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

HAIRY ROOTS AS A MODEL TO INVESTIGATE THE ROLE OF SUBERIN IN THE PHYTOPHTHORA-SOYBEAN PATHOSYSTEM

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ABSTRACT

Part of the resistance mechanism in soybean (*Glycine max* [L.] Merr.) to *Phytopthora sojae* Kauf. & Gerd. is pre-formed root suberin. In order to investigate the role of suberin in this pathosystem, I characterized hairy roots, formed as a result of *Agrobacterium rhizogenes* (Riker) Conn infection, as a model to be used as a reliable soybean transformation system. I established hairy root cultures and demonstrated that they were the result of *A. rhizogenes* infection. The anatomy and suberin deposition in soybean hairy roots was found to be very similar to that of wild-type roots. In hairy roots, the amount of suberin (quantified using gas chromatography-mass spectrometry) increased in both epidermal and endodermal cells along the root axis as in wild-type. Finally, the response of soybean hairy roots to *P. sojae* infection was investigated and shown to be similar to that of wild-type roots.

Keywords

Suberin, *Glycine max*, *Phytophthora sojae*, hairy roots, *Agrobacterium rhizogenes*, transformation.

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CHAPTER 1: INTRODUCTION

1.1 Soybean

Soybean [*Glycine max* (L.) Merr.], a legume that is intricately link human life. Due to its high protein and oil content, soybea consumption, animal feed and various industrial products. have allowed the cultivation of soybean throughout Canada from Ontario since the mid 1970's. This has led to an increase in soybean production as noted in 2006 by Agriculture Canada (Dorff, 2009). today, soybean is grown in about six extensively cultivated in Prince Edward Island, Quebec, Alberta and approximately 2 over the world (Canadian Grain Commission, 2012). In oth important agricultural crops; cultivated, it is also not free from *Phytophthora sojae*. Initial outbreaks were in 1948 in Indiana (U.S) (Kaufmann and Gerdemann, 1958) and numerous outbreaks continue to plague producing countries (Wrather and Koenning, 2006; Dorrance et al., 2003).

1.2 *Phytophthora sojae*: life cycle and infection process

Phytophthora sojae is one of 80 species from the gro *Phytophthora*. Oomycetes differ from true fungi in that hyphae ar cell walls consisting cellulose as opposed to chitin, with zoospores a 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 soybea
amount-2 to 1 billion dollars and maintenance costs are several
Riberio, 1996; Wrather et al., 2001a,b). Oomycete pathoge
mainly because they infect underground plant parts and where
difficult and uneconomical (Tyler, 2007).

Zoospores, or flagellated spores, are the primary infective spo
P. sojae
water-borne in the spring when the soil is moist and warm, zoos
form-10 zoospores, which are attracted to soybean root
chemotaxis
exudates (Morris and Ward, 1992). Once at the root surfac
outer epidermal cell wall, encyst, germinate, and the emerging hypha p
epidermis and into the cortex. As hyphae grow between cor
pathogen can feed on host tissue through structures called appressori
haustoria
on the outer surface of root epidermal cell walls allow the fo
which penetrate root cell walls; haustoria can penetrate and grow in the en
pathogen for feeding (Tyler, 2002). Initial, biotrophic feeding leads
weakening of the host, and necrotrophic mechanisms
lesions
formation. Lesions cause above ground plant parts to wilt and die
cannot take up and transport water and nutrients. Once th
tissues
are colonized, hyphae form haploid antheridia and oogonia
the formation of diploid overwintering oospores, which can
germinating to produce hyphae that can produce sporangia
Sporangia
initial infection process involves physical penetration of th

plant resistance involves physical barriers, namely epiderm
the spread of within the root.

1.3 Plant defense pathogens and the genetics of resistance

Pathogens such as Phytophthora species cause substantial crop losse
annually and they lead to an alteration of host metabolism and
Dehne, 2007). To combat microbial threats, plants have evolved two kinds of defense t
constitutive and inducible defenses. Constitutive defenses include physical
such as suberized and lignified cell walls as well as antimicrobial
compounds such as isoflavonoids, flavonoids, and terpenoids. Recognition of a
pathogen and induction of host specific and/or host specific responses
known as pathogen associated (PAMP) triggered immunity (formerly
known as horizontal or basal resistance) and immunity (formerly
known as resistance) (Boller and Felix, 2009).
In the former, specific microbial protein such as flagellin mig
the host and in the latter an associated factor (e.g., an
avirulence gene product) might be recognized. In the early stages
localized necrosis may result, whereas, the effector mediated
the presence of pathogen and induction of a series of interactions can
lead to the hypersensitive response. The hypersensitive response
response called systemic acquired resistance (SAR) gains
increased immunity from initial infection through the up regulation
(reviewed in Coll et al., 2011).

