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Hairy roots as a model to investigate the role of suberin in the *Phytophthora sojae*-soybean pathosystem

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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HAIRY ROOTS AS A MODEL TO INVESTIGATE THE ROLE OF
PHYTOPHTHORA-SOYBEAN PATHOSYSTEM

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by

PooSharma

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A thesus submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

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ABSTRACT

Part of the resistance mechanism of soybean (*Glycine max*) to the root-knot disease caused by *Phytophthora sojae* is the formation of root suberose. To investigate the role of suberose in this disease, hairy roots, formed as a result of *Agrobacterium rhizogenes* infection, as a model to be used as a reliable soybean host for *P. sojae* infection, were cultured and demonstrated to be highly resistant to *P. sojae* infection. The anatomy and suberin deposition in soybean hairy roots were compared with those of wild-type roots. The amount of suberin was quantified using gas chromatography-mass spectrometry in both epidermal and endodermal cells along the root axis. Finally, the response of soybean hairy roots to *P. sojae* infection was investigated and compared with that of wild-type roots.

Keywords

Suberin, Glycine, *Phytophthora sojae*, *Agrobacterium rhizogenes*, transformation.

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CHAPTER 1: INTRODUCTION

1.1 Soybean

Soybean (*Glycine max* Meisn.), legume that is intricately linked to human life. Due to its high protein and oil content, soybean is used for human consumption, animal feed and various industrial products. Soybean production have allowed the cultivation to spread throughout Canada from Ontario since the mid 1970s. To this day, soybean production as noted in 2006 by Agriculture Canada (Dorland et al., 2009) today, soybean is grown almost exclusively in Ontario, Prince Edward Island, Quebec, Alberta and approximately 2% of the world over the world (Canadian Grain Commission, 2012). In other important agricultural crops, soybean is also a root rot infection caused by *Phytophthora sojae*. General outbreaks were first reported in the U.S (Kaufmann and Gerdemann, 1958) and outbreaks continue to plague soybean producing countries (Wraight, Detrahnce et al., 2003).

1.2 Phytophthora life cycle and infection process

Phytophthora is a saprophyte and one of 80 species from the group Oomycetes. Phytophthora oomycetes differ from true fungi in that hyphae are aseptate and cell walls containing cellulose as opposed to chitin. Zoospores are biflagellate with an anterior tinsel flagellum and a posterior whiplash flagellum throughout much of their life cycle (Dick, 1969; Heffer Link)

P. sojae has a very narrow host range, infecting mainly soybean amount-2 billion dollars and maintenance costs are several Riberio, 1996; Wrather et al., 2001a,b). Oomycete pathogens mainly because they infect underground plant parts and where difficult and uneconomical (Tyler, 2007).

Zoospores, or flagellated spores, are released in water-borne in the spring when the soil is moist and warm, zoospores form 30 zoospores, which are attracted to soybean root exudates (Morris and Ward, 1992). Once at the root surface outer epidermal waxy, germinate, and the emerging hypha penetrate epidermis and into the cortex. As hyphae grow between cortical pathogen can feed on host structures called appressoria on the outer surface of root epidermal cell walls allow the formation which penetrate root cell walls; haustoria penetrate the epidermal pathogen (Tyler 2002) feeding is obligate, however, feeding leads to weakening of the host and necrotrophic which then leads to wilting and plant death above ground plant parts to wilt and die cannot take up and transport water and nutrients. Once the roots are colonized, hyphae form haploid antheridia and oogonia and the formation of diploid overwintering oospores, which can germinate to produce hyphae that can produce sporangia. The initial infection process involves physical penetration of the

plant resistance involves physical barriers, namely epidermal and the spread of pathogens within the root.

1.3 Plant defense pathways and the genetics of resistance

Pathogens such as Phytophthora cause a substantial crop loss annually and they lead to an alteration of host metabolism (Danko and Dehne, 2004) to combat microorganisms there are two kinds of defense: constitutive and inducible. Constitutive defenses include physical barriers such as suberized and lignified cell walls and chemical compounds such as isoflavonoids. Inducible defenses include induction of a pathogen and/or host specific and/or host specific responses known as pathogen associated molecular patterns (PAMPs) (formerly known as horizontal or basal resistance) and acquired immunity (formerly known as resistance) (Boller and Felix, 2009). In the former case, a microbial protein such as flagellin might be recognized by the host and induce a specific response, e.g., an avirulence gene product) might be recognized and induce localized necrosis may result, whereas, here recognition of the presence of a pathogen and induction of a series of responses can lead to the hypersensitive response (HR) and the systemic acquired response (SAR) gains increased immunity from initial infection through the up regulation (reviewed in Coll. et al., 2011)

In soybean, resistance is based on both qualitative and quantitative genes, where the former is triggered and the latter is inherited as partial resistance. Qualitative resistance, also known as field resistance, is based on a few major genes, including *Rps* genes, 15 of which have been identified (Anderson and Polzin et al., 1994; Dorrance et al., 2003; Smith and Tetrick, 2004; 2010). The effectiveness of these genes depends on the presence of a corresponding avirulence gene in the pathogen whose product can be recognized by the plant's R protein. However, these genes are not always successful for long periods of time because this pathogen has a short life cycle and can overcome resistance genes in a relatively short period (Burnham et al., 2003; Dorrance et al., 2003). Quantitative resistance is targeted against a wide range of structural and/or antimicrobial targets and is inherited quantitatively (Dorrance et al., 2003; Ranathunge et al., 2010). As the incidence of *P. vitivora* continues to increase in area production, it is essential to increase and incorporate partial resistance cultivars for effective disease management.

