathogen recognition (Wendehenne *et al.* 2004). Previous studies suggest that NO has several roles in disease resistance, including regulating defence gene expression and interfering with ROS signalling pathways to modulate cell death (Perchepied *et al.* 2010). Host ROS mediated defence plays a key role through cross-talk with other mechanisms to reduce its spread to further pant tissues. However, overproduction of ROS in plant cells can become a serious threat by causing oxidative damage to major macromolecules such as DNA, lipids and proteins, when production and eradication of ROS are disturbed (Das and Roychoudhury 2014). Plants produce different anti-oxidative scavengers to cope with the excessive accumulation of ROS (Alscher *et al.* 1997). The enzymatic ROS scavengers include superoxide dismutases (SODs), peroxidases, glutathione-S-transferases (GSTs) and glutathione reductases (GRs) (Kumar *et al.* 2009). Understanding the mechanisms involved in chickpea responses against *S. sclerotiorum* is important in designing chickpea resistant breeding strategies.

RNA sequencing is an essential means of understanding the molecular defence mechanisms in hosts against pathogens. Following such studies, the selection of candidate chickpea genes to further study their functional roles in resistance through gene editing can reduce the required time required to develop highly resistant genotypes. In the current study, we hypothesised that two chickpea cultivars (PBA HatTrick and Kybra) will differentially regulate genes involved in the response to *S. sclerotiorum* infection. The analysed significant transcriptional changes of

moderately resistant and highly susceptible chickpea genotype. Our study provides an understanding of the molecular mechanisms underlying partial resistance against *S. sclerotiorum* found in chickpea (CHAPTER 2; Mwape *et al.* 2021a).

5.3. Materials and Methods

This section followed similar procedures as described in CHAPTER 4 (Mwape *et al.* 2021b).

5.3.1. Plant material

This study used two chickpea desi cultivars with varying susceptibility levels to *S. sclerotiorum*. The moderately resistant (PBA HatTrick) and highly susceptible (Kyabra) chickpea seeds were sown in 4L pots with an all-purpose potting mix (UWA mix, Richgro, Perth, Australia) and grown as described in CHAPTER 4 (Mwape *et al.* 2021b). These two lines are referred to as the MR line (moderately resistant PBA HatTrick) and S line (susceptible Kyabra) in the current study.

5.3.2. Fungal material preparation and inoculation

An aggressive *S. sclerotiorum* isolate CU8.20 that was previously reported to be aggressive in *B. napus* (Denton-Giles *et al.* 2018) and chickpea (CHAPTER 2; Mwape *et al.* 2021a) was used in the study. The isolate CU8.20 culture was prepared by cutting sclerotia in half, placing side down on potato dextrose agar (PDA) (Becton Dickinson, USA) and incubated at 20°C for 5-7 days in the dark plates. Consequently, a mycelial plug was cut from actively growing edges of the PDA culture and sub-cultured for two days in a fresh PDA plate at 20°C. Infection was conducted as described in CHAPTER 2 (Mwape *et al.* 2021a).

5.3.3. Sample collection and RNA extraction

The stem samples were harvested at 0 (un-inoculated plants), 6, 12, 24, 48, and 72 hours post inoculation (hpi) and processed as described in CHAPTER 4 (Mwape *et al.* 2021b). There was a mock-inoculated replicate (stem inoculated with a PDA plug non-inoculated plants samples that represented time point 0). Each treatment (time point /cultivar) consisted of three biological replicates. Six stem sections were collected from four individual plants and pooled for one biological replicate.

5.3.4. Bioinformatics pipeline

The quality checks followed a similar protocol as described in CHAPTER 4 (Mwape *et al.* 2021b). Trimmomatic v0.38 was used to trim low-quality bases calls and filtering adapters and low-quality reads (Bolger *et al.* 2014). The trimmomatic parameters used were as described in CHAPTER 4 (Mwape *et al.* 2021b). The trimmed reads were split between the pathogen (*S. sclerotiorum* strain 1980 genome, Bioproject PRJNA348385, assembly ASM185786v1) (Derbyshire *et al*., 2017) and the host (Bioproject PRJNA190909, assembly ASM33114v1) (Varshney *et al.* 2013) using BBSplit tool v38.12 (https://sourceforge.net/projects/bbmap/). Quality filtered *C. arietinum* reads were aligned using HISAT2 v2.1.0 (Kim *et al.* 2015), and result from alignments in SAM format converted to BAM format using SAMtools v0.1.19 (Li *et al.* 2009).

5.3.5. Differential gene expression analysis

Differential gene expression analysis was conducted using edgeR and Limma Bioconductor packages in R (Robinson *et al.* 2010, Ritchie *et al.* 2015). Pairwise contrasts were performed using quasi-likelihood F tests (Lun *et al.* 2016). A false discovery rate (FDR) cut-off of 0.05 was applied. A log2 (fold change) of ≥ 1 indicated upregulation and ≤ -1 indicated down-regulation. Differentially expressed genes (DEGs) at each time point relative to un-inoculated samples (time 0) were examined.

Functional enrichment analysis (KEGG and Gene Ontology) was conducted using DAVID V6.8 (Data for Annotation, Visualization and Integrated Discovery) and the inputs were the locus tags of the respective genes (Huang *et al.* 2007). All transcript locus tags from the RNA sequencing study were used as the background population, and a minimum count of five hits was established to find categories with a *p* value of <0.05. The similarity or divergence in the expression of genes between time points and genotypes was generated using the online tool jvenn v1.6 (Bardou *et al.* 2014).

5.3.6. Reverse transcription quantitative real-time PCR

RNA sequencing expression results were confirmed by conducting a reverse transcription quantitative real-time PCR (RT-qPCR) on samples collected 0, 24 and 72 hpi on three replicates per time point. RNA from these samples were reverse transcribed into cDNA using the AMV First Strand cDNA Synthesis Kit (NEB Inc., Ipswitch, MA, USA) and oligo-DT primer following the manufacturer's instructions. RT-qPCR was performed using the iTaq

universal SYBR green one-step kit (Bio-Rad, Hercules, CA, USA). Each reaction consisted of 10 µL of iTaq universal SYBR green mix, 1 µL of 1:10 – fold diluted cDNAs, and 4 µL of gene-specific forward and reverse primers in the final volume of 20 µL. Primers were designed using Geneious software (https://www.geneious.com/) to amplify gene fragments that were approximately 100-250 bp in length and with a melting temperature of 60 ºC. The primer list is presented in Table 5.1. RT-qPCR was performed on a CFX 384 real-time PCR system (Bio-Rad, Hercules, CA, USA). The cycling conditions: 95 ºC for 2 min, then 95 ºC for 15 sec, 60 ºC for 30 sec and 72 ºC for 15 sec, repeated 40 cycles, followed by 72 ºC for 2 min. The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with the chickpea Ubiquitin gene (AJ001901) used as an endogenous control.

Table 5.1: Primers used in the RT-qPCR validation analysis.

Gene ID	Function	Primer sequence	
LOC101499159	serine decarboxylase-like	forward	ATCACTGGTAGCCGTTGTGG
		reverse	TGCACCAATTCCAGCATCAC
LOC101501659	Shikimate-o-hydroxycinnamoyl transferase	forward	ATCAACGGAGGAGCTGGTTG
		reverse	TAAATCCTCGCCAACCCTGG
LOC101513347	Spermidine-hydroxycinnamoyl transferase	forward	GCTAAGTTAGCGCGTGGAGA
		reverse	CAAATCACGCCCCAACCAAC
LOC101493509	peroxidase 5 -like	forward	TACAATCAGGGCGCAGAGAC
		reverse	GAGCTCCAGAAAGTGTCACCA
LOC101508209	pectinase 2 like	forward	TGAACGCTGCCCCAAAGAAT
		reverse	CGACACTTTTGCTACCGGTG
LOC101501627	Cyanogenase-beta- glucosidase-like	forward	ACTGGTTTGTGCCACTCTCG
		reverse	ACTTTGGTAACCGTGCTCCA
LOC101514256	Peroxidase	forward	CACAAGCCAAACCTCCTCCT
		reverse	AGTGAAGACGAAGCAAGCCA
LOC101510953	glucose-1-phosphate adenylytransferase	forward	ACGATCTGCTACACCTGCTG
		reverse	TATGTGCGAGCGATGTGACG
AJ001901	Ubiquitin	forward	CGCAGACTCCGTGCAGAAC
		reverse	CGAACATTGCATCGATCTCAT

5.4. Results

5.4.1. Mapping and overview of RNA sequencing data

To investigate the molecular responses involved in the chickpea- *S. sclerotiorum* interaction, a time course of infection was conducted between 0 and 72 hours post inoculation (hpi) on a moderately resistant (MR line) chickpea cultivar PBA HatTrick and a susceptible (S line) cultivar Kyabra. These two parental chickpea lines were previously shown to have differential responses to *S. sclerotiorum* infection in terms of lesion development over time (Mwape *et al.* 2021a; Mwape *et al.* 2021b; CHAPTER 2 and 3). The molecular signatures on the pathogen side of the interaction were described in CHAPTER 4 (Mwape *et al.* 2021b). Here we

investigate the molecular responses of the MR and S line by comparing RNA sequencing data of infected samples relative to uninfected time point zero (Table 5.2). Sequence reads were aligned to both the plant and fungal genomes, and between 31.9 and 99.2 % of reads mapped back to the host reference genome and 0.7 to 68.1% mapped back to the *S. sclerotiorum* genome (Table 5.2).

Table 5.2: Summary of the sequence metrics of RNA–seq.

Host	Hours post inoculation (hpi)	Total raw read pairs	Trimmomatic reads retention (%)	BBSplit reads separation	
				S. sclerotiorum	*C. arietinum*
MR	6	67,354,385	98.7	1,207,201 (1.8%)	66,147,184 (98.2%)
	12	68,680,857	98.76	5,929,006 (8.6%)	62,751,851 (91.4%
	24	61,114,985	98.91	28,078,637 (45.9%)	33,036,348 (54.1%)
	48	56,616,306	98.85	40,566,767 (71.7%)	16,049,538 (28.3%)
	72	63,109,260	98.92	39,012,318 (61.8%)	24,096,941 (38.2%)
S	6	58,025,893	98.4	414,371 (0.7%)	57,611,521 (99.2%)
	12	72,896,961	98.4	1,851 043 (2.5%)	71,045,918 (97.5)
	24	54,049,381	98.4	18,414,027 (31%)	35,635,354 (65.9%)
	48	60,727,165	98.5	36,714,863 (60.4%)	24,012,301 (39.5%)
	72	57,636,636	98.5	39,273,084 (68.1%)	18,363,551 (31.9%)

**averages number of reads [a]MR: moderately resistant cultivar PBA HatTrick [b]S: susceptible cultivar Kyabra

5.4.2. Principle Component Analysis

The relatedness of the three biological replicates and the RNA sequencing analysis consistency were confirmed by multidimensional scaling (MDS) plots (Fig. 5.1). Of the early time point samples, 6 hpi was the most closely related to un-inoculated control samples (time zero), with successive time points showing greater differences with time zero. There was a clear distinction between the *C. arietinum* transcriptome between the two early time points (6-12 hpi), 24 hpi (mid-stage) and late time points (48-72 hpi). The S line samples at 72 hpi also were more closely related to the 48 hpi samples of both the S and MR line than the 72 hpi MR samples (Fig. 5.1).

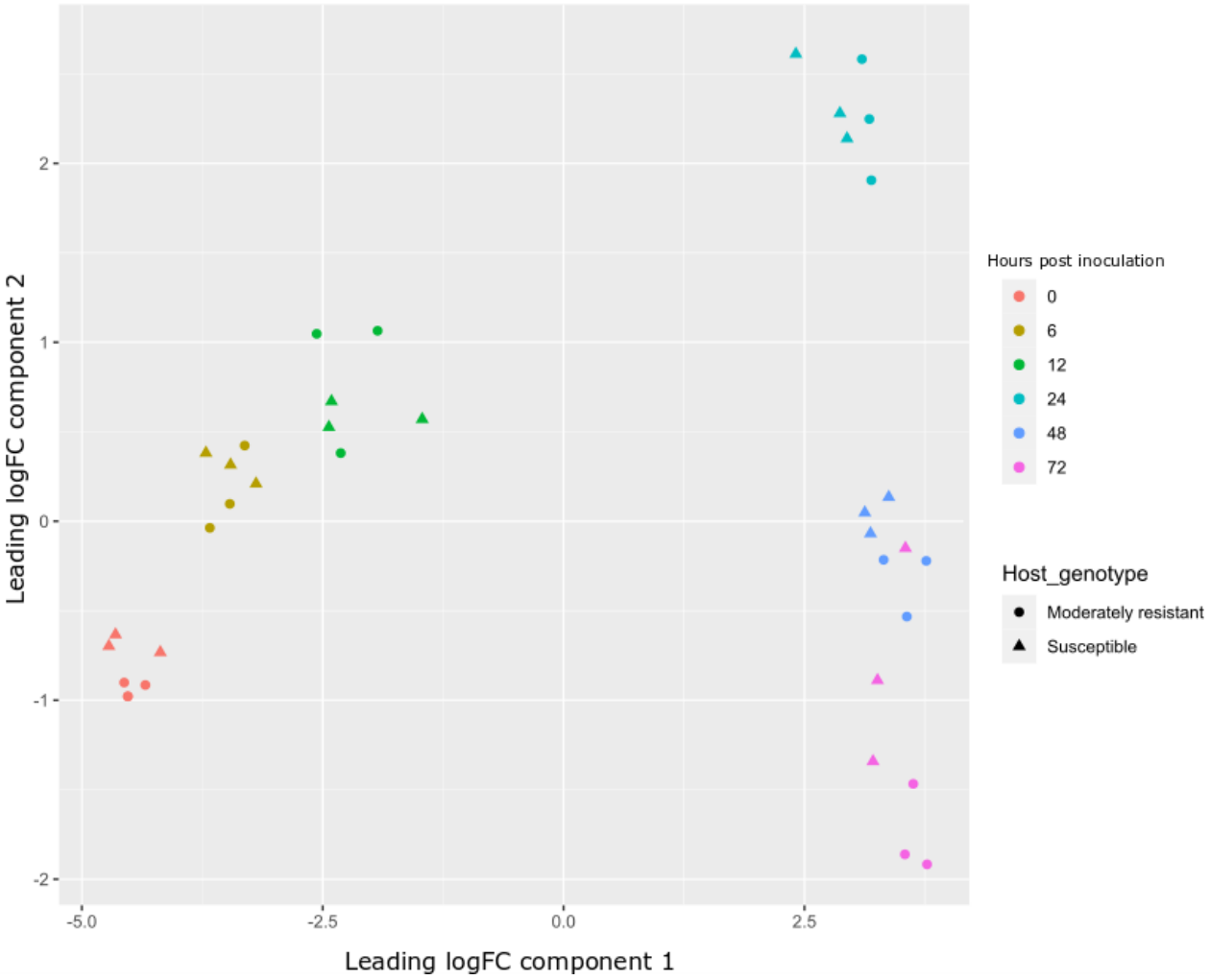


Figure 5.1: A multidimensional scaling (MDS) plot showing the similarity among the genes expression patterns of moderately resistant (MR) and susceptible (S) lines at 0 (uninfected stems), 6, 12, 24, 48 and 72 hours post-inoculation (hpi). The distinct colours signify each treatment, and individual circles and triangles signify individual samples for MR and S lines, respectively.

