Physiological, anatomical and molecular characterization of partial resistance against Sclerotinia sclerotiorum in soybean

by

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Abstract

Evelyn Valera Rojas, Advisors:
University of Guelph, 2014

Professor Dr. Istvan Rajcan
Professor Emeritus Dr. Greg J. Boland

Sclerotinia sclerotiorum is the causal agent of Sclerotinia stem rot (SSR), which affects over 400 plant species including economically important crops. The genetic and physiological basis of partial resistance of soybean to SSR needs to be characterized before it can be incorporated effectively into new soybean cultivars. This thesis explored the physiological, anatomical and molecular characterization of the defense responses against this necrotrophic fungal pathogen observed in a susceptible cultivar OAC Shire and partially resistant cultivar OAC Salem. Measurements of area under canker progress curve, number of days for visible disease-related symptoms, stomatal conductance (gs), dry and fresh matter, and accumulation of starch grains were analyzed comparatively between the two cultivars for a period up to 12 days after inoculation. Two days after inoculation, susceptible plants exhibited significantly greater starch accumulation than partially-resistant plants. A significant increase in gs was observed in the susceptible plants only. Disease related symptoms, such as severity of wilting and number of
days to plant death were significantly lower in OAC Salem than in OAC Shire. Light microscopy analyses on stem and detached leaf samples of both genotypes showed that direct penetration of the fungal hyphae through the cuticle using the base of non-glandular trichomes was observed exclusively in the susceptible cultivar. Cytoplasm disorganization and reinforced cell walls were observed in epidermal and cortical cells of OAC Salem causing a delay in tissue maceration. RNA-Sequencing analyses at several stages of infection were carried out using Next Generation Sequencing. Genes related to PAMP-triggered Immunity (PTI) were identified, including respiratory burst oxidases and mitogen activated protein kinases. In addition, other genes related to PTI such as jasmonic acid/ethylene biosynthesis and regulation were differentially expressed as well. A transient activation of those mechanisms was observed only at 3 days post-inoculation (dpi) with a shutdown of several processes at 5 dpi in the susceptible cultivar, OAC Shire. The results obtained in this thesis may contribute to a better understanding of the plant defense mechanisms against necrotrophic pathogens and lead to development of breeding strategies for incorporating partial resistance to SSR into commercial cultivars using gene expression-based markers in soybeans and potentially other hosts.
CONTENTS

ACKNOWLEDGEMENTS.................................................................................................................. vi
LIST OF TABLES .............................................................................................................................. vi
LIST OF FIGURES ........................................................................................................................... vii
LIST OF ABBREVIATIONS ................................................................................................................. xi
CHAPTER 1 LITERATURE REVIEW ................................................................................................ 1
  1.1. Introduction .............................................................................................................................. 1
  1.2. Soybean, origin and biology ................................................................................................... 3
  1.3. Sclerotinia sclerotiorum .......................................................................................................... 4
    1.3.1. Taxonomy ........................................................................................................................... 4
    1.3.2. Life cycle of S. sclerotiorum ............................................................................................. 4
    1.3.3. Disease symptoms ............................................................................................................ 6
  1.4. Plant Immunity ....................................................................................................................... 11
  1.5. Uncovering plant-pathogen molecular interactions ............................................................... 16
    1.5.1. Use of complementary DNA (cDNA) libraries to elucidate plant defense mechanism .............................................................................................................................................. 16
    1.5.2. Microarrays as a useful platform for the study of different pathosystems ....................... 18
    1.5.3. RNA-sequencing analyses aid deciphering PTI and ETI immune responses .................. 21
    1.5.4. Sclerotinia sclerotiorum-soybean pathosystem .............................................................. 24
  1.6. Conclusions ............................................................................................................................. 31
  1.7. Thesis objectives ..................................................................................................................... 33
CHAPTER 2 PHYSIOLOGICAL CHANGES DURING INFECTION OF SUSCEPTIBLE AND PARTIALLY- RESISTANT SOYBEAN (GLYCINE MAX (L) MERR.) CULTIVARS BY SCLEROTINIA SCLEROTIORUM ................................................................. 34
  2.1. Abstract ..................................................................................................................................... 34
  2.2. Introduction .............................................................................................................................. 35
  2.3. Materials and Methods .......................................................................................................... 37
  2.4. Results ...................................................................................................................................... 40
  2.5. Discussion ............................................................................................................................... 49
CHAPTER 3 ANATOMICAL RESPONSES OF SOYBEAN PLANTS TO SCLEROTINIA SCLEROTIORUM INFECTION ........................................................................................................................................... 55
  3.1. Abstract ..................................................................................................................................... 55
  3.2. Introduction .............................................................................................................................. 56
  3.3. Materials and Methods .......................................................................................................... 60
  3.4. Results ...................................................................................................................................... 63
  3.5. Discussion ............................................................................................................................... 77
CHAPTER 4 GENE EXPRESSION PROFILES OF OAC SHIRE AND OAC SALEM IN RESPONSE TO S. SCLEROTIORUM INFECTION ................................................................................................................. 83
  4.1. Abstract ..................................................................................................................................... 83
  4.2. Introduction .............................................................................................................................. 84
  4.3. Materials and Methods .......................................................................................................... 88
  4.4. Results ...................................................................................................................................... 93
  4.5. Discussion ............................................................................................................................... 125
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER 5 GENERAL DISCUSSION AND CONCLUSION</td>
<td>133</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>137</td>
</tr>
<tr>
<td>APPENDIX A-CHAPTER 2 ANOVA TABLES</td>
<td>163</td>
</tr>
<tr>
<td>APPENDIX B-CHAPTER 4 RNA-SEQ TABLES</td>
<td>166</td>
</tr>
<tr>
<td>APPENDIX C-CHAPTER 4 qPCR PRIMERS</td>
<td>168</td>
</tr>
<tr>
<td>APPENDIX D-CHAPTER 4 FIGURES</td>
<td>170</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

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LIST OF TABLES

Table 1.1. Top eight soybean producing countries from 2008 to 2013, in million metric tons. (Source: ©Statista.com)……………………………………………………………………………………………………2

Table 1.2. Taxonomic classification of *Sclerotinia sclerotiorum* (Lib.) de Bary………………...6

Table 2.1. Number of days for visible development of lesion (VL), time to wilt (TW), severity of wilting (SW) and number of days to plant death (PD), for OAC Salem and OAC Shire after inoculation with *S. sclerotiorum* 1980…………………………………………………………………………………42

Table 2.2. Area under canker progress curve (AUCPC) of plants from cultivars OAC Salem and OAC Shire at 8 days post-inoculation (dpi) with Ss 1980……………………………………………………………42

Table 2.3. Fresh weight (g) of plants of cultivars OAC Salem and OAC Shire at 5 and 12 days post-inoculation (dpi) with Ss 1980 compared to non inoculated plants (Control). Bolded LSMEANS represent those of inoculated plants…………………………………………………………………………………………44

Table 2.4. Dry weight (g) of plants of cultivars OAC Salem and OAC Shire at 5 and 12 days post-inoculation (dpi) with Ss 1980 compared to non inoculated plants (Control). Bolded LSMEANS represent those of inoculated plants……………………………………………………………………………………………………45

Table 2.5. LSMEANS values for stomatal conductance (mMm$^2$s$^{-1}$) with genotype as the main effect…………………………………………………………………………………………………………………………45

Table 4.1. Pair-wise comparisons performed in the susceptible OAC Shire and partially-resistant OAC Salem cultivars inoculated with *S. sclerotiorum*…………………………………………………………………………………………91
LIST OF FIGURES

Figure 1.1. *S. sclerotiorum* life cycle (Source: http://www.potatodiseases.org/whitemold.html) ........................................................................................................................................................................................................................................7

Figure 1.2. Sclerotinia stem rot symptoms in soybean. (A) White fluffy mycelia along the main stem of soybean plants, note the sharp edges of lesion contrasting with the green stem. (B) Brown and yellow lesions on non-mature pods, stem and petiole of a soybean plant. (C) and (D) Soybean field infected with *S. sclerotiorum*, note infected plants in contrast with healthy green plants........................................................................................................................................................................................................................................10

Figure 1.3. Representation of PTI (horizontal) and ETI (vertical resistance) in plants in response to fungal and bacterial pathogens, adapted from Wirthmueller *et al.* (2013) ...........................................13

Figure 1.4. Schematic representation of the three models that describe the receptor-effector interactions in ETI........................................................................................................................................................................15

Figure 1.5. Microarray experiment depicting the “on chip synthesis” technology (Source: http://angerer.swissbrain.org/archive/2002/11/) ..................................................................................................................................................................................................................20

Figure 1.6. Steps included in a RNA-Seq experiment, from RNA and cDNA isolation through gene quantification (Source: http://home.cc.umanitoba.ca/~zhangx39/PLNT7690/presentation/presentation.html) ..22

Figure 2.1. Disease related symptoms in OAC Shire and OAC Salem soybean plants infected with *S. sclerotiorum*. A) OAC Shire plants showing bleaching of stems after 2 dpi and (B) severe wilting symptoms at 6 dpi. C) OAC Salem plants with less severe wilting symptoms at 4 dpi and (D) 6 dpi........................................................................................................................................................................................................................................41

Figure 2.2. Stomatal conductance of OAC Salem (partially-resistant) and OAC Shire (susceptible) plants during the first three days of infection with *S. sclerotiorum*; non-inoculated plants from both genotypes were use as control. Points represent LSMEANS of experiments, bars indicate standard errors and (*) indicates significant differences between inoculated plants of OAC Shire and OAC Salem (P<0.05)........................................................................................................................................................................................................................................47

Figure 2.3. Wilting symptoms in SSR infected soybean plants at 3 days post-inoculation. A) OAC Shire cultivar, B) OAC Salem cultivar ..................................................................................................................................................................................................................48

Figure 2.4. Starch grains accumulate differentially on epidermis and cortex of stem tissue of inoculated plants for both susceptible OAC Shire and partially-resistant OAC Salem. Each point represents LSMEANs for each treatment. Starch accumulation was calculated by counting number of cells with starch grains on epidermis and cortex of three samples in 5 fields. (*) represents significant differences for P < 0.05........................................................................................................................................................................................................................................50
**Figure 3.1.** Light microscopy of *S. sclerotiorum* infected soybean stems of cultivar OAC Shire (susceptible) during early to advanced (A-H) and late (I) stages of infection.

(A) Strong accumulation of phenolic compounds on cell walls of epidermis and cortex at 1 dpi. (B) Granular intracellular hyphae invading cortical and epidermal tissue and diminished accumulation of phenolic compounds at 2 dpi. (C) Cytoplasmic disorganization on areas closed to the infection site and granular infection hyphae at 3 dpi. (D) Appressorium at 3 dpi. (E) Infection cushion at 3 dpi, note the maceration of tissues surrounding the inoculation site. (F) Granular cytoplasm on cells surrounding inoculation sites at 2 dpi. (G) Oxalate crystal-like structures (arrow) on xylem cells of susceptible at 2dpi. (H) Hyphae running parallel and vertical to longitudinal axis of plant, note granular infection hyphae and small ramifying hyphae at 3 dpi. (I) Single appressoria at 9 dpi (arrows), scale bars: A=30µm, B-C=20µm, D-E=30µm, F-G=20µm, H=30µm, I=10µm

**Figure 3.2.** Light microscopy of *S. sclerotiorum* infected soybean stems of cultivar OAC Salem (partially-resistant) during early/advanced (A-E) and late (F) stages of infection. A-C and E, cross sections, stained with Toluidine Blue (A-C) and Safranine O (E). D and F, longitudinal sections, stained with Toluidine Blue (D) and cotton blue in lactophenol (F). (A) Accumulation of phenolic compounds on cell walls of epidermis and cortex at 1dpi. (B) Strong accumulation of phenolic compounds and cytoplasmic disorganization at 2pi. (C) Granular intercellular and intracellular hyphae invading cortex at 3 dpi. (D) Epidermal and cortical cells with thickened cell walls and granular cytoplasmic contents at 3dpi. (E) Granular infection hyphae invading inter and intracellular spaces, note the integrity of the cuticle at 3 dpi. (F) Dichotomous branching of hyphae at 9 dpi. Scale bars: A=10 µm, B-D and F=30 µm, E=20 µm

**Figure 3.3.** Late stages of SSR on stems of soybean plants of cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant); arrows indicate sclerotia on OAC Shire, and growth of new branches below inoculation site on OAC Salem

**Figure 3.4.** Early stages of *S. sclerotiorum* infection on detached leaves of soybean plants, cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant), at one, two and three days post-inoculation (dpi)

**Figure 3.5.** Light microscopy of *S. sclerotiorum* infected detached leaves of soybean plants from cultivar OAC Shire (susceptible) stained with cotton blue in lactophenol. (A) Infection cushion on leaf surface at 1 dpi. (B) Infection cushion around base of non-glandular trichome at 2 dpi. (C) Parallel orientation of sub-cuticular hyphae on advancing fronts at 5 dpi. (D) Sclerotial primordia on leaf surface tissue at 7 dpi. (E) Hyphal penetration around the base of non-glandular trichome at 7 dpi. (F) Hyphal strand emerging from stoma and re-infecting the leaf tissue with single appressorium at 9 dpi. Tri: trichome. Scale bars: A-E= 20 µm, F=10 µm
Figure 3.6. Light microscopy of *S. sclerotiorum* infected detached leaves of soybean plants from cultivar OAC Salem (partially-resistant) stained with cotton blue in lactophenol. (A) Advancing infection fronts with single appressoria at 2 dpi. (B) Vein invasion of sub-cuticular and aerial hyphae at 2 dpi. (C) Appresorium at 5 dpi. (D) Protoplasm extrusions on tips of advancing hyphae at 7 dpi. (E) Hyphal strand apparently exiting stomatal aperture (arrow) for secondary infection at 7 dpi. Scale bars: A-C, E=30 µm, D=10 µm.

Figure 3.7. Late stages of *S. sclerotiorum* infection on detached leaves of soybean plants, cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant) at 5, 7 and 9 dpi, note that sclerotia are present at 9 dpi (arrows) only in OAC Shire.

Figure 4.1. Venn diagrams of up- and down-regulated genes in partially-resistant cultivar OAC Salem compared to susceptible OAC Shire at 0, 3 and 5 days post-inoculation with *S. sclerotiorum*.

Figure 4.2 Cluster analysis of differentially expressed genes [P<0.001 and log₂(fold change) < -2 or > 2] in partially-resistant OAC Salem cultivar compared to susceptible OAC Shire at 0, 3 and 5 dpi with *S. sclerotiorum*, organized into ten k-means clusters. Each line represents a gene that was differentially expressed at least in one of the treatments assessed. Scale on the right indicates levels of expression related to the colors of the figure.

Figure 4.3. Functional classification of up-regulated genes in control (0 dpi) and inoculated (3 and 5 dpi) stems of partially-resistant OAC Salem compared to susceptible OAC Shire plants. A) Biological process, B) cellular component and C) molecular function.

Figure 4.4. Functional classification of down-regulated genes in control (0 dpi) and inoculated (3 and 5 dpi) stems of partially-resistant OAC Salem compared to susceptible OAC Shire plants. A) Biological process B) cellular component and C) molecular function.

Figure 4.5. PTI-related genes differentially expressed (P<0.001, log₂(fold change) < -2 or >2) in partially-resistant OAC Salem compared to susceptible OAC Shire at 0, 3 and 5 days post-inoculation with *S. Sclerotiorum*.

Figure 4.6. Heat map of PTI-related genes differentially expressed (P<0.001, log₂(fold change) < -2 or >2) in susceptible OAC Shire (S) and partially-resistant OAC Salem (PR) at 3 and 5 dpi with *S. sclerotiorum* compared to their controls. Increasing shades of colors represent increasing levels of expression.

Figure 4.7. Differentially expressed peroxidases homologues (P< 0.001 and log₂(fold change) < -2 or >2) in susceptible OAC Shire (S) and partially-resistant OAC Salem (PR) cultivars inoculated with *S. sclerotiorum*. First three columns indicate comparisons between genotypes, the rest of the columns indicate comparisons of the cultivars at 3 and 5 dpi with their respective controls (0 dpi).
**Figure 4.8.** Differentially expressed genes (P< 0.001 and log₂ (fold change) < -2 or >2) involved in the biosynthesis of lignins in susceptible OAC Shire (S) and partially-resistant OAC Salem (PR) cultivars inoculated with *S. sclerotiorum*. First three columns indicate comparisons between genotypes, the rest of the columns indicate comparisons of the cultivars at 3 and 5 dpi to their respective controls (0 dpi). PAL: phenylalanine ammonia lyase, C4H: cinnamate 4-hydrolase, 4CL: 4-coumarate: CoA ligase, CCR: cinnamoyl-CoA reductase and CAD: cinnamoyl alcohol dehydrogenase.  

**Figure 4.9.** Levels of gene expression related to biosynthesis of JA in the partially-resistant OAC Salem (PR) and the susceptible OAC Shire (S) cultivars inoculated with *S. sclerotiorum*.

**Figure 4.10.** Levels of gene expression related to the regulation of JA-induced responses in the partially-resistant OAC Salem (PR) and the susceptible OAC Shire (S) cultivar inoculated with *S. sclerotiorum*.

**Figure 4.11.** Levels of gene expression related to the biosynthesis of ethylene in the partially-resistant OAC Salem (PR) and the susceptible OAC Shire (S) cultivars inoculated with *S. sclerotiorum*.

**Figure 4.12.** Levels of gene expression related to regulation of ethylene-induced responses in the partially-resistant OAC Salem (PR) and the susceptible OAC Shire (S) cultivars inoculated with *S. sclerotiorum*.

**Figure 4.13.** Validation of RNA-Seq data using qRT-PCR eight differentially expressed genes in the partially-resistant cultivar OAC Salem compared to the susceptible OAC Shire accession.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
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<tr>
<td>µl</td>
<td>microliter</td>
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<tr>
<td>µm</td>
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<td>abscisic acid</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>AP2</td>
<td>apetala 2</td>
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<td>AUCPC</td>
<td>area under canker progress curve</td>
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<td>BLP</td>
<td>bacterial leaf pustule</td>
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<td>CC-NBS-LRR</td>
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</tbody>
</table>

a: Abbreviations used throughout the text and listed in alphabetical order
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definition</th>
</tr>
</thead>
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<tr>
<td>R</td>
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<td>SBS</td>
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<td>SSR</td>
<td>Sclerotinia stem rot</td>
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<td>SW</td>
<td>severity of wilting</td>
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<td>transcription factor</td>
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<td>TIR</td>
<td>toll interleukin 1 receptor</td>
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<tr>
<td>TW</td>
<td>time to wilt</td>
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<tr>
<td>VIT</td>
<td>vacuolar iron transporter</td>
</tr>
<tr>
<td>VL</td>
<td>visible lesion</td>
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CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Since its domestication, soybean \([Glycine\ max\ (L.)\ Merril]\) has been important to the world’s economy. The seeds contain approximately 40% protein and 20% oil and, for that reason, soybeans are processed for these components (Cober et al., 2010). Examples of soybean use includes soy milk, tofu, miso and soy granola bars. In general, soybean oil composition includes about 22% oleic (18:1), 54% linoleic (18:2), 10% palmitic (16:0), 4% stearic (18:0), and 10% linolenic (18:3) acid (Wilson, 2004). Consequently, soybean oil is considered one of the healthiest vegetable oils available for human consumption (Cober et al., 2010).

In 2012, soybean represented 57% of the world oilseed production (SoyStats, 2013), with Brazil being the largest exporter (83.5 million (M) metric tons), followed by the USA with 82.1 M metric tons (Table 1.1). In the same year, soybean was planted on 77.2 million acres in the USA, resulting in a crop value of $43 billion dollars (SoyStats, 2013). Canada was the sixth largest exporter of soybeans in 2012 with 4.9 M metric tons (Statista.com). In Ontario, the seeded area for 2013 was 2.55 M acres with the majority of the area seeded by May 20th and approximately 3.075 M metric tons produced (Grain Farmers of Ontario, 2014).

*Sclerotinia sclerotiorum* (Lib) de Bary is one of the most devastating and cosmopolitan of plant pathogens (Bolton et al., 2006). The disease has received more than 60 common names including cottony rot, watery soft rot, stem rot, and the widely used, white mold (Bolton et al., 2006). The fungus infects more than 400 species of plants worldwide, including important crops
Table 1.1. Top eight soybean producing countries from 2008 to 2013, in million metric tons. (Source: © Statista.com)

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such as sunflower and soybean (Boland and Hall, 1994). In soybean, the disease is called Sclerotinia stem rot (SSR) and its incidence causes significant damage to yield and grain quality (Cunha et al., 2010). Annual losses from S. sclerotiorum infection in the United States are severe and exceeded $500 million dollars in 2009 (Peltier et al., 2012). According to Danielson et al. (2004), yield losses per 10% disease incidence of SSR varied from 83.2 to 229 kg/ha. A model has been presented to describe the relationship between percentage of yield loss (Y) and number of apothecia (X): $Y = -4.5499 + 2.313X$ ($r= 0.84$) (Saharan and Mehta, 2008).

1.2. Soybean, origin and biology

The domesticated soybean [G. max (L.) Merrill] is a member of the Fabaceae family, subfamily Papilionoideae, tribe Phaseoleae (Hymowitz, 2004). The genus Glycine is composed of two subgenera, Glycine (perennials) and Soja (annuals). The subgenus Soja includes G. soja, the wild annual soybean relative and the cultivated soybean G. max, a true domesticate that arose as a consequence of human intervention (Cober et al., 2010).

Soybean originated in China and its domestication took place ~1500-1100 BC (Palmer and Hymowitz, 2004). It was introduced to Southeast Asia during the 15\textsuperscript{th} century, to Europe around 1713, and to North America in 1765 (Cober et al., 2010).

The basic chromosome number for Phaseoleae has a probable general pattern of $x=11$ with an aneuploid reduction of $x=10$ prevalent through the Papilionoideae subfamily (Goldblatt, 1981). A putative ancestor of the genus Glycine with $2x=n=20$ originated in Southeast Asia as hypothesized by Hymowitz (2004). This progenitor has not been collected to date or is extinct (Cober et al., 2010). A more complete path of migration from the ancestral region to China was proposed by Singh et al. (2001) with the assumption that the common progenitor is a wild
perennial (2n=4x=40, unknown) that afterwards gave origin to the wild annual *G. soja* (2n=4x=40) and lastly to the domesticated soybean *G. max* (2n=4x=40). Soybean chromosomes are smaller than chromosomes of most crop plants; thereby, studies conducted on them are limited and difficult (Cober et al., 2010). Due to their high number (2n=40) and their small and similar sizes, soybean is not considered a model plant for cytogenetic studies (Singh *et al*., 2007).

### 1.3. *Sclerotinia sclerotiorum*

#### 1.3.1. Taxonomy

*S. sclerotiorum* was first described in 1837 as *Peziza sclerotiorum* (Libert, 1837; cited by Bolton *et al*., 2006). This name was valid until the specie was transferred to the new genus *Sclerotinia* (Fuckel, 1870; cited by Bolton *et al*., 2006) and renamed *Sclerotinia libertiana* in honor of Madame M.A. Libert (Purdy, 1979). Scientists accepted and used *S. libertiana* Fuckel but, in 1924, Wakefield discovered a disagreement of the name with the International Code of Botanical Nomenclature “as a species that is transferred from one genus to another must retain the original specific name, unless the resulting combination is already occupied” (Bolton *et al*., 2006). For *Sclerotinia sclerotiorum*, the name was available at that time. Wakefield (1924) then incorrectly reported that the combination of *S. sclerotiorum* was first used by G.E. Massee in 1895, citing *S. sclerotiorum* (Lib.) Massee. However, Purdy (1979) observed that de Bary used the name in 1884. For that reason the name and authority for the fungus should be *Sclerotinia sclerotiorum* (Lib.) de Bary (Table 1.2).

#### 1.3.2. Life cycle of *S. sclerotiorum*

Sclerotia are the long-term survival structures of the pathogen (Willetts and Wong, 1980). A sclerotium can remain viable for up to eight years (Adams and Ayer, 1979) and consists of a
hyphal aggregate covered with an outer layer of melanized mycelia (Bell and Wheeler, 1986). Sclerotia formation depends on a number of environmental and nutritional factors, including temperature, pH and availability of carbohydrates and amino-acids in the host tissues (Chet and Hennis, 1975). According to Townsend and Willetts (1954), sclerotia development can be summarized into three stages: i) initiation (hyphae aggregate into sclerotial primordia), ii) development (aggregates increased in size), and iii) maturation (melanin deposition on the outer rind of cells, consolidation of the interior of the structure and surface delimitation).