1.4 Suberin: physiological roles and structure

Suberin functions as a barrier to water loss and also as a barrier to pathogen attack and ingress mainly because of its molecular structure. Suberization of cell walls occurs mainly in the epidermis and part of the endodermis) and exodermis of underground plant parts.

molecule is characterized by a poly(aliphatic) domain (SPAD) and a poly(phenolic) domain (SPPD) (Figure 1), which differ in their location and are covalently linked to one another. The SPPD is hypothesized to be located in the primary cell wall, whereas the SPAD, often appearing as rings (known as suberin lamellae), is found between the primary cell wall and the plasma membrane (reviewed by Bernards, 2002).

A) SPPD

B) SPD

Figure: The hypothesized structure of the upper (phenolic) domain covalently attached to the primary cell wall (aliphatic) domain between primary cell wall and plasma membrane by a SPPD via ester linkages appearing as dark and light bands (C = carbohydrate, S = sugar from Bernards, 2002).

The SPAD is made up of aliphatic monomers specific to example hydroxy fatty acids, and diacids generally form the major aliphatic substituents, and can be found in a wide range of plants (Pereria, 1997; Barr-Timmmons, 2010). Other components include other fatty acids, glycerol and hydroxy acids (e.g. p-coumaric and ferulic acids) (Grac Pereria, 1997). The SPPD comprises a covalently linked network of polymers from hydroxycinnamic acids and alcohols (hydroxycinnamyl alcohols) (Kollatukudy, 1984; Zeier and Schreiber, 1997; Mat 2009).

In epidermal cells, suberin is deposited in radial and tangential walls enclosing the cells (reviewed in Bernards, 2002 and Enstone, 2002). In the endodermis, suberization is characterized in three distinct stages in the root tip both aliphatic and phenolic suberin is found in the radial walls of endodermal cells and forms the Casparian band which can be visualized with the use of lipophilic dyes. Within the Casparian band, the deposition of suberin in the radial walls of endodermal cells results in a contiguous wall thickening that resembles a fishnet surrounding the stele when the underlying cortex is removed (Figure 2). Further along the root axis additional suberin is deposited in all walls of endodermal cells (Casperian band), however not in all endodermal cells, finally, iii) far from the root tip further deposition of suberin in radial and tangential walls of endodermal cells thickenings of endodermal cells (Wilson and Peterson, 1984).

Similarly, heavier, although (non., diffuse) suberin deposition
epidermal cells along the root axis, moving away from the

Figure 2 Longitudinal scanning electron micrograph of
 minia Reg. Suberin forms a net like base as shown by the
 arrowhead shows suberized cell walls remaining after cell
 enzymatically digested). Bar = 100µm (modified from Sch
 permission from Oxford) University Press

1.5 Suberin and resistance

The physical nature of suberin is the principal factor in the inhibition of pathogen penetration into plant tissues that can be demonstrated in soybean (Khalil et al., 2002; Viljevac et al., 2009; Khalil (2007) et al.). A strong correlation between high root poly(aliphatic) suberin and partial resistance to *P. sojae* in the soybean cultivar Conrad, with lower rates of infection in field trials, was demonstrated by the root suberin compared to cultivar OX760 and lower amounts of both pre-aliphatic suberin and field level resistance. Specifically, fatty acid and dicarboxylic acids of the SPAD were directly related to soybean field level resistance. Furthermore, Ranathunge et al. (2007) showed that *P. sojae* hyphal penetration and growth was inhibited to a greater extent in OX760 compared to OX760 hyphal progress was especially impaired at endodermal layers.

The role of the phenolic suberin in resistance to pathogens is also important as it not only anchors the suberin molecule to the cell wall via hydroxycinnamic acid monomers but also serves as a barrier (Lai and Collier, 1998; Pomar et al., 2004; Bajji et al., 2005). Collectively, the suberin barrier and some of the molecules that make up its core, is being studied when studying constitutive defense mechanisms, increased to shift soybean resistance.

1.6 Manipulating suberin levels part of enhanced partial resistance

In essence, suberin not only acts to prevent water loss but also restricts pathogen growths at the suberinized epidermal cell wall. In one word based upon the above mentioned hypothesis, it is hypothesized that suberin plays a role in plant partial resistance against many pathogens. One possible strategy to manipulate suberin levels in plants is to increase the synthesis of suberin. This may be achieved, for example, by enhancing the synthesis of suberin synthesizing enzymes, which can lead to increased deposition of suberin. The hydroxy fatty acids which form much of the SPAD, which is directly linked to an inhibition of pathogen ingress. Thus, it would be expected that the hydroxylation of fatty acids expressed by an attempt to enhance suberin biosynthesis in roots.

1.7 SPAD monomer synthesizing enzymes

Fatty hydroxylase is a part of the cytochrome P450 monooxygenase family of enzymes that catalyze the hydroxylation of the terminal carbon of fatty acids (Serrano et al., 2003; Pinot and Beisson, 2006). More specifically, the CYP86A and CYP94B monooxygenase members that can catalyze the hydroxylation of fatty acids, respectively, from fatty acids in a NADPH-dependent reaction (Serrano et al., 2009). According to Serra et al. (2009), RNAi knockdown of a CYP86A tuberous leads to lower retention of water by the epidermal cells (WT) because of a changed suberin composition in Arabidopsis.

genes, and numerous other related pseudogenes, and the expression of these has been shown in soybean roots (Koteles, 2012).