5.4.3. Expression profile of *C. arietinum* genes during infection

During the interaction of the host and the pathogen, genes implicated in host defence responses may be observed at different stages of pathogen infection. Comparative analysis of differentially expressed genes (DEG's) was performed during infection in both MR and S lines and between these two lines at a single time point. In total, 9339 and 7382 genes (logFC down- $\leq 1 \geq$ up-) were significantly (Adj. $P \leq 0.05$) differentially expressed in the MR and S lines, respectively, in at least one time point during infection, relative to the control (Supplementary

Table S5.1). The number of genes upregulated at 6, 12, 24, 48, and 72 hpi were 105, 565, 3681, 3657, 3850 and 134, 379, 3078, 3509, 3338 in MR and S lines respectively (Fig. 5.3; Supplementary Table S5.1). The number of genes downregulated at 6, 12, 24, 48, and 72 hpi were 96, 164, 1211, 1560, 1410 and 22, 112, 986, 1128, 896 in MR and S lines. There were 47 and 78 upregulate genes differentially expressed at all time points in the MR and S lines, respectively (Fig.5.3A and B).

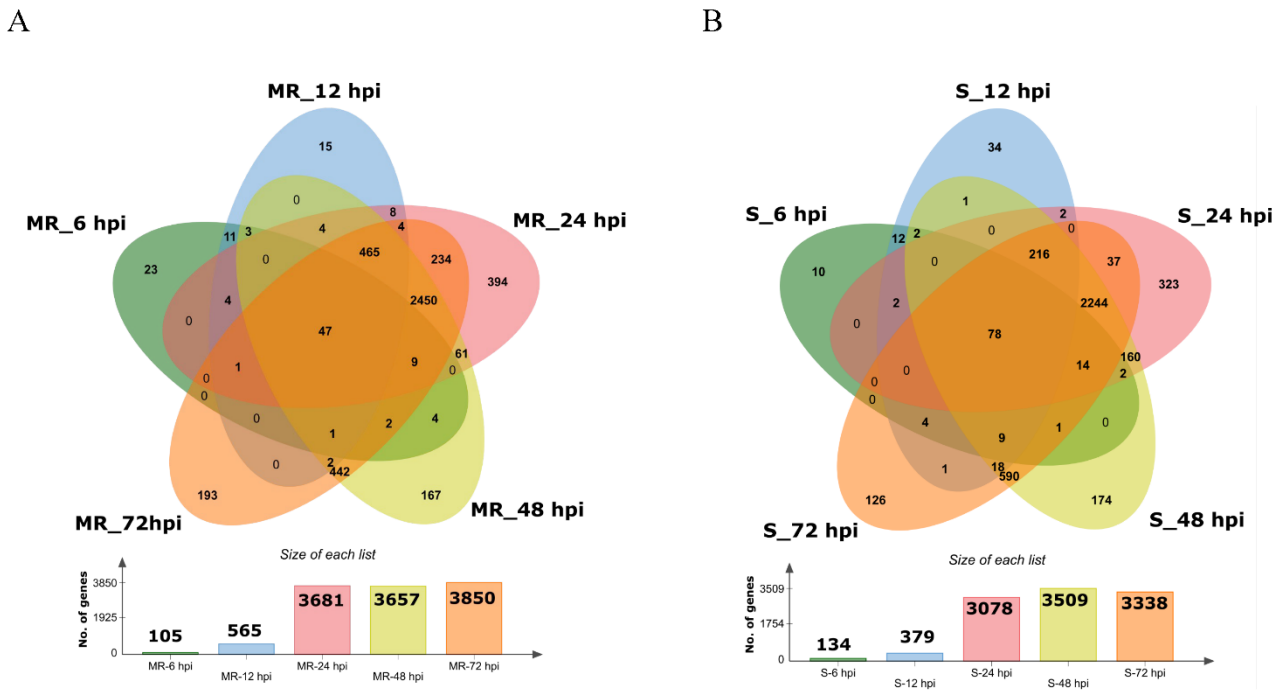
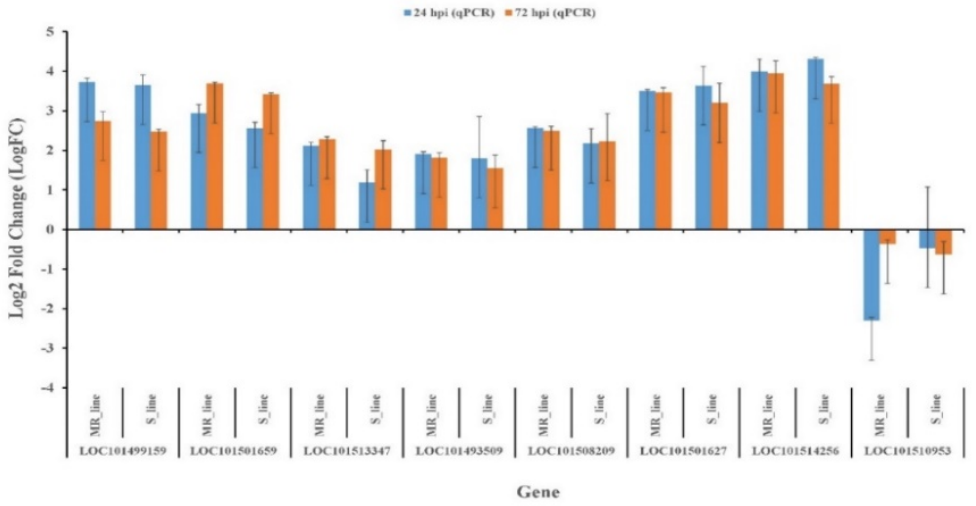


Figure 5.2 Venn diagram showing upregulated chickpea genes during *S. sclerotiorum* infection at time points 6, 12, 24, 48, and 72 hpi in (A) moderately resistant (MR) and (B) susceptible (S) chickpea lines.

5.4.4. Validation of RNA sequencing data using RT-qPCR

To validate the RNA sequencing data's accuracy, seven upregulated genes and one downregulated gene showing distinct expression patterns in both MR and S lines compared to un-inoculated samples (time-0) were selected randomly and quantified using SYBR Green-based RT–qPCR (Table 5.1). Compared to the control (uninfected sample), the expression patterns of all eight genes were similar between RNA sequencing and RT-qPCR (Fig. 5.2).

A



B

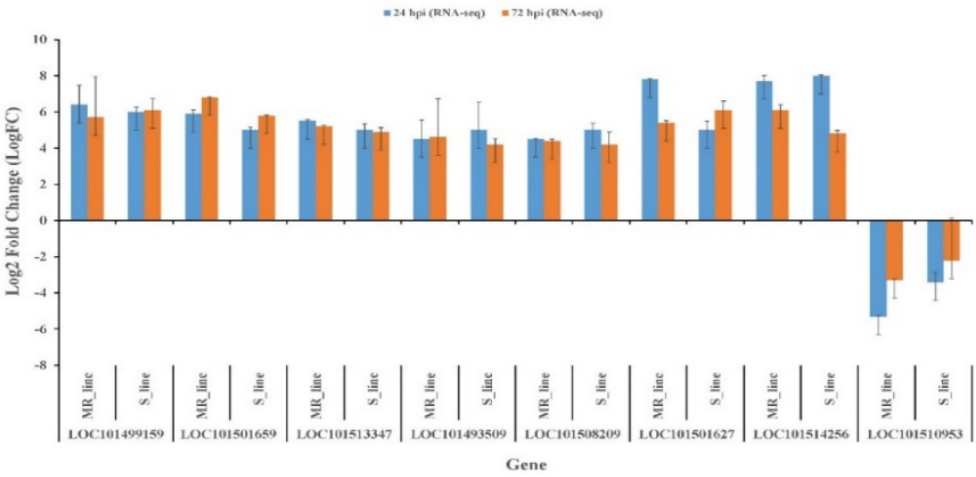


Figure.5.3: qRT-PCR validation of relative expression levels of selected chickpea genes differentially expressed during *Sclerotinia sclerotiorum* interaction. Log_2 fold change (LogFC) values were generated for qPCR samples by comparing the expression of genes at each time point of infection vs the uninfected (0 - time) sample (Fig. A). LogFC values were generated for RNA sequencing samples by comparing the average raw read counts at each time point of the infection versus the uninfected stem sample (Fig. B). The MR represent moderatetely resistant line (PBA HatTrick) and S represents susceptible line (Kyabra).

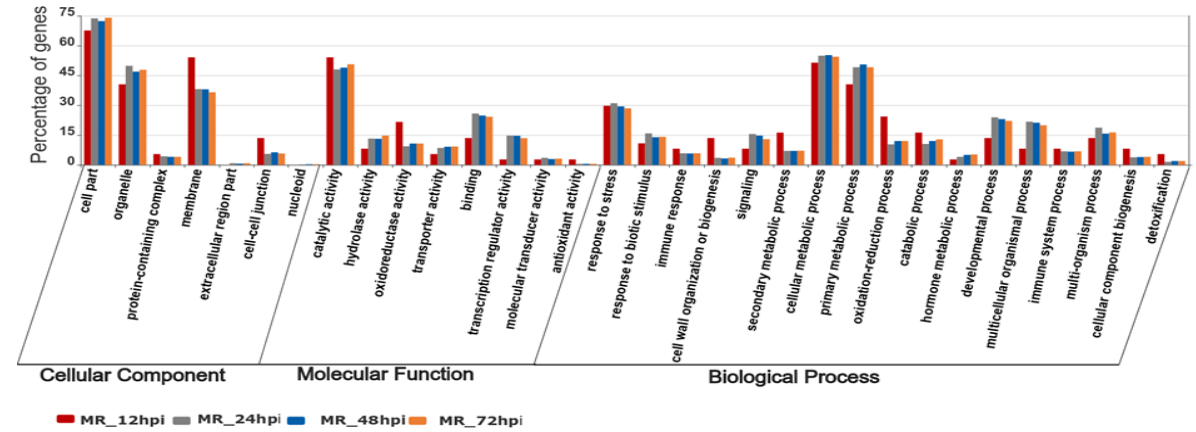
5.4.5. Gene Ontology classification

Many of the downregulated genes did not appear to have significant functional categories associated with plant defence. Therefore the focus was on gene ontology (GO) analysis for differentially upregulated genes at 6, 12, 24, 48 and 72 hpi was performed to functionally categorise the transcripts into three principal categories: biological processes (BP), molecular function (MF) and cellular component (CC) (Fig. 5.4). There were no significant GO categories enriched at 6 hpi in both genotypes. In the BP category, response to stress, oxidation-reduction process, immune response, primary metabolic processes, secondary metabolic process, cellular metabolic process and catabolic process were the most enriched categories in both genotypes at 6-72 hpi (Fig. 5.4). The most enriched MF categories in both chickpea genotypes at 6-12 hpi were catalytic activity, binding, transcription regulator activity and oxidoreductase activity (Fig. 5.4). Generally, the MR line showed a higher number of upregulated genes in some BP categories such as response to stress, cellular metabolic process and primary metabolic process, and MF categories such as catalytic activities and hydrolase activities (Fig. 5.4 A). The BP categories, response to stimulus, response to stress and cellular metabolic processes showed a high number of upregulated genes at 12 hpi, followed by fewer genes as the infection progressed in the S line (Fig 5.4 B).

5.4.6. Functional classification of DEGs shows early upregulation of defence-associated genes occurs in the moderately resistant chickpea cultivar during *S. sclerotiorum* infection

The identified significantly upregulated genes were assigned to specific plant pathways in which they may function, as described in the KEGG database (Kanehisa and Goto 2000). The KEGG categories with the highest number of expressed transcripts in both genotypes included metabolic pathway, biosynthesis of secondary metabolites, glutathione metabolism, biosynthesis of antibiotics and phenylpropanoid biosynthesis (Supplementary Table S5.2). The analysis showed differences in expression of genes involved in plant-pathogen interaction pathway, with upregulation occurring earlier in the MR line at mid-stage of infection (12-24 hpi) and later (48 -72 hpi) in the S line (Supplementary Table S5.2). Similarly, the expression of genes involved in the plant hormone transduction pathway showed upregulation at 12 – 72 hpi in the MR line and downregulation at 12, 48 and 72 hpi in the S line (Supplementary Table S5.2). At 24 hpi, the analysis showed 47 transcripts involve in plant hormone transduction pathways were upregulate and 41 downregulated in the S line (Supplementary Table S5.2).

A



B

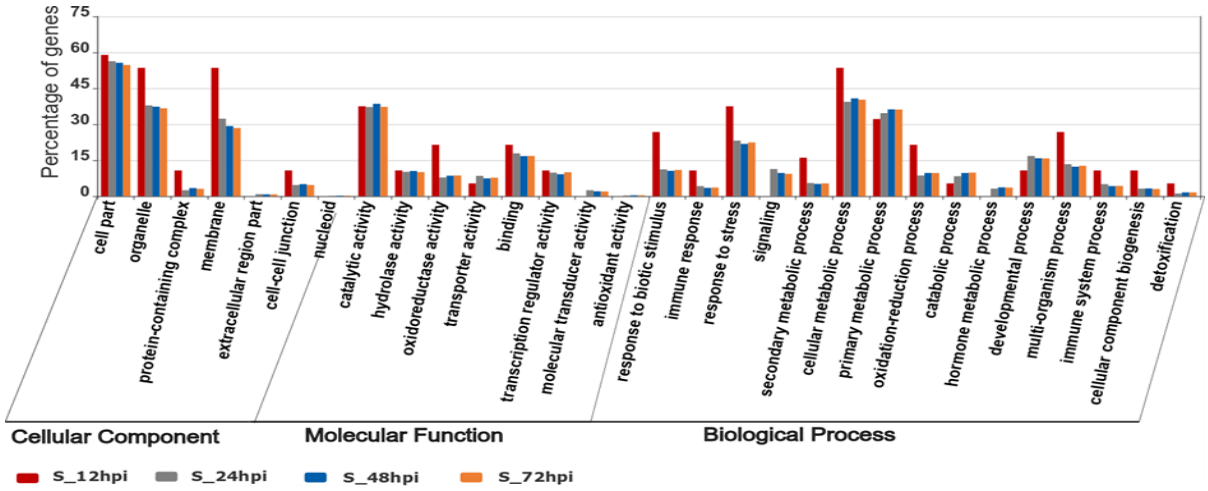


Figure 5.4 Significantly enriched gene ontology (GO) categories of the upregulated chickpea genes in (A) MR line PBA HatTrick and (B) S line Kyabra at different time points (12, 24, 48 and 72 hpi) following infection with *S. sclerotiorum*. The y-axis indicates the percentage of genes annotated to each process, and the x-axis represents GO processes in the cellular component, molecular function, and biological process enriched (FDR < 0.05).