Germination of sclerotia usually occurs via apothecia formation (carpogenic germination); however, myceliogenic germination is sometimes reported (Grogan, 1979; Renuka et al., 2013; Huang and Chang, 2003). Pre-conditioning of sclerotia before germination is necessary, and some studies have revealed that newly-formed sclerotia are incapable of germination without a period of exposure to cool and moist conditions (Abawi and Grogan, 1979). There is no particular length of time required for carpogenic germination as this varies depending primarily on environmental conditions ensuring production of ascospores when the circumstances for germination are favorable (Willets and Wong, 1980; Hao et al., 2003; Garg et al., 2010c).

Apothecia require ~98 % relative humidity (RH) to stay viable and able to produce ascospores (Partyka and Mai, 1962). Low humidity dehydrates the structures, which diminishes them as a source of inocula (Kruger, 1974). Optimum temperature for ascospore discharge ranges between 4-32 °C, lower (≤ 4 °C) and higher (≥32 °C) temperatures may cause no release of spores at all (Clarkson et al., 2003).
Released ascospores land on dying or dead tissues (e.g. flower petals in nodes of stems) where they germinate (Purdy, 1979) if environmental conditions are favorable (Figure 1.1). Mycelia move through the infected tissue and reach healthy parts of the plants. Once the plant is dying and the nutrient supply is diminished, new sclerotia are formed in- and outside of the infected tissues (Figure 1.1). These sclerotia are the resting and overwintering structures of *S. sclerotiorum* and, after the following winter, they are ready to germinate and initiate infection and a new life cycle.

The germination of sclerotia directly into mycelia has been reported in crops such as sunflower (Purdy, 1958; Saito, 1975). This myceliogenic germination consists in the development of individual hyphae which emerge through the rind of the sclerotium (Adams and Tate, 1976; Huang, 1991) and generally infect the base of the stems (Figure 1.1).

### 1.3.3. Disease symptoms

*S. sclerotiorum* is a necrotrophic, homothallic, fungal pathogen (Bolton *et al.*, 2006). The most common signs are the appearance of white fluffy mycelia covering stems and leaves that then develop into sclerotial bodies (Saharan and Mehta, 2008). Water-soaked lesions are commonly observed on leaf tissue as well, usually expanding rapidly and moving down the petiole into the stem. By crop maturity, stem tissues are white with a shredded appearance. At harvest, diseased stems are white, with poor pod development and an abundant number of sclerotia are found within the pith (Saharan and Mehta, 2008). Pods harvested from diseased plants can contain sclerotia in place of the seeds, and seeds, when present are flattened bearing ruptured and wrinkled testa (Saharan and Mehta, 2008).
Figure 1.1. Life cycle of *S. sclerotiorum* (Illustration by the Iowa State University Agronomy Development lab -  
http://masters.agron.iastate.edu/classes/514/lesson05/images/white_mold_life_cycle.jpg)
In soybean particularly, symptoms of SSR are visible during the early stages of maturity R3-R4 (Fehr, 1971) and include chlorosis, wilting and presence of white mycelia observed under the canopy level (Fig 1.2). Once the disease has been initiated in the host, infection can spread to adjacent plants through plant-to-plant contact (Purdy, 1979; Bolton et al., 2006). Curly leaves remain attached to the plants at late stages of maturity (Saharan and Mehta, 2008).

Penetration of the host cuticle by the pathogen hyphae is achieved by mechanical pressure and enzymatic action (Lumsden and Dow, 1972; Bolton et al., 2006; Heller and Witt-Geiges, 2013). Disorganization of tissues is rapidly observed after pathogen penetration due to the action of cell-wall degrading enzymes (CWDEs), affecting the middle lamella (Lumsden and Dow, 1972). Water-soaked lesions are often the first symptoms observed on infected plants (Purdy, 1979). Other hosts might present “dry” lesions on stems, twigs and branches with a sharp differentiation between damaged and healthy tissue (Purdy, 1979).

Typically, these lesions enlarge during the days following infection. With severe infection the plants turn brown and die (Lumsden, 1979; Purdy, 1979). The harvest of parts of plants infected with *S. sclerotiorum* results in postharvest disease from damage to healthy seeds in storage or shipping containers (Purdy, 1979).

Sclerotia are often found inside infected tissue, such as in the stem pith, but may also form on the surface of tissues during high humidity conditions (Purdy, 1979; Hegedus and Rimmer, 2005). Sclerotia can also be observed on or in flowering and seed-producing portions of the plant and, therefore, found in harvest samples (Bolton et al., 2006). Seed size and appearance are affected on infected pods. Flattened, wrinkled testa and dark color are some of the characteristics observed during harvest of infected seeds (Saharan and Mehta, 2008).
Table 1.2. Taxonomic classification of *Sclerotinia sclerotiorum* (Lib.) de Bary

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<td><em>Sclerotinia</em></td>
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Figure 1.2. Sclerotinia stem rot symptoms in soybean. (A) White fluffy mycelia along the main stem of soybean plants, note the sharp edges of lesions, contrasting with the green stem. (B) Brown and yellow lesions on non-mature pods, stem and petiole of a soybean plant. (C) and (D) Soybean crop infected with \textit{S. sclerotiorum}, note infected plants in contrast with healthy green plants.
1.4. Plant Immunity

Challenges in the increase of agricultural production together with changes in climatic conditions and environments have made possible the appearance of new diseases (Boyd et al., 2013). Evolution of pathogens, including biotrophic and necrotrophic fungi, bacteria and viruses has caused the loss of resistance that was previously present in several crop species due to changes in their colonization and infection strategies (Nicaise et al., 2009). Different from animals, plant immunity relies on the ability of the cells to recognize pathogens, and respond with different mechanisms (Nicaise et al., 2009). The first layer of defense is pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) or horizontal resistance; recognition of molecules of pathogen origin (PAMPs) is accomplished by receptors located at the host cell membrane (Medhitov and Janeway, 1997) referred to as pattern recognition receptors (PRRs). Examples of PAMPs include bacterial flg22, elongation factor Tu (EF-Tu), fungal chitin, ergosterol, and β-glucans. The recognition of those molecular patterns triggers a series of cascade events that eventually lead to the production of reactive oxygen species (ROS) including superoxide and hydrogen peroxide molecules (Mishina and Zeier, 2007), expression of defense-related genes and callose deposition in cell walls (Brown et al., 1998). In general, PTI responses are considered the first line of defense in plants and, in some cases, are weaker and more transient compared to the second layer of defense called effector-triggered immunity (ETI) (Tsuda and Katagiri, 2010).

Defense signaling includes fluxes of Ca^{2+}, H^+, K^+ and Cl^- are detected minutes after the pathogen comes into contact with the surface of the host (Nurnberger et al., 2004) and are responsible for the activation of NADPH oxidases, also known as respiratory burst oxidases (RbOH) elevating the toxicity of the environment with the production of corresponding reduced
oxygen forms (Garcia-Brugger et al., 2006). At the same time, Ca\(^{2+}\) fluxes activate a series of mitogen-activated protein kinases (MAPKs), including MPK4 and MPK6 that contribute to the activation of transcription factors (WRKY transcription factors) and the expression of pathogenesis-related proteins (e.g. PR-1, chitinases and glucanases) (Figure 1.3). There is not enough evidence of the position of RbOH in the cascade of events of PTI, but some results have indicated that it is independent of MAPK cascade (Zhang et al., 2007). Also, callose deposition may be downstream of ROS production, since Arabidopsis RbOH mutants exhibit less callose accumulation after treatment with the bacterial PAMP flg22 (Zhang et al., 2007).

Defense responses that are common to both PTI and ETI, as well as to general defense, that serve only to limit the rate of disease progression but do not lead to an immune reaction, include the activation of genes related to phytoalexin metabolism. Phytoalexins are low-weight secondary metabolites synthesized “de novo” as a response to biotic and environmental stresses. Their antimicrobial activity contributes to their common use as biochemical marker for expression of defense responses (Ahuja et al., 2102) and they are considered one of the most effective defense responses of plants against the attack of necrotrophic pathogens.

Plant pathogens, including some fungi, have evolved to suppress PTI by releasing effector proteins into the host plant cells where they interfere with PTI signalling or function. Plants have evolved to recognize effector proteins either directly or indirectly through proteins encoded by R genes resulting in the activation of ETI or vertical resistance (Ausubel, 2005) (Figure 1.3). The most common form or ETI studied to date occurs when an effector (or host protein modification caused by an effector) is perceived in the cytoplasm of the host cells by the leucine rich repeats (LRR) domain of an R protein. These R proteins are also often characterized by the presence of a nucleotide-binding site (NBS) that contributes to signal activation and requires exchange of ATP
Figure 1.3. Representation of PTI and ETI in plants in response to fungal and bacterial pathogens (Reproduced from Wirthmueller et al., 2013, License Number: 3517821059553). RLK: receptor-like kinases or PRRs, T3SS: type 3 secretion systems, ePG: endopolygalacturonases, DAMPs: damaged-associated molecular patterns, MAMPs: microbial associated molecular patterns (PAMPs), PGIPs: polygalacturonase inhibiting proteins.
and ADP (Belkhadir et al., 2004). Two different types of NBS-LRR proteins have been described in plants, toll interleukin (TIR)-NBS-LRR, which presents a homology with the TIR domain present in mammalian toll interleukin-1 receptor, and coil coiled (CC)-NBS-LRR receptors with the CC domain for protein-protein interaction, also called the leucine zipper (LZ) (Belkhadir et al., 2004).

Three hypotheses have been presented to describe the interaction between pathogen effectors and host R gene products (Figure 1.4). The “receptor-ligand hypothesis” describes the direct interaction between an effector and its respective immune receptor triggering a series of signal transduction events and the expression of ETI. A clear example of this is the fungal effector AvrPita, a small metallo-protease that can modify plant proteins involved in PTI, interacting with its corresponding plant immune receptor Pi-ta in the cytoplasm of the host cell, triggering the expression of ETI responses (Jia et al. 2000). The “decoy hypothesis” states that a mimic molecule acts as an alternate target of the effector in the host cell. Once the effector binds the decoy, the associated NB-LRR protein is activated and triggers resistance. A well-known example of this is the interaction between AvrPto with the decoy Pto. When the effector phosphorylates Pto, conformational changes occur in the immune receptor Prf expressing ETI-related defense responses (Oh and Martin, 2011). The last hypothesis is known as the “guard
1. Receptor-ligand hypothesis.

\[ \text{AvrPi-ta} \quad \text{NBS-LRR receptor} \]

2. Guard hypothesis

\[ \text{AvrRpt2} \quad \text{Guardee} \quad \text{Guard (NBS-LRR receptor)} \]

3. Decoy hypothesis

\[ \text{AvrPto} \quad \text{Host} \quad \text{Decoy} \quad \text{NBS-LRR receptor} \]

Figure 1.4. Schematic representation of the three hypotheses describing the receptor-effector interactions in ETI.
hypothesis”. Plant immune receptors guard the proteins that are target of effectors. Once the effector binds the target protein, a receptor-target-effector complex is formed. This binding causes conformational changes in the immune receptor triggering signaling transduction and expression of ETI responses. For example, the *Arabidopsis* protein RIN4 is a target of AvrRpt2 and AvrRpm1 and is guarded by two NB-LRR receptors, RPS2 and RPM1 (Day *et al.*, 2002; Kim *et al.*, 2005).

Activation of ETI occurs in similar ways to that in PTI; Ca\(^{2+}\) fluxes, ROIs and MAPK cascade activation contribute to the activation of defense responses, leading in general to the activation of the hypersensitive response (HR), which is basically a very rapid and robust expression of defense determinants (Zou *et al.*, 2005). Vertical resistance is an old term that has been used to describe an ETI induced immune response against the attack of biotrophic pathogens.

### 1.5. Uncovering plant-pathogen molecular interactions

In past years, notable innovations in several platforms for genomic and transcriptomic research has provided scientists with potent resources to promote investigations in different plant species (Mochida and Shinozaki, 2010) and biological processes, including plant-pathogen molecular interactions. A number of technologies such as microarrays, quantitative PCR (qPCR) and RNA-sequencing (RNA-Seq) are being used currently to answer many questions related to plant immunity.

#### 1.5.1. Use of complementary DNA (cDNA) libraries to elucidate plant defense mechanisms

Complementary DNA libraries are a combination of cloned cDNA fragments inserted into a collection of host cells, that reflect gene expression at a particular time for the specific cell type
or tissue (Ying, 2003; Mochida and Shinozaki, 2010) comprising the partial transcriptome of the studied organism. cDNA obtained from fully transcribed mRNA contains only the expressed genes of an organism or a specific tissue. Spliced mRNA can be readily expressed in a bacterial cell (Mochida and Schinozaki, 2010). The construction of a full-length cDNA library is essential for screening of known and novel genes (Yang et al., 2007). These expressed genes are responsible for the characteristics of the organisms. Therefore, the study of specific genes such as defense-related signaling and biosynthesis pathways are important for distinguishing the phenomena occurring in different species under various conditions (Sasaki et al., 1994; Seki et al., 2002; Calla et al., 2009; Kim et al., 2011).

Sequencing of Populus nigra full-length (PnFL) cDNA libraries generated about 116,000 expressed sequence tags (ESTs) corresponding to 19,841 non-redundant clones. This population of PnFL cDNA clones represents 44% of the predicted genes in the Populus genome (Nanjo et al. 2007). Ralph et al. (2008) generated a full-length cDNA library collection using the biotinylated CAP trapper method. The tissues used in that study represented RNA from xylem, phloem, cambium, and green shoot tips and leaves from the P. trichocarpa and insect-attacked leaves of the P. trichocarpa × P. deltoides hybrid. Results revealed that 11.5% of the poplar FL-cDNAs displayed no significant sequence similarity to other plant proteins.

cDNA libraries constructed on S. sclerotiorum-challenged oil-seed rape stem and leaf tissues were screened for expression of polygalacturonase-inhibiting proteins (PGIPs) (Wang et al., 2008). PGIPs are known for their activity as inhibitors of endo-PGs, virulence factors produced by S. sclerotiorum. PGIPs also elicit the defense response in plants when long chain oligogalacturonides (OGs) are produced by the interaction of PGs with them (Cervone et al., 1989). PGIPs are located in the apoplast and apparently they interact with PGs using the same
region that attach them to the cell wall (De Lorenzo and Ferrari, 2002) (Figure 1.3). Results from the study conducted by Wang et al., (2008) showed that the interacting proteins had a predicted Ca$^{2+}$ binding domain and shared ~ 80% of homology with a C2 domain protein in Arabidopsis. Their levels of expression were elevated when leaves and stems of oil-seed rape plants were inoculated with *S. sclerotiorum*.

1.5.2. Microarrays as a useful platform for the study of different pathosystems

In general, biosynthesis-related pathways include the regulation of gene expression in many cellular processes (Aharoni and Vorst, 2001). It is known that the final activity of a gene is determined by the encoded protein; however measurements of mRNA levels have been shown to offer valuable inputs in deciphering several biological processes (Aharoni and Vorst, 2001). Together, the availability of complete genome sequences and large sets of expressed sequence tags (ESTs) from model organisms and in crop species have enabled the use of genome-wide and gene expression analyses (Aharoni and Vorst, 2001).

DNA microarray technology has been an important element in functional genomics for the last decade. Different characteristics offered by this method such as its automation and large-scale processing have contributed to its wide use (Aharoni and Vorst, 2001). To date, genotyping of mutations and polymorphisms have been favored by the use of microarray technologies. Also, a wide range of biological processes in plants are being investigated using this technique, including plant defense to Sclerotinia (Calla et al., 2009; Calla et al., 2014), environmental stress responses (Rabbani et al., 2003) and fruit ripening (Koia et al., 2012).

As a hybridization-based method, microarrays combine miniaturization and the use of fluorescent dyes for labeling (Aharoni and Vorst, 2001). With this approach, a combination of
two differently-labeled samples in a single hybridization experiment can be studied. The competitive hybridization established at the time of the experiment reduces experimental errors, resulting in relative expression levels of large numbers of genes (Mochida and Shinozaki, 2010). There are two microarray-based technologies described to date, the ‘spotting type’, consisting of spotting a DNA solution onto a glass slide, and the ‘on chip synthesis’ usually known as the *in situ*, oligonucleotide-based hybridization (Mochida and Shinozaki, 2010).

With the use of microarrays, researchers are able to explore the response of thousands of genes at once during a given experiment (Wan *et al.*, 2002) (Figure 1.5). Using an Affimetrix full genome chip, Thilmony *et al.* (2006) were able to study the transcriptome of *Arabidopsis* associated with basal defense in plants challenged with *Pseudomonas syringae pv tomato* DC3000 and *Escherichia coli*. Their findings summarized a total of 2800 genes with differential expression patterns, including peroxidases, small GTPases and those related to jasmonic acid (JA) and cell wall changes.

* Zhao *et al.* (2007) investigated the gene expression changes associated with *S. sclerotiorum* infection in a partially-resistant and a susceptible genotype of *Brassica napus* using a whole genome microarray from *Arabidopsis*. Their results showed 686 and 1,547 genes to be differentially expressed after infection in the resistant and susceptible genotypes, respectively. The number of differentially expressed genes increased over time with the majority being up-regulated in both genotypes. The functional analyses of those genes revealed groups of PR
Figure 1.5. Microarray experiment depicting the “on chip synthesis” technology. (Reproduced with permission from: http://www.cfgbiotech.com/microarray/genechip_expression.htm)
proteins, proteins involved in the oxidative burst, protein kinases, as well as proteins with unknown functions.

Other studies using a *B. napus* oligonucleotide microarray at 48 hpi with *S. sclerotiorum*, has revealed increased transcript levels of defense-associated proteins. Those included chitinases, glucanases, osmotins and lectins, as well as genes encoding transcription factors belonging to the zinc finger group, WRKY TFs, APETALA2 (AP2) and MYB classes (Zhao *et al.*, 2009).

### 1.5.3. RNA sequencing analyses aid in deciphering defense responses

RNA-sequencing (RNA-Seq) is a newly developed technology for transcriptome profiling using deep-sequencing technologies. This method is based on cDNA sequencing and allows the quantification and mapping of the transcriptomes analyzed (Wang *et al.*, 2009).

RNA-Seq offers advantages over the hybridization methods, e.g. microarray and EST sequencing that have been used in the last decade (Figure 1.6). This method is based on high-throughput sequencing, with the resolution of a single base and with low background noise (Wang *et al.*, 2009). By using RNA-Seq, researchers are able to map transcribed regions and quantify gene expression at once. Also, this technology can distinguish different isoforms and allele expression of a particular gene (Wang *et al.*, 2009). On the practical side, RNA-Seq requires low amounts of RNA and the cost for mapping transcriptomes to large genomes is relatively low (Wang *et al.*, 2009).
Figure 1.6. Steps included in a RNA-Seq experiment, from RNA isolation and cDNA synthesis through gene quantification (Reproduced with permission from Wang et al., 2009, License Number: 3517831340041)
Recently, studies on creeping bentgrass inoculated with the pathogen *Sclerotinia homeocarpa* were conducted using RNA-Seq to determine the basal and induced defense responses in this particular pathosystem (Orshinski *et al.*, 2012). Transcript sequence data were obtained using Illumina’s sequence-by-synthesis (SBS) method in the creeping bentgrass cv. Crenshaw inoculated with *S. homeocarpa* over a 96 hr period. The genes of interest that were identified in these analyses included germin, ubiquitin transcripts related to proteasome degradation and cinnamoyl reductase, this latter enzyme is involved in lignin biosynthesis.

Analyses conducted on soybean seed development have also benefited from the application of RNA-Seq. Jones and Vodkin (2013) examined seed development using seven physiological stages, from a few days after fertilization to complete maturation. Results indicated an early accumulation of proline-rich proteins and histones as well as storage proteins such as glycinins and β-conglycinins. During the late or dry stages of seed development, late embryogenesis abundant (LEA) proteins and dehydrins were actively produced indicating the importance of these types of proteins in the preservation of cellular structures and nutrients within the seed during desiccation (Jones and Vodkin, 2013).

*Xanthomonas axonopodis pv. glycines* is an important pathogen of soybean. The study of the soybean-bacterial leaf pustule (BLP) pathosystem has been of interest since it was found that the reduction of yield caused by the incidence of this pathogen could be as high as 40% in high humidity conditions. Basal defense mechanisms have been studied using near-isogenic lines infected with BLP through RNA-Seq analysis (Kim *et al.*, 2011). PAMPs and damage-associated molecular patterns (DAMPs)-related genes (receptors) were detected as being differentially expressed in both the susceptible and resistant lines. Pathogenesis-related proteins (PR-1, PR-14, PR-3 and PR-6) were found up- or down-regulated at the different time x genotype treatments
studied. Other genes that showed significant differences were JA-signaling components, MYC2 and the JASMONATE ZIM-motif (JAZ) genes (Kim et al., 2011).

1.5.4. Sclerotinia sclerotiorum-soybean pathosystem

There has been extensive molecular genetic characterization of *S. sclerotiorum*, which have demonstrated that oxalic acid (OA) production and CWDEs are key agents of pathogenicity for this pathogen (Guimarães and Stotz, 2004; Bolton *et al*., 2006; Noyes and Hancock, 1981; Kolkman and Kelly, 2000). Guimarães and Stotz (2004) tested the hypothesis that oxalate causes foliar dehydration by disturbing guard cell function. Evidence has indicated that oxalate alters guard cell osmoregulation and interferes with abscisic acid (ABA)-induced stomatal closure. The precise mechanism of OA action during infection is not completely understood but it has been proposed that OA removes Ca\(^{2+}\) bonds to pectins, thereby, exposing host cell walls to catabolic enzymes of fungal origin, including PGs (Bateman and Beer, 1965; Favaron *et al*., 1993). High-throughput gene expression analyses also support that OA might be chelating iron away from plant host proteins, leading to rapid cell death and redox imbalance (Calla et al. 2014).

Oxalic acid also favors plant cell wall degradation by shifting the pH of infected plant tissues close to the optimum of CWDEs, such as PGs and glucanases (Bateman and Beer, 1965). In addition, OA suppresses the defense-related oxidative burst of soybean and tobacco cells (Cessna *et al*., 2000) and inhibits the activity of plant polyphenol oxidase (Magro *et al*., 1984). Recently, Heller and Witts-Geiges (2013) described a detoxifying role for OA that includes the removal of calcium precipitates to old parts of the fungal hyphae and the conversion of these molecules into stable, non-toxic oxalate crystals. Studies have shown that OA-deficient strains of *S. sclerotiorum* (OA\(^{-}\)) were nonpathogenic when inoculated on stems, leaves and pods of bean plants (Jeff Rolling, personal communication). In contrast, wild and revertant isolates of the pathogen developed water-
soaked and necrotic lesions up to 1 cm long after 30 h post-inoculation (hpi). Sclerotial development was also observed after approximately 3 dpi and the extension of the lesion occurred down- and upwards from the inoculation point, causing extensive wilting symptoms. None of these symptoms were observed on bean plants inoculated with OA strains (Godoy et al., 1990).

CWDEs also constitute pathogenicity factors of *S. sclerotiorum*. There is a vast array of enzymes and their isozymes that attack the middle lamella and disrupt host tissues (Hancock, 1966; Lumsden, 1969; Riou et al., 1991). Glucanases, pectinases, cellulases and glycosidases are included in the group of CWDEs found in *S. sclerotiorum* (Annis and Goodwin, 1997). Differences in isoelectric points, molecular weights and transcriptional processes that characterize these enzymes provide the pathogen with great flexibility for host penetration and tissue colonization (Annis and Goodwin, 1997). Environmental factors such as pH and availability of carbon/nitrogen supplies affect the level of expression of the CWDEs as well (Cotton et al., 2003).

Pectinases are the first set of CWDEs released by the pathogen when it comes into contact with the plant surface (Alghisi and Favaron, 1995). The action of pectinases or pectin degrading enzymes accounts for the rapid and extensive degradation of cell walls and maceration of host tissues. Generally the actions of these enzymes are responsible for the appearance of the first disease-related symptoms, especially those of pathogens producing soft rots (Alghisi and Favaron, 1995).

Polygalacturonases (PGs) are one group of pectin-degrading enzymes responsible for the water-soaked lesions produced by *S. sclerotiorum* (Hancock, 1966; Favaron et al., 1993). Some studies have shown a positive correlation between PG activity and low pH in the host
environment, probably created by the secretion of OA by the pathogen (Favaron et al., 2004; Zuppini et al., 2005). Studies conducted on soybean hypocotyls revealed that the major basic endo-PG (PGb) is stimulated by OA at pH ~ 5, whereas the acidic endo-PG (PGA) appears late in the infection of host tissue and is active even at a low pH of 3.6 (Favaron et al., 2004). Oxalic acid concentration and PGIP expression also modulate the activity of endo-PGs (Favaron et al., 2004). PGIPs are shown to have high affinity to pathogen endo-PGs and also be active in environments with low pH and high concentrations of OA during the fungal infection (Sella et al., 2005). Studies conducted on soybean seedlings also revealed a delay in expression of PGIP compared to the induction of endo-PGs expression (Sella et al., 2005).