1.8 Soybean transformation

In order to begin to work on changing gene expression of GmFAE1 genes, a method of plant transformation must be selected. The insertion of suberin biosynthetic genes of interest into the genome for analysis of their changed expression in suberin deposition affected root tissue. Traditional transformation techniques using *Soybean* & Towns (1981) *Rhizobium radiobacter* (Boivin et al., 1998) Young et al. (1998) a bacterium that can be engineered to carry foreign genes. However, transformation efficiency and long plant regeneration times make transformation is not always feasible (Cao et al., 2009; Tsai et al., 2009). An alternative strategy is to use *Rhizobium* (Rizkogre et al., 2001) *Rhizobium rhizogenes* (Rizkogre et al., 2001) Young et al. (2001) transformation and generation of transgenic hairy roots.

1.9 *Agrobacterium* hairy roots

Agrobacterium *rhizogenes* species is a member of the family Rhizobiaceae leads to extensive adventitious root growth (root syndrome at the site of infection) (Rizkogre et al., 2001). Hairy root formation is dependent upon the successful transformation of plant cells with bacterial genes. Although the transformation mechanism is a

tumefaciens mediated transformation of adventitious root formation rather than the formation of undifferentiated cells (Nilsson and Olsson, 1997). Ri (root inducing) plasmids are 250 kb in size and of several hundred base pairs in length. The T-DNA region may contain auxin biosynthetic genes as well as genes that lead to increased sensitivity of the plant tissue (White et al., 1991). Expression of the particular gene is what causes increased sensitivity to endogenous auxin which is formation. Consequently, hairy root genes lead to adventitious root formation (White et al., 1985). These oncogenes lead to neoplastic growth (adventitious root growth), and lead to the formation of opines. The purpose of this is to provide a growth substrate (opines) for bacteria, thus giving bacteria a competitive advantage over other species of bacteria in the soil (Nilsson and Olsson, 1997). The engineered plasmids can be used to knock down or over express native plant genes, or to introduce genes from bacteria, the relative proportion of transformed tissue is greater than in traditional transformation since transformed cells differentiate into adventitious roots. It is important to note that hairy roots are transformed because of local phytohormones which induce cell differentiation into root tissue.

Since hairy roots are neoplastic and adventitious in nature (unlike roots in the traditional sense), it might be anticipated that

deposition might be affected. Hairy root cultures have been more than 100 species, their anatomy and structure have been studied by all kinds of plants (Cancha and Vargas, 1999) such as *Californisaccharalis* (Cancha and Vargas, 1999) and *Poppy* (Munier-Ponpey and Fanchini, 2000) and tomato (*Solanum lycopersicon*) (Picus et al., 2004). The differences in cell arrangement structure between transgenic roots have sometimes been observed. In particular, poppy hairy roots had a loose cortical arrangement whereas tomato hairy roots had a higher number of vascular bundles. This suggests that hairy roots differ between species. Thus, to study the anatomy and chemistry of hairy root cultures of different plants to understand the impact those differences may have on functional properties.

Soybean hairy roots can be induced and cultured (Chou et al., 2000). Induction of hairy roots involves wounding plant material, such as hypocotyls, stems or young leaves, and simultaneously infecting with an Agrobacterium strain. Usually within a few days root growth begins and small roots appear at the site of inoculation. Once established, roots can be cultured in solid or liquid nutrient media. The stability and ease of regeneration are important to plant biologists. (2009) have found that hairy roots retain morphological stability in culture and thus can be cultured relatively easily and efficiently. Thus, studying plant hairy root culture is simpler and more efficient than traditional plant culture. Hairy root culture, as a fast growth rate, is stable and can be viewed in a flask.

Hairy roots have been a wide variety of plant biology topics including gene expression (Ding et al., 1999), nodule development (Ding et al., 2001). They have also been used to study the reactions between plant roots and microbes. For instance, an approach has been used to study the interaction of soybean with *Fusarium oxysporum* (Mandal and Mitra, 2004). In a similar system, the synthesis of flavonoid compounds in soybean roots was measured using HPLC. In particular, glyceollin and coumestrol synthesis was measured using HPLC in two different cultivars of soybean after infection with *F. oxysporum* (Liszovaya et al., 2004).

1.10 Rationale: why hairy roots?

Since *Phytophthora sojae* pathogenesis leads to the hairy root phenotype, it seems that using soybean hairy root system is an excellent option. In a hairy root transformation system, suberin levels can be manipulated using appropriate constructs to alter the suberin content. This experiment further allows to test whether suberin plays a vital role in the pathogen growth within soybean roots. Generating a whole hairy root system and cells that may or may not be transformed can take a few weeks. However, transformed hairy roots are generated within a few weeks.

pathogen preferentially attacks roots, a whole plant need not be infected. As mentioned earlier, the aliphatic domain of suberin is closely associated with the partial resistance in roots. Therefore, it becomes imperative to study whether or not the deposition of suberin in hairy roots mimics that in WT. For this reason, hairy roots must be investigated more carefully by using a hairy root model to study the role of suberin in soybean partial resistance. This study fits into my thesis objectives.