The plant-pathogen interaction pathway plays a key role in the recognition of specific pathogens and initiates programmed response to counteract infection. In the current study, 30 genes involved in this pathway were upregulated in both genotypes (Fig. 5.7). Two genes

encoding WRKY33 were induced earlier upon inoculation in the MR (12-72 hpi) as compared to the S line (24-72 hpi) (Fig. 5.7). Similarly, four genes involved in plant-pathogen interaction pathways upregulated earlier in MR includes probable LRR receptor-like kinase, disease-resistant protein RPM1-like,calmodulin-like protein 3, calcium-dependent protein kinase 26-like, and one calcium-binding protein CM38-like was upregulated only in the MR line. Two genes encoding calcium-binding proteins (LOC101510992, LOC101505073) were upregulated within 24 hpi in the MR line and later stage of infection in both lines.

Phytohormones are central regulators of plant defence. In the current study, 23 genes involved in the plant hormone signal transduction pathway were expressed in both genotypes during *S. sclerotiorum* infection (Fig. 5.8). Two auxin-responsive proteins (LOC101511547 and LOC101503044) were downregulated at 12 -72 hpi (Fig. 5.8). Two putative indole -3-acetic acid protein and an ethylene receptor, upregulated in MR line only while two ethylene-responsive transcription factors, two auxin-responsive proteins and an auxin-induced protein, were upregulated earlier in MR (12-72 hpi) and later in S line (24-72 hpi) (Fig. 5.8).

Exposure of chickpea genotypes to *S. sclerotiorum* resulted in earlier upregulation of genes involved in the phenylpropanoid pathway, including chorismate mutase (CM), chalcones synthase (CHS), cinnamoyl-CoA-reductase (CCR), isoflavone reductase (IFR), Caffeoyl-CoA *O*-methyltransferase (COMT and CCOoAOMT), and phenylalanine ammonia-lyase (PAL). The study showed earlier upregulation of a gene encoding PAL (LOC101496077), Flavonoid3'-monooxygenase-like (LOC101510693 and LOC101505076), CM (LOC101513066) and CCR (LOC101496560) in MR line only. We observed upregulation of PAL, Isoflavone4'-*O*-methyltransferase, Isoflavone reductase, and CHS at an earlier stage (12-24 hpi) in MR and at a later stage (24-72 hpi) in the S line.

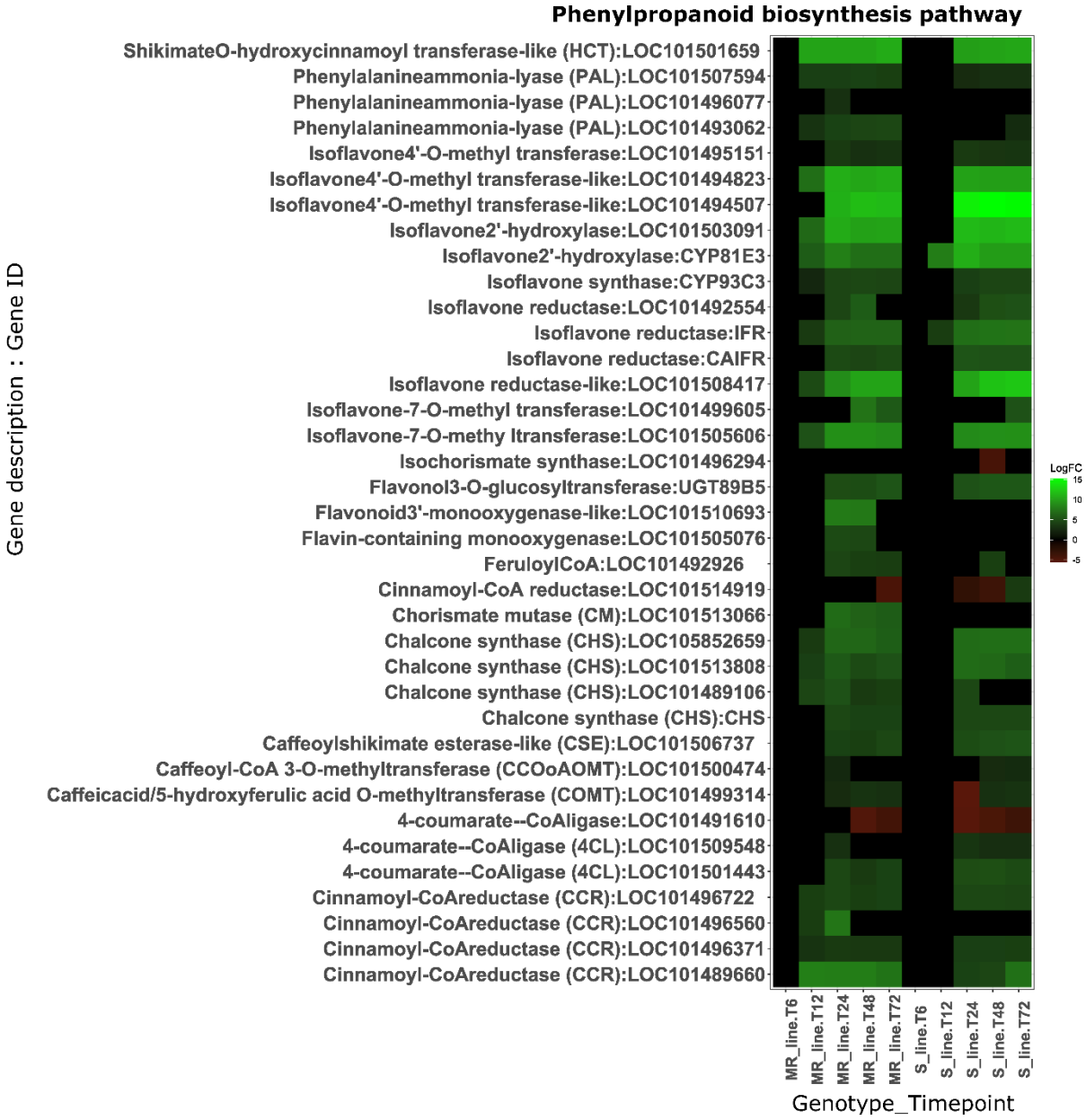


Figure 5.5: Heat maps showing the expression pattern of chickpea genes involved in plant-pathogen interaction pathway in a moderately resistant (MR) and susceptible (S) chickpea line after infection with *S. sclerotiorum*. The data represents treatments at 6, 12, 24, 48 and 72 hours post-infection. The colour gradient represents the logFC in gene expression, upregulated (green), and no change (black) compared to uninfected control.

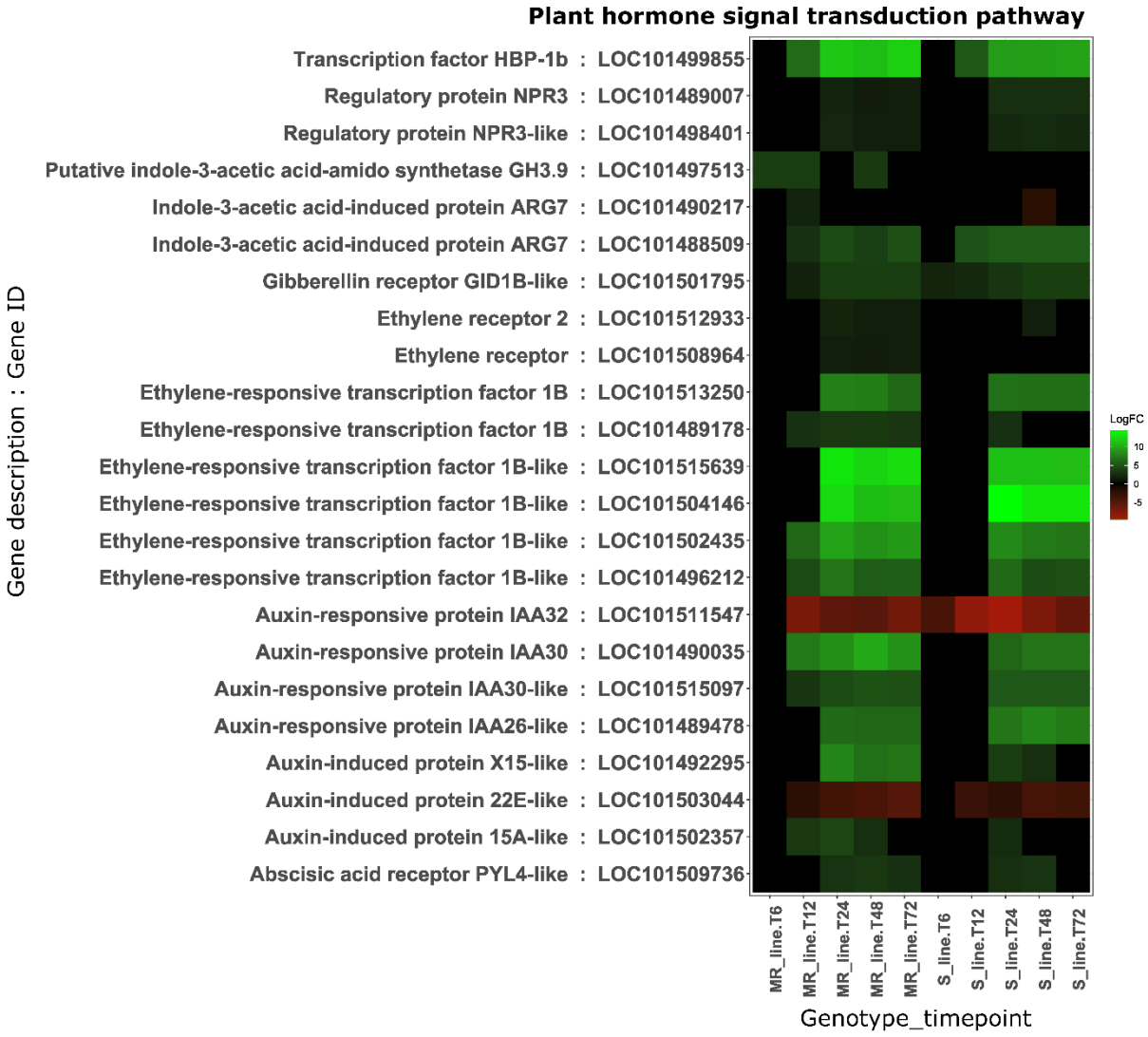


Figure 5.6: Heat maps showing the expression pattern of chickpea genes involved in plant hormone signal transduction pathway in a moderately resistant (MR) and susceptible (S) chickpea line after infection with *S. sclerotiorum*. The data represents treatments at 6, 12, 24, 48 and 72 hours post-infection. The colour gradient represents the logFC in gene expression, upregulated (green), downregulated (red), and no change (black) compared to uninfected control.

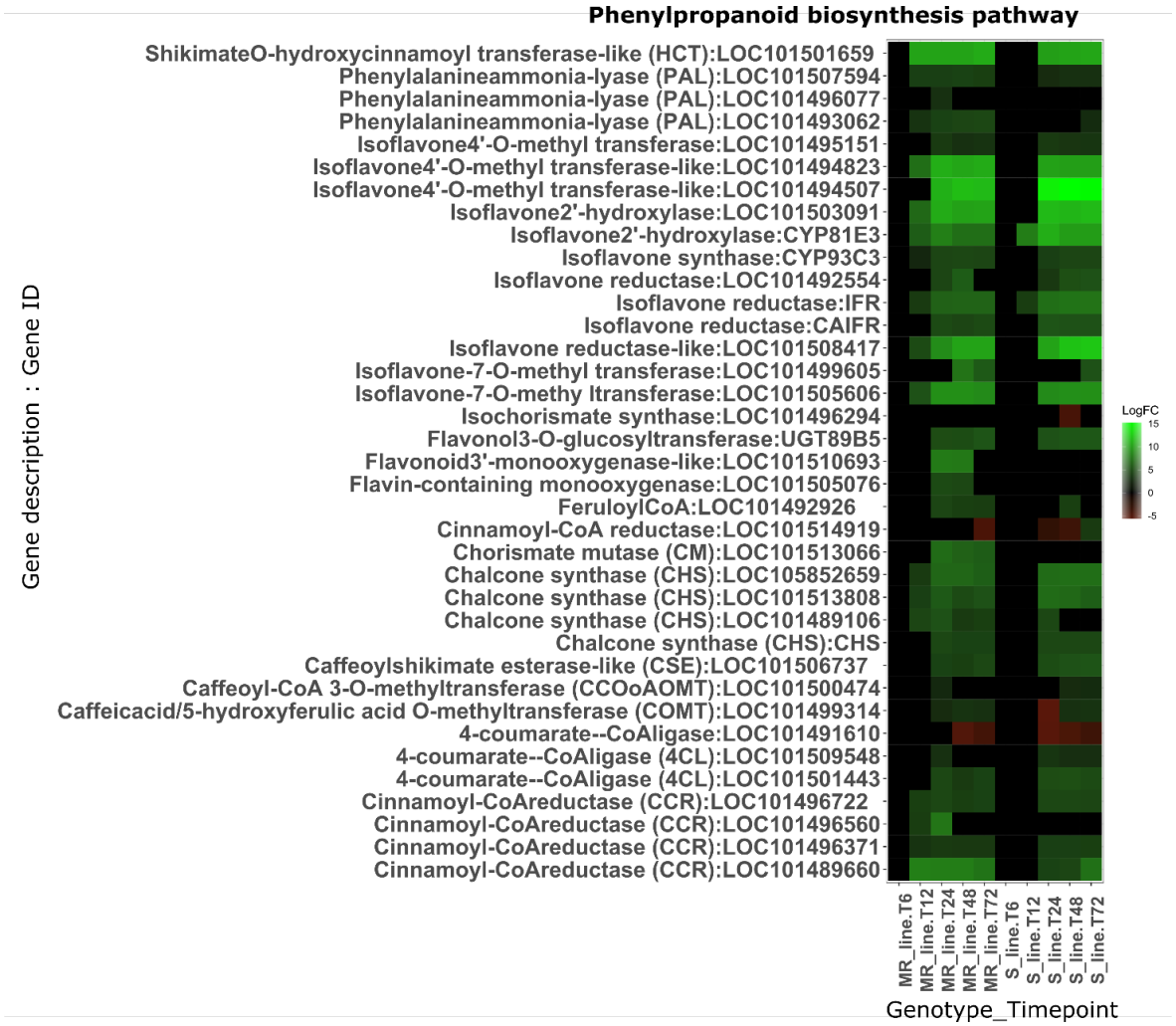


Figure 5.7: Heat maps showing the expression pattern of chickpea genes involved in phenylpropanoid pathway in a moderately resistant (MR) and susceptible (S) chickpea line after infection with *S. sclerotiorum*. The data represents treatments at 6, 12, 24, 48 and 72 hours post-infection. The colour gradient represents the logFC in gene expression, upregulated (green), downregulated (red), and no change (black) compared to uninfected control.