The interactions between S. sclerotiorum and several of its hosts at the tissue and cellular levels have always been of great interest since the early days of its discovery by De Bary (Lumsden and Dow, 1972). Anatomical studies on these phenomena have been reported since the 1880s. Direct penetration of the host surface by exertion of mechanical forces was first described by Boyle (1921). He observed that the mycelia strongly adhered to the host surface by a mucilaginous substance and “changes” occurred when the tip of the hyphae came into contact with the host. Those changes were the action of ‘ferments’, now known as CWDEs. This study also detailed the formation of infection vesicles inside the host cells once the hyphae entered the plant tissue, as well as some chemical changes that occurred in those affected host cells. The germination of ascospores was described by Purdy (1958), confirming the dependence on several nutrients such as dead flowers and a firm surface for appresorium formation. Purdy (1958) also described the myceliogenic germination of sclerotia when certain organic nutrients were available.
Changes in the cytoplasm of host cells due to pathogen infection have been described (Mansfield and Richardson, 1981; Pring et al., 1981). Ultra-structure studies on faba bean (*Vicia faba* L.) infected with different species of *Botrytis* revealed extensive vesicle formation in the cytoplasm of host cells as well as swollen cell walls that later collapsed (Mansfield and Richardson, 1981). Pear fruits infected with *Monilinia fructigena* Honey were also observed under light and electron microscopy, and revealed inter- and intracellular colonization and collapse of the host protoplasts (Pring et al., 1981).

Studies conducted in bean (Lumsden and Dow, 1972), soybean (Sutton and Deverall, 1983), sunflower (Davar et al., 2012) and canola (Jamaux et al., 1995) have described compatible interactions between the pathogen and hosts. Modes of penetration, invasion and sclerotia formation have been studied, offering insights on single and complex appressoria formation, hyphal types and sclerotia development. In bean hypocotyls, penetration structures were observed as early as 6 hpi (Lumsden and Dow, 1972). Those penetration structures were likely infection cushions with three main types of hyphae distinguished; i) at the top of the cushion, were darkly red stained hyphae, ii) the middle portion was described as a light area, with inflated hyphae and iii) the closest area to the host surface was characterized by a dark red in color and fungal hyphae filled with vesicles (Lumsden and Dow, 1972). Development of penetration pegs piercing the surface of the hypocotyls, and evidence of the considerable force exerted by the pathogen during penetration, were observed in this study as well.

Direct penetration of sunflower stem tissues was described when 4-week old susceptible plants were inoculated with *S. sclerotiorum* (Davar et al., 2012). Appressoria were observed 12 hpi, when the hyphae came into contact with the host surface. Intra- and intercellular colonization
of host tissue was prominent at 24 hpi with a total collapse of stems at 48 hpi. Branching of infection hyphae, as well as secondary colonization using stomatal apertures was also described.

Ascospores of *S. sclerotiorum* are known to germinate on dying or dead tissues (Purdy, 1979). Infected flower petals then fall onto leaf surfaces and infect healthy tissue. A scanning microscopy study was conducted on *B. napus* leaves infected with *S. sclerotiorum*-colonized petals (Jamaux *et al*., 1995) described the processes involved in ascospore adhesion and germination, penetration of the host surface by the germ tubes and collapse of epidermal cells surrounding the inoculation site. Results also showed the failure of ascospore germination when deposited directly on healthy tissues, suggesting the importance of a source of nutrients for germination and development of germ tubes and appressoria (Jamaux *et al*., 1995).

No source of complete resistance, such as R genes, has been described against *S. sclerotiorum* in soybean. However, a number of QTL sources for resistance were identified (Kim and Diers, 2000; Arahana *et al*., 2001; Bolton *et al*., 2006; Hyuhn *et al*., 2010). Twenty-eight QTLs were identified on 15 linkage groups in 500 recombinant inbred lines (RILs) from five populations (Arahana *et al*., 2001). The inoculation method used in this study was a detached leaf assay since it showed the greatest correlation with field results (Kim *et al*., 2000). Of these 28 QTLs, seven were identified in more than one population and were located on different linkage groups including regions with mapped resistance genes and resistant genes analogs (Arahana *et al*., 2001). The numerous resistance gene analogs mapped to these clusters suggested that the molecular basis of these QTLs resembles that of major R genes. Phenotypic variation explained by individual QTLs varied from 4-10%, and several alleles implicated in lesion size reduction came from the susceptible parent (Arahana *et al*., 2001).
Two putative QTLs for SSR resistance were mapped in the soybean plant introduction PI194639 (Vuong et al., 2008). The inoculation and disease evaluation followed the methods described previously by Vuong et al. (2004). The amount of phenotypic variability explained by these two QTLs ranged from 11 to 12% and the resistant allele for the LG B2 QTL came from the susceptible parent Merrit. Linkage groups related to these two QTLs were LG A2 and B2, linked to markers Sat_138 and Satt126, respectively. Other QTLs of minor effect on resistance against S. sclerotiorum were detected as well. Located on LG K and LG L, these two QTLs when combined with the previous two, explained up to 27 % of the phenotypic variation of the lesion length in the plants studied (Vuong et al., 2008).

Resistance against S. sclerotiorum has been developed by introducing the wheat germin gene (gf-2.8), an oxalate oxidase (OxO), into transgenic soybean lines (Donaldson et al., 2001; Cober et al., 2003). The OxO enzyme catalyses the oxidation of oxalic acid to carbon dioxide (CO$_2$) and hydrogen peroxide (H$_2$O$_2$) serving as scavenger of the fungal pathogenicity factor and also as a defense response by means of the release of H$_2$O$_2$ (Donaldson et al., 2001; Cober et al., 2003). The transgenic plants resulted from these studies had a 35S-gf-2.8 gene expressing a protein of approximate 130 kD, which was very similar to that of wheat. OxO activity was localized at cell walls close to the proximity of the inoculation site and a corresponding decrease on disease progression and lesion length were observed (Donaldson et al., 2001; Cober et al., 2003).

Studies conducted on soybean plants transformed with the decarboxylase gene (oxdc) from Flammulina spp. showed a delay in disease establishment and progression on T$_2$ plants (Cunha et al., 2010). Oxalate decarboxylase can degrade OA into CO$_2$ and formate without the requirement of any cofactor. Its high affinity to OA and its activity even in low or neutral pH
environments (Kesarwani et al., 2000) make this enzyme a great candidate for SSR resistance. The reduction of infection on detached leaves from T2 plants ranged between 61-96%, with some plants showing no symptoms even 92 hpi (Cunha et al., 2010).

Functional genomics analyses using microarrays have been used (for) during the last decade. Calla et al. (2009) reported the use of soybean microarrays to study host gene expression following inoculation with S. sclerotiorum in the susceptible cultivar Williams 82 and the partially-resistant line PI194.639. The time-course analyses showed a wide range of responses against the attack of the pathogen; changes in cell wall composition, activation of signaling pathways and biosynthesis of secondary metabolites were the most important ones. An increase in the amount of transcripts related to expansins, isoflavonoids and inositol-related genes was also observed (Calla et al., 2009).

Recently, studies conducted on an oxalate-degrading (OxO) soybean line were conducted by Calla et al. (2014). The response of the transgenic line and its parent AC Colibri used in microarray studies showed a faster establishment of basal defense responses in the transgenic line than in the non-transformed plants. Other than that, the results support the hypothesis that S. sclerotiorum induces cell death at least partially, since several peroxidases were differentially expressed in both the susceptible and the OxO line, and these peroxidases share a similar expression pattern as that observed during a Pseudomonas syringae induce HR. Lignin biosynthesis genes and G-proteins, also identified in the research showed a differential pattern of expression between the transgenic line and its parent.

As mentioned above, high-throughput gene expression studies using OA-infiltrated leaves of soybean suggested that OA chelates iron away from host proteins, leading to a disruption of
the iron homeostasis during SSR establishment (Calla et al., 2014). Some of the genes detected by included a ferric reductase and two ferredoxins that were down-regulated in AC Colibri. RNA-Seq analysis was also conducted on some of the samples. Results indicated that several iron-related genes were differentially expressed including vacuolar iron transporters (VITs), stabilizers of iron transporter (SufD), iron-dependent oxygenases and ferritins. As OA has shown a capacity to free iron from plant proteins (Marcur et al., 1991), the authors concluded that *S. sclerotiorum* could benefit from this, as it would help to induce cell death as well as free up iron for uptake of iron by the pathogen for its own metabolism.

1.6. Conclusions

As a necrotrophic pathogen, *S. sclerotiorum* has a wide arsenal of pathogenicity factors including OA and CWDEs. Oxalic acid has been shown responsible for the weakening of cell walls by chelation of Ca\(^{2+}\) even 3-5 cell layers in advance of fungal invasion (Bateman and Beer, 1965). Deregulation of guard cells in stomata causing wilting symptoms has been attributed to OA as well (Guimaraez and Stotz, 2004). Also, the action of CWDEs, including glucanases and endo-PGs is crucial for establishment and development of SSR. In the other hand, plants have developed a group of apoplastic proteins, PGIPs, to neutralize the action of these enzymes and activate plant defense mechanisms.

In summary, *S. sclerotiorum* affects a wide range of plant species including important crops such as soybean and canola. SSR disease in soybeans accounted for yield losses of approximately 500 M dollars in the United States in 2009 (Peltier et al., 2012), and no complete resistance is described for soybean against *S. sclerotiorum*. However, several studies have described QTLs located close to resistance genes and resistance gene analogues (Kim et al.,
2000; Arahana et al., 2001; Vuong et al., 2008). Transgenic lines (Donaldson et al., 2001; Cunha et al., 2010), and microarray analyses (Calla et al., 2009; Calla et al., 2014) have been used to elucidate physiological resistance strategies. Arahana et al. (2001) showed that the molecular bases of the described QTLs resemble those of major R genes.

Little is known about physiological mechanisms of defense response in soybean. Studies have shown that partial resistance exists in some cultivars (Auclair et al., 2004a; Auclair et al., 2004b), that was derived from the susceptible parent that originated the line. The purpose of this thesis was to gain more insight on physiological resistance against SSR in the partially-resistant soybean cultivar OAC Salem, comparing its behavior to the susceptible cultivar OAC Shire. It was hypothesized that important physiological, anatomical, and molecular changes related to plant defenses against the attack of the necrotrophic pathogen occur at earlier stages of the infection in the partially-resistant cultivar compared to the susceptible one.
1.7. Thesis Objectives

The specific objectives of this thesis were:

1. To describe the physiological responses in susceptible OAC Shire and partially-resistant OAC Salem during *S. sclerotiorum* infection;
   a) using stomatal conductance, fresh and dry matter measurements,
   b) determining starch grain accumulation levels in host cells and,
   c) evaluating disease-related symptoms.

2. To illustrate changes that occur at cellular and tissue levels on leaves and stems of OAC Shire and OAC Salem at early to advanced stages of SSR disease using light microscopy by;
   a) analyzing pathogen strategies for penetration and colonization of susceptible and partially-resistant tissues and,
   b) observing defense responses in host cells at cytoplasmic and cell wall levels.

3. To analyze the transcriptomic profiles of *S. sclerotiorum*-inoculated stems using next generation sequencing (RNA-Seq) at early to advanced stages of infection by
   a) isolating total RNA from inoculated and mock inoculated plants at different times,
   b) constructing and sequencing the cDNA libraries,
   c) determining relative gene expression in each of the times sampled and,
   d) conducting functional genomics analyses.
CHAPTER 2

PHYSIOLOGICAL CHANGES DURING INFECTION OF SUSCEPTIBLE AND PARTIALLY-RESISTANT SOYBEAN (GLYCINE MAX (L) MERR.) CULTIVARS BY SCLEROTINIA SCLEROTIORUM

2.1. Abstract

Sclerotinia stem rot (SSR) is a major disease of soybean, caused by Sclerotinia sclerotiorum. The genetic and physiological basis of partial resistance of soybean to SSR needs to be characterized before it can be incorporated effectively into new soybean cultivars. The objective of this study was to describe physiological responses in susceptible and partially-resistant soybean cultivars infected by S. sclerotiorum. Variations in host-pathogen interactions were examined by comparing the responses of soybean cultivars, OAC Salem (partially-resistant) and OAC Shire (susceptible) to S. sclerotiorum. Measurements of area under canker progress curve (AUCPC), number of days for visible disease-related symptoms, stomatal conductance ($g_s$), dry and fresh matter, and accumulation of starch grains were analyzed for both cultivars for a period up to 12 days after inoculation. After day 2, susceptible plants presented significantly greater starch accumulation than partially-resistant plants. No significant differences were found between the cultivars for AUCPC or fresh matter. A significant increase in $g_s$ was observed in the susceptible plants only. Disease related symptoms, such as severity of wilting and number of days to plant death, were significantly lower in OAC Salem than in OAC Shire. Physiological characterization of the soybean - S. sclerotiorum pathosystem may facilitate the development of novel strategies to incorporate partial resistance to SSR into commercial soybean cultivars and help to develop breeding strategies for other host crops.
2.2. Introduction

Soybean *Glycine max* (L) Merr is one of the most important crops in the world, and its production has been steadily increasing because it is an important source of protein and vegetable oil for human and animal nutrition (Cober et al., 2010). In 2012, approximately 77 million acres of soybean were planted in the United States, producing 82 M metric tons (Soy Stats, 2013). Soybean can be seriously affected by Sclerotinia stem rot (SSR), a disease also called white mold, caused by the wide host range necrotroph *Sclerotinia sclerotiorum* (Lib) de Bary. The severity of SSR causes significant damage to yield and seed quality (Cunha et al., 2010), and annual losses caused by SSR in the United States exceeded $500 million dollars in 2009 (Peltier et al., 2012). Yield losses due to SSR could be reduced using agronomic practices such as crop rotation and tillage (Gracia-Garza et al., 2002), fungicides (Mueller et al., 2004) and the best available germplasm resistant to the disease (Peltier et al., 2012).

SSR is characterized by several symptoms and signs such as bleaching of stems, wilting of leaves, white and fluffy mycelia covering the external parts of the infected plants and the presence of sclerotia (Bolton et al., 2006). *S. sclerotiorum* is similar to other phytopathogenic fungi, e.g. *Botrytis cinerea*, that produce millimolar concentrations of OA in infected tissues (de Bary, 1886; cited by Bolton et al., 2006; Ferrar and Walker, 1993) and secretes cell-wall-degrading enzymes (CWDEs) as essential virulence factors.

When the pathogen releases oxalic acid, it causes wilting symptoms in infected plants (Noyes and Hancock, 1981; Kolkman and Kelly, 2000). The precise mechanism of OA action during infection is not completely understood but it has been proposed that OA removes Ca $^{2+}$ bonds to pectins, thereby exposing host cell walls to catabolic enzymes of fungal origin (Bateman and Beer, 1965).
Guimaraes and Stotz (2004) suggested that oxalate causes foliar dehydration by altering osmoregulation of guard cells that interferes with abscisic acid (ABA)-induced stomatal closure. Oxalic acid also favors plant cell wall degradation by shifting the pH of infected plant tissues close to the optimum of CWDEs, such as polygalacturonases (Bateman and Beer, 1965), and suppresses the defense-related oxidative burst of soybean and tobacco cells (Cessna et al., 2000), perhaps through binding of iron molecules leading to redox imbalance and cell death (Calla et al. 2014).

In soybean, SSR is difficult to control. There is no evidence of total resistance in the host against this pathogen (Hoffman et al., 2002), although the use of partial resistance (Hoffman et al., 2002) has been applied. Several studies indicated that partial resistance to S. sclerotiorum may be present in selected breeding lines or cultivars, with reports showing a quantitative basis for inheritance (Kim et al., 1999; Vuong and Hartman, 2003) with up to 27% of the variation being explained by quantitative trait loci (QTLs) (Vuong et al., 2008).

OAC Salem and OAC Shire cultivars were chosen for this study based on greenhouse and field performance results. OAC Shire was ranked the most susceptible cultivar in studies conducted in controlled environment using the straw inoculation method with disease severity ratings of 5.2 and 7.6, measured at one and two weeks post-inoculation, respectively (Auclair et al., 2004a). Field experiments using the barley kernel method also showed an elevated disease severity index (DSI) of 42.75 and inoculation severity index (ISI) of 2.45 in OAC Shire (Auclair et al., 2004b). Reports by Cober et al., (2003) showed high DSI and ISI for the susceptible cultivar OAC Shire whereas DSI and ISI values were low for the partially-resistant cultivar OAC Salem. DSI of 4.2 and 5.7 were reported in the latter cultivar at one and two weeks after inoculation, respectively, following the straw inoculation method (Auclair et al., 2004a). Also, Auclair et al.
(2004b) reported DSI of 0 and 0.2 in OAC Salem in experiments conducted in 1998 and 1999, respectively.

The objective of this study was to describe the physiological responses in susceptible OAC Shire and partially-resistant OAC Salem during S. sclerotiorum infection. Evidence of the potential for using artificial inoculation methods and physiological measurements for characterizing disease tolerance has been presented. Although no distinction could be established between OAC Salem and OAC Shire cultivars in terms of lesion length or area under the canker progress curve (AUCPC), other variables such as starch accumulation and fresh weight offered valuable insights for future studies in terms of physiological characterization of the different responses in susceptible and partially-resistant genotypes.

2.3. Materials and Methods

Plant material

The cultivars used in this study were OAC Salem as partially-resistant and OAC Shire as susceptible (Cober et al., 2003; Auclair et al., 2004; Auclair et al., 2004b). Seeds were planted in Sunshine Mix LA4 soil medium (SunGro, Vancouver, BC) in 3.5 inch square pots, one seed per pot. Three growth chambers set at 25 °C with a photoperiod of 16/8 h (day/night) and light intensity of ~ 350 µmol photons ms\(^{-1}\) were used for a period of 4-5 weeks covering growth of plants to the V4 stage (Fehr et al., 1971).

Inoculation of plants with S. sclerotiorum

Growth medium for culturing S. sclerotiorum consisted of potato dextrose agar (PDA) prepared from potato broth supplemented with 20 g L\(^{-1}\) dextrose and 10 g L\(^{-1}\) agar. Approximately
800 ml of distilled water was boiled with 200 g of potatoes for 20 min. In an autoclave-resistant glass bottle, 500 ml of potato broth were mixed with the agar and the dextrose until complete dissolution. Growth medium was then autoclaved and plated into 20 ml Petri dishes (Thermo Fisher Scientific Inc, USA) and inoculated with fungal cultures of isolate 1980 (Auclair et al., 2004a; Auclair et al., 2004b) two days prior to inoculation. Fungal cultures were obtained from the germination of sclerotia in PDA medium at room temperature. Up to two subcultures were obtained from each plate containing germinated sclerotia.

Before inoculation, growth chambers were set to 18 °C. Five-millimeter-diameter plugs of PDA medium from the edge of the growing mycelia were collected with the back side of 30 µl pipette tips. Agar plugs were positioned below the third node on the stem of plants and secured with Parafilm™ following the method described by Lone Buchwaldt (personal communication). Each replication consisted of 60 plants, 30 from each of the two cultivars (partially-resistant and susceptible). A different set of 20 plants for each cultivar was also arranged in the growth chambers to collect data on fresh and dry matter. The experiment followed a randomized complete block design (RCBD) with three replications.

**Measurements**

Lesion length was measured with calipers every 24 h during a period of eight days on a total of 180 plants (90 plants per cultivar). Data were used to calculate the AUCPC applying the formula developed by Shaner and Finney (1977). Disease symptoms were rated at the V4 stage. Ratings included number of days to plant death (PD), start of wilting symptoms (TW), severity of wilting (SW) and number of days to observe bleaching of stems (VL). Both fresh and dry weights (g) of shoots were measured with a Metler Toledo scale (Mississauga, ON) in non-inoculated and
inoculated plants at 5 and 12 days post-inoculation (dpi). For dry weight, plants were kept in a drying oven set at 60 °C for a period of five days.

Stomatal conductance measurements were conducted in 4-week old plants at one, two and three dpi using a LI-6400 Portable Photosynthesis System (LI-COR, Nebraska, USA) on the middle leaflet just above the inoculation site in both partially-resistant OAC Salem and susceptible OAC Shire plants. Leaf temperature was maintained at 25 °C and the sample chamber CO₂ concentration was set at 380 ppm. Portions of the leaflets were sealed within the 2 x 3 cm clear chamber to determine the water vapor exchange between the leaf sample and the measuring chamber. Sufficient time was allowed to reach near–steady state photosynthesis and stomatal conductance, before data were recorded. Measurements were conducted on a total of 60 inoculated and 60 non-inoculated plants, 30 plants per cultivar in each case.

Sample preparation for microscopy

Stem samples of inoculated and non-inoculated plants from genotypes OAC Shire and OAC Salem were collected for observation with a Nikon Eclipse 50i light microscope equipped with a Nikon Digital Sight DS-Fi2 camera (Nikon Corporation, Japan). Stem pieces of approximately 0.5 cm containing the infection front were cut from the plants and placed in 70% ethanol. Samples were then immersed in formalin for 30 min (twice), 70 % isopropanol for 30 min, 95 % isopropanol for 60 min and 100% isopropanol for 60, 90 and 90 min. Samples were then immersed in xylene for 60 and 90 min and paraffin prior to sectioning. Sections were 4µm thick. Xylene, isopropanol and water wash were carried out prior to staining with Periodic Acid-Schiff Reagent (PAS). Microscopy slides were prepared by the Animal Health Laboratory, University of
Cells containing starch grain deposits were counted in five fields of epidermal and cortical tissues.

**Statistical analysis**

Within each experiment, plants were arranged in a randomized complete block design (RCBD), with each growth chamber considered as a block. Before the statistical analyses were conducted, all data sets were tested for assumptions of homogeneity and normal distributions of residuals. Statistical analyses included ANOVA and LSMEANS comparisons using proc glm (SAS package, Cary, NC). Pair-wise comparisons for each variable evaluated genotypes to detect differences using Tukey’s procedure at the 5% level of significance.

**2.4. Results**

**Disease related symptoms**

Susceptible OAC Shire and partially-resistant OAC Salem genotypes had equal wilting symptoms and visible bleaching of stems but differed in severity of SSR disease (Figure 2.1). No significant differences were found between the two genotypes for number of days for visible development of lesion or time to wilt (Table 2.1., refer to Appendix A, tables A.1 and A.2 for ANOVA tables). However, OAC Shire plants died at a significantly faster rate than those of OAC Salem ($P <0.0001$, Table 2.1, refer to Appendix A, table A.4 for ANOVA table). Severity of wilting, which consisted of the number of leaves wilted above the inoculation site at 5 dpi, was significantly higher for OAC Shire ($1.54±0.128$) than for OAC Salem ($0.56±0.13$, $P <0.0001$, Table 2.1, refer to Appendix A, table A.3 for ANOVA table).
Measurements of lesion lengths on stems were taken and the AUCPC was estimated for inoculated plants 8 dpi. No significant difference was found between OAC Salem and OAC Shire (Table 2.2; refer to Appendix A, table A.5 for ANOVA table).

Figure 2.1. Disease related symptoms in OAC Shire and OAC Salem soybean plants infected with *S. sclerotiorum*. A) OAC Shire plants showing bleaching of stems 2 days post-inoculation (dpi) and (B) severe wilting symptoms at 6 dpi. C) OAC Salem plants with less severe wilting symptoms at 4 dpi and (D) 6 dpi.
Table 2.1. Number of days for visible development of lesion (VL), time to wilt (TW), severity of wilting (SW) and number of days to plant death (PD), for OAC Salem and OAC Shire after inoculation with *S. sclerotiorum*.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>VL (days)</th>
<th>TW (days)</th>
<th>SW (days)</th>
<th>PD (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAC Salem</td>
<td>1.85±0.08</td>
<td>6.77±0.34</td>
<td>0.56±0.13</td>
<td>12.93±0.168</td>
</tr>
<tr>
<td>OAC Shire</td>
<td>2.02±0.08</td>
<td>6.20±0.33</td>
<td>1.54±0.128*</td>
<td>11.35±0.162*</td>
</tr>
</tbody>
</table>

Note: Values represent LSMEANS±SE for each treatment. Experiments were repeated three times and each replication was considered as a block. *: Significantly different at *P*<0.05 within a column following Tukey’s procedure. Refer to Appendix A for ANOVA tables.