1.11 Objectives

1. Establish soybean hairy root cultures from soybean cultivar 'Pioneer 606'.
2. Confirm that established hairy roots are representative of the whole plant by investigating the expression of the hairy root marker gene using PCR.
3. Study the anatomy and suberin deposition pattern in hairy root and WT root cultures using histochemical techniques.
4. Characterize the aliphatic suberin (quantitatively and qualitatively) in hairy roots.
5. Investigate the infection by *Phytophthora sojae* in hairy roots and compare to WT roots.

By fulfilling these objectives, I hope to gain a clear picture of the structure and functional similarity between hairy roots to WT roots, thus establish a hairy root model tool to investigate the role of suberin in the soybean partial resistance system.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant materials

Seeds of *Glycine max* var. Conrad and Firmex 760 grown plants (year of culture 2009) were obtained from Agriculture and Agri-Food Canada (London, Ontario). Seeds were surface sterilized by 10% available bleach, Clorox Co. (Brampton, Canada) for 20 minutes and rinsed with sterile water. Surface sterilized seeds were then placed in petri dishes. Petri dishes were sealed using parafilm and placed in a 25°C incubator (light/dark) period. Once the cotyledons emerged, they were excised and used to generate hairy roots as described below.

2.2 Agrobacterium strain

Agrobacterium strain LBA9402, a gift from Dr. Patrick (National Research Council Canada) was used to generate hairy roots. The strain was maintained on YMA (4HR) 0.8 mM MgSO₄ 1.7 mM NaCl, 1.5% mannitol, 0.4 g yeast extract, and 15% sucrose, pH 7.0 at 4°C in the dark.

2.3 Generation of hairy roots

Hairy roots were generated using a protocol developed by Cho et al. with slight modifications. Briefly, soybean cotyledons were inoculated on the abaxial side by wounding the major vein using a scalpel.

coated Avicel with zooglone for 24 h. Explants were grown overnight on medium at 30 °C inoculated explants were placed on a medium containing MS (Sigma) (4 g L⁻¹), sucrose (3%) and cefotaxime (500 mg L⁻¹), adjusted to pH 5.8 with NaOH. The antibiotic is used to prevent the growth of rhizoglyphs but the site of inoculation. The inoculated explants were placed in a 16 h light/8 h dark period, 25 °C).

2.4 Maintenance of roots

Once hairy roots emerged (approx. 5 cm length) they were excised from the parent explants and placed on a medium with same concentration of cefotaxime to prevent bacterial growth. They were plated on MS medium without antibiotic. Plates with hairy roots were placed in an incubator at 25 °C in a 16 h light/8 h dark period. The first 4 cm of root was subcultured every few weeks onto a fresh plate as described above to propagate and maintain the hairy roots.

2.5 PCR analysis

In order to confirm the origin of adventitious root growth, DNA was amplified from genomic DNA obtained from established hairy roots by polymerase chain reaction. Genomic DNA was extracted from both Conrad and modified (CTAB-trimethyl Ammonium Bromide) protocols and Yang (2004) by briefly, roots were ground

in liquid. Using a sterile mortar and pestle and approximately 100 mg of leaf tissue was suspended in 600 µL of CTAB buffer (1M NaCl, 100 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 2% CTAB) along with 0.2% v/v mercaptoethanol and 2 µL of β-mercaptoethanol. The suspension was heated at 65°C for 1 hour. To the suspension, 600 µL of Chloroform:Isoamylalcohol (24:1) was added and centrifugation at 13,000 rpm for 10 minutes. The supernatant was transferred to a fresh microcentrifuge tube and 0.7 volumes of isopropanol were added. The mixture was mixed by inversion and cooled at 4°C for 30 minutes. The mixture was centrifuged again at 13,000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 700 µL of 70% ethanol. The pellet was dried at 60°C for 1 hour. The dried pellet was suspended in 50 µL of DNAse-free water.

The *OsDRE1* gene was amplified using primers designed by Zeng et al. (2011). The forward primer (F) sequence is 5'-GCTCTTGCAGTGCTAGATGAGGTTGCAGCTACCTACTC-3' and the reverse primer (R) sequence is 5'-GCTCTTGCAGTGCTAGATGAGGTTGCAGCTACCTACTC-3'. A 430 bp fragment was amplified from hairy root and WT roots from cultivars was used as a control. PCR products were run on 1% agarose gel. A bacterial preparation as a positive control. The 25 µL PCR reaction mixture contained 1.25 µL MgCl₂ (50 mM), 2.5 µL 10 X PCR buffer (100 mM), 0.3 µL DMSO, 0.5 µL Taq polymerase (5000 U/ml), 100 ng of genomic DNA from hairy root bacterial preparation and 100 ng of WT root DNA. The enzyme and primers were obtained from Qiagen. The PCR reaction was conducted at a ratio of 94°C for 30 s, 55°C for 45 s, 72°C for 1 min for 30 cycles. The annealing temperature was 55°C and final extension was 72°C for 10 minutes.

The PCR products were run on a 1.0% agarose gel at a voltage of 100 V and stained with ethidium bromide. Gel bands were visualized on a UV transilluminator (Universal Hood II, S76S/01511).