5.4.7. Reactive oxygen species-related genes upregulated earlier in moderately resistant chickpea genotype during *Sclerotinia sclerotiorum* infection

In the current study, both chickpea lines upregulated genes involved in ROS scavenging enzymes, including L-ascorbate oxidase, Glutathione S-transferase (GST), peroxidases, and linoleate 13S-lipoxygenase (LOX) (Table 5.3). Our study showed early expression of genes encoding GST (LOC101508320) and peroxidases (LOC101514256) in MR compared to the S

line (Table 5.3). A gene encoding a GST (LOC101499254) was upregulated in the MR line only (Table 5.3).

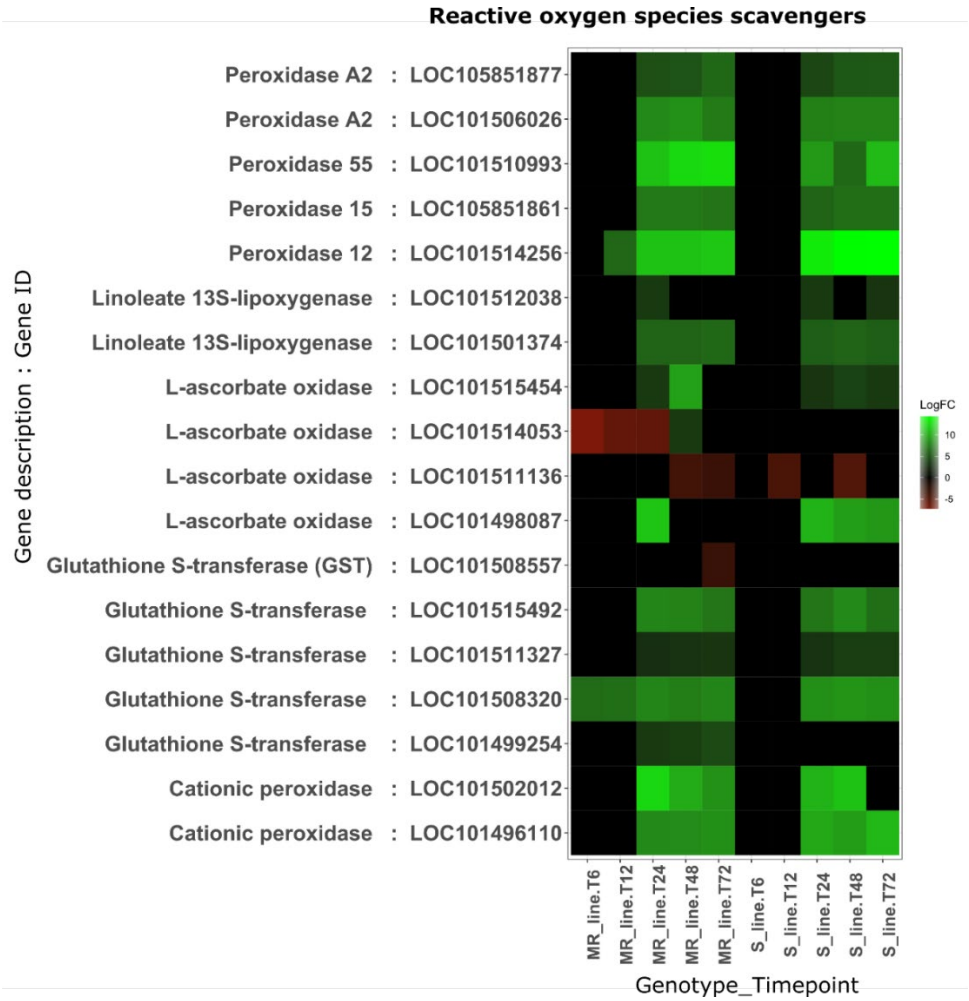


Figure 5.8: Upregulated chickpea genes that are involved in reactive oxygen species scavenging and antioxidant activities in response to *Sclerotinia sclerotiorum* infection in a moderately resistant (MR) and susceptible (S) chickpea line after infection with *S. sclerotiorum*. The data represents treatments at 6, 12, 24, 48 and 72 hours post-infection. The colour gradient represents the logFC in gene expression, upregulated (green), downregulated (red), and no change (black) compared to uninfected control.

5.5. Discussion

Host gene expression upon infection with pathogens has been broadly studied in several agricultural plant species using various transcriptomic approaches (Casassola *et al.,* 2013). Identifying differentially expressed genes in response to infection can contribute to uncovering

critical components of defence responses, knowledge of which is essential for the development of disease-resistant crops. This study reveals several biological processes induced in MR and S chickpea lines upon *S. sclerotiorum* inoculation, which we can infer are involved in response to *S. sclerotiorum*.

Plants resist pathogen infection by activating several defence mechanisms. The plant immunity system relies on the ability to recognise the pathogen, execute signal transduction and respond through defence related pathways that involve many genes (Andersen *et al.* 2018). In the current study, the two chickpea genotypes upregulated plants defence related pathways such as plant hormone signal transduction, phenylpropanoid biosynthesis and plant-pathogen interaction. Signal transduction pathways, including calcium ion signalling, hormone production, and transcriptional factor activity, inhibit pathogen reproduction and make further infection activities difficult (Antolín-Llovera *et al.* 2012). The speed that a plant detects and respondto pathogen invasion is reported as the basis of resistance mechanisms (Andam *et al.* 2020). In the current study, early upregulation of defence-related genes in the MR line was observed compared to the S line. Previous studies demonstrated that resistance to necrotrophic pathogens depends on the regulation of different hormones such as ethylene and auxins (Glazebrook 2005). In the current study, genes involved in hormonal signal transduction, including auxin-responsive protein, auxin-induced proteins, and ethylene-responsive transcriptional factors 1 (ERF1), showed early upregulation in MR compared to the S line. General auxin signalling was found to be involved in resistance to *Plectospaerella cucumerina* and *Botrytis cinerea* in Arabidopsis (Llorente *et al.* 2008). Similarly, expression of ERF1 in Arabidopsis conferred resistance upon *B. cinerea* infection (Berrocal-Lobo *et al.* 2002). The earlier upregulation of ERF1 and auxin-related genes in the MR genotype may suggest an early response to *S. sclerotiorum,* which activates basal immunity in the MR genotype.

Receptors activate signalling mechanisms such as calcium fluctuation to trigger responses to pathogenic microbes (Poovaiah *et al.* 2013). Calmodulin like proteins (CaM), calcium-binding proteins (CBPs) and calcium-dependent protein kinases (CDPKs) detect calcium to trigger a range of transcriptional factors such as calmodulin-binding transcription activator, which is involved in ROS production. Genes encoding CaM, CBPs and CDPKs were upregulated in both genotypes, an indication of calcium signalling and hormone activation in responses to *S. sclerotiorum* infection. A CDPK encoding gene was upregulated earlier in the MR line compared to the S line (Fig. 5.7). CDPKs play a key role in the phosphorylation of WRKY transcription factors, which are involved in ETI. Similarly, two probable WRKY33 were

induced at an early stage of infection in the MR line (Fig. 5.7). Zhao *et al.* (2009) demonstrated that *S. sclerotiorum* infection of a partially resistant *B. napus* cultivar resulted in rapid induction of a WRKY33 orthologue. In *A. thaliana*, *wrky33* mutants were found to be more susceptible to *B. cinerea* and *Arabidopsis brassicicola*, corresponding to reduced expression of the plant defence genes (Zheng *et al.* 2006).

In chickpea, multiple enzymes are involved in the phenylpropanoid pathway and the genes encoding these enzymes are induced in response to pathogen attack (Kavousi *et al.* 2009). The phenylpropanoid metabolism is initiated by phenylalanine ammonia-lyase (PAL) and gives rise to compounds such as including lignin, phytoalexin, and antioxidants flavonoids and isoflavonoids (Dixon and Paiva 1995; Yadav *et al.* 2020). PAL encoding genes play a critical role in cell wall-mediated immunity and broad-spectrum disease resistance through the high production of lignin, phenyl and phytoalexins (Yadav *et al.* 2020). The current study demonstrated the upregulation of PAL in both genotypes. It is interesting to note the early induction of PAL in MR compared to S line, with one gene encoding PAL induced exclusively in MR line (Fig 5.7).Thus, PAL may play a role in the moderate resistance characteristic of the MR chickpea genotype. These results show that the production of PAL leading to phenylpropanoid biosynthesis in MR line is an early defence response against *S. sclerotiorum*. Two other genes, IFR and CHS, were induced earlier in MR. These results are similar to previous studies that reported genes encoding IFR and CHS enzymes to be induced to a higher degree in resistant chickpea and *Medicago sativa* lines than in susceptible lines (Tiemann *et al.* 1991) and during infection of *Medicago truncatula* with the foliar necrotrophy *Phoma medicaginis* (Kamphuis *et al.* 2012). Rapid and high accumulation of antifungal compounds such as phytoalexins medicarpin and maackiain were found to be significant traits during chickpea resistance to *Ascochyta rabiei* (Weigand *et al.* 1986). Upon infection, the extent and timing of expression of defence-related genes are two vital factors affecting the outcome of the interaction (Sun *et al.* 2019). Therefore, the earlier upregulation of the phenylpropanoid pathway enzymes in the MR line compared to the S line may indicate that the phenylpropanoid pathway plays a key role in the defence response to *S. sclerotiorum*.

Reactive oxygen species (ROS) play an essential role in plants, including immune signalling during biotic stress (Das and Roychoudhury 2014b). *S. sclerotiorum* is reported to produce genes encoding necrosis and ethylene- inducing peptides that induce host cell death, an outcome that is favourable for the necrotrophic lifestyle of *S. sclerotiorum* (Liang and Rollins 2018). To overcome such stress, plants secrete enzymes that detoxify ROS and maintain redox

homeostasis. The glutathione metabolism pathway, known to play a critical role in detoxification of ROS and signalling in plant defence against biotic stress (Hasanuzzaman *et al.* 2017), was upregulated at an early stage in both lines (Table 5.4). Our study shows an early upregulation of ROS scavenging genes, including peroxidases and glutathione S-transferases (GSTs). Some GST and peroxidases were upregulated at an early stage of infection in MR compared to the S line, suggesting that early response to oxidative stress may be a resistance strategy employed by the MR chickpea line. Further, proteins that play a role in calcium signalling transduction were detected in the MR line but not the S line at 6 - 24 hpi. Calcium-binding proteins play a crucial role in downstream signalling by regulating the plant responses to abiotic and biotic stress (Sanders *et al.* 2002). These findings show activation of ROS scavengers and antioxidants in the resistance to *S. sclerotiorum* to offset the pathogen's production of an oxidative state. Therefore, this study proposes that the early pathogen recognition in the MR line is the principals behind a timely response and an overall partial resistance to *S. sclerotiorum*.

5.6. Conclusion

The current study highlights the regulation of defence response in chickpea following *S. sclerotiorum* infection, thus providing valuable insight into the molecular interaction between chickpea and *S. sclerotiorum*. Importantly, this study highlights numerous differentially expressed proteins controlling host responses to pathogen infection and reveals that in the moderately resistant line, transcripts related to phenylpropanoid pathway, hormonal homeostasis and plant defence signals appear to contribute to a successful defence against *S. sclerotiorum*. Functional characterisation of these chickpea genes would validate their role in disease resistance, the knowledge that may prove useful for the development of SSR resistant chickpea cultivars.

The data discussed herein leads us to the conclusion that moderate resistance to *S. sclerotiorum* observed in the PBA HatTrick cultivar is linked to early pathogen recognition and prompt initiation of plant-pathogen interaction and signalling genes. Overall, RNA sequencing analysis revealed critical factors that are potentially involved in chickpea resistance and highlights the importance of early response and redox regulations upon *S. sclerotiorum* infection.

5.7. References

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

Alscher RG, Donahue JL, Cramer CL (1997) Reactive oxygen species and antioxidants: Relationships in green cells. *Physiologia Plantarum* **100**, 224–233.

Amselem J, Cuomo CA, Kan JAL van, Viaud M, Benito EP, Couloux A, Coutinho PM, Vries RP de, Dyer PS, Fillinger S, Fournier E, Gout L, Hahn M, Kohn L, Lapalu N, Plummer KM, Pradier J-M, Quévillon E, Sharon A, Simon A, Have A ten, Tudzynski B, Tudzynski P, Wincker P, Andrew M, Anthouard V, Beever RE, Beffa R, Benoit I, Bouzid O, Brault B, Chen Z, Choquer M, Collémare J, Cotton P, Danchin EG, Silva CD, Gautier A, Giraud C, Giraud T, Gonzalez C, Grossetete S, Güldener U, Henrissat B, Howlett BJ, Kodira C, Kretschmer M, Lappartient A, Leroch M, Levis C, Mauceli E, Neuvéglise C, Oeser B, Pearson M, Poulain J, Poussereau N, Quesneville H, Rascle C, Schumacher J, Ségurens B, Sexton A, Silva E, Sirven C, Soanes DM, Talbot NJ, Templeton M, Yandava C, Yarden O, Zeng Q, Rollins JA, Lebrun M-H, Dickman M (2011) Genomic Analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *PLoS Genetics* **7**,1002230.