Table 2.2. Area under canker progress curve (AUCPC) of plants from cultivars OAC Salem and OAC Shire at 8 days post-inoculation (dpi) with *S. sclerotiorum*.

<table>
<thead>
<tr>
<th></th>
<th>OAC Salem</th>
<th>OAC Shire</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>27.99±0.99</td>
<td>26.29±0.96 (ns)</td>
</tr>
</tbody>
</table>

Values represent LSMEANS±SE for each genotype. (ns): no significant difference. Experiments were repeated 3 times and each replication was treated as a block. Refer to Appendix A for ANOVA table.
Fresh and dry weights

Fresh weight reduction of inoculated as compared to non-inoculated plants of susceptible OAC Shire occurred 12 dpi. In contrast, no significant differences were found in partially-resistant OAC Salem, between inoculated and non-inoculated plants at time intervals of 5 or 12 dpi ($P=0.24$ and $0.96$, respectively) (Table 2.3, refer to Appendix A, table A.6 for ANOVA table).

Weights of inoculated and non-inoculated plants of OAC Shire did not differ statistically at 5 dpi; however, at 12 dpi, fresh weight of non-inoculated plants was significantly greater than that of inoculated plants (Table 2.3, $P<0.0001$).

Dry weight of both susceptible and partially-resistant genotypes was affected by SSR. Dry weights of plants inoculated with *S. sclerotiorum* were collected at 5 and 12 dpi. Plants from both genotypes had significantly smaller dry weights than their respective controls at 12 dpi (Table 2.4, refer to Appendix A, table A.7 for ANOVA table). At 5 dpi, OAC Salem had significantly lower dry weight than OAC Shire (1.22±0.08 vs. 1.65±0.07, $P=0.008$). The same result could be observed at 12 dpi (1.33±0.08 vs. 1.79±0.08, $P=0.0023$) (Table 2.4).

Stomatal conductance ($g_s$) in susceptible OAC Shire and partially-resistant OAC Salem plants

In susceptible OAC Shire, LSMEANS values for stomatal conductance show a significant difference ($P=0.002$) between the two genotype (Table 2.5, refer to Appendix A, table A.8 for ANOVA table). OAC Salem showed similar stomatal conductance during the time course of the experiment both when compared with non-inoculated controls and within inoculated plants (Figure 2.2). However, for OAC Shire, stomatal conductance was shown to increase over the first 3 dpi following *S. sclerotiorum* inoculation.
Table 2.3. Fresh weight (g) of plants of cultivars OAC Salem and OAC Shire at 5 and 12 days post-inoculation (dpi) with *S. sclerotiorum* compared to non-inoculated plants (Control). Bold fonts represent LSMEANS of inoculated plants.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>5 dpi</th>
<th>12 dpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAC Shire Control</td>
<td>5.03±0.56 (a)</td>
<td>9.15±0.53 (b)</td>
</tr>
<tr>
<td>OAC Shire</td>
<td><strong>5.48±0.35 (a)</strong></td>
<td><strong>5.07±0.53 (a)</strong></td>
</tr>
<tr>
<td>OAC Salem Control</td>
<td>3.17±0.58 (a)</td>
<td>5.03±0.54 (a)</td>
</tr>
<tr>
<td>OAC Salem</td>
<td><strong>4.04±0.38 (a)</strong></td>
<td><strong>3.43±0.42 (a)</strong></td>
</tr>
</tbody>
</table>

Values represent LSMEANS±SE for each treatment. Treatments were repeated 3 times and each replication was treated as a block. Different letters indicate significant differences at $P <0.05$ within a column.
Table 2.4. Dry weight (g) of plants of cultivars OAC Salem and OAC Shire at 5 and 12 days post-inoculation (dpi) compared to non-inoculated plants (Control). Bolded LSMEANS represent those of inoculated plants.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>5 dpi</th>
<th>12 dpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAC Shire Control</td>
<td>1.47±0.11(a)</td>
<td>2.20±0.10(b)</td>
</tr>
<tr>
<td>OAC Shire</td>
<td>1.65±0.07(a)</td>
<td>1.79±0.08 (a)</td>
</tr>
<tr>
<td>OAC Salem Control</td>
<td>0.84±0.11(c)</td>
<td>1.80±0.11(a)</td>
</tr>
<tr>
<td>OAC Salem</td>
<td>1.22±0.08(c)</td>
<td>1.33±0.08(d)</td>
</tr>
</tbody>
</table>

Values represent LSMEANS for each treatment. Treatments were repeated 3 times and each replication was treated as a block. Different letters indicate significant differences at $P<0.05$ within a column.

Table 2.5. LSMEANS values for stomatal conductance (mM m$^2$ s$^{-1}$) on two soybean genotype

<table>
<thead>
<tr>
<th>OAC Salem</th>
<th>OAC Shire</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.9±2.33</td>
<td>61.5±2.39 *</td>
</tr>
</tbody>
</table>

(* ) significant differences for $P < 0.05$
Significant differences were found at 2 and 3 dpi \((P = 0.003,\) and \(< .0001,\) respectively) in infected plants (Figure 2.2). OAC Shire showed a significant increase in stomatal conductance at 3 dpi \((84.15\pm 4.56)\) compared to OAC Salem in the same time frame \((58.45\pm 4.38)\) and also compared to the non-inoculated OAC Shire controls \((67.52\pm 5.82)\), resulting in visible wilting symptoms (Figure 2.3).

**Starch accumulation**

Starch grains accumulated differentially in susceptible cultivar OAC Shire and partially-resistant OAC Salem during the first three days post-inoculation. No significant differences were found for genotypes as the main effect but a statistically significant difference was found for time points \((P < 0.0001,\) Figure 2.4, refer to Appendix A, table A.9 for ANOVA table)

Genotypes did not differ statistically for starch accumulation in control plants. For partially-resistant OAC Salem, the amount of cells with starch deposits did not change during the time course of the trial. For OAC Shire, the number of cells with starch grains increased significantly from 1dpi to 2 dpi \((P=0.004)\). Significant differences were found also between plants collected at 1 dpi and 3 dpi \((P=0.02)\).
Figure 2.2. Stomatal conductance of OAC Salem (partially-resistant) and OAC Shire (susceptible) plants during the first 3 dpi with *S. sclerotiorum*; non-inoculated control plants from both genotypes were used as controls. Points represent LSMEANS of experiments, bars indicate standard errors and (*) indicates significant differences between inoculated plants of OAC Shire and OAC Salem ($P<0.05$).
Figure 2.3. Wilting symptoms in SSR infected soybean plants at 3 days post-inoculation. A) OAC Shire cultivar, B) OAC Salem cultivar
2.5. Discussion

To date, no source of complete resistance against SSR has been identified in soybean. However, partial resistance has been reported in several soybean lines (Hoffman et al., 2002; Auclair et al., 2004a; Auclair et al., 2004b; Cober et al., 2003). This study provided a detailed characterization of physiological responses to \textit{S. sclerotiorum} infection in susceptible and partially-resistant genotypes. A simple and reliable inoculation method to achieve the goal was used.

Several infection characteristics, including time to visible lesion and time to wilt were described in this study. For both susceptible and partially-resistant genotypes, no differences were found and lesions appeared within approximately three days after inoculation. Similar results were found when soybean plants were artificially and naturally infected with \textit{S. sclerotiorum} (Voung et al. 2004; Auclair et al. 2004a; Auclair et al. 2004b; Cober et al., 2003). The development of necrotic and water-soaked lesions after 20 hr of infection of tomato plants with the necrotroph \textit{Botrytis cinerea}, the causal agent of grey mold, has been reported as well (Benito et al., 1998). Lesions on detached leaves of oilseed-rape plants inoculated with \textit{S. sclerotiorum} were visible after 36 hr of inoculation (Rahmanpour et al., 2011) but a significant difference between susceptible and partially-resistant cultivars could not be established at that time. These results suggested that perhaps smaller time frames between measurements than the ones used are necessary to capture subtle differences in those cultivars.
**Figure 2.4.** Starch grains accumulate differentially in epidermis and cortex of stem tissue of inoculated plants for both susceptible OAC Shire and partially-resistant OAC Salem. Each point represents LSMEANs for each treatment. Starch accumulation was calculated by counting number of cells with starch grains in epidermis and cortex of three samples in five fields. (*) represents significant differences for $P < 0.05$
Severity of wilting and number of days to plant death were among the characteristics measured during soybean-\textit{S. sclerotiorum} interaction in this study. At 5 dpi, susceptible OAC Shire showed a higher rate of wilting severity in comparison with partially-resistant OAC Salem. This result may suggest that OA, which causes wilting on leaves (Kolkman and Kelly, 2000; Guimaraez and Stotz, 2004), moved faster systemically in susceptible than in partially-resistant plants. Also the two cultivars might be differentially sensitive to OA. At day 12, 52.2% of OAC Shire plants were dead whereas partially-resistant OAC Salem had an approximately 2.2% death rate. The number of days to plant death was recorded as well, and results showed that OAC Shire plants died within a shorter period of 11 days than the period of time needed for OAC Salem plants to die. Similar results were reported using several inoculation methods to test soybean-\textit{S. sclerotiorum} interaction, that caused higher death rates for susceptible cultivars at the end of the experiments (Cline, 1981).

Lesion length on infected plants was recorded every 24 h for a period of eight days and AUCPC was calculated for each of the two genotypes. No differences were found between susceptible and partially-resistant cultivars concurring with results found by Kim \textit{et al} (2000) in naturally- and artificially-infected soybean plants. Corsoy 79, one of the cultivars used in their study, showed consistent tolerance across different infection methods, while Resnik showed consistent susceptibility. Disease rates for those two cultivars were based on the DSI; however, values for both cultivars for the AUCPC did not differ significantly (Kim \textit{et al.}, 2000). Studies developed in several canola cultivars also showed no differences for AUDPC between susceptible and partially-resistant plants inoculated using the petiole inoculation technique (PIT) (Bradley \textit{et al.}, 2005).
SSR caused fresh weight loss on day 12 after inoculation in the susceptible cultivar OAC Shire, while no losses were found on partially-resistant OAC Salem plants at the same time. The loss of fresh weight in OAC Shire measured at the last time point (12 dpi) could suggest that there was a faster movement of oxalic acid in the susceptible plants than in partially-resistant ones, resulting in greater water loss for the susceptible genotype. Dry weights of both susceptible and partially-resistant cultivars were affected by \textit{S. sclerotiorum} infection. These results are in agreement with the report by Guimarães and Stotz (2004), in which different strains of \textit{S. sclerotiorum} were used to test pathogenicity in \textit{Vicia faba}. Decreases in dry matter were related indirectly with the ability of the fungus to utilize plant–derived nutrients at its convenience (Guimarães and Stotz, 2004) and, thereby, influence plant growth (Edwards \textit{et al.}, 2007).

OA constitutes the main virulence factor of \textit{S. sclerotiorum} (Cessna \textit{et al.}, 2000). Recent studies have shown that OA moves systemically throughout the infected plant and causes deregulation of guard cells of stomata by interacting with ABA, breaking down starch, and potassium accumulation (Noyes and Hancok, 1981; Guimarães and Stotz, 2004). In this study, stomatal conductance measurements indicated that susceptible OAC Shire plants had higher \( g_s \) at 2 and 3 dpi, indicating a rapid decrease of water potential for these plants. Increased stomatal conductance was also found on plants inoculated with wild-type \textit{S. sclerotiorum} when compared with mutant type (no production of oxalic acid) or mock inoculation (Guimarães and Stotz, 2004). The present study also showed specifically increased \( g_s \) in the susceptible cultivar OAC Shire which was related to wilting symptoms that were severe for this cultivar at 5 dpi. Reports of studies conducted in grapes infected with the hemibiotroph oomycete \textit{Plasmopara viticola} revealed that deregulation in guard cells could be a result of products secreted by the pathogen or host plant and lack of pressure exerted by surrounding epidermal cells (Allègre \textit{et al.}, 2007).
Deregulation of guard cells of stomata contributes to increased transpiration (Allègre et al., 2007) and consequently wilting symptoms. Our results agree with the report by Edwards et al. (2007) who found grapevines infected with the biotrophic pathogen Phaeomoniella chlamydospora presented increased stomatal conductance and diminished water potential compared with uninfected plants. This condition results in a clear impact on physiological responses of infected plants to water stress. Importantly, no increase in $g_s$ was found for partially-resistant OAC Salem plants in comparison with non-inoculated controls.

Starch accumulation in both susceptible and partially-resistant cultivars behaved differentially during the first three days of $S$. sclerotiorum infection. Starch constitutes the main storage polysaccharide in plants. It is composed of D-glucose residues, and characterized by $\alpha$-1,4 bonds and a helical conformation (Mischnick and Momcilovic, 2010). Studies conducted by Martel et al. (2002) demonstrated that $S$. sclerotiorum secreted glucoamylase, an enzyme responsible for cleavage of $\alpha$-1,4 bonds. For partially-resistant OAC Salem, no changes in number of cells with starch deposits were observed between inoculated and non-inoculated plants or within the inoculated plants themselves. For susceptible OAC Shire, an increase of cells with starch grain deposits was observed after 2 dpi. This observation in susceptible plants supports the results from the study of barley plants infected with the fungal biotroph Erysiphe graminis (Minarcic and Janitor, 1994). Fungal amylase activity was found to be responsible for the decrease of starch deposits during the first hours of infection when the pathogen was provided with nutrients. Afterwards, a decrease of amylase activity generated accumulation of starch grains in host cells close to the infection site (Minarcic and Janitor, 1994). Similar results were reported in wheat plants inoculated with Puccinia striiformis (McDonald and Strobel, 1970). Scanning electron microscopy revealed starch accumulation in chloroplasts of host cells very close to fungal hyphae,
with accumulation of this polysaccharide decreasing from day 5 to day 9 after inoculation followed by an increase from day 9 to day 12 (McDonald and Strobel, 1970). The results in this study also concur with those reported by Garg et al. (2010). Two canola cultivars were inoculated with \textit{S. sclerotiorum} and light microscopy analyses of samples stained with PAS showed a greater number of cells with starch grain deposits in the susceptible cultivar used, RQ001-02M2. Other studies have shown that bacterial and fungal pathogens manipulated metabolic pathways of host plants to accumulate starch close to infection sites such as citrus greening on orange trees (Etzeberria et al., 2009) and \textit{Kabatiella caulivora} in subterranean clover (Bayliss et al., 2001) to obtain nutrients from degraded starch molecules.

In conclusion, physiological characterization of soybean-\textit{S. sclerotiorum} pathosystem was conducted between a susceptible and a partially-resistant cultivar. The results from this study may contribute to the understanding of the defense strategies used by partially-resistant soybean cultivars against white mold, e.g. control of stomatal closure/opening mechanisms and re-growth of new branches below the infection site, and help in the development of breeding strategies for this and other host crops as well.
CHAPTER 3
ANATOMICAL RESPONSES OF SOYBEAN PLANTS TO SCLEROTINIA SCLEROTIORUM INFECTION

3.1. Abstract

*Sclerotinia sclerotiorum* is the causal agent of Sclerotinia stem rot (SSR), also known as white mold, which affects over 400 species of plants. Compatible interaction studies on cotton, bean, sunflower, canola and pea have been reported; however, little is known about anatomical changes that occur in host plants in incompatible interactions. The aim of this study was to describe changes that occur at cellular and tissue levels in susceptible OAC Shire and partially-resistant OAC Salem cultivars of soybean infected with *S. sclerotiorum*, using stem and leaf inoculation techniques. Light microscopy analyses on stem and detached leaf samples were conducted. Stains used for microscopic observations included toluidine blue, methylene blue, cotton blue in lactophenol and safranine O. Direct penetration of the fungal hyphae through the cuticle using the base of non-glandular trichomes was observed exclusively on susceptible tissues. Single and complex appressoria characterized the early stages of tissue colonization in OAC Shire, whereas small infection cushions were observed on OAC Salem only during the late stages of colonization. Cytoplasm disorganization and reinforced cell walls were present in epidermal and cortical cells of partially-resistant OAC Salem causing a delay in tissue maceration. The mechanisms of infection described in this study may contribute to the understanding of *S. sclerotiorum* infection strategies and anatomical responses observed in partially-resistant soybean cultivars. Also, these results could aid in the improvement of markers for screening of resistance against white mold in several other crops.
3.2. Introduction

*Sclerotinia sclerotiorum* (Lib.) de Bary is a necrotrophic homothallic pathogen capable of infecting more than 400 species of plants including important crops such as sunflower, cotton and soybean (Boland and Hall, 1994). In the latter, the disease is also called Sclerotinia stem rot (SSR) and its incidence causes significant damage to yield and grain quality (Cunha et al., 2010). Reported annual losses from SSR have exceeded $500 million dollars in 2009 in the United States (Peltier et al., 2012) and could be estimated at approximately 83.2 to 229 kg/ha per 10% disease incidence (Danielson et al., 2004).

Sclerotia as the long-term survival structures of *S. sclerotiorum* could be viable for up to eight years in soil (Adams and Ayer, 1979; Willetts and Wong, 1980). Their formation depends on a number of environmental and nutritional factors including temperature, pH and availability of carbohydrates and amino-acids (Chet and Hennis, 1975). Also, sclerotial germination usually occurs via apothecia formation (carpogenic germination), although myceliogenic germination has also been reported (Purdy, 1958; Saito, 1975; Grogan, 1979; Huang, 1991; Huang and Chang, 2003; Renuka et al., 2013). There is no particular length of time required for carpogenic germination; it varies depending mainly on environmental conditions ensuring production of ascospores when the circumstances for germination are favorable (Willetts and Wong, 1980; Hao et al., 2003; Garg et al., 2010c).

Released ascospores land on dying or dead tissues (e.g. flower petals in nodes of stems) where they germinate (Purdy, 1979). Mycelia move through the dead/dying tissue and invade healthy parts of the plants. Once the plant is dying and the nutrient supply is diminished, new sclerotia are formed in and on infected tissues and become the new survival structures for disease.
development for the following years (Saharan and Mehta, 2008). The germination of sclerotia directly into mycelia is rare and consists of the development of individual hyphae which emerge through the rind of the sclerotium (Adams and Tate, 1976) and generally infect the base of the stems of sunflower (Purdy, 1958; Saito, 1975; Davar et al., 2012).

*S. sclerotiorum* has developed a set of structures and strategies to penetrate host tissues. These strategies vary according to the type of inoculum, the characteristics of the host, the availability of different nutrients and the surrounding environments (Lumsden, 1979; Hegedus and Rimmer, 2005; Mengden et al., 1996; Muthamilarasan and Prasad, 2013). Penetration structures that include single and complex appressoria have been described, as well as the requirements needed by the pathogen to grow and invade host tissue (Lumsden and Wergin, 1980). Germinated ascospores can produce single appressoria that form infection pegs penetrating the epidermis of the host giving origin to infection vesicles (Abawi et al., 1975). A complex penetrating structure, a multicellular infection cushion, is also observed during the infection process of *S. sclerotiorum*. Infection cushions or complex appressoria usually originate from hyphal strands that branch dichotomously forming compact and multicellular barrel-shape structures on the host surfaces (Lumsden and Dow, 1972; Abawi et al., 1975; Lumsden, 1979). The penetration pegs originating from these cushions give origin to infection vesicles that later develop into infection hyphae with intercellular movements (Abawi et al., 1975; Lumsden, 1979; Orshinski et al., 2012). However, intra- and intercellular hyphal movements have been described for *S. sclerotiorum* infection in an indiscriminate manner (Purdy, 1958; Abawi et al., 1975; Garg et al., 2010a).

The compatible interactions between *S. sclerotiorum* and several of its hosts have always been of interest since the early days of its discovery by De Bary (Lumsden and Dow, 1972,
Bolton et al., 2006). Studies conducted on bean (Lumsden and Dow, 1971), soybean (Sutton and Deverall, 1983), sunflower (Davar et al., 2012) and canola (Jamaux et al., 1995; Garg et al., 2010a) have described the requirements of senescent tissues for ascospore germination and infection progress on healthy plants, however, little is known of interactions with partially-resistant and resistant cultivars. Resistance against S. sclerotiorum in plant species such as Scarlet runner bean (Phaseolus coccineus) and some cultivars of dry white beans has been found (Dow and Lumsden, 1975; Schawrtz et al., 2006; Gilmore and Myers, 2000; Gilmore et al., 2002). The growth of S. sclerotiorum in runner bean tissue is limited by a physical barrier or middle lamellae of the plant cells that prevents the penetration and infection of host tissues (Dow and Lumsden, 1975). Histopathology studies revealed great differences compared to the inoculated susceptible dry bean tissue. For example, the penetration of the cuticle of P. coccineus often is impeded; the infection cushions often develop beneath the cuticle; and the infection hyphae in P. coccineus are often small, distorted and not subcuticular (Dow and Lumsden, 1975). Resistance has been also described in wild sunflower, Helianthus resinosus, with pre-formed defense mechanisms including sclerified fibre cells in cortical tissues, glandular hairs and flavonoids secreted in the epidermis (Mondolot-Cosson et al., 1994). Natural resistance observed in several plant species like onion (Allium cepa), potato (Solanum tuberosum) and cultivars of Brassica juncea has its origin in pre-formed defenses as well (Echandi and Walker, 1957; Rai et al., 1979; Uloth et al., 2013).

In soybean, SSR is difficult to control. Currently, sources of resistance against S. sclerotiorum are limited within the soybean germplasm and complete resistance against this pathogen has not been reported (Hoffman et al., 2002). In spite of good agronomic practices such as crop rotation and tillage (Gracia-Garza et al., 2002) and fungicide application (Mueller et al.,
SSR can still cause great yield damage. The development of partially-resistant lines and the identification of partially-resistant plant introductions are a research focus nowadays. Recently, AxN-1-55 was registered as a partially-resistant line developed by the Illinois Agricultural Experiment Station at the University of Illinois and the Michigan Agricultural Experiment Station at Michigan State University (Diers et al., 2006). This line offers a significantly low disease severity index (DSI) of 17, compared to its parents S19-90 and A2506 with DSI of 29.3 and 34.7, respectively, after greenhouse and field evaluations. The study of plant introductions (PIs) has offered a valuable insight on partial resistance against *S. sclerotiorum* on soybean germplasm as well. A total of 68 PIs were identified as partially-resistant after small plot evaluations in the greenhouse and field with two inoculation techniques (Hoffman et al., 2002).

Several soybean cultivars have been described as partially-resistant after greenhouse and field evaluations. OAC Salem was ranked the most tolerant cultivar in the group of lines and varieties studied in controlled environments using the straw inoculation method, with DSI of 4.2 and 5.7 at one and two weeks after inoculation, respectively (Auclair et al., 2004a). Similar results were obtained during field inoculations using the barley kernel method, with inoculation severity index (ISI) of 1.2 and 2.8 at one and two weeks post-inoculation, respectively (Auclair et al., 2004b). Field experiments in natural infested soils also showed a significantly low DSI of 2 for OAC Salem (Cober et al., 2003).

The hypothesis of this study was that partially-resistant OAC Salem triggered defense-related mechanisms against *S. sclerotiorum* earlier than susceptible OAC Shire. Thereby, the aim of this research was to describe changes that occur at cellular and tissue levels in susceptible OAC Shire and partially-resistant OAC Salem cultivars of soybeans infected with *S. sclerotiorum*, using...
stem and leaf inoculation techniques. Strategies of pathogen penetration and plant defense were studied and evidence of direct penetration using the base of non-glandular trichomes was found only in the susceptible line. However, only direct penetration of the cuticle was present in partially-resistant plants. Inter- and intracellular invasion of plant tissues by fungal hyphae was also found during the infection process in both soybean cultivars. The mechanisms of infection described in this study might contribute to the understanding of *S. sclerotiorum* infection strategies and anatomical responses observed in partially-resistant soybean cultivars. Also, these results could aid in the improvement of markers for screening of resistance against white mold in several other crops.

### 3.3. Materials and Methods

#### Plant Material

Seeds of soybean cultivars OAC Salem (partially-resistant) and OAC Shire (susceptible) were planted in Sunshine Mix LA4 soil medium (SunGro, Vancouver, BC) in 3.5 inch square pots following the methods described in Chapter 2. Growth chambers set at 25 °C with a photoperiod of 16/8 h (day/night) and light intensity of ~ 350 μmol photons ms⁻¹ were used for a period of 4-5 weeks for the required growth period of plants (to the V4 stage) (Fehr *et al.*, 1971).