A relatively easy method of determining whether or not a plant was transformed was also employed, which allowed for a more confident analysis. In vitro grown cotyledons (obtained as described above) were divided into two groups. One group was mock inoculated as described above, whereas, the second group was inoculated with the Agrobacterium strain. On days 10, 15, and 20 the presence or absence of hairy root formation was noted.

2.6 Histochemical analysis

In order to view the pattern of suberin deposition and the presence of fluorescent dyes were employed. Sections of hairy roots (cultivar 4-12 in length) were cut at intervals of 1.5 cm along the length of the root. Sections were viewed using a Zeiss Axioimager Z1 Microscope. The presence of a band in the endodermis was determined using Toluidine Blue (TBO) (Sigma) according to Lulai and Morgan (1992). free hand cross sections were stained with fresh TBO (0.05% in 0.1 M phosphate buffer, pH 6.5) for 1 minute followed by rinsing with 0.1 M phosphate buffer, pH 6.5. Stained sections were then rinsed several times in water. Sections were viewed with blue light (490 nm) through a dichroic mirror (510 nm) with a suberin Caspary band fluorescing yellow. Images were taken with a Nikon 3. Suberin lamellae were

viewed using fluorol yellow (FY) (Brundrett et al., 1991). The sections were then rinsed several times in distilled water and viewed (filter 560 nm, excite 400 nm, barrier 585 nm), barrier image taken with a CAM R3. Under these conditions, staining suberin lamellae gave bright yellow fluorescence. Suberin was detected using Sudan Red VII B (fast red) dissolved in 0.1% (w/v) (in 1: polyethylene glycerol) for 2 hours (Brundrett et al., 1991). The sections were rinsed with distilled water in 75% glycerol and viewed with bright field. Images were taken with a CAM R3. Suberized cell walls stain pink.

2.7 GGMS analysis

Suberin is a polymeric network and in order to quantify it in plant tissue, it has to be depolymerised. Both roots and isolated epidermal and endodermal tissues were analysed. For whole shooty roots of 14 cm (m) were inflorescence and ground a powder using a mortar and pestle.

To isolate epidermal and endodermal tissues, separate root inoculations were used, for epidermal tissue from both cultivars. Roots of 14 cm length were separated into 3 segments of 4-8 cm. Any lateral branching was removed. Root cross-sections were made; the epidermis was removed; three separate endodermal cross-sections

for any root sections that were dried. This was done so that the surface area of the roots could be calculated and suberin area per mm^2 value. The epidermis was then removed using a wire which was pulled through the wire which stepped the epidermis and few cells left behind the endodermis, stele and a few cortical cells. removed, it was frozen in liquid nitrogen. Ground tissue was placed in a microcentrifuge kept frozen at -80°C until extraction. Root sections were made of endodermis and its diameter measurements were made at different locations. The endodermal tissue was also removed from the epidermis and placed in a separate microcentrifuge.

In order to quantify the suberin in the root tissue was first extracted in chloroform:methanol (Meyer et al., 2011). Briefly, frozen hairy root tissue from both tissue layers and cellulose thimbles and extracted in chloroform:methanol (2:1) for 3.5 hours. The solution was removed and the thimbles were cleaned and the remaining tissue was extracted for 3.5 hours in fresh chloroform:methanol (2:1). This solution was added to the first and the tissue was extracted in 100% chloroform overnight. The third aliquot was combined with the previous extracts and removed from the thimbles, weighed and placed in 5 mL solvent. The components were obtained by drying the chloroform:methanol mixtures using a rotary evaporator. The amount of suberin was determined by the weight of the residue.

bottom flasks, the solutions were removed using a glass pipette and weighed into clean screw cap glass vials. A 100 µL of a 1:1 (v/v) chloroform:methanol was added and the solutions were evaporated using a steady stream of nitrogen and the mass was weighed again.

Suberin monomers were obtained by using a methylation protocol according to M. S. Campbell et al. (2011). Separated hairy roots (approximately 6 mg for intact hairy roots) were incubated with 100 µL of 3 N HCl (Supelco) in 1 mL of dry methanol at 80°C for 24 hours. Once samples were brought to room temperature, deionized water was added to stop the reaction after which 10 µL of a 100 µg/mL standard was added. Samples were extracted three times with hexane and the vial was sealed. The upper layer was then removed and a clean screw cap glass vial and extraction with hexane was performed. Hexane solubles were dried under a steady stream of nitrogen and 10 µL of pyridine and 100 µL of 1% TMSO in 1% bis(trimethylsilyl)trifluoroacetamide were added and incubated at 70°C for 40 minutes to derivatize remaining hydroxyl and carboxylic acid groups. The final sample was transferred to a clean vial and analyzed on a GC (Agilent) concentrations of esterified monomers and any TMSO were analyzed on a GC/FID and an ion trap mass spectrometer were connected in series. A 5 µL sample was injected on a WCOT silica 30 m X 0.25 mm column.

Hairy roots were incubated directly in zoospore suspension and 20 hours. -hFarrad c-sections were made 1 cm from the root tip for microscopy. ~~phycostogol~~ described by Ranathunge et al., 200 were cleared using 10% aniline stained with 2% crystal blue water for 2 (Sigma) and stained with a solution of 1:1:1 of glycerol and 10% for 5 min. Cross sections were mounted in 75 and viewed under bright light using the microscope described taken using CamICc1.