Andam A, Azizi A, Majdi M, Abdolahzadeh J (2020) Comparative expression profile of some putative resistance genes of chickpea genotypes in response to ascomycete fungus, *Ascochyta rabiei* (Pass.) Labr. *Brazilian Journal of Botany* **43**, 123–130.

Andersen EJ, Ali S, Byamukama E, Yen Y, Nepal MP (2018) Disease resistance mechanisms in plants. *Genes* **9**,9070339.

Antolín-Llovera M, Ried MK, Binder A, Parniske M (2012) Receptor kinase signalling pathways in plant-microbe interactions. *Annual Review of Phytopathology* **50**, 451–473.

Bardou P, Mariette J, Escudié F, Djemiel C, Klopp C (2014) jvenn: an interactive Venn diagram viewer. *BMC Bioinformatics* **15**, 293.

Berrocal-Lobo M, Molina A, Solano R (2002) Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. *The Plant Journal: For Cell and Molecular Biology* **29**, 23–32.

Boland GJ, Hall R (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology* **16**, 93–108.

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120.

Brooks KD, Bennett SJ, Hodgson LM, Ashworth MB (2018) Narrow windrow burning canola (*Brassica napus* L.) residue for *Sclerotinia sclerotiorum* (Lib.) de Bary sclerotia destruction. *Pest Management Science* **74**, 2594–2600.

Cao J-Y, Xu Y-P, Cai X-Z (2016) TMT-based quantitative proteomics analyses reveal novel defense mechanisms of *Brassica napus* against the devastating necrotrophic pathogen *Sclerotinia sclerotiorum*. *Journal of Proteomics* **143**, 265–277. doi:10.1016/j.jprot.2016.03.006.

Casassola A, Brammer SP, Chaves MS, Martinelli JA, Grando MF, Denardin ND (2013) Gene expression: A review on methods for the study of defence-related gene differential expression in plants. *American Journal of Plant Sciences* **4**, 64–73.

Cessna SG, Sears VE, Dickman MB, Low PS (2000) Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *The Plant Cell* **12**, 2191–2200.

Dallal Bashi Z, Rimmer SR, Khachatourians GG, Hegedus DD (2012) Factors governing the regulation of *Sclerotinia sclerotiorum* cutinase A and polygalacturonase 1 during different stages of infection. *Canadian Journal of Microbiology* **58**, 605–616.

Dangl JL, Horvath DM, Staskawicz BJ (2013) Pivoting the plant immune system from dissection to deployment. *Science (New York, NY)* **341**,1236011.

Das K, Roychoudhury A (2014a) Reactive oxygen species (ROS) and the response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science* **2**, 00053.

Denton-Giles M, Derbyshire MC, Khentry Y, Buchwaldt L, Kamphuis LG (2018) Partial stem resistance in *Brassica napus* to highly aggressive and genetically diverse *Sclerotinia sclerotiorum* isolates from Australia. *Canadian Journal of Plant Pathology* **40**, 551–561.

Derbyshire M, Denton-Giles M, Hegedus D, Seifbarghy S, Rollins J, van Kan J, Seidl MF, Faino L, Mbengue M, Navaud O, Raffaele S, Hammond-Kosack K, Heard S, Oliver R (2017) The complete genome sequence of the phytopathogenic fungus *Sclerotinia sclerotiorum* reveals insights into the genome architecture of broad host range pathogens. *Genome Biology and Evolution* **9**, 593–618.

Dixon RA, Paiva NL (1995) Stress-Induced phenylpropanoid metabolism. *The Plant Cell* **7**, 1085–1097. doi:10.1105/tpc.7.7.1085.

Ferrari S, Galletti R, Pontiggia D, Manfredini C, Lionetti V, Bellincampi D, Cervone F, De Lorenzo G (2008) Transgenic expression of a fungal endo-polygalacturonase increases plant resistance to pathogens and reduces auxin sensitivity. *Plant Physiology* **146**, 669–681.

Ferrari S, Savatin DV, Sicilia F, Gramegna G, Cervone F, Lorenzo GD (2013) Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. *Frontiers in Plant Science* **4**, 00049.

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology* **43**, 205–227.

Guo X, Stotz HU (2007) Defense against *Sclerotinia sclerotiorum* in Arabidopsis is dependent on jasmonic acid, salicylic acid, and ethylene signaling. *Molecular plant-microbe interactions: MPMI* **20**, 1384–1395.

Hasanuzzaman M, Nahar K, Anee TI, Fujita M (2017) Glutathione in plants: biosynthesis and physiological role in environmental stress tolerance. *Physiology and Molecular Biology of Plants* **23**, 249–268.

Huang DW, Sherman BT, Tan Q, Collins JR, Alvord WG, Roayaei J, Stephens R, Baseler MW, Lane HC, Lempicki RA (2007) The DAVID gene functional classification tool: a novel

biological module-centric algorithm to functionally analyze large gene lists. *Genome Biology* **8**, R183.

Jones JDG, Dangl JL (2006) The plant immune system. *Nature* **444**, 323–329. d

Jukanti AK, Gaur PM, Gowda CLL, Chibbar RN (2012) Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. *British Journal of Nutrition* **108**, S11–S26.

Kabbage M, Yarden O, Dickman MB (2015) Pathogenic attributes of *Sclerotinia sclerotiorum*: Switching from a biotrophic to necrotrophic lifestyle. *Plant Science* **233**, 53–60.

Kamphuis LG, Williams AH, Küster H, Trengove RD, Singh KB, Oliver RP, Ellwood SR (2012) *Phoma medicaginis* stimulates the induction of the octadecanoid and phenylpropanoid pathways in *Medicago truncatula*. *Molecular Plant Pathology* **13**, 593–603.

Kanehisa M, Goto S (2000) KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Research* **28**, 27–30.

Kavousi HR, Marashi H, Mozafari J, Bagheri AR (2009) Expression of phenylpropanoid pathway genes in chickpea defense against race 3 of *Ascochyta rabiei*. *Plant Pathology Journal* **8**, 127-132.

Kim D, Langmead B, Salzberg SL (2015) HISAT: a fast spliced aligner with low memory requirements. *Nature Methods* **12**, 357–360.

Kim KS, Min J-Y, Dickman MB (2008) Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Molecular Plant-Microbe Interactions*® **21**, 605–612.

Kumar M, Yadav V, Tuteja N, Johri AK (2009) Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. *Microbiology,* **155**, 780–790. doi:10.1099/mic.0.019869-0.

Lane D, Denton-Giles M, Derbyshire M, Kamphuis LG (2019) Abiotic conditions governing the myceliogenic germination of *Sclerotinia sclerotiorum* allowing the basal infection of *Brassica napus*. *Australasian Plant Pathology* **48**, 85–91.

Lewis LA, Polanski K, de Torres-Zabala M, Jayaraman S, Bowden L, Moore J, Penfold CA, Jenkins DJ, Hill C, Baxter L, Kulasekaran S, Truman W, Littlejohn G, Prusinska J, Mead A, Steinbrenner J, Hickman R, Rand D, Wild DL, Ott S, Buchanan-Wollaston V, Smirnoff N, Beynon J, Denby K, Grant M (2015) Transcriptional dynamics are driving mamp-triggered immunity and pathogen effector-mediated immunosuppression in arabidopsis leaves following infection with *Pseudomonas syringae* pv tomato. *The Plant Cell* **27**, 3038–3064.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome project data processing subgroup (2009) the sequence alignment/map format and SAMtools. *Bioinformatics (Oxford, England)* **25**, 2078–2079.

Liang X, Rollins JA (2018) Mechanisms of Broad Host Range Necrotrophic Pathogenesis in *Sclerotinia sclerotiorum*. *Phytopathology®* **108**, 1128–1140.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods* **25**, 402–408.

Llorente F, Muskett P, Sánchez-Vallet A, López G, Ramos B, Sánchez-Rodríguez C, Jordá L, Parker J, Molina A (2008) Repression of the auxin response pathway increases Arabidopsis susceptibility to necrotrophic fungi. *Molecular Plant* **1**, 496–509.

Lun ATL, Chen Y, Smyth GK (2016) It's DE-licious: A Recipe for differential expression analyses of RNA-seq experiments using quasi-likelihood methods in edgeR. *Methods in Molecular Biology (Clifton, NJ)* **1418**, 391–416.

Mwape VW, Khentry Y, Newman TE, Denton-Giles M, Derbyshire M, Chen K, Berger J, Kamphuis L (2021a) Identification of sources of *Sclerotinia sclerotiorum* resistance in a collection of wild *Cicer* germplasm. *Plant Disease*. doi:10.1094/PDIS-02-21-0367-RE.

Mwape VW, Mobegi FM, Regmi R, Newman TE, Kamphuis LG, Derbyshire MC (2021b) Analysis of differentially expressed *Sclerotinia sclerotiorum* genes during the interaction with moderately resistant and highly susceptible chickpea lines. *BMC Genomics* **22**, 333.

Newman TE, Derbyshire MC (2020) The evolutionary and molecular features of broad host-range necrotrophy in plant pathogenic fungi. *Frontiers in Plant Science* **11**,591733.

Ni W, Fahrendorf T, Ballance GM, Lamb CJ, Dixon RA (1996) Stress responses in alfalfa (*Medicago sativa* L.). XX. Transcriptional activation of phenlpropanoid pathway genes in elicitor-induced cell suspension cultures. *Plant Molecular Biology* **30**, 427–438.

Oliveira MB, Nascimento LB, Junior ML, Petrofeza S (2010) Characterization of the dry bean polygalacturonase-inhibiting protein (PGIP) gene family during *Sclerotinia sclerotiorum* (Sclerotiniaceae) infection. *Genetics and molecular research: GMR* **9**, 994–1004.

Perchepied L, Balagué C, Riou C, Claudel-Renard C, Rivière N, Grezes-Besset B, Roby D (2010) Nitric oxide participates in the complex interplay of defense-related signaling pathways controlling disease resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions®* **23**, 846–860. doi:10.1094/MPMI-23-7-0846.

Piffanelli P, Devoto A, Schulze-Lefert P (1999) Defence signalling pathways in cereals. *Current Opinion in Plant Biology* **2**, 295–300.

Poovaiah BW, Du L, Wang H, Yang T (2013) Recent advances in calcium/calmodulin-mediated signaling with an emphasis on plant-microbe interactions1. *Plant Physiology* **163**, 531–542.

Pulse Australia - APB Chickpea IDM strategies http://www.pulseaus.com.au/growing-pulses/bmp/chickpea/idm-strategies. Accessed 10 July 2020.

Ranjan A, Westrick NM, Jain S, Piotrowski JS, Ranjan M, Kessens R, Stiegman L, Grau CR, Conley SP, Smith DL, Kabbage M (2019) Resistance against *Sclerotinia sclerotiorum* in soybean involves a reprogramming of the phenylpropanoid pathway and up-regulation of antifungal activity targeting ergosterol biosynthesis. *Plant Biotechnology Journal* **17**, 1567–1581.

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research* **43**, e47–e47.

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)* **26**, 139–140.

Sanders D, Pelloux J, Brownlee C, Harper JF (2002) Calcium at the crossroads of signaling. *The Plant Cell* **14**, S401–S417.

Sun J, Cao L, Li H, Wang G, Wang S, Li F, Zou X, Wang J (2019) Early responses given distinct tactics to infection of *Peronophythora litchii* in susceptible and resistant litchi varieties. *Scientific Reports* **9**, 2810.

Tan K-C, Oliver RP, Solomon PS, Moffat CS (2010) Proteinaceous necrotrophic effectors in fungal virulence. *Functional Plant Biology* **37**, 907–912.

Tiemann K, Inzé D, Montagu MV, Barz W (1991) Pterocarpan phytoalexin biosynthesis in elicitor-challenged chickpea (*Cicer arietinum* L.) cell cultures. *European Journal of Biochemistry* **200**, 751–757.

Uloth MB, You MP, Finnegan PM, Banga SS, Banga SK, Sandhu PS, Yi H, Salisbury PA, Barbetti MJ (2013) New sources of resistance to *Sclerotinia sclerotiorum* for crucifer crops. *Field Crops Research* **154**, 40–52.

Varshney RK, Song C, Saxena RK, Azam S, Yu S, Sharpe AG, Cannon S, Baek J, Rosen BD, Tar'an B, Millan T, Zhang X, Ramsay LD, Iwata A, Wang Y, Nelson W, Farmer AD, Gaur PM, Soderlund C, Penmetsa RV, Xu C, Bharti AK, He W, Winter P, Zhao S, Hane JK, Carrasquilla-Garcia N, Condie JA, Upadhyaya HD, Luo M-C, Thudi M, Gowda CLL, Singh NP, Lichtenzveig J, Gali KK, Rubio J, Nadarajan N, Dolezel J, Bansal KC, Xu X, Edwards D, Zhang G, Kahl G, Gil J, Singh KB, Datta SK, Jackson SA, Wang J, Cook DR (2013) Draft genome sequence of chickpea (*Cicer arietinum*) provides a resource for trait improvement. *Nature Biotechnology* **31**, 240–246.

Wang Z, Ma L-Y, Cao J, Li Y-L, Ding L-N, Zhu K-M, Yang Y-H, Tan X-L (2019) Recent Advances in mechanisms of plant defense to *Sclerotinia sclerotiorum*. *Frontiers in Plant Science* **10**, 01314.

Wang Z, Mao H, Dong C, Ji R, Cai L, Fu H, Liu S (2009) Overexpression of *Brassica napus* MPK4 Enhances Resistance to *Sclerotinia sclerotiorum* in Oilseed Rape. *Molecular Plant-Microbe Interactions®* **22**, 235–244.

Wang Z, Tan X, Zhang Z, Gu S, Li G, Shi H (2012) Defense to *Sclerotinia sclerotiorum* in oilseed rape is associated with the sequential activations of salicylic acid signaling and jasmonic acid signaling. *Plant Science* **184**, 75–82.

Weigand F, Köster J, Weltzien HC, Barz W (1986) Accumulation of phytoalexins and isoflavone glucosides in a resistant and a susceptible varieties of *Cicer arietinum* during infection with *Ascochyta rabiei*. *Journal of Phytopathology* **115**, 214–221.

Wendehenne D, Durner J, Klessig DF (2004) Nitric oxide: a new player in plant signalling and defence responses. *Current Opinion in Plant Biology* **7**, 449–455.

Yadav V, Wang Z, Wei C, Amo A, Ahmed B, Yang X, Zhang X (2020) Phenylpropanoid pathway engineering: an emerging approach towards plant defense. *Pathogens* **9**, 312.

Yu Y, Xiao J, Zhu W, Yang Y, Mei J, Bi C, Qian W, Qing L, Tan W (2017) Ss-Rhs1, a secretory Rhs repeat-containing protein, is required for the virulence of *Sclerotinia sclerotiorum*. *Molecular Plant Pathology* **18**, 1052–1061.