Growth medium for culturing *S. sclerotiorum* consisted of potato dextrose agar (PDA) prepared from 1 liter potato broth supplemented with 20 g dextrose and 10 g agar and was inoculated with fungal cultures of isolate 1980 (Auclair *et al.*, 2004a; Auclair *et al.*, 2004b) two days prior to inoculation. Media preparation and sub-culturing followed the methods described in Chapter 2.
**Stem inoculations**

Prior to inoculation, growth chambers were set to 18 °C. Five-millimeter-diameter plugs of PDA medium with growing mycelia from the leading edge were collected with the back side of 30 µl pipette tips. Agar plugs were positioned below the third node on the stem of plants and secured with Parafilm™ (Thermo Fisher Scientific Inc, USA) following the method described by Lone Buchwaldt (personal communication). Stem samples of 0.5 -1.0 cm were collected every 24 hr during a period of nine days. Each day, five samples were collected per genotype for a total of approximate 90 samples. The experiment followed a randomized complete block design (RCBD) with two replications (each growth chamber constitute a replication).

**Detached leaf assay**

Entire leaves from the third node of OAC Shire and OAC Salem plants were collected on wet paper towel and immediately placed on 50 x 45 cm plastic trays in the laboratory. Inoculations followed the method described by Cunha *et al.* (2010). Five millimetre mycelia plugs were collected from the growing margins of the inoculated plates and placed on the adaxial surface of the middle leaflet of the youngest fully-expanded trifoliate leaf at V4 stage. Leaves were collected every 24 hr for a period of nine days. Each day, three samples per genotype were collected for a total of 54 leaves. The experiment followed a randomized complete block design (RCBD) with three replicates (each growth chamber constitutes a replication).
Sample preparation for light microscopy

Stem tissue

Stem samples of inoculated and non-inoculated plants from cultivars OAC Shire and OAC Salem were observed using a Nikon Eclipse 50i light microscope equipped with a Nikon Digital Sight DS-Fi2 camera (Nikon Corporation, Japan). Stem pieces from early to advanced stages (1 dpi-3 dpi) were collected in 60% alcohol and processed at the Animal Health Laboratory, University of Guelph. Samples were immersed in formalin for 30 min (twice), 70% isopropanol for 30 min, 95% isopropanol for 60 min and 100% isopropanol for 60, 90 and 90 min. Samples were then immersed in xylene for 60 and 90 min and paraffin prior to sectioning. Sections were 4µm thick. Xylene, isopropanol and water washes were carried out prior to staining. Stains included toluidine blue and saffranin O for detection of phenolic compounds and lignified cell walls (Garg et al., 2010), respectively, and methylene blue for detection of fungal structures (Sinclair and Dhingra, 1995).

Samples from late stages of infection (5 dpi-9 dpi) were cleared with ethanol: acetic acid: water (2: 2: 1) solution at 70ºC for 4h in a water bath, then rinsed twice with de-ionized water. Wet mounted tissues on glass slides were stained with 1% cotton blue in lactophenol or trypan blue for observation of fungal structures.

Leaf tissue

Leaves from both susceptible and partially-resistant cultivars were cleared in ethanol: acetic acid: water (2: 2:1) solution at 65 ºC for 3h in a water bath and then rinsed three times in de-ionized water. Samples were mounted on glass slides and stained with 1% cotton blue in lactophenol and trypan blue as described for stem tissues.
3.4. Results

**Disease development on stem tissues**

*1 dpi.* For susceptible OAC Shire plants, strong accumulation of phenolic compounds was observed only in cortical cells. Epidermal cells showed only partial lignification of the cell walls (Figure 3.1A). For partially-resistant OAC Salem plants, stem tissues showed a strong accumulation of phenolic compounds in the epidermis and the outer two cell layers of cortex (Figure 3.2A). Cells beneath the cortex were characterized by granular structures in their cytoplasm, also known as cytoplasm disorganization (Figure 3.2A).

*2 dpi.* Accumulation of phenolic compounds in susceptible OAC Shire plants did not increase during this time; however, some intracellular hyphae of approximate 5 μm diameter were observed (Figure 3.1B). Several cells close to the proximity of the lesion showed cytoplasmic disorganization in susceptible tissues (Figure 3.1B, F). Invasion of granular infection hyphae extended through the cortex and reached the proximities of phloem tissue. Hyphae were oriented parallel and vertical to the longitudinal axis of plants. Cortical cells collapsed and some structures resembling oxalate crystals were observed in the lumen of xylem cells (Figure 3.1G). Accumulation of phenolic compounds extended throughout the cortex and reached the phloem tissue in partially-resistant plants. Several cells near the infection site showed cytoplasmic disorganization (Figure 3.2B). At this time, no colonization of vascular tissue was observed for either genotype.

*3 dpi.* Stem tissues of susceptible plants had extensive hyphal invasion and a diminished accumulation of phenolic compounds. Great damage in epidermal and cortical cells was observed as well (Figure 3.1C). Granular infection hyphae invading the interior of cortical cells were
apparent with diameter of approximately 8 \( \mu m \). Formation of simple (Figure 3.1D) and complex appressoria (Figure 3.1E) was evident for the susceptible genotype OAC Shire at 3dpi. Three types of hyphae as part of the infection cushions were found in the susceptible line: I) darkly-stained, dome-shaped hyphae at the top, II) lightly-stained hyphae in areas close to the host surface, and III) granular hyphae, similar in color to the top of the cushion (Figure 3.1E). Attempts to invade vascular tissue were observed from 2 dpi in the form of barrier-like clumps of hyphae that in some cases resembled infection cushions (Figure 3.1D). Invasion of phloem and xylem tissues occurred after 3 dpi (Figure 3.1H). For partially-resistant plants, a partial maceration of epidermal and cortical tissues was observed. However, around these macerated areas the cuticle remained intact (Figure 3.2C, E). Some granular intracellular and intercellular infection hyphae were observed also with approximately 10 \( \mu m \) and 3-4 \( \mu m \) diameter, respectively (Figure 3.2C). For those areas close to the infection site, a strong deposition of phenolic compounds as well as cytoplasmic disorganization in nearby cells were noted (Figure 3.2D).
Figure 3.1. Light microscopy of *S. sclerotiorum*-infected soybean stems of cultivar OAC Shire (susceptible) during early to advanced (A-H) and late (I) stages of infection.

A-C, F and G, cross sections stain with Toluidine Blue (A-C) and Safranine O (F and G). D, E H and I are longitudinal sections stain with Toluidine Blue (D and H), methylene Blue (E) and cotton blue in lactophenol. (A) Strong accumulation of phenolic compounds on cell walls of cortex at 1 dpi. (B) Granular intracellular hyphae invading (arrows) cortical and epidermal tissue and diminished accumulation of phenolic compounds at 2 dpi. (C) Cytoplasmic disorganization on areas close to the infection site (*) and granular infection hyphae (arrows) at 3 dpi. (D) Appresorium (arrow) at 3 dpi. (E) Infection cushion at 3 dpi, note the maceration of tissues surrounding the inoculation site (*). (F) Granular cytoplasm on cells surrounding inoculation sites at 2 dpi (arrows). (G) Possible oxalate crystal-like structures (arrow) in xylem cells of susceptible at 2 dpi. (H) Hyphae running parallel and vertical to longitudinal axis of plant, note granular infection hyphae and small ramifying hyphae at 3 dpi. (I) Single appressoria at 9 dpi (arrows), scale bars: A=30 µm, B-C=20 µm, D-E=30 µm, F-G=20 µm, H=30 µm, I=10 µm.
Figure 3.2. Light microscopy of *S. sclerotiorum*-infected soybean stems of cultivar OAC Salem (partially-resistant) during early to advanced (A-E) and late (F) stages of infection.

A-C and E, cross sections, stained with Toluidine Blue (A-C) and Safranine O (E). D and F, longitudinal sections, stained with Toluidine Blue (D) and cotton blue in lactophenol (F). (A) Accumulation of phenolic compounds on cell walls of epidermis and cortex at 1 dpi (arrows). (B) Strong accumulation of phenolic compounds (arrows) and cytoplasmic disorganization (*) at 2 dpi. (C) Granular inter- and intracellular hyphae invading cortex (arrows) at 3 dpi. (D) Epidermal and cortical cells with thickened cell walls and granular cytoplasmic contents at 3 dpi. (E) Granular infection hyphae invading inter- and intracellular spaces, note the integrity of the cuticle at 3 dpi. (F) Dichotomous branching of hyphae at 9 dpi. Scale bars: A=10 µm, B-D, E=20 µm and F=30 µm.
Late stages of infection

During late stages of infection (5 dpi–9 dpi), deterioration of host tissues was significant in both cultivars (Figure 3.3). Invasion of vascular tissues was present; with both direct penetration of the cuticle (Figure 3.1I) and penetration of tissue at the base of non-glandular trichomes at 9 dpi in the susceptible cultivar (Data not shown). However, only direct penetration was found for partially-resistant plants, with emergence of lateral branches from aerial hyphae (Figure 3.2F) that could possibly develop into single appressoria. Macroscopic observations indicated the presence of sclerotia on susceptible plants while no sclerotia were found on stems of partially-resistant plants at 9 dpi (Figure 3.3). Growth of new branches on the nodes below the inoculation sites was observed in partially-resistant OAC Salem plants (Figure 3.3).
Figure 3.3. Late stages of SSR on stems of soybean plants of cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant); arrows indicate sclerotia on OAC Shire, and growth of new branches below inoculation site on OAC Salem.
Disease development on detached leaf tissues

1 dpi and 2 dpi. During the first two days of infection, no differences were found between the two cultivars on macroscopic examination (Figure 3.4). Under the light microscope, complex (Figure 3.5A) and single appressoria were observed in the susceptible cultivar. Similar structures were observed in the partially-resistant cultivar and a large hyphal accumulation in areas proximal to veins was evident at this time (Figure 3.6A and B). Advancing sub-cuticular granular hyphae with 3 to 5 µm diameter were also observed in OAC Salem at 2 dpi (Figure 3.6B). Formation of infection cushions around the base of non-glandular trichomes was observed exclusively in susceptible OAC Shire as early as 2 dpi (Figure 3.5 B) and also at 7 dpi (Figure 3.5 D) whereas direct penetration of host tissues characterized *S. sclerotiorum* infection in partially-resistant OAC Salem (Figure 3.6A, C).

Day 5. Detached leaves of susceptible OAC Shire showed greater lesion expansion than partially-resistant OAC Salem (Figure 3.7). In OAC Shire, the lesion extended to the apical part of the leaf, covering almost the entire surface (Figure 3.7). Light microscopy observations on leaf tissue revealed extensive hyphal invasion of veins for both cultivars with the presence of complex and single appressoria. Aerial hyphae approximately 3 µm in diameter were observed in OAC Shire (Figure 3.5A); however, 2 µm diameter hyphae were present on partially-resistant tissue (Figure 3.6C). Sub-cuticular hyphae on detached leaves were observed as well with diameters of 5 µm and 3 µm, in the susceptible (Figure 3.5C) and partially-resistant lines (Figure 3.6E), respectively. Subcuticular infection hyphae oriented parallel to one another were observed at this time in susceptible OAC Shire (Figure 3.5C).
Figure 3.4. Representative images of the early stages of *S. sclerotiorum* infection on detached leaves of soybean plants, cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant), at one, two and three days post-inoculation (dpi).
**Figure 3.5.** Light microscopy of *S. sclerotiorum*-infected detached leaves of soybean plants from cultivar OAC Shire (susceptible) stained with cotton blue in lactophenol. (A) Infection cushion on leaf surface at 1 dpi (arrow). (B) Infection cushion around base of non-glandular trichome at 2 dpi. (C) Parallel orientation of sub-cuticular hyphae on advancing fronts at 5 dpi. (D) Sclerotial primordia on leaf surface tissue at 7 dpi. (E) Hyphal penetration around the base of non-glandular trichome at 7 dpi. (F) Hyphal strand emerging from stoma and re-infecting the leaf tissue with single appresorium (arrow) at 9 dpi. Tri: trichome. Scale bars: A-E= 20 µm, F=10 µm.
**Figure 3.6.** Light microscopy of *S. sclerotiorum* infected detached leaves of soybean plants from cultivar OAC Salem (partially-resistant) stained with cotton blue in lactophenol (A) Advancing infection fronts with single appressoria at 2 dpi (arrows). (B) Vein invasion of sub-cuticular and aerial hyphae at 2 dpi. (C) Appresorium at 5 dpi. (D) Protoplast extrusions on tips of advancing hyphae at 7 dpi (arrows). (E) Hyphal strand apparently exiting stomatal aperture (arrow) for secondary infection at 7 dpi. Scale bars: A-C, E=30 µm, D=10 µm.
Day 7. Penetration of host tissue using the base of non-glandular trichomes was observed in susceptible OAC Shire at this time point (Figure 3.5E). Advancing fronts in a fan-shaped manner were also present in this genotype together with the presence of sclerotial primordia (Figure 3.5D). For the partially-resistant OAC Salem, protoplasm extrusions from tips and/or intercalary hyphal cells of advancing hyphae were observed as well as swelling of the cells (Figure 3.6D). Some hyphae passing through stomatal openings were observed, possibly exiting to re-infest the host tissue or to form sclerotial primordia (Figure 3.6E).

Day 9. Complete deterioration of tissue with some sclerotia was observed in infected detached leaves of OAC Shire (Figure 3.7). No differences were found between susceptible and partially-resistant cultivars macroscopically, except for the lack of sclerotia in OAC Salem. Secondary colonization of leaf tissue using the stomatal aperture was observed in OAC Shire, at this stage of infection (Figure 3.5F).

3.5. Discussion

*S. sclerotiorum* is responsible for white mold disease in more than 400 species of plants (Boland and Hall, 1984). This study describes anatomical changes during infection of susceptible and partially-resistant soybean plants by this pathogen. Observations on stem and detached leaves of these two cultivars were conducted and several anatomical characteristics and changes were described.
Figure 3.7. Late stages of *S. sclerotiorum* infection on detached leaves of soybean plants, cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant) at 5, 7 and 9 dpi, note that sclerotia are present at 9 dpi (arrows) only in OAC Shire.
Direct penetration of leaf tissue by means of infection cushions was a characteristic observed in both cultivars analyzed as early as 2 dpi. Dome-shaped structures, likely infection cushions, on stems of susceptible OAC Shire at 3 dpi were observed. The description of types of hyphae observed by Lumsden and Dow (1973) in white mold-infected bean hypocotyls is consistent with the observations made on susceptible plants in this study. Other reports have described infection cushions on host-fungal pathogen interactions, with mycelial aggregates exerting pressure on the cuticle, but without any enzymatic action (Abawi et al., 1975; Armentrout and Downer, 1986; Smith et al., 1986; Garg et al., 2010a). Direct penetration using single appressoria on infected leaf and stem tissues on both susceptible and partially-resistant cultivars was also observed. Flattened hyphal tips with granular content were noteworthy in susceptible stems and leaves (Figure 3.2 and 3.5). This result is in agreement with those found in studies conducted on creeping bentgrass infected with Sclerotinia homoeocarpa, where infection vesicles derived from appressoria on the surface of the host colonized the interior of the plant (Orshinsky et al., 2012). Studies conducted in canola hypocotyls infected with S. sclerotiorum also described the presence of single appressoria after 2 dpi followed by development of infection cushions at 6 dpi (Garg et al., 2010a).

Aggregation of hyphae at 2 dpi and penetration of host surfaces using the base of non-glandular trichomes of susceptible plants, were observed at 7 dpi. Clumps of aerial hyphae accumulated around the trichomes and penetrated the cuticle of OAC Shire plants at this time (7dpi). In contrast, no penetration via base of trichomes was observed in tissues of partially-resistant OAC Salem plants. Studies conducted in Arabidopsis thaliana plants infected with either B. cinerea or R. solani demonstrated the influence of foliar trichomes on infection by providing adhesion of fungal hyphae onto the host surface (Calo et al., 2006). Also, S.
*homoeocarpa* has been found to penetrate the leaf surface of creeping bentgrass using marginal leaf trichomes (Orshinsky et al., 2012).

After extensive colonization of susceptible host tissue, emerging ramifying hyphae were found organized in barrel-shape clumps and formed sclerotial primordia at 7 dpi. However, no sclerotial primordia were found in OAC Salem at this time. Several nutritional and environmental factors affect the formation of sclerotia (Chet and Henis, 1975; Clarkson et al., 2003; Clarkson et al., 2004). Promotion of sclerotia formation based on nutrient-limitation has been described (Christias and Lockwood, 1973). Susceptible cultivar OAC Shire might have suffered a rapid depletion of nutrients, which triggered the formation of sclerotial primordia on leaf tissue at 7 dpi followed by appearance of sclerotia at 9 dpi. These results concur with those reported on bean hypocotyls (Lumsden and Dow, 1973), bean stems (Lumsden and Wergin, 1980), oilseed rape (Garg et al., 2010a), sunflower (Davar et al., 2012) and faba bean plants (Guimaraes and Stotz, 2004) infected with *S. sclerotiorum* where the formation of sclerotial primordia was followed by formation of sclerotia in a period of approximately seven days.

Ramifying hyphae were observed to exit the infected tissue using open stomata on both susceptible and partially-resistant cultivars. Other studies have described the use of open stomata for secondary infection on bean hypocotyls (Lumsden and Dow, 1972), bean tissue (Lumsden and Wergin, 1980) and faba beans (Guimarãez and Stotz, 2004) and formation of sclerotial primordia (Lumsden and Dow, 1973; Lumsden and Wergin, 1980; Davar et al., 2012).

Observations made on stems infected by *S. sclerotiorum* revealed changes in cells close to the infection site. Accumulation of phenolic compounds on cell walls of epidermal and cortical cell layers was prominent for the partially-resistant cultivar OAC Salem as early as 1
dpi (Figure 3.2 A, B). In contrast, changes in tissues of the susceptible cultivar OAC Shire plants were less dramatic and extended only a few cell layers of cortical tissue later in the infection (Figure 3.1A, B). These results suggested a strong response to pathogen attack in the partially-resistant cultivar and, therefore, less damage to cortical and epidermal tissues by preventing penetration of fungal hyphae during early to advanced stages of the infection (Figure 3.2B). These results are in accordance with those reported in tomato infected with *B. cinerea*, where phenolic accumulation was found on cell walls of epidermis and cortex around 24 hpi (Asselbergh *et al.*, 2007). Studies on root tissue of *Eucalyptus marginata* infected with *Phytophthora cinnamomi* (Cahill *et al.*, 1988) and cauliflower inoculated with *Rhizoctonia* anastomosis groups (Pannecouque and Höfte, 2009) revealed the same results of strengthened cell walls of inner cortical cells to prevent penetration of fungal hyphae. Granular contents of cytoplasmic materials also characterized this stage of infection in OAC Salem and OAC Shire plants. Cytoplasmic disorganization has been described as a defense response in host plants (Mansfield and Richardson, 1981; Garg *et al.*, 2010a) since accumulation of phenolic compounds and phytoalexins have been detected after fungal infection in other pathosystems such as *B. napus-Leptosphaeria maculans* (Hua Li *et al.*, 2007). These metabolites have been implicated in the defense response of plants against the attack of a wide range of pathogens (Beckman, 2000). In both susceptible and partially-resistant tissues, cells around infection hyphae presented granular contents and in the case of the susceptible line, those areas stained dark purple with toluidine blue, resembling an HR-like reaction (Figure 3.1 B). Recently, studies conducted in *Nicotiana benthamiana* plants infected with *S. sclerotiorum* and *B. cinerea* revealed a manipulation of the host redox environment caused by OA secreted by the pathogens leading to programmed cell death (PCD) (Williams *et al.*, 2011). These necrotic tissues could
provide *S. sclerotiorum* with the necessary nutrients to expand the infection and ultimately kill the plants.

The present study has described the characteristics of the soybean-*S. sclerotiorum* pathosystem (i.e. sites of pathogen penetration, intra- and intercellular hyphal invasion and host cellular defense responses) associated with the susceptible OAC Shire and the partially-resistant cultivar OAC Salem. Several differences found between the two cultivars studied, including a possible manipulation of certain defense responses to favor pathogen colonization in the susceptible line. Studies in the accumulation of lignins, phenolic compounds and phytoalexins in *S. sclerotiorum*-challenged tissues could provide valuable insights into the anatomical changes that occur at different stages of infection and, therefore, aid in the development of markers for screening of resistance against SSR not only in soybean but in several other crops.
CHAPTER 4
GENE EXPRESSION PROFILES OF OAC SHIRE AND OAC SALEM IN RESPONSE TO S. SCLEROTIORUM INFECTION

4.1. Abstract

*Sclerotinia sclerotiorum* is one of the most devastating and cosmopolitan of plant pathogens and the causal agent of Sclerotinia stem rot (SSR) in soybean. No source of complete resistance has been described against this pathogen in this crop; however, a number of studies have examined identification of QTL for partial resistance, introduction of oxalic acid-degrading enzymes into soybean germplasm, and functional genomics analyses, and have offered insights into the quantitative nature of the resistance. Two soybean cultivars, susceptible OAC Shire and partially-resistant OAC Salem, were studied for genome-wide gene expression using RNA-sequencing (RNA-Seq) at 1 day post-inoculation (dpi) to advanced stages (3 and 5 dpi) of infection with *S. sclerotiorum*. Differentially expressed genes were classified into functional categories and pathways, and analyzed for differences in the transcriptomic profiles of the two lines. A total of 2316 genes were differentially expressed in at least one of the treatments, of which 712 were up-regulated and 1604 were down-regulated. Genes related to PAMP-triggered immunity (PTI) were identified, including receptor-like kinases, respiratory burst oxidases and mitogen-activated protein kinases. Additional defense genes, such as peroxidases, PR proteins, PGIPs, lignin and jasmonic acid/ethylene biosynthesis and regulation, also were differentially expressed. In general, several mechanisms related to defense responses that were activated in both cultivars at similar times during the experiment were found. However, for the susceptible line, a transient activation of these mechanisms was observed only at 3 dpi with a shutdown of several processes at 5 dpi compared to its control at 0 dpi, while the activation of those responses was stronger and
lasted to 5 dpi in the partially-resistant line compared to its control at 0 dpi. These results contribute to the understanding of the molecular defense mechanisms against necrotrophic pathogens and the development of strategies to incorporate partial resistance to SSR into commercial soybean cultivars and possibly other crops affected by this pathogen.

4.2. Introduction

*S. sclerotiorum* affects more than 400 species of plants worldwide including important crops such as sunflower and soybean (Boland and Hall, 1994). In soybean, the disease is called Sclerotinia stem rot (SSR) and its incidence causes significant economic damage to yield and grain quality (Cunha et al., 2010), estimated at $500 M dollars in the United States in 2009 (Peltier et al., 2012). In 2014, weather conditions were ideal for SSR development across the province of Ontario, Canada and severely impacted fields were widespread across the province and yields were expected to be reduced significantly (Ministry of Agriculture, food and Rural Affairs, 2014). Yield losses per 10% disease incidence of SSR vary from 83.2 to 229 kg/ha (Danielson et al., 2004). *S. sclerotiorum* is a necrotrophic homothallic pathogen and there are no unique symptoms that belong to all plants infected by this fungus (Bolton et al., 2006). However, the most common signs of disease are the appearance of white fluffy mycelia covering stems and leaves that then develop into sclerotial bodies (Saharan and Mehta, 2008). Water-soaked lesions are observed on leaf tissue as well, usually expanding rapidly and moving down the petiole into the stem (Bolton et al., 2006).

Plant resistance relies on the ability of the host to recognize pathogens, and different layers of a defensive response exist. The first line of defense is pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) or horizontal resistance, in which recognition of several molecules of pathogen origin (PAMPs) is accomplished by receptors located in the membrane of the host cells (Medhitov and Janeway, 1997) known as pattern recognition
receptors (PRRs). Examples of PAMPs include bacterial flg22, bacterial elongation factor Tu, fungal chitin, ergosterol and β-glucans (Boller and Felix, 2009; Kunze et al., 2004; Klemptner et al., 2014). The recognition of those molecular patterns triggers a series of cascade events that lead to the production of reactive oxygen species (ROS), including superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) molecules (Mishina and Zeier, 2007), expression of defense-related genes and callose deposition in cell walls (Brown et al., 1998).