2.9 Statistical analysis

Student's t test was used to compare poly(aliphatic) suberin of intact hairy roots from both cultivars. Analysis of Variance to compare mean amount of poly(aliphatic) suberin of isolated cultivars. ANOVA was also done to compare mean amount of suberin distributed by chain length in intact hairy roots of both cultivars. waxes of isolated hairy roots. In the case of significant differences denoted by ANOVA, tukey's post hoc test was used to determine. All analysis was done using SigmaStat® 3.5.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Hairy root generation

Hairy roots from *C. xanthocoma* were generated in the lab using a protocol of Cho et al. (2000) where inoculation with *Agrobacterium rhizogenes* 14929s surmounting inoculation became successful and approximately one week inoculation callus growth appeared. Hairy roots emerged from the callus region in approximately 14 days (Figure 3.1). Previous studies report similar timeline for hairy root generation in different al., 2009; Fernandez et al., 2010). Although many roots were generated from 14 days root growth was also noticed before a time range. This could be attributed to the inherent differences which in turn affect the level of endogenous auxin in the tissue. This affects the hairy root growth is abundant (Takahashi, 1997). Furthermore, the insecticide also affect the amount and frame of hairy root (Seki et al., 2005). Hairy roots cultured in media growth rates of approximately 2k.

Figure 3 Time course of hairy root induction in *Arabidopsis thaliana* by *Agrobacterium tumefaciens* strain C58. A, Necrosis on wounded cotyledons and growth 7 days pi, C, roots emerging from callus 10 days pi and D, Two-week-old hairy roots in nutrient rich agar medium supplemented with sucrose and cefotaxime (500 mg⁻¹).

Cotyledons obtained from in vitro germination of soybean root production, proved to be a more efficient system rather than cotyledons from soybean plants germinated in greenhouse. Cotyledons from greenhouse soybean followed by inoculation with bacteria resulted in necrosis and eventually embryonic leaf death before any hairy root formation.

Once putative hairy root cultures were established it was determined whether or not they were *A. rhizogenes* mediated transformation. For this analysis was done using genomic DNA isolated from hairy roots along with WT roots. The presence of *B. (430 bp) Ro* confirmed a hairy root genotype. This is important because in order to manipulate suberin levels in a system is required that will alter the soybean genome, and hormonal changes. However, successful generation of hairy roots visually. That is, cotyledons wounded with a scalpel but not inoculated with *A. rhizogenes* did not produce any roots whereas those inoculated with *A. rhizogenes* formed roots at the site of inoculation (data not shown). It is the necessity of conducting PCR analysis on hairy roots, because a genetically transformed hairy root only has the hairy root form plant tissue with a native soybean hairy root also transformed with a secondary plasmid, thus is efficient as a binary vector system through the use of a binary vector system transformed soybean as well as gene encoding a protein product which fluoresces under any generated hairy root which fluoresces red is truly transformed.

reported that 34% of roots generated *in vitro* were initiated due to hormonal effects.

Figure 1 PCR analysis of genomic DNA Ausrihig pgrifnges for the A. rhizogenes whole bacterial DNA preparation (6 WT) and Conrad WT = genomic DNA isolated from wild soybean (negative control) and Conrad HR and CX7 DNA isolated from soybean hairy roots. PCR run on 1% agarose gel. Selected ladder (bp) are labeled on the left hand side of the gel. T 430 bp.

One major reason why hairy roots form the basis of an system is that they are cultured on nutrient rich media almost in true for WT roots (Fernandez et al., 2009). Morphologically WT roots, however there were some differences, namely the (Figure 3D), which is where hairy roots obtain their name. Ex and a fast growth rate can be attributed to increased DNA (as of high mentioned above) or the increased sensitivity of hairy roots which influences growth (Shen et al. 1988, Van Sluys and 1991, Mallol et al. 2001, Peres et al. 2001, Lee et al. 2004).

It was observed that hairy roots grow aerially, i.e. roots emerge callus and display negative geotropism (Fitzgerald et al. 2006). suggested that fewer amyloplasts are found in hairy roots possibly leading to a weak gravitational response (Kim et al. 2009). Furthermore, large air spaces were observed in the aerial (Figure 5). Teakle et al. (2011) reported that roots growing in areas, such as agar, develop an aerenchymatous morphology in root tissue limiting conditions. These features were found in both COX760.

3.2 Hairy root anatomy

Hairy roots are anatomically similar to WT roots as can be seen. However in addition to the air spaces found in hairy roots, the vascular anatomy of hairy roots seems different than WT. In particular, in some

three xylem poles whereas, WT roots have four (Figure 5). (1957), auxin levels can increase in roots removed from decapitated roots placed in auxin medium. Thus, since higher levels of endogenous auxin (or are more sensitive to) that the number of xylem poles may in hairy roots.

I also noted that hairy roots did not appear to have the same mechanical strength as WT roots. This was especially evidenced during sectioning of hairy roots. This is also a characteristic of soybean hairy roots. The air spaces present in the cortex (Figure 5) may also contribute to the mechanical integrity. Additionally, due to the influence of the moist media in which hairy roots are first grown and then propagated under the conditions can weaken the epidermal cell walls making them more prone to damage. Hairy roots are widely grown in liquid cultures and used as tools and model systems for questions involving plant tissue culture. It seems that while media may have the robustness it does not preclude their use as an experimental system.



Figure C5: Cross section of a *Ox760* (top image) and a *Ox760* (bottom image) stained with farrus. Left hand side shows the cortex and central stele.