Zhao J, Buchwaldt L, Rimmer SR, Sharpe A, McgregoR L, Bekkaoui D, Hegedus D (2009) Patterns of differential gene expression in *Brassica napus* varieties infected with *Sclerotinia sclerotiorum*. *Molecular Plant Pathology* **10**, 635–649.

Zheng Z, Qamar SA, Chen Z, Mengiste T (2006) Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. *The Plant Journal* **48**, 592–605.

Zipfel C (2014) Plant pattern-recognition receptors. *Trends in Immunology* **35**, 345–351.

CHAPTER 6

General discussion

A major focus in global agriculture in the past decade is oriented towards increased pulse production. Chickpea (*Cicer arietinum* L.) ranks third among the pulse crops after dry beans and peas in terms of production globally (FAOSTAT, 2019) and belongs to a group of pulses that are a source of food to the global population (Smýkal *et al.* 2018). Chickpea is known for its importance in sustainable agriculture due to its potential as a major source of human nutrients and medicinal benefits that include reducing cancer, cardiovascular ailments and diabetes risks (Jukanti *et al.* 2012). Further, chickpea is important as a rotational crop with cereal crops due to its nitrogen-fixing ability.(Aslam *et al.* 2003).

Counteracting chickpea nutritional and yield benefits is the presence various biotic stresses including *Sclerotinia sclerotiorum*, the causal agent of Sclerotinia stem rot (SSR). SSR is a destructive disease with the potential of causing substantial yield losses as an emerging disease in chickpea (Pulse Australia, 2020). Lack of effective disease control measures and inadequate resistance in the cultivated germplasm limits the management of the disease. Poorly characterised resistance mechanisms against this pathogen further confine the strategies that can be undertaken to design durable resistance or effective disease control measures. Furthermore, *S. sclerotiorum* is a host generalist phytopathogen (Kabbage *et al.* 2013), thus making cultural control options through crop rotations difficult if host crops are being grown in succession.

There is a growing interest in broadening the genetic diversity of cultivated crops using their wild relatives to break linkage drag and/or introduce new adaptive traits. Owing to the narrow genetic diversity of chickpea, it is important to understand adaptive differences between wild and domesticated chickpea to develop an understanding of how wild traits can be exploited to improve crop performance (Varshney *et al.* 2013; Abbo *et al.* 2003). Several research studies have shown that wild *Cicer* species offer superior sources of genetic resistance and diversity, and therefore are a novel resource for chickpea improvement (Abbo *et al.* 2003; Reen *et al.* 2019; Newman *et al.* 2020; Muñoz *et al.* 2017). The purpose of this PhD research was (i) to explore wild and domestic *Cicer* responses upon *S. sclerotiorum* infection, (ii) to identify valuable loci for resistance to SSR, (iii) to study *S. sclerotiorum* transcriptome during chickpea

infection and (iv) to define the underlying molecular mechanism(s) of resistance in chickpea against SSR.

A literature review (Chapter 1) reveals one of the main limitations for chickpea production in Australia is the lack of resistance to pests and diseases such as Ascochyta blight, Botrytis grey mould, Phytophthora root rot, root-lesion nematodes and SSR (Pulse Australia, 2020). There is also a knowledge gap in the current resistance status of Australian chickpea cultivars to SSR, and there is limited knowledge on the molecular interplay between *S. sclerotiorum* and chickpea. This issue has partly arisen due to low research resources directed to pulse grains studies compared to their counterpart, cereal grains. Also, the low genetic diversity of domesticated chickpea is a limitation in the breeding programs (Abbo *et al.* 2003), which is reflected in the recent effort by researchers to visit the ancestral origin of chickpea in Turkey to collect wild relatives as a source of novel genetic alleles (von Wettberg *et al.* 2018). Addressing this disease-based limitation in the chickpea industry is difficult. The novel genetic and phenotypic diversity of wild and domesticated chickpea has not yet been extensively explored to identify germplasm resistance and valuable loci for resistance to *S. sclerotiorum* infection.

Furthermore, there are no registered fungicides in Australia to control SSR in chickpea (Pulse Australia, 2020). Expanding our knowledge in these areas is, therefore, of utmost importance. In addition, the wild *Cicer* collection is a valuable germplasm resource to explore for any favourable alleles responsible for resistance to biotic and abiotic stresses affecting the chickpea industry (Croser *et al.* 2003). Consequently, dissection and characterisation of *S. sclerotiorum* resistance in the wild *Cicer* collection and domesticated elite cultivars may also make important contributions to the broader knowledge of *S. sclerotiorum* management in pulse crops and other hosts.

With these conclusions in mind, the research in this thesis aimed to comprehensively explore the physiological resistance and genetic resistance of a collection of wild *Cicer* and elite cultivated chickpea cultivars to *S. sclerotiorum*. In addition, this thesis aimed to characterise the molecular mechanisms used by this pathogen to infect chickpea and the genetic and molecular defence responses employed by chickpea upon invasion by *S. sclerotiorum*. To fulfil this overall aim, several key research objectives were completed, including:

1) In addition, this thesis aimed to characterise the molecular mechanisms used by this pathogen to infect chickpea and the genetic and molecular defence responses employed by chickpea upon invasion by *S. sclerotiorum* (Chapter 2; Mwape *et al.* 2021a).
2) The identification of quantitative trait loci (QTLs) responsible for *S. sclerotiorum* resistance in a segregating population derived from crosses of Australian elite cultivars PBA HatTrick and Kyabra (Chapter 3).
3) Investigation of the global transcription changes of *S. sclerotiorum* genes during chickpea infection (Chapter 4; (Mwape *et al.* 2021b).
4) Exploration of the responses of chickpea upon infection by *S. sclerotiorum* and identification of defence-related genes expressed during interaction with the pathogen (Chapter 5).

Here, is a general discussion of the implications of the current research on knowledge development in research, focusing on identifying SSR resistance in chickpea. In addition, the summary provides an outlook of several experiments that could be carried out in future to build on the research reported in this thesis.

6.1. Characterisation of *S. sclerotiorum* isolates and development of a chickpea inoculation assay

A pathogen's aggressiveness is its relative ability to colonise the host and cause damage (Miorini *et al.* 2019). It is well documented that isolates of *S. sclerotiorum* worldwide vary in aggressiveness on different hosts (Ge *et al.* 2012; Taylor *et al.* 2015; Willbur *et al.* 2017). The research in this thesis reports a study of the relative aggressiveness of nine *S. sclerotiorum* isolates, previously collected in regions of Western Australia (WA) (Denton-Giles *et al.* 2018), on wild and domesticated chickpea accessions (Mwape *et al.* 2021a). The nine isolates were found to be pathogenic on all *Cicer* germplasm; however, there were significant differences in the level of their aggressiveness. Until the current report in this thesis, little was known about the variation of aggressiveness in the *S. sclerotiorum* isolates collected in WA concerning chickpea as a host. This permitted selection of the highly aggressive and moderately aggressive isolates for subsequent SSR resistance screening.

Screening methods for SSR need to show consistent results, discriminatory power and be both cost and time effective. Stem inoculation with a mycelial plug has been reported in previous studies and is designed to simulate the natural *S. sclerotiorum* infection in the field (Denton-Giles *et al.* 2018; Taylor *et al.* 2015). The results of the host resistance assay can be influenced by the pathogen population-specific traits and the effectiveness of the inoculation method. In

Chapter 2, a stem inoculation method was successfully employed to assess the aggressiveness of *S. sclerotiorum* isolates and perform a resistance evaluation assay (Mwape *et al.* 2021a). This project successfully developed a reproducible, rapid, and reliable method of screening chickpeas for SSR resistance that can identify the reaction of chickpea genotypes to SSR. The development of this assay was a prerequisite of this project to quickly screen a large number of *Cicer* lines and establish resistance levels of the genotypes against SSR. The resulting stem inoculation assay thus proved to be invaluable, especially as it led to the discovery of varying responses of wild *Cicer* and domesticated germplasm to SSR (Chapter 2; Mwape *et al.* 2021a). Further, the developed assay was a useful tool in identification of QTLs responsible for SSR resistance (Chapter 3), in the study of *in planta S. sclerotiorum* gene expression during chickpea infection (Chapter 4; Mwape *et al.* 2021b) and resistance responses of chickpea after SSR infection (Chapter 5). Therefore, this thesis makes substantial contributions towards addressing knowledge gaps in relation to screening techniques in the search for SSR resistance in chickpea germplasm.

6.2. Wild *Cicer* as a potential source of high resistance to sclerotinia stem rot

Perhaps the most significant scientific contribution of this thesis was the advances made in identifying sources of SSR resistance in a wild *Cicer* germplasm collection. The improvement of chickpea cultivars is limited by a lack of genetic diversity caused by genetic bottlenecks with domestication; however, approximately 95% of the missing genetic variation is present in wild chickpea progenitors (Abbo *et al.* 2003; von Wettberg *et al.* 2018). Many wild *Cicer* accessions (67 *C. reticulatum* and 19 *C. echinospermum*) were screened for resistance to highly aggressive and moderately aggressive *S. sclerotiorum* isolates, using a stem inoculation assay developed in the initial stage of this project. The search for resistant sources in the wild *Cicer* species to test whether the wild germplasm can provide better sources of resistance to chickpea diseases in comparison to cultivated species has been demonstrated in previous studies (Newman *et al.* 2020; Reen *et al.* 2019; Knights *et al.* 2008; Amalraj *et al.* 2019). It is noteworthy that the two domesticated genotypes (PBA HatTrick and Kyabra) showed consistent responses irrespective of the isolate's aggressiveness, suggesting that they should be preferentially used as standards in disease screening programs and commercial breeding programs for SSR resistance. On average, the wild *Cicer* accessions exhibited a higher level of resistance to SSR than the cultivated cultivars (*C. arietinum*) assessed in this study. The current study revealed a range of resistance to both isolates within wild *Cicer* accessions and therefore

we could confidently accept our hypothesis that the wild chickpea germplasm hold some resistance to *S. sclerotiorum* compared to domesticated cultivars (PBA HatTrick and Kyabra).

Further, this demonstrates the benefit of screening chickpea genotypes with a cultivar of *S. sclerotiorum* isolates to precisely identify the responses of different host genotypes. Whereas such a spectrum of isolates is not readily available, it is best to use a highly pathogenic isolate, such as the one used in this research project (CU 8.20). This concept is also demonstrated in previous research during screening various *S. sclerotiorum* hosts (Denton-Giles *et al.* 2018; Taylor *et al.* 2015; Miorini *et al.* 2019; Viteri *et al.* 2015). Therefore, this study supports our hypothesis that the wild *Cicer* germplasm can provide a better source of resistance to SSR than those available in commercial cultivars.

Accessions screened in this study originated from a narrow geographical area that nevertheless covered diverse physical and environmental scales based on soil type, elevation and climate that affect genetic differences more than geographical sites (von Wettberg *et al.* 2018). It is noteworthy that analysis of this new collection for resistance against SSR based on the 21 wild *Cicer* collection sites in Turkey (von Wettberg *et al.* 2018) demonstrated some important inferences. Karalbahce and CudiB showed the greatest resistance, while those from Savur and Sirnak showed high susceptibility to SSR. This study indicates that future research should consider wild *Cicer* accessions' collection sites when evaluating *S. sclerotiorum* resistance. Chapter 2 reports, for the first time, the assessment of this new wild *Cicer* collection from Turkey for resistance to SSR (Mwape *et al.* 2021a). While only 89 accessions of the wild *Cicer* collection have been evaluated in this project, there are 335 available accessions in Australia currently. Future work should thus focus on evaluating the remaining accessions to identify additional accessions that harbour partial resistance. Such an extensive dataset could be employed to conduct a genome-wide association study (GWAS) or to determine if genomic selection can be utilised to incorporate SSR resistance into future chickpea cultivars. Nevertheless, the knowledge and data achieved in this project offer breeders the chance to choose from resistant wild *Cicer* accessions and use this germplasm in their crossing program to aid the development of future SSR resistant cultivars.

6.3. Identification of quantitative trait loci controlling resistance

A significant achievement of this study was the identification of quantitative trait loci (QTLs) controlling resistance to *S. sclerotiorum* in chickpea using an F_7 population derived from crosses of elite chickpea cultivars (PBA HatTrick x Kyabra), as described in Chapter 3. Based

on the linkage mapping, novel QTLs were identified on three different chromosomes. QTLs with major effects were identified in Ca4 and Ca7, and two QTLs with minor effects were located on Ca1 and Ca7. These genomic regions can be targeted for developing SSR resistant chickpea cultivars through marker-assisted breeding (MAB). Further work to identify plausible candidate genes for *S. sclerotiorum* resistance should be a high priority for future research work. We therefore accept out hypothesis that the RIL population generated from crossing the two chickpea cultivars (PBA HatTrick and Kyabra) will show higher resistance levels to S. *sclerotiorum* compared to their parents.

Using the available chickpea reference genome sequences and physical locations identified for the major SSR partial resistance QTL, candidate genes linked to disease resistance in chickpea were identified. A set of these disease resistance genes was found to be located within Ca4 and Ca7. The chickpea candidate genes identified in both genomic locations are involved in pathways such as plant-pathogen interactions, phenylpropanoid biosynthesis, and signalling. Genes involved in signalling pathways, plant development or presently uncharacterised genes may govern partial resistance loci in a plant pathosystem (Poland *et al.* 2009; Nivedita *et al.* 2017). In this study, several genes co-located in Ca4 and Ca7 may play roles that are related to the manifestation of partial resistance against SSR in chickpea. This includes pathogenicity related proteins (PR-1), ethylene-responsive transcriptional factors (ERF), leucine-rich repeat receptor-like proteins (LRR), glutathione reductase (GR) and serine/threonine–protein kinase. Therefore, these genomic regions could be significant in understanding disease resistance in chickpea, and the generation of crosses using the wild materials may be important in introgressing complementary sources of resistance. However, as this is the primary genetic study of SSR resistance in chickpea, further studies on validation of molecular markers associated with SSR resistance QTLs in a large collection of Australian chickpea breeding germplasm is recommended.