No source of complete resistance, such as R genes, mediated resistance, has been described against S. sclerotiorum in soybean. However, a number of QTLs have been identified (Kim and Diers, 2000; Arahana et al., 2001; Bolton et al., 2006; Huynh et al., 2010). Twenty-eight QTLs on 15 linkage groups in 500 recombinant inbred lines (RILs) from five populations were described by Arahana et al. (2001). Of these, seven QTLs were identified in more than one population and were located on different linkage groups including regions with mapped resistance genes and resistance gene analogs (Arahana et al., 2001). Phenotypic variation explained by individual QTLs varied from 4-10%, and several alleles implicated in lesion size reduction came from the susceptible parent (Arahana et al., 2001). In a different study, two QTLs were identified; explaining 11-12% of the variation, and one of the resistant alleles also came from the susceptible parent used (Vuong et al., 2008). Two minor QTLs were also identified and, together with the previous two QTLs, explained up to 27% of the phenotypic variation in lesion length in the plants studied (Vuong et al., 2008). Recently, Bastien et al. used GBS to identify four QTLs for resistance to Sclerotinia (Plant Genome doi:10.3835/plantgenome2013.10.0030)

Resistance against S. sclerotiorum has been achieved by introducing the wheat germin gene (gf-2.8) into transgenic soybean lines (Donaldson et al., 2001; Cober et al., 2003). This
oxalate oxidase (OxO) catalyses the oxidation of oxalic acid to carbon dioxide (CO₂) and hydrogen peroxide (H₂O₂) which serves as a defense activator as well (Donaldson et al., 2001). Studies have also been conducted on soybean plants transformed with the decarboxylase gene (oxdc) from Flammulina spp. Second generation transformed plants (T2) showed a delay in disease establishment and progression (Cunha et al., 2010). Oxalate decarboxylase can degrade OA into CO₂ and formate without the requirement of any cofactor. Its high affinity for OA and its activity even in low or neutral pH environments (Kesarwani et al., 2000) make this enzyme a great candidate for SSR resistance. The reduction of infection on detached leaves from T₂ plants ranged between 61-96%, with some plants showing no symptoms even after 92 hpi (Cunha et al., 2010).

In past years, notable innovations in several platforms for genomic and transcriptomic research have provided scientists with potent resources for investigations in different plant species (Mochida and Shinozaki, 2010) and biological processes including plant-pathogen molecular interactions. A number of technologies, such as microarrays, quantitative PCR (qPCR) and RNA-sequencing (RNA-Seq), are currently being used to answer many questions related to plant resistance to diseases.

DNA microarray technology has been an important element in functional genomics for the last decade. Different characteristics offered by this method such as its automation and large-scale processing have contributed to its widespread use (Aharoni and Vorst, 2001). Several biological processes in plants have been investigated using this technique, including plant defense to Sclerotinia (Calla et al., 2009; Calla et al., 2014), environmental stress responses (Rabbani et al., 2003) and fruit ripening (Koia et al., 2012). Calla et al. (2009) reported the use of soybean microarrays to study host gene expression following inoculation of
a susceptible cultivar (Williams 82) and a partially-resistant accession (PI194.639) with *S. sclerotiorum*. The time course analyses showed a number of responses against the attack of the pathogen, including changes in cell wall composition, activation of signaling pathways and biosynthesis of secondary metabolites. An increase in the amount of transcripts related to expansins, isoflavonoids and inositol-related genes were also observed in these experiments (Calla *et al.*, 2009).

Next-generation sequencing, including the widely-used Illumina platform, provided researchers with powerful tools and strategies to explore the transcriptome of several organisms (Mortazavi *et al.*, 2008; Yang *et al.*, 2011; Ozsolak and Milos, 2011). RNA-Seq, as a newly developed technology, is used for transcriptome profiling through deep-sequencing technologies (Wang *et al.*, 2009). This method, based on cDNA sequencing, allows the quantification and mapping of the transcriptomes analyzed (Wang *et al.*, 2009). RNA-Seq offers a set of advantages over the hybridization methods, such as microarray and EST sequencing that have been used in the last decade. Researchers using RNA-Seq are able to map transcribed regions and quantify gene expression with the same set of data. Also, this technology enables discrimination of different isoforms and allele expression of a particular gene (Wang *et al.*, 2009).

Currently, little is known about the mechanisms underlying partial resistance against *S. sclerotiorum* in soybean. Therefore, the objective of this study was to use RNA-Seq to study the defense responses that characterized this phenomenon. Susceptible OAC Shire and partially-resistant OAC Salem plants were inoculated with this pathogen and their transcriptomic profiles analyzed at 1 to 5 dpi. Several defense-related genes were identified and their patterns of expression evaluated. In general, a transient up-regulation of the defense
mechanisms up to 3 dpi was observed in the susceptible line. However, for the partially-resistant cultivar, activation of those defense responses was observed over the entire time course of the experiment in infected plants compared to control, non-infected ones.

4.3. Materials and Methods

Plant material

The cultivars used in this study were OAC Salem as a partially-resistant treatment and OAC Shire as a susceptible treatment (Colber et al., 2003; Auclair et al., 2004). Seeds were planted in Sunshine Mix LA4 soil medium (SunGro, Vancouver, BC) in 9 cm square pots. Three growth chambers set at 25 °C with a photoperiod of 16/8 h (day/night) and light intensity of ~350 µmol photons m⁻¹s⁻¹ were used for a period of 4-5 weeks required for growth of plants to the V4 stage (Fehr et al., 1971).

Inoculation of plants with S. sclerotiorum

Growth medium for culturing S. sclerotiorum consisted of potato dextrose agar (PDA) prepared from potato broth supplemented with 20 g L⁻¹ dextrose and 10 g L⁻¹ agar and inoculated with fungal cultures of isolate 1980 (Auclair et al., 2004a; Auclair et al., 2004b) two days prior to inoculation.

Before inoculation, growth chambers were set to 18 °C. Five-millimeter-diameter plugs of PDA medium from the leading edge with growing mycelia were collected with the back side of 30 µl pipette tips. Agar plugs were positioned 1 to 2 cm below the third node on the stem of plants and secured with Parafilm™ (Thermo Fisher Scientific Inc, USA) following the method described by Lone Buchwaldt (personal communication). One agar plug, containing the actively growing edge of the colony, was placed on each stem section. Three biological replications were used; each of them consisted of 60 plants, 30 from each of the two cultivars (susceptible OAC
Shire and partially-resistant OAC Salem) in each growth chamber and the experimental design followed a randomized complete block design (RCBD). Five plants per time point per genotype were sampled and pieces of inoculated stems of ~2 cm long were collected at 1, 3 and 5 dpi for each genotype. Controls plants (0 dpi) were Parafilm™ wrapped only, and 2 cm stem pieces were collected as well, shortly after applying the Parafilm. Samples were immediately immersed in liquid nitrogen (N₂). The rest of the plants (10 per genotype per replication) were kept in the growth chambers to confirm disease signs and symptoms.

**RNA isolation, cDNA preparation and sequence analysis**

Total RNA was isolated from pooled samples using the PureLink® RNA Mini Kit (Life Technologies™, USA) and treated with DNase using the on-column PureLink® DNase Set (Life Technologies™, USA) according to the manufacturer’s instructions. The quality of the isolated RNA was determined using a NanoDrop 2000 (Thermo Fisher Scientific Inc, USA) and a bioanalyzer 2100 (Agilent Technologies Inc, USA).

cDNA libraries were prepared using the Illumina TruSeq RNA sample preparation kit v2 (Illumina Inc, San Diego, California, USA) for the Illumina platform. Samples were sequenced using the Illumina HiSeq 2500 with TruSeq SBS kit v3-HS chemistry (Illumina Inc, San Diego, California, USA), using the multiplex paired-end protocol (2x101-bases). Insert sizes are summarized in Appendix 4.1 and a standard deviation of +/- 25-base pairs (bp) was accounted for in the analysis. Base calling was performed with the Illumina pipeline 1.8.2 software.

Reads were pre-processed for removing Illumina specific adapters and quality trimming using Trim Galore! v 0.2.8 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Parameters were adjusted to trim low-quality ends from reads (Phred score <20), to remove one
bp from the 3’ end and to retain only reads that still had a valid pair after processing. Appendix B shows tables with the number of read pairs before and after pre-processing.

Sequencing data were mapped to the publicly available *G. max* Williams 82 genome (GMAX-189, ftp://ftp.jgi-psf.org/pub/compgen/phytozome/v9.0/Gmax/annotation/). Files were generated using Bowtie2 v 2.1.0 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) and for each sample, processed reads were aligned to the reference genome using TopHat v 2.0.9 (http://tophat.cbcb.umd.edu/). Bam files (accepted_hits.bam) generated by TopHat were sorted for downstream analysis.

Raw read counts were generated for each gene using HTSeq software v 0.5.4p1, with the HTSeq counting mode “union” (http://www.huber.embl.de/users/anders/HTSeq/doc/count.html). Raw read counts were analyzed for differential expression using the R/Bioconductor package DESeq (http://www.bioconductor.org/packages/release/bioc/html/DESeq.html). The analyses and comparisons performed in the study are shown in Table 4.1. The Benjamini-Hochberg false discovery rate (FDR) was used for nominal p-value correction (Benjamini and Hochberg, 1995) and estimates of absolute expression, reads per kilobase per million reads (RPKM) were generated using the edgeR function RPKM. Transcripts with expression values of log$_2$ (fold change) > 2 or < -2 and $P < 0.001$ were selected as differentially expressed and were considered for further analyses. The experiments and bioinformatic analyses were conducted at The Centre for Applied Genomics (TCAG), Hospital for Sick Children (SickKids), Toronto, Canada.

**Clustering and functional analyses**

K-means clustering analyses were performed using Cluster 3.0 and visualized using TreeView (Hoon *et al.*, 2004). Ten groups of differentially expressed genes in at least one of
the treatments were obtained based on their expression patterns. Gene annotation and ontology analyses were performed using SoyBase Analysis Tools (http://soybase.org/tools.php). Genes were then classified into three categories, i.e. biological process, cellular component and molecular function.

**Validation of RNA-Seq data using qRT-PCR**

qRT-PCR analyses were performed for eight genes to validate the results of the RNA-Seq analyses. These genes were randomly selected from the pool of up- and down-regulated groups that were found differentially expressed ($P<0.001$ and $\log_2$ (fold change) $<-2$ or $>2$) in the study.

First strand cDNA was synthesized using SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, USA) following manufacturer’s instructions. Up to 2.5 µg of RNA was mixed with 4 µl of 5X VILO reaction mix, 2 µl of 10X SuperScript enzyme mix and up to 20 µl of DEPC-treated water. Tubes were gently mixed and incubated for 10 min at 25ºC. cDNA was then diluted with water and qRT-PCR was run in a StepOnePlus Real Time System (Life Technologies, USA). For a total volume of 20 µl reaction, 5 µl of template, 0.8 µl of forward + reverse primers (200 nM), 4 µl of water and 10 µl of 2X PerfeCta SYBR Green FastMix, ROX (Quanta BioScience, USA) were used. The running conditions were as follows: 95 ºC for 30 s to activate the enzyme, 95 ºC for 3 s, 60 ºC for 30 s, and the cycle was repeated 40 times. A melting curve was run after each PCR run for each gene.

Primers were designed using Primer Express® software v2.0 (Thermo Fisher Scientific Inc, USA). Sequences of those primers are provided in Appendix 4.4. Expression levels of selected genes were normalized to cons4 expression (Libault et al., 2008).
Table 4.1. Pair-wise comparisons performed in the susceptible OAC Shire (S) and partially-resistant OAC Salem (PR) cultivars inoculated with *S. sclerotiorum*

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Label</th>
<th>Control (0 dpi)</th>
<th>OAC Shire (dpi)</th>
<th>OAC Salem (dpi)</th>
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<tr>
<td>OAC Shire</td>
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<td>vs</td>
<td>(3 dpi)</td>
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<tr>
<td>OAC Shire</td>
<td>vs</td>
<td>(5 dpi)</td>
<td>OAC Salem</td>
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<td>OAC Shire Control (0 dpi)</td>
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<td>OAC Salem</td>
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<td>Control (0 dpi)</td>
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4.4. Results

**Differentially expressed genes in partially-resistant OAC Salem**

Changes in gene level expression at 0, 1, 3 and 5 dpi were analyzed on susceptible OAC Shire and partially-resistant OAC Salem using RNA-Seq. Read count on the 12 cDNA libraries generated for each cultivar were approximately 136 and 125 million reads, respectively. Reads were then mapped to the soybean reference genome (GMAX-189) with 78.1 % alignments on average for OAC Shire and 77.8% for OAC Salem. Of the 73,320 predicted transcripts in the soybean genome, 54,175 (73.9 %) were quantified in this study based on sequence reads. Raw read counts were then imported and analyzed for differential expression and differentially expressed genes were selected based on a criteria of $P < 0.001$ and $\log_2$ (fold change) $> 2$ or $< -2$.

Four reference genes (Libault *et al.*, 2008) were used for quality control of the RNA-Seq data. The absolute levels of expression of the ATP-binding cassette gene *cons4* (Glyma12g02310), the F-box protein gene *cons6* (Glyma12g05510), the CDPK-related kinase *cons15* (Glyma02g21350) and the ATP-citrate B2 gene *cons5* (Glyma07g36840) were evaluated. Their absolute fold change values ranged from 0.48 to 3.08 ($P > 0.001$) in all cases. These results indicate that no changes in the expression levels of the four reference genes across all treatments in the susceptible OAC Shire or the partially-resistant OAC Salem were observed. Therefore, the RNA-Seq data obtained in this experiment is considered trustworthy and appropriate for further transcriptome analysis (Libault *et al.*, 2008; Kim *et al.*, 2011).
Cluster and gene ontology (GO) analyses

The number of differentially expressed genes in OAC Salem compared to OAC Shire across all treatments in the experiments was estimated ($P<0.001$ and $\log_2$ (fold change) $>2.0$ or $<-2.0$). No differentially expressed genes were found when the cultivars were compared at 1 dpi. However, a total of 2316 genes were identified to be differentially expressed in at least one of the rest of the treatments. Of those, 712 were up-regulated at 0, 3 and/or 5 dpi whereas 1604 were down-regulated (Figure 4.1). The group of up-regulated genes (Figure 4.1, left diagram) contained 14 genes that were over-expressed at all times, including Glyma02g33660 (calcium-binding EF-hand family protein) and Glyma11g11560 (cytochrome P450). Thirty-four genes were up-regulated at 0 dpi. Of those, four genes were exclusively found up-regulated at this time, including Glyma03g04140, which is a leucine rich repeat (LRR) and nucleotide-binding (NB) domains-containing disease resistance protein. At 3 dpi, 441 genes were found to be up-regulated and, of these, 420 were found only at this time point. Four genes were found up-regulated at 0 dpi and 3 dpi, and 3 genes were found up-regulated at 3 dpi and 5 dpi. A total of 237 genes were up-regulated at 5 dpi. Of these, 208 genes were exclusively up-regulated at this time, whereas 12 genes were also found up-regulated at 0 dpi (Fig 4.1, left diagram).

The number of down-regulated genes and their distribution across the treatments are shown on Figure 4.1, right diagram. A total of 16 genes were exclusively observed at 0 dpi, whereas 10 and 1558 genes were observed at 3 and 5 dpi, respectively. For this last time, important genes involved in photosynthesis, metabolism and defense-responses were identified. Several protein kinases and Ca$^{2+}$ dependent protein kinases, such as Glyma04g38150 and the stabilizer of iron transporter SufD, were also found down-regulated at 5 dpi.
Figure 4.1. Venn diagrams of up- and down-regulated genes in partially-resistant cultivar OAC Salem compared to susceptible OAC Shire in at least one of the treatments assessed (0, 3 and 5 days post-inoculation with *S. sclerotiorum*).
Cluster analysis was conducted using Cluster 3.0 software. K-means clustering on differentially expressed genes are shown in Figure 4.2. Groups 1, 3 and 9 showed a similar pattern of expression with up-regulated genes at the beginning of the experiment (0-3 dpi) and a down-regulation at 5 dpi. However, groups 4, 5 and 6 showed no changes on the pattern of expression at 0 dpi, an up-regulation of the genes at 3 dpi followed by a down-regulation at 5 dpi. The rest of the groups (2, 7, 8 and 10) followed independent expression patterns, e.g. genes in group 7 were up-regulated at the beginning of the experiment (0 dpi), down-regulated at 3 dpi, and up-regulated again at 5 dpi. Also, group 8 showed a down-regulation during the first three days of infection followed by an up-regulation of these genes at 5 dpi.

The differentially expressed genes in OAC Salem and OAC Shire were then analyzed and classified according to their function using the SoyBase GO enrichment tool. Genes were grouped according to the categories: biological process, cellular component and molecular function (Figures 4.3 and 4.4). Annotation of the 712 up-regulated and 1604 down-regulated genes was conducted using the Genome Feature Annotation on SoyBase. The report table showed the GlymaID from Gmax version 1.1, the protein match determined via BLASTP of Glyma1.1 primary proteins against Uniref100 (version 11/26/2012) with a minimum e-value <10^{-6} and the protein match determined via BLASTP of Glyma1.1 primary proteins against Arabidopsis thaliana proteins (TAIR10) with a minimum e-value <10^{-6} (Appendix 4.5 and 4.6).
Figure 4.2 Cluster analysis of differentially expressed genes [P<0.001 and log$_2$(fold change) < -2 or > 2] in partially-resistant OAC Salem cultivar compared to susceptible OAC Shire at 0, 3 and 5 dpi with *S. sclerotiorum*, organized into ten k-means clusters. Each line represents a gene that was differentially expressed at least in one of the treatments assessed. Scale on the right indicates levels of expression related to the colors of the figure.
Figure 4.3. Functional classification of up-regulated genes in control (0 dpi) and inoculated (3 and 5 dpi) stems of partially-resistant OAC Salem compared to susceptible OAC Shire plants. A) biological process, B) cellular component and C) molecular function.
Figure 4.4. Functional classification of down-regulated genes in control (0 dpi) and inoculated (3 and 5 dpi) stems of partially-resistant OAC Salem compared to susceptible OAC Shire plants. A) biological process B) cellular component and C) molecular function.
A total of 613 gene ontologies (GOs) were identified for the up-regulated group of genes identified in control (0 dpi), and inoculated (3 and 5 dpi) stems of partially-resistant OAC Salem plants compared to susceptible OAC Shire including GO: 0009873 (ethylene mediated signaling pathway), GO: 0009817 (defense response to fungus) and GO: 0009753 (response to jasmonic acid stimulus). There were genes found in more than one gene ontology. Also, several genes could not be classified into any group. A total of 107 genes were grouped in the biological process category, 472 genes were identified for the cellular component and 270 were allocated to molecular function (Figure 4.3). The largest percentages of genes for cellular component were grouped in the plasma membrane (GO: 0005886), thylakoid (GO: 0009579) and nucleus (GO: 0005634). In the biological process category, photosynthesis (GO: 0015979) and translation (GO: 0006412) were the largest groups whereas sequence-specific DNA binding transcription factors (GO: 0003700) and protein binding (GO: 0005515) were the largest clusters for the molecular function category (Figure 4.3).

Of the total of 1604 genes found down-regulated in OAC Salem compared to OAC Shire, the GO analysis revealed 967 GOs. There were genes found in more than one GO and some genes could not be classified into any gene ontology. A group of 169 genes were grouped under biological process, 873 genes under the cellular component category, and 620 under molecular function term (Figure 4.4). Transport (GO: 0006810) and carbohydrate metabolic process (GO: 0005975) were the biological processes with the greatest number of genes. Sequence-specific DNA-binding TFs (GO: 0003700) and kinase activity (GO: 0016301) were the molecular functions most affected. The plasma membrane (GO: 0005886) and membrane (GO: 0016020) GOs grouped the largest amount of genes in the cellular component category (Figure 4.4).
Defense-related genes

The interactions between plants and pathogens involve a two-way communication system; the plant should be able to recognize a potential pathogen at its surface and the pathogen needs to overcome and manipulate the plant biology for successful infection (Boyd et al., 2013). One of the two levels of immune responses characterized to date is pathogen associated molecular patterns (PAMP)-triggered immunity (PTI). It is the first line of active plant defenses, involves the recognition of PAMPs (Kang et al., 2003; Ausubel et al., 2005) and is key in initial recognition and defense against necrotrophic pathogens (Ausubel et al., 2005). Details on levels of expression of several genes involved in PTI responses are presented below.

PTI-related genes

Recognition of PAMPs by pattern recognition receptors (PRRs) in the host plant is an important and first step for triggering PTI (Monaghan and Zipfel, 2012). Receptor-like kinases (RLKs) are plasma membrane localized PRRs known for their interaction and recognition of PAMPs (Ausbel et al., 2005; Monaghan and Zipfel, 2012). A receptor-like kinase, containing a cysteine-rich domain (Glyma10g40001) was found up-regulated around 5-fold in the partially-resistant OAC Salem compared to the susceptible OAC Shire at 5 dpi (Figure 4.5). Other RLK genes were identified as differentially expressed in both susceptible and partially-resistant cultivars at 3 and 5 dpi compared to their respective controls (Figure 4.6 and Appendix 4.6).

Plants use secondary messengers to perceive and process the information received (Chebal et al., 2013). One of these messengers is the calcium ion (Ca$^{2+}$). High levels of free Ca$^{2+}$ in a cell compartment could act as signals carrying information that is later translated into
Figure 4.5. Putative PTI-related genes differentially expressed (P<0.001, log₂ (fold change) < -2 or >2) in partially-resistant OAC Salem compared to susceptible OAC Shire at 0, 3 and 5 days post-inoculation with *S. sclerotiorum*. 
Figure 4.6. Heat map of putative PTI-related genes differentially expressed (P<0.001, \( \log_2 \) (fold change) <-2 or >2) in susceptible OAC Shire (S) and partially-resistant OAC Salem (PR) at 3 and 5 dpi with \textit{S. sclerotiorum} compared to their controls. Increasing shades of colors represent increasing levels of expression. High levels of expression are represented in red, low levels of expression are represented in yellow, (*) represents significant differences.
| Glyma06g47771 | 6.32 | 2.15 | 22.38 | 6.14 |
| Glyma11g39961 | *3673.00 | *1622.00 | 280.93 | *385.89 |
| Glyma14g08810 | 841.20 | 626.17 | 158.99 | 363.57 |
| Glyma07g10440 | 209.75 | 414.88 | 20.57 | 8.57 |
| Glyma09g07470 | *17.68 | 33.32 | 21.53 | 10.59 |
| Glyma07g30990 | 8.13 | 6.05 | 9.56 | 3.35 |
| Glyma08g12100 | 47.62 | 29.64 | 52.80 | 20.33 |
| Glyma11g24410 | 64.66 | 101.03 | 70.64 | 33.50 |
| Glyma16g03875 | *7.74 | 11.18 | 12.57 | 4.39 |
| Glyma15g18640 | *168.44 | *36.03 | *135.69 | *121.09 |
| Glyma11g06150 | *3627.83 | *1122.66 | 348.13 | 304.68 |
| Glyma18g07140 | *194.76 | 87.52 | 542.73 | 657.02 |
| Glyma05g34760 | 51.13 | 16.22 | 210.12 | 282.31 |
| Glyma08g04920 | *12.55 | 3.02 | 52.48 | 47.88 |
| Glyma08g20420 | 1239.33 | 672.06 | 977.52 | 1705.36 |
| Glyma07g00630 | *6.91 | 5.77 | 7.25 | 2.00 |
| Glyma07g23760 | *4.86 | 4.05 | 7.25 | 1.78 |
| Glyma09g05970 | *113.23 | *49.86 | *532.34 | *613.06 |
| Glyma15g42220 | *1117.65 | *543.80 | 118.87 | *1.34 |
| Glyma02g39610 | *140.69 | 55.13 | *99.03 | *1.82 |
| Glyma01g43190 | *3.69 | *1.43 | *19.58 | *2.49 |
| Glyma08g15620 | 8.78 | 3.37 | 8.56 | 8.48 |
| Glyma06g12960 | 12.73 | 7.04 | 38.93 | 139.00 |
| Glyma07g07270 | 26.37 | 10.47 | 21.88 | 21.33 |
| Glyma08g30300 | *8.76 | 3.03 | 9.41 | 11.31 |
| Glyma07g11910 | *8.27 | 3.03 | 5.82 | 6.00 |
| Glyma01g31921 | *218.44 | *77.61 | *200.10 | *222.69 |
| Glyma02g39870 | *35.39 | 15.30 | *35.24 | *44.70 |
| Glyma03g05220 | 180.37 | 47.58 | *88.23 | *84.97 |
| Glyma18g44030 | 42.96 | 6.24 | *151.57 | *53.64 |
| Glyma02g12490 | 8.50 | 12.79 | 4.63 | 2.77 |
| Glyma07g02630 | *103.96 | *147.10 | *181.72 | *46.37 |
| Glyma08g23380 | 23.61 | 31.27 | 66.37 | 24.30 |
| Glyma13g44730 | 158.16 | 164.25 | *133.60 | *43.05 |
| Glyma14g11920 | *156.98 | 478.18 | 170.06 | 94.17 |
| Glyma15g00570 | 36.98 | 18.70 | *22.68 | *4.13 |
| Glyma02g04820 | *5710.96 | *2777.07 | *357.66 | *330.13 |
| Glyma13g42210 | 4324.30 | 1934.21 | *220.42 | 849.27 |
| Glyma10g06880 | 4476.53 | 2574.72 | *223.53 | *823.97 |
| Glyma18g07050 | 4707.35 | 2228.71 | *273.23 | *602.16 |
| Glyma11g14970 | 1.64 | 18.06 | 2.29 | 1.25 |
| Glyma01g37020 | 0.50 | 0.10 | 0.08 | 0.60 |
| Glyma04e02580 | *0.16 | *0.04 | *0.01 | *0.52 |
| Glyma11g08250 | 0.28 | 0.13 | 0.05 | 0.68 |
| Glyma18g05890 | *9.86 | 5.82 | *8.06 | *8.71 |

**OAC Shire**

**S 3dpi** | **S 5dpi**
--- | ---
**RLKs**
**CaM/CML**
**ACA9**
**ACA11**
**RbOH**
**MKS1**
**MPK4**
**MPK9**
**WRKY33**
**WRKY4**
**WRKY40**
**PR-3**
**PR-5**
**PR-14**

- 104 -
specific biological responses (Chebal et al., 2013). Several Ca\(^{2+}\) pumps, including auto-
inhibited Ca\(^{2+}\)-ATPases (ACAs) and Ca\(^{2+}\) sensors [calmodulin (CaM) and calmodulin-like (CML) genes] were detected in this study. Glyma09g35970 (ACA11) showed increased levels of expression in the partially-resistant cultivar at 5 dpi compared to the susceptible line with levels as high as 5-fold (Figure 4.5). Transcript levels at 3 dpi and 5 dpi in both cultivars were compared to their respective controls at time 0 dpi. Two genes were found up-regulated in the partially-resistant OAC Salem, including Glyma07g10440 at 3 and 5 dpi and Glyma09g07470 at 5 dpi, both containing a calmodulin-binding domain (Figure 4.6). Glyma07g10440 was also found up-regulated in susceptible OAC Shire, but only at 3 dpi compared to its control at 0 dpi. Four calmodulin binding-like genes (Glyma07g30990, Glyma08g12100, Glyma11g24410 and Glyma16g03875) and two ACA homologues (Glyma07g00630 and Glyma08g23760) were found up-regulated, only at 3 dpi in OAC Shire (Figure 4.6). An additional group of ACAs and CaM/CML genes that were found differentially expressed in both susceptible and partially-resistant tissues compared to their respective controls (0 dpi), are shown in Figure 4.6 as well.