3.3 Hairy root suberin histochemical analysis

In order to use hairy roots to test the hypothesis that soybean pathosystem, it is imperative that suberin deposition resembles that of WT roots. Through the use of fluorescence microscopy, it was shown that cell wall suberin histochemistry of hairy roots (Figure 6B). Previous research using WT roots documented that clover root has a state I endodermis, in other words, a suberized barrier and solute transport between the cortex and the stele in the root (Thomas et al., 2007). In a transverse section, the Casparian band is evident as points of fluorescence in endodermal cell walls stained with a fluorescent dye, and this same pattern can be seen in hairy roots (Figure 6C). At the root tip, endodermal cell walls of hairy roots were more suberized than in WT roots. However, not every endodermal cell was suberized resulting in a characteristic suberin deposition pattern where regions of no lamellae were present (Figure 6C). This may be to allow the passage of water and solutes into the stele even at these young distal parts (Charko et al., 1975; and Robards, Peterson and Enstone, 1996; Waduwara et al., 2008). Importantly, hairy roots still had a Casparian band. Normally as roots mature, suberin deposition also increases (Paterson, 1978). In hairy roots we found increased suberin deposition in epidermal cell walls distal to the tip along the length of the root (Figure 6E; this was evident in greater staining of epidermal cell walls close to the root tip at approximately 10 cm and this was also observed in WT roots (Thomas et al., 2007).

Figure 5 Suberin deposition patterns in soybean hairy roots (A, C, E, G) and nodules (B, D, F, H) of *Albizia julibrissin* (Raymond Thomas), free hairy root cross sections, stained with toluidine blue O and neutral red, 2 cm from the root tip. C, Section stained with toluidine blue O, 2 cm from the root tip. E, Section stained with toluidine blue O, 4-6 cm from the root tip. G, Section stained with toluidine blue O, 8 cm from the root tip. B, Section stained with toluidine blue O, 2 cm from the root tip. D, Section stained with toluidine blue O, 4-6 cm from the root tip. F, Section stained with toluidine blue O, 8 cm from the root tip. H, Section stained with toluidine blue O, 8 cm from the root tip. Arrows indicate lamellar suberin in the endodermis. Scale bars = 50 μ m.

Hairy roots from both cultivars displayed similar suberin in root axis and similar morphology and anatomy compared to important because use of a root model hinges on the tenet actual system are comparable.

3.4 Hairy root suberin analysis

After establishing suberization patterns of hairy roots, poly(aliphatic) suberized tissues (1.5 cm x 0.5 cm x 0.2 cm long, see materials and methods for details) were analyzed for amount and amount.

3.4.1 Quantitative analysis

Quantitative analysis of hairy roots from both cultivars compared to Conrad (Figure 7). Although this difference is in contrast to published literature Conrad had a relatively amount of aliphatic suberin (Thomas et al., 2007), this difference to both cultivars responding differently to the growth conditions and/or the medium in which they are grown. It has been shown that suberin deposition is influenced by the environment. It has been shown that has greater partial resistance to higher amounts of suberin in suberizing tissues (Dorrance et al., 2003; Thomas et al., 2008; Ranathunge et al., 2008). Anderson (1982) found that a 2% mortality rate was observed in susceptible cultivar Ar

had 40% mortality rates. However, interestingly, the percentage of plants surviving in the fields changes between years, leading to the fact that a change in the environment can influence level of suberin deposition and thus resistance to pathogens. In other words, hairy roots may be more plastic to a changing environment, thus have higher suberin levels compared to control plants grown in a growth medium as opposed to growing in soil may contribute to this difference. Root nodule insertion may also influence expression of genes, (Baker et al., 2010) thus cause hairy roots to have a different phenotype.

Figure 7 Quantification of poly(aliphatic) suberin and intact so
 Conrad) hairy Tissue was subjected to esterification to rele
 poly(aliphatic) suberin. Total poly(aliphatic) suberin was me
 data are expressed on basis of amount of tissue (mg) trans
 average \pm SE of three replicates. No significant difference

Quantitative differences in aliphatic suberin were found in hairy roots when the endodermis and epidermis were analyzed. Differences were not always statistically significant, but suberin was highest farthest away from the endodermis. Two important aspects of suberin deposition in hairy root dermal poly(aliphatic) suberin is higher in hairy roots than in hairy root cultivars, and 2) poly(aliphatic) suberin amounts increased. Furthermore, there are quantitative differences between WT (publ. al., 2007) and hairy roots, where hairy roots have aliphatic suberin approximately 8 times lower than WT. In other words, amounts of aliphatic suberin in hairy roots are a per m comparable to roots in the case of hairy roots. This is important because when developing a root model system it is necessary to understand the model may have from the original system. With this data and histochemical data it is possible to determine appropriate in vitro model compared to Conrad.

Figure 8 Quantification of insoluble poly(aliphatic) suberin in root tissues. Epidermal and endodermal tissues were isolated and separated into 0.4, 0.8 and 2.8 cm. ~~Boils~~ tissues were subjected to MeOH-HCl transesterification to release poly(aliphatic) suberin. suberin was measured using GC-MS and data are expressed on basis of mg endodermal suberin per g dry weight. Data are average \pm SE of three replicates. Letters with different letters are significantly different (P = 0.001).