The QTLs identified in Chapter 3 combined explain a relatively small percentage (19.5 %) of the SSR resistance phenotype. This is in line with observations made in other crops such as soybean (Guo *et al.* 2008) and canola (Behla *et al.* 2017), where many loci explain SSR resistance in the genome with relatively small effects. The latter has deterred breeding programs from using molecular markers linked to small effect loci. Still, modern breeding programs, including the Australian chickpea breeding program at the Department of Primary Industries (New South Wales), are adopting a technique termed genomic selection that is

particularly amenable to introgression of traits that are polygenic such as yield and resistance to SSR and Ascochyta blight. Nevertheless, some breeding programs might still adopt molecular markers associated with the major QTLs identified in this thesis. If such an approach is taken, a locus with a major effect is the most likely to be chosen in breeding schemes using a MAB approach. Quantitative or partial resistance in plants is governed by genes linked with QTL regions with either major or minor effects (Poland *et al.* 2009). Both major and minor QTLs' combined action is more robust as polygenic resistance is complex for pathogen selection to withstand (Amalraj *et al.* 2019). Hence, a combination of located candidate genes with both major and minor effects for SSR resistance could be employed to apply molecular markers in chickpea breeding programs.

The low level of allelic diversity in the *C. arietinum* gene pool has hindered efforts by breeders to produce greater resistance to biotic and abiotic stresses (Singh and Ocampo 1997). Introducing a range of traits from wild relatives of chickpea can improve the cultivated gene pool and increase disease resistance. Developing an F_7 population from crosses of the partially resistant wild *Cicer* reported in Chapter 2 and elite cultivars will enable further QTL analysis. This population will present a novel source of diverse traits introgressed from a partially resistant wild parent, which could reveal molecular markers for SSR resistance and potentially for other adaptable traits between the two parents. Whilst the challenges related to including wild relatives in crop improvement are important, the findings in this study and future experiments will provide comprehensive information on the degree of phenotypic differences and total variances in genetic complexity of SSR resistance in wild and domesticated species.

6.4. *Sclerotinia sclerotiorum* infection strategies

In addition to identifying QTLs involved in *S. sclerotiorum* resistance, transcriptome studies in this research project have laid the foundation for understanding and analysing the interaction between *C. arietinum* and *S. sclerotiorum* at a molecular level through the generation of *in planta* transcriptome sequence resources (Chapter 4; Mwape *et al.* 2021b and Chapter 5). This thesis provides the examination of gene expression in *S. sclerotiorum* during infection of chickpea genotypes with varying susceptibility levels. The findings from this study emphasise the role of cell wall degrading enzymes (CWDEs) and the detoxification of host metabolites as an important process underlying the pathogenesis of *S. sclerotiorum* in chickpea. In this regard, some of the reported *in planta* expressed genes in *S. sclerotiorum* would be good candidates

for further functional analysis and determination of their possible role in pathogenesis through RNA interference approaches such as gene silencing.

Furthermore, changes in the expression pattern of numerous genes overtime involved in reactive oxygen species (ROS) scavenging at an early stage and late-onset of necrosis inducing effectors such as *SsSSVP1* provided support for the two-phase infection approach comprising of a short biotrophic phase followed by a necrotrophic phase (Kabbage *et al.* 2015). Several other predicted (Derbyshire *et al.* 2017) but uncharacterised *S. sclerotiorum* putative effectors were also induced during chickpea infection. We therefore accept our null hypothesis that *S. sclerotiorum* uses different strategies during the infection of the two cultivars (PBA HatTrick and Kyabra).

Follow-up functional analysis using knockout or gene silencing could help determine predicted effectors' role during infection and disease development. Functional analysis of these genes would provide a further understanding of disease development events and colonisation of host tissues.

6.5. Mechanisms of resistance

Identifying beneficial traits in a species aims at translating them into crop improvement programs. Valuable insight into the resistance mechanisms that are operational in the moderately resistant (MR) cultivar (PBA HatTrick) compared to the susceptible (S) cultivar Kyabra in response to *S. sclerotiorum* infection was obtained using RNA-seq analysis (Chapter 5). Many genes were differentially regulated after infection in both MR and S lines, with a higher number of genes upregulated at a later stage of infection. At an early stage of infection (6-24 hpi), genes related to metabolic pathways, including the one involved in the production of reactive oxygen species, were significantly upregulated in the MR line but not in the S line, and they could have been responsible for mediating defence responses against the pathogen. Early expression of sugar metabolism in the MR line indicated that carbon reserves such as sucrose and starch are accessed and reshuffled earlier in the MR line compared to the S line. The sugar metabolism activities lead to the formation of glyoxylate and alter the TCA cycle, which may form a part of the defence responses (Hückelhoven *et al.* 1999)

Several genes, especially those known to play important roles in the phenylpropanoid and (iso)flavonoid pathways, were differentially expressed in the moderately resistant and susceptible chickpea lines. Recruitment of these pathways leads to the production of antifungal compounds. Cultivars that naturally produce higher levels of such antifungal compounds might

harbour higher levels of resistance to SSR infection. Validation of the expression of these genes and the antifungal metabolites they produce in response to SSR infection could aid in the breeding of resistant cultivars. The findings in Chapter 4 (Mwape *et al.* 2021b) and 5 add to the knowledge of *S. sclerotiorum* and *C. arietinum* genes that are involved in chickpea infection, which can be helpful for further studies to exploit genes in the pathogen and host and establish a sustainable management strategy for SSR in chickpea. Other histology studies microscopically examine the events occurring soon after the initial interaction between *S. sclerotiorum* and chickpea is recommended. In addition, metabolomics approaches to identify the secondary metabolites with antifungal activity are highly recommended.

6.6. Practical applications for chickpea breeding and future research work

A key goal of this thesis was to get an improved understanding of the phenotypic and genetic resistance of wild and domesticated chickpea germplasm to *S. sclerotiorum* with the end goal to develop resistance to this pathogen in future cultivars. Before the current study, there were no known wild or domesticated chickpea accessions with partial resistance to *S. sclerotiorum*. At present, the wild *Cicer* germplasm harbours partial resistance to *S. sclerotiorum* (Chapter 2; Mwape *et al.* 2021a). This study has opened a spectrum of possibilities, especially from an agronomic point of view. For instance, the introgression of identified partial resistance in wild *Cicer* accession via hybridisation to develop recombinant inbred lines is underway. Future work focusing on further enhancing the population to the F7 generation and identifying and mapping genes governing resistance offer significant potential to enhance SSR management in chickpea further. It will also be helpful to investigate the genetic and molecular responses in the new introgressed lines upon *S. sclerotiorum* infection compared to those found in this study.

In addition, we have identified genetic loci that contribute to partial resistance in cultivar PBA HatTrick and identified candidate genes located in these regions (Chapter 3). These genomic regions provide a strong basis for further investigation and fine mapping and validation of the determined QTLs responsible for SSR resistance. This study also provides breeders with a tangible tool to develop breeder friendly marker assays for use in selecting among populations segregating for SSR reaction by converting the DArTseq markers into PCR-based marker assays of a breeding program's choice. Comparing QTLs among future studies and sharing markers and sequence information will help develop *S. sclerotiorum* resistant chickpea

cultivars. With further validation, the reported markers in this thesis could be used in marker-assisted selection in breeding and enhance the efficiency with which the resistance to *S. sclerotiorum* is fixed in elite cultivated cultivars. Other than genetic resources, SSR resistance in other hosts is also reported to be influenced by other plant developmental factors such as the host flowering time and stem width (Wei *et al.* 2014). Therefore, future studies could also focus on chickpea developmental traits such as flowering time and growth stage to investigate if they may affect resistance.

Given the high economic importance of the SSR for growers in Australia and elsewhere, the isolation of underlying resistance genes is crucial for developing SSR resistant cultivars. However, this research can be replicated with more mapping populations to identify small effect genes that may have been masked due to a genetic relationship between cultivars PBA HatTrick and Kyabra. Thus, future studies should exploit RIL mapping populations derived from parents with wide genetic distances; for instance, a population developed from crosses of a wild *Cicer* accession with a domesticated cultivar (Chapter 2). Further, saturating the QTL regions with closely linked markers will be important for breeders in the introgression of resistance QTLs into commercial chickpea cultivars by marker-assisted selection and help reduce linkage drag. The advancement of wide-crossing methods such as embryo culture and applying new methods of reducing generation period and breeding cycle (Croser *et al*. 2016) will quicken resistance introgression and enable the advancement of robust resistance to this important chickpea disease.

Development of RIL populations by crossing the partially resistant lines such as *Cicer echinospermum* Karab_084 with highly susceptible domesticated cultivars such as Kyabra will provide additional resources to investigate the genetic control of SSR resistance. Mapping for QTLs conferring resistance to *S. sclerotiorum* using a population derived from Karab_084 and Kyabra currently under development (Chapter 2), will potentially provide additional loci for SSR resistance or confirm the contribution of resistance loci identified in the current study. A combination of marker-assisted selection and developing genomic prediction models for SSR resistance in chickpea probably the most effective method to develop new chickpea cultivars with greater resistance to *S. sclerotiorum*. However, there is still a gap for firm genetic sources of resistance against *S. sclerotiorum*. Further study is recommended to achieve complete genetic control over SSR in the chickpea production industry.

6.7. Final remarks

In the current PhD project, a concerted effort has been made to expand our level of understanding of the responses of the current wild *Cicer* collection to SSR, the genetic basis of chickpea resistance to SSR, the molecular mechanisms employed by *S. sclerotiorum* during chickpea infection, and the molecular pathways expressed in chickpea in response to *S. sclerotiorum*. These findings provide important information to current and future chickpea breeding programs to understand the conserved/divergent alleles in the current domesticated cultivars and in the current collection of wild *Cicer* that can be exploited to improve SSR resistance chickpea. This project provides the first evaluation of SSR resistance in the new collection of wild *Cicer* accessions. Our results showed some accessions were more resistant than two popular Australian chickpea cultivars. Additionally, the current data provide an information repository that will allow linkage with the genetic diversity studies and offer novel insight into SSR resistance. Introgression of resistance identified in wild *Cicer* accessions into cultivated chickpea could ultimately lead to cultivars with enhanced resistance and increased yields.

The current research identifies *S. sclerotiorum* resistance loci in domesticated germplasm. It provides a preliminary report on *S. sclerotiorum* pathogenesis during chickpea infection and pathways involved in relation to chickpea defence against *S. sclerotiorum* infection. Investigating the transcriptional changes of candidate genes underlying the identified QTLs and determining sequence difference in these genes could lead to the development of perfect molecular markers for a breeding program. The outcomes of this research will thus be a first step to tackle the losses caused by this *S. sclerotiorum*, especially knowing that this PhD study was steered by the current lack of registered chemical control options and high variability in disease outbreaks (depending on environmental conditions) of this cosmopolitan pathogen.

6.8. References

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

Abbo S, Berger J, Turner NC (2003) Viewpoint: Evolution of cultivated chickpea: four bottlenecks limit diversity and constrain adaptation. *Functional Plant Biology* **30**, 1081–1087.

Amalraj A, Taylor J, Bithell S, Li Y, Moore K, Hobson K, Sutton T (2019) Mapping resistance to Phytophthora root rot identifies independent loci from cultivated (*Cicer arietinum* L.) and wild (*Cicer echinospermum* P.H. Davis) chickpea. *Theoretical and Applied Genetics* **132**, 1017–1033.

Aslam M, Mahmood IA, Peoples MB, Schwenke GD, Herridge DF (2003) Contribution of chickpea nitrogen fixation to increased wheat production and soil organic fertility in rain-fed cropping. *Biology and Fertility of Soils* **38**, 59–64.

Behla R, Hirani AH, Zelmer CD, Yu F, Fernando WGD, McVetty P, Li G (2017) Identification of common QTL for resistance to *Sclerotinia sclerotiorum* in three doubled haploid populations of *Brassica napus* (L.). *Euphytica* **213**, 260.

Croser J, Ahmad F, Clarke H, Siddique K (2003) Utilisation of wild *Cicer* in chickpea improvement - progress, constraints, and prospects. *Australian Journal of Agricultural Research* **54**, 429–444.

Croser JS, Pazos-Navarro M, Bennett RG, Tschirren S, Edwards K, Erskine W, Creasy R, Ribalta FM (2016) Time to flowering of temperate pulses in vivo and generation turnover in vivo–in vitro of narrow-leaf lupin accelerated by low red to far-red ratio and high intensity in the far-red region. *Plant Cell, Tissue and Organ Culture (PCTOC)* **127**, 591–599.

Denton-Giles M, Derbyshire MC, Khentry Y, Buchwaldt L, Kamphuis LG (2018) Partial stem resistance in *Brassica napus* to highly aggressive and genetically diverse *Sclerotinia sclerotiorum* isolates from Australia. *Canadian Journal of Plant Pathology* **40**, 551–561.

Derbyshire M, Denton-Giles M, Hegedus D, Seifbarghy S, Rollins J, van Kan J, Seidl MF, Faino L, Mbengue M, Navaud O, Raffaele S, Hammond-Kosack K, Heard S, Oliver R (2017) The complete genome sequence of the phytopathogenic fungus *Sclerotinia sclerotiorum* reveals insights into the genome architecture of broad host range pathogens. *Genome Biology and Evolution* **9**, 593–618.

Ge XT, Li YP, Wan ZJ, You MP, Finnegan PM, Banga SS, Sandhu PS, Garg H, Salisbury PA, Barbetti MJ (2012) Delineation of *Sclerotinia sclerotiorum* pathotypes using

differential resistance responses on *Brassica napus* and *B. juncea* genotypes enables identification of resistance to prevailing pathotypes. *Field Crops Research* **127**, 248–258.

Guo X, Wang D, Gordon SG, Helliwell E, Smith T, Berry SA, Martin SKS, Dorrance AE (2008) Genetic mapping of QTLs underlying partial resistance to *Sclerotinia sclerotiorum* in soybean PI 391589A and PI 391589B. *Crop Science* **48**, 1129–1139.

Hückelhoven R, Fodor J, Preis C, Kogel K-H (1999) Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. *Plant Physiology* **119**, 1251–1260.

Jukanti AK, Gaur PM, Gowda CLL, Chibbar RN (2012) Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. *British Journal of Nutrition* **108**, S11–S26.

Kabbage M, Williams B, Dickman MB (2013) Cell Death Control: The interplay of apoptosis and autophagy in the pathogenicity of *Sclerotinia sclerotiorum*. *PLoS Pathogens* **9**,.1003287.

Kabbage M, Yarden O, Dickman MB (2015) Pathogenic attributes of *Sclerotinia sclerotiorum*: Switching from a biotrophic to necrotrophic lifestyle. *Plant Science* **233**, 53–60.

Knights EJ, Southwell RJ, Schwinghamer MW, Harden S (2008) Resistance to P*hytophthora medicaginis* Hansen and Maxwell in wild *Cicer* species and its use in breeding root rot resistant chickpea (Cicer arietinum L.). *Australian Journal of Agricultural Research* **59**, 383–387.