The production of reactive oxygen species (ROS) is considered an important step in defense signaling (Wojtasezek, 1997). It is known that NADPH oxidases or respiratory burst oxidases (RbOH) are activated via second messenger Ca\(^{2+}\) (McAinsh and Pittman, 2009). Three RBOH–like genes were identified as differentially expressed in the cultivars compared to their controls. Glyma19g42220 was particularly up-regulated in both cultivars but mostly in the susceptible OAC Shire (Figure 4.6). In general, RBOH genes had high levels of expression at 3 dpi that were reduced at 5 dpi in the partially-resistant cultivar whereas the high levels of expression of those genes were steady throughout the entire experiment in the susceptible cultivar.
Ca\textsuperscript{2+} influxes also activate a series of mitogen-activated protein kinases (MAPKs), leading to the activation of transcription factors (WRKY transcription factors) and defense related gene expression (Chebal et al., 2013). The role of MAPK cascades is to serve as signal transducers using protein phosphorylation/de-phosphorylation cycles to channel information (Hamel et al., 2012). In this study several MAPKs were detected as differentially expressed genes. Glyma07g07270, a MPK4, was found up-regulated approximately 26-fold in the susceptible cultivar at 3 dpi compared to the control, but this up-regulation was transient since the expression levels were reduced by approximately 60% at 5 dpi (Figure 4.6). MKS1 is a known substrate for MPK4, and together they form a multi-protein complex necessary for the activation of WRKY TFs (Rispail et al., 2009). The transcript levels of MKS1-like genes such as Glyma06g12960 were elevated at 5 dpi in the partially-resistant accession compared to its control (139-fold, Figure 4.6). However, for the susceptible genotype, no changes in the level of expression were observed (Figure 4.6).

Previous studies have shown that infection by \textit{S. sclerotiorum} deregulates guard cells in infected plants and, thus, causes wilting symptoms (Guimaraes and Stotz, 2004). Genes related to guard cell closure mechanisms were surveyed in this study. MPK9 is a known MAPK preferentially expressed in guard cells and functions as a positive regulator downstream of ROS in ABA-signaling stomatal closure (Jammes et al., 2009), therefore, contributing to the first line of defense against pathogens (Jammes et al., 2011). Glyma07g11910, a MPK9-like encoding gene was found up-regulated approximately 8-fold in the susceptible accession at 3 dpi compared to its control (Figure 4.6). However, no changes in the levels of expression of this gene were observed in the partially-resistant cultivar. Glyma09g30300, also a MPK9, was
found up-regulated in OAC Salem approximately 4-fold compared to OAC Shire (Figure 4.5), and 11-fold compared to its control at 5 dpi (Figure 4.6).

WRKY transcription factors (WRKY TFs) contribute to the activation of defense-related genes via MAPKs cascades (Zhen et al., 2006). Recent studies have shown the interaction between WRKY33 and MPK4, a kinase that is a repressor of SA-dependent resistance, allowing the expression of JA/ethylene-related genes (Zhen et al., 2006). WRKY33 homologues were found up-regulated in both partially-resistant and susceptible cultivars (Figure 4.6). Interestingly, the up-regulation of Glyma02g39870 and Glyma18g44030 in the susceptible cultivar only occurred at 3 dpi, no changes were detected at 5 dpi compared to its control.

Pathogenesis-related proteins are part of the defense responses against plant pathogens (Stintzi et al., 1993). Their physicochemical properties enable them to resist harsh environments, such as acidic pH and proteolytic cleavage, which characterize the cellular component in which they are produced (Stintzi et al., 1993). Several PR-like genes were examined in both OAC Shire and OAC Salem cultivars, including PR-3, PR-5 and PR-14 (Figure 4.6). Glyma12g25740, a chitinase A was found up-regulated at 5 dpi in OAC Salem compared to OAC Shire (Figure 4.5). Also, two PR-3 gene homologues, Glyma02g04820 and Glyma13g42210, were found differentially expressed in both cultivars at both times compared to their controls at 0 dpi (Figure 4.6).

Five transcripts encoding a thaumatin-like protein or PR-5 were identified as differentially expressed in OAC Salem compared to OAC Shire, three of them at 3 dpi (Glyma01g37020, Glyma04g02580 and Glyma11g08250) and two at 5 dpi (Glyma10g07050 and Glyma11g14970). These genes were also analyzed in both cultivars at 3 and 5 dpi versus
their controls. Only Glyma10g06880, Glyma10g07050 and Glyma04g02580 were found up-regulated during the course of the experiment in both genotypes compared to their controls (Figure 4.6).

Lipid transfer proteins have been described for their role in plant defenses (Ge et al., 2003). Transcripts encoding a PR-14, a lipid transfer protein, were surveyed in this study. Glyma18g05890 was found up-regulated at 5 dpi in OAC Salem compared to OAC Shire. High levels of expression of this gene were found as well in both cultivars at 3 and 5 dpi compared to their respective controls. Notably, the up-regulation of PR-14 in the susceptible OAC Shire was transient as it was reduced between 3 and 5 dpi, while in OAC Salem the up-regulation remained stable over 3 and 5 dpi (Figure 4.6).

Peroxidases are involved in several cellular processes due to their peroxidative or hydroxylic properties (Lüthje et al., 2010). These enzymes play an important role in the detoxification of the cellular environment during oxidative stress by cleavage of several molecules in the presence of H₂O₂, converting it to water (Lüthje et al., 2010). Twenty-six peroxidases homologues were identified with differential expression in one or both cultivars, in at least one of the treatments evaluated (Figure 4.7). Three genes, including Glyma02g15280, Glyma07g39020 and Glyma17g01720, were found up-regulated in the partially-resistant line compared to the susceptible cultivar at 3 dpi (Figure 4.7). Also, a different group of seven genes, including Glyma01g36780, Glyma07g39020, Glyma08g17850, Glyma09g00480, Glyma11g05300, Glyma11g08520, Glyma13g38310 and Glyma17g01720 were found down-regulated in the partially-resistant cultivar compared to the susceptible line at 5 dpi (Figure 4.7). Sixteen genes were found significantly up-regulated in susceptible OAC Shire at 3 dpi compared to its control at 0 dpi, including Glyma02g05930, Glyma02g40040,
Glyma03g04710, Glyma03g04740, Glyma03g04760, Glyma04g39860, Glyma06g15030, Glyma09g02600, Glyma09g41440, Glyma09g41450, Glyma14g38210, Glyma15g13500, Glyma15g13560, Glyma16g06030, Glyma16g27900 and Glyma20g31190.

Some of these genes were also found up-regulated at 5 dpi compared to 0 dpi in the same susceptible line (Figure 4.7). In the partially-resistant line, a total of eight genes were found up-regulated during the entire experiment, including Glyma02g05930, Glyma02g40040, Glyma04g39860, Glyma06g15030, Glyma15g13500, Glyma16g06030, Glyma16g27900 and Glyma20g31190 (Figure 4.7). Glyma20g31190 and Glyma15g13500, two peroxidase super-family members, and Glyma16g27900, a peroxidase 55 were found up-regulated throughout the experiment in both cultivars compared to their respective controls.

Lignin biosynthesis

Lignins are the result of oxidation of 4-hydroxyphenylpropanoids and contribute to the secondary structure of the cell wall (Vanholme et al., 2010). Lignins are deposited throughout the developmental stages of plant growth, but are also involved in plant defense responses against pathogens since their biosynthesis can be induced under certain biotic stresses (Vanholme et al., 2010).

Several enzymes involved in different steps of lignin biosynthesis were surveyed in this study, including enzymes common to multiple branches of the phenylpropanoid pathway such as phenylalanine ammonia lyase (PAL), cinnamate 4-hydrolase (C4H), and 4-coumarate: CoA ligase (4CL) as well as enzymes specific to the lignin branch: cinnamyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). PAL is the enzyme catalyzing the conversion of
**Figure 4.7.** Differentially expressed peroxidase homologues (P< 0.001 and log₂ (fold change) < -2 or >2) in susceptible OAC Shire (S) and partially-resistant OAC Salem (PR) cultivars inoculated with *S. sclerotiorum*. First three columns indicate comparisons between genotypes, the rest of the columns indicate comparisons of the cultivars at 3 and 5 dpi with their respective controls (0 dpi).
phenylalanine into cinnamic acid, the first step in the phenylpropanoid pathway and the biosynthesis of lignins (Vanholme et al., 2010). Two PAL-like genes (Glyma03g33890 and Glyma02g47940) were up-regulated in susceptible OAC Shire at 3 dpi. However, both transcripts were also found up-regulated up to 7-fold at 3 and 5 dpi in OAC Salem compared to its control (Figure 4.8). C4H is responsible for converting cinnamic acid into ρ-coumaric acid and Glyma20g24810, a C4H-like encoding gene, was found up-regulated in both genotypes at 3 and 5 dpi compared to their controls. The induced expression of C4H in OAC Shire was transient, high at 3 dpi but waning at 5 dpi while in OAC Salem expression was induced at 3 dpi and further increased at 5 dpi.

Further in the phenylpropanoid/lignin biosynthesis pathway, ρ-coumaric acid is then converted into ρ-coumaroyl CoA by means of 4CL. Five 4CL-like transcripts were identified in the pool of genes. One of these, Glyma17g07170 was found down-regulated in the susceptible OAC Shire at 3 dpi and in OAC Salem at both 3 and 5 dpi. However, Glyma01g44270 and Glyma11g01240 were found up-regulated in OAC Shire at 3 dpi. High transcript levels of these two genes were observed throughout the experiment in OAC Salem (Figure 4.8).

Later in the biosynthesis pathway, substrates such as ρ-coumaroyl CoA and feruloyl CoA are converted into their respective aldehydes with the involvement of CCR, the first committed step of the lignin branch off the phenylpropanoid pathway. A total of 17 CCR-like genes were predicted in this study. Due to the homology between CCR and dihydroflavonol-4-reductase (DFR), an enzyme involved in anthocyanin biosynthesis, and also because of the ambiguity of their annotations (Lacombe et al., 1997), these sequences were aligned to a well annotated CCR in Eucaliptus gunnii (Lacombe et al., 1997). Of them, only Glyma08g23310 was found with high levels of homology to EgCCR. These two genes share sequence homologies and also the protein sequence that describes the catalytic centre in E. gunnii,
NWYCY, is similar to that in Glyma08g23310 (Appendix 4.7). This gene was found down-regulated in the susceptible cultivar at 3 dpi approximately 3-fold compared to its control at 0 dpi (Figure 4.8).

The final step in the synthesis of lignin is the conversion of sinapaldehyde, conyferaldehyde and/or ρ-coumaraldehyde into their respective alcohols. The enzyme responsible for this step is cinnamoyl alcohol dehydrogenase (CAD). Glyma08g15420, which is a CAD-like gene, was found down-regulated in both cultivars but by approximately 5-fold in the susceptible cultivar; whereas the expression of another CAD gene, Glyma14g40170, was increased in both cultivars, but much more (6-fold) in the partially-resistant cultivar (Figure 4.8).

**JA-related genes**

It is known that the biosynthesis of JA is induced either as a response to wounding or the attack of necrotrophic pathogens (Farmer and Ryan, 1992). Recent studies have confirmed the role of known components in JA signaling presented by Farmer and Ryan (1992). Also other studies have described the role of E3 ubiquitin ligase activity in the degradation of certain signaling components (Turner et al., 2002). The release of linolenic acid from membrane phospholipids is the first step in the synthesis of JAs and the enzymes involved in the process are phospholipases (PLs). Several transcripts encoding genes for PLs were analyzed in this study. Glyma01g00541 and Glyma20g08070 were found down-regulated at 3 dpi in the susceptible line compared to 0 dpi. Also Glyma07g38890 was found significantly up-regulated at 3 dpi (Figure 4.9). However, no significant changes were found when the partially-resistant cultivar was compared to its control or the susceptible cultivar (Figure 4.9).
Figure 4.8. Differentially expressed genes (P< 0.001 and log$_2$ (fold change) < -2 or >2) of the phenylpropanoid pathway, leading up to the biosynthesis of lignins in susceptible OAC Shire (S) and partially-resistant OAC Salem (PR) cultivars inoculated with *S. sclerotiorum*. First three columns compare the genotypes; the remaining four columns show gene expression of the cultivars at 3 and 5 dpi compared to their respective controls (0 dpi). PAL: phenylalanine ammonia lyase, C4H: cinnamate 4-hydrolase, 4CL: 4-coumarate: CoA ligase, CCR: cinnamoyl-CoA reductase and CAD: cinnamoyl alcohol dehydrogenase.
As a result of PLs actions, linolenic acid is released and then converted into 13-hydroperoxy-octadecatrienoic acid (13-HPOT) by lipoxygenases (LOXs). This step was also investigated in the study. The transcript levels of Glyma13g42330, a LOX1, were elevated 8-fold in the partially-resistant accession compared to the susceptible cultivar at 3 dpi. Changes in expression of other LOX-like genes were also examined. Findings indicated that the levels of expression of Glyma08g20220 (LOX1) were not detectable in the susceptible line, however, an up-regulation up to 9-fold was observed in the partially-resistant line at 3 and 5 dpi (Figure 4.9). Interestingly, Glyma10g29490 and Glyma13g42330 were down-regulated up to 5-fold in the susceptible line at 3 dpi. Similar results were found in the partially-resistant line, but only at 5 dpi. LOX2-like homologues were also analysed, Glyma12g05840 and Glyma20g11610 were found down-regulated at 3 dpi to a lesser degree in the partially-resistant line as compared to the susceptible one. Glyma12g05840 was also found significantly down-regulated in the susceptible cultivar at 3 dpi compared with its control at 0 dpi (Figure 4.9).

Allene oxidase cyclase (AOC) participates in the stereospecific cyclization of the allene oxide to a stable phytodienoic acid (Vanholme et al., 2010). An AOC-like gene (Glyma18g51600) was found down-regulated in the susceptible line at 3 dpi whereas no significant changes in levels of expression were observed in the partially-resistant cultivar.

The following step in the biosynthesis of JAs is catalyzed by 12-oxo-phytodienoic acid reductase (OPR3). Glyma13g16950 and Glyma17g05770 were found up-regulated at 3 dpi in the susceptible and partially-resistant line, respectively. Glyma13g16950 was also significantly up-regulated in the partially-resistant line at 5 dpi (Figure 4.9).

The synthesis of JAs is regulated by a large battery of genes including jasmonate ZIM-domain (JAZ) proteins, jasmonate resistant 1 (JAR1), coronate insensitive 1 (COI1) and E3 ubiquitine ligase genes (Santner and Estelle, 2007). JAZ proteins are transcriptional regulators
that control the transcription of JA-induced genes acting as repressors by binding to MYC2, a basic helix-loop-helix (bHLH) and positive regulator of JA-related gene expression (Santner and Estelle, 2007). Glyma13g17180, a JAZ1-like gene was found up-regulated at 3 and 5 dpi in the partially-resistant line; in the susceptible line, this transcript was up-regulated to a greater degree at 3 dpi compared to 5 dpi. Furthermore Glyma09g08290 and Glyma15g09980, a JAZ1 and a JAZ8 gene, showed higher levels of expression in the susceptible line (3 and 5 dpi) than the partially resistant line (3 and 5 dpi) (Figure 4.10). These results indicate greater repression of JA-induced responses in the susceptible cultivar compared to the partially-resistant line.

MYC2-like genes were also identified in this study. No changes in expression were observed in the partially-resistant OAC Salem compared to the susceptible accession OAC Shire and no changes in levels of expression were noted within OAC Salem. However, Glyma01g12740 was down-regulated at 3 dpi in the susceptible line compared to 0 dpi.

JAR1 conjugates JA to isoleucine (Ile), promoting the interaction of JA-Ile with JAZ. As a result, JAZ is polyubiquitinated and degraded by the 26S proteasome, releasing MYC2 and, consequently, inducing the expression of JA-related defense responses (Santner and Estelle, 2007).
Figure 4.9. Levels of gene expression related to biosynthesis of JA in the partially-resistant OAC Salem (PR) and the susceptible OAC Shire (S) cultivars inoculated with *S. sclerotiorum*
Figure 4.10. Levels of gene expression related to the regulation of JA-induced responses in the partially-resistant OAC Salem (PR) and the susceptible OAC Shire (S) cultivar inoculated with *S. sclerotiorum*. 
Glyma06g37401, a JAR1 homologue exhibited high levels of expression up to 9-fold as early as 0 dpi in the partially-resistant line compared to the susceptible OAC Shire (Figure 4.10). No changes in E3 ubiquitin ligase expression levels were detected in the cultivars at 3 and 5 dpi compared to their controls.

**Ethylene-related genes**

Ethylene regulates many aspects of the biological development in plants, including root hair development, nodulation, flower senescence, and response to abiotic and biotic stimuli (Wang et al., 2002). Several genes involved in the biosynthesis of ethylene and the regulation of the response to this hormone were examined in this study. The first step in ethylene biosynthesis is the conversion of methionine (Met) into S-adenosylmethionine (S-AdoMet) catalyzed by the enzyme S-AdoMet synthetase (SAM synthetase). Glyma10g28500, a SAM synthetase-like gene was found down-regulated in both OAC Shire and OAC Salem cultivars compared to their controls by 4-fold at 3 dpi and 5 dpi, respectively (Figure 4.11). Also, this transcript was found down-regulated in the partially-resistant line compared to the susceptible one at 5 dpi (Figure 4.11).

The other important precursor in ethylene biosynthesis is 1-aminocyclopropane 1-carboxylic acid (ACC). The synthesis of this precursor from S-AdoMet is catalyzed by ACC synthase. Two transcripts encoding ACC synthases, Glyma01g42290 and Glyma11g03070, were down-regulated in OAC Shire at 3 dpi and OAC Salem at 5 dpi (Figure 4.11). For both genes, there’s a decreased level of expression at 5 dpi in the partially-resistant compared to the susceptible line (Figure 4.11). Glyma08g02130 was found up-regulated in the susceptible line, with increases in level of expression up to 6-fold at 3 dpi and 5 dpi. The same gene was found up-regulated in partially-resistant OAC Salem close to 8-fold during the same time-points.
Figure 4.11. Levels of gene expression related to the biosynthesis of ethylene in the partially-resistant OAC Salem (PR) and the susceptible OAC Shire (S) cultivars inoculated with *S. sclerotiorum*. 
ACC is then converted into ethylene by the action of the ACC oxidase. The level of expression of Glyma07g15480, an ACC oxidase homologue, was found elevated ~ 7-fold in the partially-resistant line at 5 dpi compared to 0 dpi (Figure 4.11).

Regulation of ethylene-related responses is finely tuned by a number of receptors, positive and negative regulators and ethylene responsive transcription factors (Ecker and Davis, 1987; Lorenzo et al., 2003). Glyma10g33240, an ethylene receptor type 2 (ETR2), was found up-regulated at 3 and 5 dpi in the susceptible and partially-resistant cultivars, respectively. Furthermore, Glyma20g34420, also an ETR2, was found significantly up-regulated ($P=0.000689$) exclusively in the partially-resistant line at 5 dpi (Figure 4.12). The ethylene insensitive 3 family protein (EIN3) and several ethylene responsive element binding proteins (ERFs) were also surveyed in this study. The expression of Glyma02g44220, an EIN3-like gene, was reduced by 2-fold in the susceptible line at 3 dpi. In addition, a number of ERFs were found up-regulated in both the susceptible and partially-resistant line at either 3 dpi (e.g. Glyma01g41520) or at 3 dpi in the susceptible line and 5 dpi in the partially-resistant line (i.e. Glyma13g30720, Glyma17g15460 and Glyma17g15480). Glyma15g08560, an ERF-like gene was exclusively up-regulated in the susceptible line at 3 dpi (Figure 4.12). The levels of expression of Glyma03g26310, an AP2/ERF gene were found elevated ~ 2-fold in OAC Salem compared to OAC Shire at 5 dpi (Figure 4.12).
Figure 4.12. Levels of gene expression related to regulation of ethylene-induced responses in the partially-resistant OAC Salem (PR) and the susceptible OAC Shire (S) cultivars inoculated with *S. sclerotiorum*. 
Polygalaturonase-inhibiting proteins (PGIPs)

PGIPs are leucine-rich proteins located in extracellular compartments that bind fungal polygalacturonases and, thus, limit the action of these CWDEs (Di Mateo et al., 2006). Two PGIP-encoding transcripts were found differentially expressed in this study. Glyma05g25370 and Glyma19g32700 were found up-regulated approximately 10- and 12-fold, respectively, in susceptible OAC Shire at 3 dpi compared to its control. Increases of expression of Glyma19g32700 were also observed at 5 dpi compared to 0 dpi in this cultivar. For OAC Salem, the levels of expression of those transcripts were 10-fold higher than its control at 0 dpi and were exclusively observed at 5 dpi.

Validation of RNA-Seq results using qRT-PCR

Several differentially expressed genes detected in the partially-resistant cultivar OAC Salem using RNA-Seq were validated by qRT-PCR. Eight genes involved in different biological processes including defense and photosynthesis were evaluated (Figure 4.13). Glyma08g37070 (IAA9), Glyma03g28130 (auxin responsive family protein 1), Glyma09g05650 (S-adenosyl-methyl transferase), Glyma01g43120 (MYB103), Glyma06g00440 (MLO1), Glyma01g43420 (WRKY41), Glyma08g08770 (chlorophyll a/b binding protein 1) and Glyma02g00870 (Ethylene responsive factor 1) were shown to be up-regulated in at least in one of the time points analyzed by RNA-Seq. In most cases, results from qRT-PCR were consistent with those of RNA-Seq, indicating the same direction of expression, and with RNA-Seq showing equal or higher levels of expression that qPCR. However, in some cases, such as Glyma02g00870 and Glyma01g43120, the levels of expression were slightly higher based on qRT-PCR than RNA-Seq. These results underline the fidelity of the RNA-Seq technology for plant-pathogen molecular interactions studies.
Figure 4.13. Validation of RNA-Seq data using qRT-PCR of eight differentially expressed genes in the partially-resistant cultivar OAC Salem compared to the susceptible OAC Shire accession.
Fold change

Days post-inoculation

Fold change

Days post-inoculation

Fold change

Days post-inoculation

Fold change

Days post-inoculation

Fold change

Days post-inoculation

Fold change

Days post-inoculation

Fold change

IAA 9 (Glyma08g37070)

Auxin responsive family protein (Glyma03g28130)

S-adenosyl-Methyltransferase (Glyma09g05650)

MYB103 (Glyma01g43120)

MLO 1 (Glyma06g00440)

WRKY41 (Glyma01g43420)

Chlorophyll a/b binding protein 1 (Glyma08g08770)

Ethylene Responsive Factor 1 (Glyma02g00870)
4.5. Discussion

The immune system of plants present two layers of defense against pathogens, PTI and ETI (Kang et al. 2003; Ausubel 2005; Chisholm et al. 2006). PTI is characterized by the recognition of molecules of pathogen origin, such as PAMPs that bind to membrane receptors (PRRs) triggering a series of signaling cascades that activate defense responses, including the oxidative burst, MAPK cascades, WRKY TFs and the expression of JA/ET-related genes (Ausubel 2005). Some pathogens, including most biotrophs, have developed mechanisms to counteract this first layer of immune responses through the secretion of effectors that inhibit the activation of PTI (Boyd et al., 2013). As a result of years of co-evolution, plants synthesize proteins that can recognize those effectors and trigger ETI (Boyd et al., 2013). This response is characterized by a strong expression of PR genes and, ultimately, leads in some cases to a hypersensitive response (HR) (Kang et al. 2003). In general, ETI and PTI are often uneffective in stopping necrotrophic fungi, including S. sclerotiorum, and the activated plant defense responses serve to only slow or gradually inhibit the pathogen. Delimitations between the PTI, ETI and general defense responses are often unclear (Zhang et al., 2010).