3.4. Qualitative analysis

Next, it was also important to assess the monomeric composition of poly(aliphatic) suberin in hairy root and WT roots. Qualitatively, hairy root aliphatic suberin contained fatty acids, hydroxy fatty acids, and dioic acids as in WT roots (Shore et al., 2007). In the hairy root tissue available for analysis, some suberin components were not detected at accurate detection levels and thus component analysis was not possible. However, it was found that although all fatty acids, hydroxy fatty acids, and dioic acids were present, not all chain lengths that are normally found were found (Table 1). Generally 16 and 18 carbon chain lengths were found, whereas longer chain lengths of fatty acids and alcohols were more of a minor component (reviewed in Bernards, 2002) and were not considered an important feature to take note of. Hydroxy fatty acids were the most abundant and are similar to those found in WT roots (70% of total aliphatic suberin in WT roots). In hairy root, it is interesting to note that there is a high correlation with resistance to pathogens (Thomas et al., 2007). Again, hairy root not only has greater suberin composition, amounts are also higher than in WT roots. Interestingly, Conrad et al. (2007) found that although this difference was not significant.

Table 1 shows the length distribution of hairy root aliphatics by substrate. The hairy roots of both COX760⁻⁵ and COX160⁻⁵ were expressed as mg/mg of hairy root tissue subjected to transesterification and measured using GC-MS. The data are presented as average of three replicates \pm SE. Means followed by different letters are significantly different (P = 0.006).

3.4.3 Soluble wax analysis

With respect to soluble wax components, the pattern of published data on WT roots (Thomas et al. 2007) for 60XF760 (where the soluble waxes increased, although the increases were not statistically significant) regardless of the lack of soluble wax contains unpolymerized alkanes, and although the actual amount of which in the hairy roots may vary between distances from the root tip. Earlier, soybean WT roots with higher amounts of insoluble wax were more resistant to infection, therefore, it can be expected that roots growing in an artificial medium may not have the same amount and may be considered more plastic to a changing environment.

Figure 9 Quantification of soluble waxes in isolated soybean hair
 Epidermal and endodermal tissues were isolated from soybean
 3 segments, 4, 8 and 28 cm. Soluble waxes were isolated by solvent
 Total suberin was measured using GC-MS. Data are expressed on
 root or endodermal surface area. Bars represent average \pm
 significant differences were found ($P = 0.235$).

3.5 Hairy root pathogen interactions

As a final step in investigating the appropriate effects of soybean WT analyzed the interaction between root- and pathogen. 6 hairy roots had higher levels of suberin, which were more structurally important to Conrad, 6 hairy roots were used for this study. Hairy roots were exposed to zoospores at different times that at 2 h zoospores attached to the epidermal cells in preparation for germination. This is in contrast to where zoospore attachment was observed (Rendall et al., 2008) but in hairy roots, zoospores had attached and germinated (Figure 10A). Finally, within 22 h zoospores had penetrated the epidermal cells and growth was seen between cortical cells (Figure 10B, C). This is in contrast to wherein 24 h post inoculation has been reported to penetrate the endodermis (Rendall et al., 2008). This difference could be due to the fact that hairy roots have gaps between cortical cells (Figure 5), which may impede the pathogen despite this it was observed that infection of hairy roots followed a similar path to that in WT roots, albeit it was slower. This is important to note the effect of the change in suberin levels obtained through germination require a pathogenicity test, which in this case will involve a baseline infection progress we can assess any change in infection when manipulated.

Figure 1 Development of *Phytophthora sojae* on fresh and cross sections of long hairy roots taken from the root tip. After incubation zoospores attached and began to germinate. After incubation zoospore germinated and hyphae with diaspores progressing between cortical cells (arrows). Bars = 50 μ m.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Through this experiment and flow work that hairy roots are similar to where root anatomy, suberin histochemistry, suberin composition and response to pathogen attack are concerned. Even though hairy root cultivars Conrad and Oxytropis with those found in WT roots, hairy root stable used a system mimicking WT roots since the anatomy, suberin composition and pathogen response has been documented experimentally. Various suberin levels can be manipulated by the expression of *GmMYB176*, changed. For this, RNA interference (RNAi) knockdown experiments which can down regulate transcription and hence the production of the SPAD. For example, Yi et al. (2010) have down regulated *GmMYB176* in soybean hairy roots via RNAi leading to decreased SPAD product. Conversely, SPAD levels could potentially be enhanced by upregulating *GmFAH* genes.

Lin et al. (2011) developed a new plant transformation vector for hairy roots, which can be used to study the relationship between hairy root growth and suberin formation. Transformed roots appear red under ultraviolet light due to the presence of suberin. Essentially, hairy roots have been engineered with the capacity to carry foreign genes separately. This can potentially be used to study the function of *GmMYB176* in hairy roots. A strong promoter, such as Cauliflower mosaic virus promoter, can be used to upregulate it.

Additionally, a different pathogenicity test could be employed. A test of the effect of changed suberin levels on hairy root resistance that relies on sectioning and a microscope, e.g. fluorescently labeled hairy roots with zoospore suspensions and monitoring root damage caused by *P. sojae* could be an alternate method of testing the effect of suberin manipulation. Such work has previously been done and further manipulation of other root specific genes have been manipulated.

In essence, if manipulated levels of suberin affect soybean hairy roots, then this will further support the hypothesis that suberin contributes to soybean partial resistance against this pathogen.

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APPENDICES

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