Miorini TJJ, Kamvar ZN, Higgins RS, Raetano CG, Steadman JR, Everhart SE (2019a) Differential aggressiveness of *Sclerotinia sclerotiorum* isolates from North and South America and partial host resistance in Brazilian soybean and dry bean varieties. *Tropical Plant Pathology* **44**, 73–81.

Miorini TJJ, Kamvar ZN, Higgins RS, Raetano CG, Steadman JR, Everhart SE (2019b) Differential aggressiveness of *Sclerotinia sclerotiorum* isolates from North and South America and partial host resistance in Brazilian soybean and dry bean varieties. *Tropical Plant Pathology* **44**, 73–81.

Muñoz N, Liu A, Kan L, Li M-W, Lam H-M (2017) Potential uses of wild germplasms of grain legumes for crop improvement. *International Journal of Molecular Sciences* **18**,.18020328.

Mwape VW, Khentry Y, Newman TE, Denton-Giles M, Derbyshire M, Chen K, Berger J, Kamphuis L (2021a) Identification of sources of *Sclerotinia sclerotiorum* resistance in a collection of wild *Cicer* germplasm. *Plant Disease*. doi:10.1094/PDIS-02-21-0367-RE.

Mwape VW, Mobegi FM, Regmi R, Newman TE, Kamphuis LG, Derbyshire MC (2021b) Analysis of differentially expressed *Sclerotinia sclerotiorum* genes during the interaction with moderately resistant and highly susceptible chickpea lines. *BMC Genomics* **22**, 333.

Newman TE, Jacques S, Grime C, Kamphuis FL, Lee RC, Berger J, Kamphuis LG (2020) Identification of novel sources of resistance to ascochyta blight in a collection of wild *Cicer* accessions. *Phytopathology®*. **111**(2),339-379.

Nivedita, Verma PK, Upadhyaya KC (2017) Lectin protein kinase is induced in plant roots in response to the endophytic fungus, P*iriformospora indica*. *Plant Molecular Biology Reporter* **35**, 323–332.

Poland JA, Balint-Kurti PJ, Wisser RJ, Pratt RC, Nelson RJ (2009) Shades of gray: the world of quantitative disease resistance. *Trends in Plant Science* **14**, 21–29.

Pulse Australia - APB Chickpea IDM strategies http://www.pulseaus.com.au/growing-pulses/bmp/chickpea/idm-strategies. Accessed 10 July 2020.

Reen RA, Mumford MH, Thompson JP (2019) Novel Sources of Resistance to Root-Lesion Nematode (*Pratylenchus thornei*) in a New Collection of Wild *Cicer* Species (C. reticulatum and C. echinospermum) to Improve Resistance in Cultivated Chickpea (C. arietinum). *Phytopathology®* **109**, 1270–1279. doi:10.1094/PHYTO-02-19-0047-R.

Singh KB, Ocampo B (1997) Exploitation of wild Cicer species for yield improvement in chickpea. *Theoretical and Applied Genetics* **95**, 418–423. doi:10.1007/s001220050578.

Smýkal P, Nelson MN, Berger JD, Von Wettberg EJB (2018) The Impact of Genetic Changes during Crop Domestication. *Agronomy* **8**, 119. doi:10.3390/agronomy8070119.

Taylor A, Coventry E, Jones JE, Clarkson JP (2015) Resistance to a highly aggressive isolate of Sclerotinia sclerotiorum in a Brassica napus diversity set. *Plant Pathology* **64**, 932–940. doi:10.1111/ppa.12327.

Varshney RK, Song C, Saxena RK, Azam S, Yu S, Sharpe AG, Cannon S, Baek J, Rosen BD, Tar'an B, Millan T, Zhang X, Ramsay LD, Iwata A, Wang Y, Nelson W, Farmer AD, Gaur PM, Soderlund C, Penmetsa RV, Xu C, Bharti AK, He W, Winter P, Zhao S, Hane JK, Carrasquilla-Garcia N, Condie JA, Upadhyaya HD, Luo M-C, Thudi M, Gowda CLL, Singh NP, Lichtenzveig J, Gali KK, Rubio J, Nadarajan N, Dolezel J, Bansal KC, Xu X, Edwards D, Zhang G, Kahl G, Gil J, Singh KB, Datta SK, Jackson SA, Wang J, Cook DR (2013) Draft genome sequence of chickpea (Cicer arietinum) provides a resource for trait improvement. *Nature Biotechnology* **31**, 240–246. doi:10.1038/nbt.2491.

Viteri DM, Otto K, Terán H, Schwartz HF, Singh SP (2015) Use of four Sclerotinia sclerotiorum isolates of different aggressiveness, three inoculations per plant, and delayed multiple evaluations to select common beans with high levels of white mold resistance. *Euphytica* **204**, 457–472. doi:10.1007/s10681-015-1366-7.

Wei D, Mei J, Fu Y, Disi JO, Li J, Qian W (2014) Quantitative trait loci analyses for resistance to Sclerotinia sclerotiorum and flowering time in Brassica napus. *Molecular Breeding* **34**, 1797–1804. doi:10.1007/s11032-014-0139-7.

von Wettberg EJB, Chang PL, Başdemir F, Carrasquila-Garcia N, Korbu LB, Moenga SM, Bedada G, Greenlon A, Moriuchi KS, Singh V, Cordeiro MA, Noujdina NV, Dinegde KN, Shah Sani SGA, Getahun T, Vance L, Bergmann E, Lindsay D, Mamo BE, Warschefsky EJ, Dacosta-Calheiros E, Marques E, Yilmaz MA, Cakmak A, Rose J, Migneault A, Krieg CP, Saylak S, Temel H, Friesen ML, Siler E, Akhmetov Z, Ozcelik H, Kholova J, Can C, Gaur P, Yildirim M, Sharma H, Vadez V, Tesfaye K, Woldemedhin AF, Tar'an B, Aydogan A, Bukun B, Penmetsa RV, Berger J, Kahraman A, Nuzhdin SV, Cook DR (2018) Ecology and genomics of an important crop wild relative as a prelude to agricultural innovation. *Nature Communications* **9**, 649. doi:10.1038/s41467-018-02867-z.

Willbur JF, Ding S, Marks ME, Lucas H, Grau CR, Groves CL, Kabbage M, Smith DL (2017) Comprehensive Sclerotinia Stem Rot Screening of Soybean Germplasm Requires Multiple Isolates of Sclerotinia sclerotiorum. *Plant Disease* **101**, 344–353. doi:10.1094/PDIS-07-16-1055-RE.

APPENDIX

This section contains supporting information for CHAPTER 2 to 5 of this thesis. Due to the large and display size of the files, a URL link and password is provided to download them from cloudstor.

URL https://cloudstor.aarnet.edu.au/plus/s/fN2rFc0AKNSsFZy

Password: VWM_Thesis2021

Appendix 1. CHAPTER 2 Supplementary files

The following supporting information is available for this chapter:

Supplementary Table S2.1. Mean area under disease progress curve (AUDPC) value for each isolate x cultivar/accession interaction.

Supplementary Table S2.2. Nested analysis of variance (ANOVA) analysis to identify collection site by isolate interactions.

Supplementary Table S2.3. Mean area under disease progress curve AUDPC for 88 *Cicer* accessions inoculated with a highly aggressive (CU8.20) and moderately aggressive (CU10.12) *S. sclerotiorum* isolate.

Supplementary Table S2.4. The 12 *Cicer* genotypes average stem lesion length measured at 6 time points for 12 days post inoculation (dpi) and their mean area under disease progress curve (AUDPC) after inoculation with a highly aggressive (CU8.20) isolate.

Appendix 2. CHAPTER 3 Supplementary files

The following supporting information is available for this chapter:

Supplementary Table S3.1: Mean area under disease progress curve (AUDPC), standard deviation (STD) and least significant difference (LSD) of Australian breeeding lines.

Supplementary Table S3.2: Mean area under disease progress curve AUDPC, standard error of difference (SED) and least significant difference (LSD) of recombinant inbred lines (RIL) and their parents (PBA HatTrick and Kyabra).

Supplementary Table S3.3: List of genes identified in the quantitative trait loci (QTL) regions of recombinant inbred lines (RIL) and their parents (PBA HatTrick and Kyabra).

Supplementary Table S3.4: Kyoto encyclopedia of genes and genomes (KEGG) pathways identified in the quantitative trait loci (QTL) regions.

Appendix 3. CHAPTER 4 Supplementary files

The following supporting information is available for this chapter:

Supplementary Table S4.1. Primers used in the validation of RNA sequencing data. (xlsx).

Supplementary Table S4.2. List of differentially expressed *S. sclerotiorum* genes during the interaction with moderately resistant (MR) and susceptible (S) chickpea lines at 6, 12, 24, 48 and 72 hours post inoculation (P. Adj. <0.05; LogFC > 2 indicated upregulated or < 2 indicate downregulated. (xlsx).

Supplementary. Table S4.3. List of common *S. sclerotiorum* genes differentially expressed during the interaction with moderately resistant (MR) and susceptible (S) at 6, 12, 24,48 and 72 post-inoculation relative to *in vitro*.

Supplementary Table S4.4. List *S. sclerotiorum* genes differentially expressed exclusively in moderately resistant (MR) only and susceptible (S) only at 6, 12, 24,48 and 72 post-inoculation relative to *in vitro* during chickpea infection.

Supplementary. Table S4.5. Enrichment analysis of *S. sclerotiorum* upregulated genes during interaction with moderately resistant (MR) and susceptible (S) chickpea lines at 6, 12, 24, and 72 hours post inoculation relative to *in vitro* control. (xlsx).

Supplementary Table S4.6. Enrichment analysis of *S. sclerotiorum* downregulated genes during interaction with moderately resistant (MR) and susceptible (S) chickpea lines at 6, 12, 24, and 72 hours post inoculation relative to *in vitro* control. (xlsx).

Supplementary Table S4.7. Description of temporal *S. sclerotiorum in planta* upregulated genes involved in cell wall degradation during chickpea infection relative to *in vitro* control (P. Adj. <0.05; LogFC ≥ 2).

Supplementary Table S4.8. Secondary metabolite synthesis, cytochrome P450 and transporter genes upregulated at some time point of the MR and S lines infection in comparison to *in vitro* control (P. Adj. <0.05; LogFC ≥ 2).

Supplementary Table S4.9. *S. sclerotiorum* transcriptional factors were upregulated at some time point of the MR and S lines infection in comparison to *in vitro* control (P. Adj. <0.05; LogFC ≥ 2).

Supplementary Table S4.10. Secreted proteins upregulated and downregulated during the course of MR and S lines infection. (xlsx).

Supplementary Table S11. Predicted *S. sclerotiorum* putative effector candidates' upregulated at some time point MR and S lines infection relative to *in vitro* control (P. Adj. <0.05; LogFC > 2). (xlsx).

Supplementary Figure S4.1. Differentially expressed genes in MR and S line at 6, 12, 24, 48 and 72 hpi based on expression pattern relative to *in vitro* (P. Adj. < 0.05; LogFC upregulated ≥ 2 and downregulated ≤ 2). The colours indicate the fold change with red = upregulated, black = regulated and green = downregulated genes.

Supplementary Figure S4.2. Differentially expressed genes exclusively in MR only and S line only at 6, 12, 24, 48 and 72 hpi based on expression pattern relative to *in vitro* (P. Adj. < 0.05; LogFC upregulated ≥ 2 and downregulated ≤ 2). The colours indicate the fold change with red = upregulated, black = regulated and green = downregulated genes.

Supplementary Figure S4.3. Heatmap showing the top 20 GO categories of upregulated genes at 6, 12, 24, 48 and 72 hpi in a moderately resistant (MR) and susceptible (S) chickpea lines based on –log(10)fold change (enrichment). The colours indicate the enrichment, with green = high enrichment and red = low enrichment.

Supplementary Figure S4.4. Heatmap showing the top 20 GO categories of downregulated genes at 6, 12, 24, 48 and 72 hpi in a moderately resistant (MR) and susceptible (S) chickpea lines based on –log(10)fold change (enrichment). The colours indicate the enrichment, with green = high enrichment and red = low enrichment.

Appendix 4. CHAPTER 5 Supplementary files

The following supporting information is available for this chapter:

Supplementary Table S5.1. List of differentially expressed genes during the infection of moderately resistant (MR; PBA HatTrick) and susceptible (S; Kyabra) *Cicer arietinum* lines with *S. sclerotiorum* at 6, 12, 24, 48, and 72 hours post inoculation (P. Adj. <0.05; LogFC > 1 indicated upregulated or < 1 indicate downregulated. (xlsx).

Supplementary Table S5.2. Kyoto encyclopedia of genes and genomes **(**KEGG) pathways upregulated during chickpea infection with *S. sclerotiorum* (MR= Moderately resistant; PBA HatTrick, S = Susceptible; Kyabra) ((xlsx).

Appendix 5. Oral and poster presentations from this thesis

International scientific conference/presentation

Presented a poster "Phenotypic evaluation *Sclerotinia sclerotiorum* resistance in a wild Cicer germplasm under greenhouse conditions" International Conference on Legume Genetics and Genomics (ICLGG), 13th to 17th May 2019- Dijon, France.

National scientific conference/presentation

Presented a poster "Phenotypic evaluation of *Sclerotinia sclerotiorum* resistance in a wild Cicer germplasm" Australasian Plant Pathology (APPS), 26th to 28th November 2019, Melbourne.

Internal presentations from this project

1. Presented a poster "Phenotypic evaluation of *Sclerotinia sclerotiorum* resistance in a wild Cicer germplasm under greenhouse conditions" Curtin HDR symposium, June 12, 2019.
2. Presented a talk "Characterization and genetic dissection of resistance to *Sclerotinia sclerotiorum* in domesticated and wild chickpea" Commonwealth Scientific and Industrial Research Organization (CSIRO). 29th September 2020.
3. Presented a talk "Transcription dynamics during *Sclerotinia sclerotiorum* – chickpea interplay" Centre for Crop Disease Management (CCDM), Curtin University. 16th October 2020.
4. Presented a talk "Transcription dynamics during *Sclerotinia sclerotiorum* – chickpea interplay" Commonwealth Scientific and Industrial Research Organization (CSIRO). 16th November 2020.
5. Presented a poster "Identification of sources of *Sclerotinia sclerotiorum* resistance in a collection of wild chickpea germplasm" GRDC crops update, 25th and 26th February 2021, Perth.

Appendix 5. Copyright Statement

I have obtained permission from the copyright owners to use any of my own published work (e.g journal articles) in which the copyright is held by another party (e.g. publisher, co-author) in the thesis.