Next generation sequencing, specifically RNA-Seq, has been used in this study to analyze the transcriptomic profile of SSR-infected soybean stems during early to advanced stages of infection in susceptible and partially-resistant cultivars. A total of 2316 genes were found differentially expressed and, of these, 712 genes were found up-regulated, whereas the log₂ (fold change) of 1604 genes were less than -2 in at least one treatment. Gene ontology and functional analyses were conducted using the SoyBase GO enrichment tool, and genes were grouped according to their molecular function, biological process and cellular component. For the up-regulated genes, 43% were classified as photosynthesis-related, 18.2 % and 15.2% of the
genes were classified as sequence-specific DNA binding TFs and protein binding, respectively, and 14.2% were plasma membrane-related genes.

Of particular interest in this study were genes related to general defense signaling responses. Most of the genes related to the oxidative burst and MAPKs cascades were found up-regulated in the partially-resistant line compared to the susceptible one. In particular, Glyma10g40001, a PRR also known as receptor-like kinase (RLK) was found up-regulated in the partially-resistant line compared to the susceptible at 5 dpi. Although an increase of expression was observed in both genotypes compared to their controls at 0 dpi, this increase was transient, occurring only at 3 dpi in the susceptible line. These results suggested that detection of PAMPs is stronger and last longer in the partially-resistant line compared to the susceptible one. The perception of PAMPs leads to an increase of ion fluxes including Ca$^{2+}$, H$^+$, Cl$^-$ and K$^+$ (Chebal et al., 2013). The study of Ca$^{2+}$ pumps was of great interest in this research, since high levels of free Ca$^{2+}$ in a cell compartment could act as specific signal transducer carrying information, in this case, activating enzymes such as RbOHs and MAPKs (Chebal et al., 2013). ACA11, an auto-inhibited Ca$^{2+}$ ATPase was found up-regulated as high as 5-fold in the partially-resistant line compared to the susceptible cultivar at 5 dpi. Several CaM and CML genes were found differentially expressed in both cultivars as well. In general a transient activation of those genes was found in the susceptible cultivar (Figure 4.6), with an increased expression at 3 dpi followed by decreased expression at 5 dpi. These results could indicate an increase of Ca$^{2+}$ fluxes only during the initial stages of infection in the susceptible cultivar. Since Ca$^{2+}$ fluxes act as a secondary messenger for RbOH and the production of ROS (McAinsh and Pittman, 2009; Bose et al., 2011) and the activation of MAPK cascades (Hamel et al., 2012), a decrease in Ca$^{2+}$ fluxes during the advanced stages of infection could affect the
expression of defense responses in this cultivar. Also, the redox homeostasis in the cellular environment in this cultivar OAC Shire could be affected.

Several studies have confirmed the detrimental function of ROS in the cellular environment since they could lead to damage in proteins, lipids and nucleic acids (Alscher et al., 1997). However, the rapid and transient production of ROS is crucial in plant defense against a wide range of pathogens (Wojtaszek, 1997). For this reason, the regulation of the redox homeostasis in the plant cell is an important factor in immune responses (Williams et al., 2011). In general, RbOH gene homologues (RbOHD, RbOHF) were found up-regulated in both cultivars at 3 dpi, with a decrease in expression at 5 dpi only in the partially-resistant line OAC Salem. On the other hand, a steady up-regulation is observed in the susceptible line suggesting a strong manipulation of host biological mechanisms related to the oxidative burst by the pathogen. These results are in accordance with recent studies in Nicotiana benthamiana, which indicated the ability of S. sclerotiorum to manipulate the oxidative burst during the establishment of infection by decreasing this defense mechanism at first, then increasing programmed cell death (PCD) processes due to secretion of OA (Williams et al., 2011), which might be killing cells and conditioning them for infection by chelating iron away from host proteins (Calla et al. 2014).

The activation of MAPK cascades as a consequence of Ca\(^{2+}\) fluxes leads to the activation of transcription factors and the expression of defense related genes. Glyma07g07270, a MPK4, was found up-regulated approximately 26-fold in the susceptible cultivar at 3 dpi compared to the control, with a reduction of the up-regulation by 60% at 5 dpi. Several studies have indicated the role of MPK4 and other MAPKs (e.g. MPK6) in the activation of some WRKY TFs, including WRKY33 (García-Pedrajas et al., 2008; Rispail et
This protein forms a multi-protein complex with its substrate MKS1 and WRKY33 that is stable in the absence of pathogen stimuli, preventing WRKY33 from activating the expression of defense-related target genes (Andreasson et al., 2005; Qiu et al., 2008). The activation of MPK4 as a consequence of pathogen perception by PRRs, results in phosphorylation of MKS1, followed by the release of WRKY33 and expression of defense-related genes (Qiu et al., 2008). The fact that the activation of MPK4 lasted only the first 72 h of infection in the susceptible line, could indicate either a manipulation by S. sclerotiorum of PTI-related processes or a progressive shutdown of several biological processes as a consequence of the high toxicity created by the OA-manipulated oxidative burst and PCD. Similar results were found for WRKY33 gene homologues. A transient up-regulation in the susceptible line at 3 dpi, followed by a decrease in its expression could indicate low to null expression of PTI-related responses in this cultivar since WRKY33 has been implicated in the positive regulation of the defense responses against necrotrophic pathogens such as Alternaria brassicicola and Botrytis cinerea (Zheng et al., 2006). Other WRKY TFs, such as WRKY40 and WRKY4 gene homologues related to necrotrophic infection, were analyzed as well showing similar results to those of WRKY33. A transient up-regulation in expression of those genes was observed in the susceptible line at 3 dpi, whereas a steady up-regulation at 3 and 5 dpi was observed in the partially-resistant line.

Peroxidases have been implicated in lignin biosynthesis (Whetten et al. 1998), the production of H$_2$O$_2$ (Halliwell and Gutteridge 1999) and oxidation of indol-acetic acid (IAA) (Lagrimini et al. 1997). Several peroxidases were found up-regulated in the partially-resistant line compared to the susceptible one at 3 dpi. However, a decrease in expression up to 5-fold of seven peroxidases, including Glyma07g39020 and Glyma13g38310, was also observed. This
last result does not agree with previous studies that reported an increase in peroxidase expression following infection with fungal pathogens (Yang et al., 2007; Calla et al., 2009). Nevertheless, some reports have shown a similar peroxidase expression pattern following inoculation with S. sclerotiorum (Calla et al., 2014) as found in this study (of the seven peroxidase genes in common to the two studies, six of them were induced or repressed in the same direction). Calla et al. (2014) compared the gene expression levels of peroxidases following inoculation with S. sclerotiorum and Pseudomonas syringae. Results indicated a similar pattern of regulation of gene expression suggesting a HR-like response in plants inoculated with S. sclerotiorum as part of a possible manipulation of host defense mechanisms by the pathogen to enhance susceptibility (Calla et al., 2014).

Several genes involved in the synthesis of lignins were evaluated in this study. Interestingly, a CCR gene homologue, an enzyme that converts substrates such as ρ-coumaroyl CoA and feruloyl CoA into their respective aldehydes, was down-regulated in the susceptible line. Other enzymes that intervene in lignin biosynthesis showed a similar pattern of expression to those genes investigated previously. For the partially-resistant cultivar, an up-regulation of the same genes was observed. Thus, accumulation of lignins is suggested to be higher in partially-resistant plants than in susceptible ones after fungal infection. Some studies have shown a negative correlation between lignin accumulation and resistance against S. sclerotiorum (Peltier et al., 2009; Calla et al., 2009). However, these results included only a limited number of cultivars. Recently, other studies have shown a positive correlation between S. sclerotiorum infection and lignin accumulation in the crucifer Camelina sativa (Eynck et al., 2012; Calla et al. 2014). Biochemical, histochemical and gene expression analyses have demonstrated the accumulation of lignins after necrotrophic infection contributing to cell wall
strengthening and a decrease in symptoms in the resistant line (Prats et al., 2003; Zhao et al., 2009; Eynck et al., 2012). A possible explanation for the discrepancies in the literature might also be related to the different tissue types used in the studies.

PTI-related responses also include the expression of ethylene and jasmonic acid related genes, and several genes involved in JA biosynthesis were evaluated in this study. In general, a down-regulation of important LOX homologues was observed in the susceptible line, whereas few of these genes were found up-regulated in the partially-resistant cultivar. The ethylene biosynthetic pathway was also studied, and results showed an up-regulation in genes encoding key enzymes such as ACC synthase in the partially-resistant cultivar OAC Salem.

Resistance against necrotrophic pathogens is positively regulated by the action of signaling components in the JA/ET pathways whereas resistance against biotrophs and hemibiotrophs is characterized in general by SA-mediated responses (Govrine and Levine, 2002; Glazebrook, 2005; Spoel et al., 2007; Tsuda et al., 2009). It is possible that the mechanisms of JA/ET synthesis were impaired in the susceptible cultivar after 3 dpi since the oxidative burst could not be controlled in this line. Therefore, an impairment of several biological functions and possibly an OA-induced PCD was present in this line after 3 dpi. An important note is that regulators of JA signaling, such as JAZ and MYC gene homologues, were found exclusively down-regulated in the susceptible line at 3 dpi. Also, a JAR1 gene homologue, another component of the regulation of JA signaling was up-regulated in the partially-resistant line as early as 0 dpi. JAR1 conjugates JA to isoleucine (Ile), promoting the interaction of JA-Ile with JAZ, which is then polyubiquitinated and degraded by the 26S Proteasome, releasing MYC2 (Trujillo et al., 2008). Consequently, an induction of the expression of JA-related defense responses is observed (Santner and Estelle, 2007). The results in this study suggest a priming of
the defense responses in the partially-resistant line contributing to the induced systemic resistance via JA signaling. As expected in the immune responses against necrotrophic pathogens, no significant changes in the levels of SA-related genes were found.

Surprisingly, only two PGIP genes were found differentially expressed in this experiment, Glyma05g25370 and Glyma19g32700. However, their high levels of expression (>10-fold) could offer great advantage to infected plants. PGs are enzymes produced by bacteria, fungi, nematodes and insect pathogens (De Lorenzo and Ferrari, 2002; Jaubert et al., 2002). Their role has been demonstrated in several pathosystems such as *B. napus*- *S. sclerotiorum* (Li et al., 2004), tomato-*R. solanacearum* (Tans-Kersten et al., 2001) and rye-*Claviceps purpurea* (Oersen et al., 2002). Extracellular located PGIPs bind to active PGs and limit their destructive potential, and lead to the accumulation of oligogalacturonides, elicitors of plant defense responses (Ridley et al., 2001). Some of the responses described as a consequence of PGIPs actions are: synthesis of phytoalexins, lignin accumulation, expression of ethylene-related and PR genes and reactive oxygen species (Ridley et al., 2001).

In summary, soybean plants with different responses after infection by *S. sclerotiorum* (partially-resistant and susceptible cultivars) were challenged with this pathogen and their transcriptome profiles analyzed. The identification and levels of expression of genes related to quantitative resistance against necrotrophs were presented, suggesting a strong manipulation of the redox homeostasis in the susceptible line OAC Shire by the pathogen. This manipulation could contribute to a general shutdown of several biological processes including lignin biosynthesis and JA/ET related defense responses in this susceptible cultivar. Since some of the elements of the JA pathway were found up-regulated as early as 0 dpi in the partially-resistant line OAC Salem, a priming of the defense responses in this cultivar is suggested as
well. These results contribute to an improved understanding of the complex molecular mechanisms underlying partial resistance against *S. sclerotiorum* and can also be used to develop molecular markers to help in the development of resistant crop varieties.
CHAPTER 5
GENERAL DISCUSSION AND CONCLUSION

There is no evidence of complete resistance in soybean to *S. sclerotiorum* (Hoffman *et al.*, 2002), although the use of partial resistance (Hoffman *et al.*, 2002; Auclair *et al.*, 2004a; Auclair *et al.*, 2004b), the introduction of the wheat germin gene (gf-2.8) (Donaldson *et al.*, 2001; Cober *et al.*, 2003) and the decarboxylase gene (oxdc) from *Flammulina spp* (Cunha *et al.*, 2010) into transgenic soybean lines, and the use of PIs (Diers *et al.*, 2006) have all been investigated. Several studies have indicated that partial resistance to *S. sclerotiorum* may be present in selected breeding lines or cultivars, with reports showing a quantitative basis for inheritance (Kim *et al.*, 1999; Vuong and Hartman, 2003; Huyhn *et al.*, 2010) with up to 27% of the variation in lesion length being explained by quantitative trait loci (QTLs) (Vuong *et al.*, 2008).

Currently, little is known about the mechanisms underlying partial resistance against *S. sclerotiorum* in soybean. Therefore, evidence of the physiological, anatomical and molecular basis underlying this partial resistance to SSR is presented in this thesis, based on studies conducted using a susceptible cultivar, OAC Shire, and a partially-resistant one, OAC Salem.

Measurements of AUCPC, number of days for visible disease-related symptoms, stomatal conductance ($g_s$), dry and fresh matter, and accumulation of starch grains were analyzed for both cultivars for a period up to 12 dpi. No differences were found between the cultivars for AUCPC or fresh matter. However, at 2 dpi susceptible plants presented greater starch accumulation than partially-resistant plants, and an increase in $g_s$ was observed in susceptible plants only. Disease related symptoms, such as severity of wilting and number of days to plant death were lower in OAC Shire suggesting that, maybe, oxalic acid, which causes
wilting on leaves (Kolkman and Kelly, 2000; Guimaraez and Stotz, 2004), moved faster systemically in susceptible than in partially-resistant OAC Salem plants and that the two cultivars were differentially sensitive to it.

Dry weight of both susceptible and partially-resistant genotypes was affected by SSR. Plants from both cultivars had significantly lower dry weights than their respective controls at 12 dpi. Interestingly, OAC Salem had significantly lower dry weight than OAC Shire at both time points analyzed, i.e. 5 and 12 dpi. Decreases in dry matter may be related indirectly to the ability of the fungus to utilize plant-derived nutrients at its convenience (Guimaraez and Stotz, 2004) and, thereby, negatively influencing plant growth (Edwards et al., 2007). Susceptible OAC Shire plants also showed a significant increase of cells with starch grain deposits close to the infection sites at 2 dpi. Numerous studies, including those involving necrotrophic pathogens, have reported the phenomenon of pathogen interference with the source-sink relationship of the host genotype. Also, reports on the ability to manipulate the host metabolic pathways, thus, obtaining nutrients from degraded starch molecules at or close to the site of infection at the expense of other parts of the host plant have been found (Minarcic and Janitor, 1994; McDonald and Strobel, 1970; Etxeberria et al., 2009; Garg et al., 2010). This might be the case in our study too.

In general, increased levels of photosynthetic products correspond to increased expression of invertase at the site of infection depending upon the type and stage of the host-pathogen interaction (Herbers et al., 2000; Roitsch et al., 2003). Simple monosaccharides such as glucose and fructose, produced from hydrolysis of sucrose by acid invertase (Chou et al., 2000), are converted into triosephosphate by means of glycolysis (Long et al., 1975). Those triosephosphates may penetrate into the chloroplasts and be metabolized into starch (Long et al., 1975). Then, it is possible that the greater accumulation of dry matter in the susceptible cultivar
OAC Shire compared to the partially-resistant OAC Salem led to increased signals of invertase activity due to an elevated photosynthate accumulation and, therefore, a greater accumulation of starch deposits compared to the partially-resistant OAC Salem.

This thesis has also described several characteristics of the soybean-\textit{S. sclerotiorum} pathosystem such as sites of pathogen penetration, intra- and intercellular hyphal invasion and host cellular defense responses. Several differences were found between the two cultivars studied including a possible manipulation of certain defense responses to favor pathogen colonization in the susceptible genotype. Sophisticated penetration strategies were used by \textit{S. sclerotiorum} to penetrate stem and leaf tissues of OAC Shire plants, including penetration of the cuticle using the base of non-glandular trichomes and complex appressoria. Small infection cushions were observed in partially-resistant OAC Salem plants and appeared at late stages of the disease only. An early accumulation of phenolic compounds in the cell wall of cortical and epidermal cells of OAC Salem plants suggested a strong response to pathogen attack in this partially-resistant cultivar and, consequently, less damage to cortical and epidermal tissues by preventing penetration of fungal hyphae during early to advanced stages of the infection. Hence, basal defense responses observed in OAC Salem earlier than in OAC Shire impeded tissue deterioration during the first 3 days of infection in the partially-resistant variety.

For the first time, RNA-Seq technology was used to characterize the transcriptome profiles of the two cultivars of soybean with different responses after infection with \textit{S. sclerotiorum}. The identification and levels of expression of genes related to quantitative resistance against necrotrophs were presented, suggesting a strong manipulation of the redox homeostasis in the susceptible cultivar OAC Shire by the pathogen. This manipulation could contribute to a general shutdown of several biological processes including lignin biosynthesis.
and JA/ET-related defense responses in this susceptible cultivar. Since some of the elements of the JA pathway presented a high constitutive expression at 0 dpi in OAC Salem, a priming of the defense responses in this cultivar was suggested as well. In general, a transient up-regulation of the defense mechanisms up to 3 dpi was observed in the susceptible OAC Shire. However, for the partially-resistant OAC Salem, activation of those defense responses was observed during the entire time course of the experiment.

The mechanisms described in this study could help in the development of novel breeding strategies to incorporate partial resistance to SSR into commercial soybean lines. Future research that could be considered may include the following:

- Determining OA concentrations and severity of disease-related signs and symptoms in susceptible and partially-resistant plants inoculated with *S. sclerotiorum* to establish a relationship between those and sensitivity to OA concentrations in both types of interactions.
- Studies in the accumulation of lignins, phenolic compounds and phytoalexins in *S. sclerotiorum*-challenged plants, which could provide valuable insights into the anatomical changes that occur at different stages of infection and, thus, aid in the development of markers for screening of resistance against SSR.
- Evaluate the molecular mechanisms underlying disease-related symptoms such as wilting symptoms, e.g. expression of stomatal closure/opening-related MAPK genes (MPK9) in infected plants.
- Evaluate defense-related genes for their use as molecular markers to detect partial resistance to SSR, including those related to the oxidative burst.
REFERENCES


response to Sclerotinia sclerotiorum infection. Molecular Plant Pathology 15 (6), 563-575.


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 Online 12(11), 2191-2199.


Donaldson PA, Anderson T, Lane BG, Davidson AL, Simmonds DH, 2001. Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2.8 (germin) gene are resistant to the oxalate-secreting pathogen Sclerotina sclerotiorum. Physiological and Molecular Plant Pathology. 59(6), 297-307.


Ferraro PH, & Walker JRL, 1993. o-diphenol oxidase inhibition—an additional role for oxalic acid in the phytopathogenic arsenal of Sclerotinia sclerotiorum and Sclerotium rolfsii. *Physiological and Molecular Plant Pathology* 43(6), 415-422


Laurie-Berry N, Joardar V, Street IH, Kunkel BN, 2006. The *Arabidopsis thaliana* JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid-


MacDonald PW, Strobel GA, 1970. Adenosine diphosphate-glucose pyrophosphorylase control
of starch accumulation in rust-infected wheat leaves. Plant Physiology 46(1), 126-135.


Plant Physiology 133(4), 1755-1767.


*Biochimie* **75**(8), 687-706.


APPENDIX A- CHAPTER 2 ANOVA TABLES

Table A.1. Analysis of variance of visible lesion (VL) in *S. sclerotiorum*-inoculated soybean plants of cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant)

<table>
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<th>Source</th>
<th>DF</th>
<th>Type I SS</th>
<th>Mean Square</th>
<th>F value</th>
<th>Pr &gt; F</th>
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</thead>
<tbody>
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<td>4.20</td>
<td>2.10</td>
<td>5.32</td>
<td>0.0062</td>
</tr>
<tr>
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<td>0.76</td>
<td>1.93</td>
<td>0.16</td>
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<td>43.82</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>48.78</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table A.2. Analysis of variance of time to wilt (TW) in *S. sclerotiorum*-inoculated soybean plants of cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type I SS</th>
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Table A.3. Analysis of variance of severity of wilting at 5 dpi (SW) in *S. sclerotiorum*-inoculated soybean plants of cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant)

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Table A.4. Analysis of variance of number of days to plant death (PD) in *S. sclerotiorum*-inoculated soybean plants of cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant)

<table>
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### Table A.5. Analysis of variance of area under canker progress curve (AUCPC) in *S. sclerotiorum*-inoculated soybean plants of cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant)

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### Table A.6. Analysis of variance of fresh weight (g) in non-inoculated and *S. sclerotiorum*-inoculated soybean plants of cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant)

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### Table A.7. Analysis of variance of dry weight (g) in non-inoculated and *S. sclerotiorum*-inoculated soybean plants of cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant)

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Table A.8. Analysis of variance of stomatal conductance in non-inoculated and *S. sclerotiorum-*
inoculated soybean plants of cultivars OAC Shire (susceptible) and OAC Salem (partially-
resistant)

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Table A.9. Analysis of variance of cells with starch granules in non-inoculated and *S. sclerotiorum-*
inoculated soybean plants of cultivars OAC Shire (susceptible) and OAC Salem (partially-resistant)

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- 165 -
## Appendix 4.1

Insert sizes of the 24 samples sequenced with the Illumina HiSeq 2500

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## Appendix 4.2. Pre-processing of the samples using TrimGalore!

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APPENDIX C - CHAPTER 4 qPCR PRIMERS

Appendix 4.4. Primers for qRT-PCR validation of the RNA-Seq data

- ATP-Binding Cassette (*cons 4*)
  Forward: GTTCATCACATGGAGCAGCAA
  Reverse: TGCCAAAGTTCTCCAGCAAA

- WRKY41
  Forward: TCCGACTTTTCCCTCGAACATGA
  Reverse: CCGGGCAAGTTTCAAGTACAG

- Ethylene Response Factor 1
  Forward: TCTTCTAGCTTTGATGGGTCTTCT
  Reverse: GCCATTTTGCCTCCTAAGGA

- Auxin Responsive Factor Protein 1
  Forward: TCCTTTGCTATGGCAGTTCGTT
  Reverse: GCCCTACTACGTATTGAAGCAACA

- Chlorophyll a/b binding protein 1
  Forward: GAAGGAACTCAAGAAGCGACGAT
  Reverse: CGGTGACAATGGCCTGAAC

- MLO 1
  Forward: AGGCAGCCAGAATACGTCAGT
  Reverse: TCGGCCAGTTTCGTAGTTCTGT

- S-adenosyl-Methyltransferase
  Forward: GACAACCCTACTCCAACGAA
  Reverse: CAGGGTGCTAGGAGGAACA
- MYB 103 -
Forward: CCACCACCAATGTACGAACAGA
Reverse: GGAGGAAGAACATGGACTTGATG

- IAA 9 -
Forward: ACTCATTTGGCCACCACCTTCAA
Reverse: CCCCCATCCATGCTGACCTT
Appendix 4.6. Comparisson of expression levels of PTI-related genes in partially-resistant OAC Salem and susceptible OAC Shire inoculated with *S. sclerotiorum*. RPKM: reads per kilobase per million reads.
Appendix 4.7. Alignments of CCR-like genes of soybean and homologues from *E. gunni* and *Medicago truncatula*. Catalytic centre NWYCG is circled in black, positive.