In soybean, resistance to *P. sojae* is of two types and includes race-specific, effector-triggered and PAMP-triggered immunity (referred to as partial resi field/quantitative resistance, tolerance and race-specific resistance includes *Rps* genes, 15 of which have been identified (Anderson Polzin et al., 1994; Dorrance and Schmitthenner, 2000; Sandhu et al., 2004; Tucker et al., 2010). The effectiveness of *Rps* genes relies on the presence of a corres avirulence gene in the pathogen whose product can be reco Furthermore, *Rps* genes recognizing compatible *P. sojae* usually remain successful for 8 to 15 years due to the fact that this pathogen has a shor and can evolve resistance to *Rps* genes in a relatively short time span (Burnham et al., 2003; Dorrance et al., 2003). For this reason, partial resistan looked to as an alternative. Partial resistance, a form of constitutive def is targeted against all *P. sojae* races, is mainly structural and/or antimicro and is inherited quantitatively (Dorrance et al., 2003; Ranathunge e et al., 2010). As virulence of *P. sojae* continues to increase in area production, it is essential to increase and incorporate part cultivars for effective disease management.

1.4 Suberin: physiological roles and structure

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

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

A) SPPD B) SPAD

Figure 1: The hypothesized structure of the suberin. A, The poly(phenolic) domain covalently attached to the primary cell wall. B, The poly(aliphatic) domain betw primary cell wall and plasma membrane, linked to SPPD via ester linkages appearing as dark and light bands, P = phenolic, C = carbohydrate, S = su from Bernards, 2002).

The SPAD is made up of aliphatic monomers specific to
example, ω-hydroxy fatty acids, α,ω-dioic acids generally form the major
aliphatic substituents, and can account for 10-40% of suberin (Graça and Pereira, 2011a, 2011b; Graça and Pereira, 1997; Barr-Tisimmons, 2010). Other components include other fatty
glycerol and hydroxycinnamic acids (e.g. coumaric and ferulic acids) (Graça and Pereira, 1997). The SPPD comprises a covalently linked m
from hydroxycinnamic acids and hydroxycinnamoyl alcohols (hydroxycinnamyl
(Kollatukudy, 1984; Zeier and Schreiber, 1997; Bernards, 1998; Matzke and Riederer, 2009).

In epidermal cells, suberin is deposited in radial and t
enclosing the cells (reviewed in Bernards, 2002; Beisson and Enstone, 2002). In the
endodermis, suberization has been characterized in three distinct stages i
root tip both aliphatic and phenolic suberin is found in the
endodermal cells and forms the Casparian band which can
the use of lipophilic tracers. Within the Casparian band, the deposition o
radial walls of endodermal cells results in a contiguous wa
fishnet surrounding the stele when the underlying tissue
away (Figure 2). Further along the root axis additional suberin
are deposited in all walls of endodermal cells (Casparian
band), however not in all endodermal cells, finally, further
further deposition of suberin in radial and outer tangential
thickenings of endodermal cells (Wilson and Peterson, 198

Similarly, heavier-metal (i.e., diffuse) suberin deposition through

epidermal cells along the root axis, moving away from the

<u>Figure 2</u>: Longitudinal scanning electron microscope image of root endodermis of *Clivia miniata* Reg. Suberin forms a net like barrier shown by the arrows. cells near the xylem (Image shows suberized cell walls remaining after cell were enzymatically digested). Bar = 100µm (modified from Schr, with permission from Oxford University Press).

1.5 Suberin and resistance

The physical nature of suberin is the principal factor in inhibition of pathogen penetration into plant tissues that c et al., 2002; Viljevac et al., 2009; Khan et al., 2007a; Khan et al., 2011). The correlation between high-temperature soybean root poly(aliphatic and partial resistance. That is, the soybean cultivar Conrad, w lower rates of infection and colonization by the root suberin compared to cultivar OX760-6, which had lower amounts of both pre aliphatic suberin and field level resistance. Specifically, fatty acids and diacids of the SPAD which were increased at soybean field level resistance. Furthermore, Ranathunge e P. sojae hyphal penetration and growth was inhibited to a grea compared to OX760-6. Hyphal progress was especially impaired at endodermal layers.

The role of the phenolic domain in resistance to pathogens important as it not only anchors the suberin molecule to th hydroxycinnamic acid monomers also possess antimicrobial properties (Liu and Co 1998; Pomar et al., 2004; Bajji et al., 2005). Collectively, barrier and some of the molecules that make up its core, is when studying constitutive defenses, which can be increased to shift soybe resistance.

1.6 Manipulating suberin levels part of enhanced partial re

In essence, suberin not only acts to prevent water loss restricts pathogen growth at the suberized cell layer endodermis. In o words, based upon the above discussion, it is hypothesized that suberin role in plant partial resistance against pathogens. Consequ strategy to manipulate suberin levels, particularly enhancing, to increase resistance. This may be achieved, for example, by enhanci synthesizing enzymes, which can lead to increased deposit the É-hydroxy acids and diacids which form much of the SPAD, which linked to an inhibition of pathogen ingress. Thus, it would lead to É-hydroxylation of fatty acids expressed in an attempt to enhance suberin biosynthesis in soybean roots.

1.7 SPAD monomer synthesizing enzymes

Fatty É-hydroxylases (FAHs) are a part of the P450 cytoch monoxygenase family enzymes that catalyze the hydroxylation of the te carbon of fatty acids (Benveniste et al., 1998; Pinot and Beisson, More specifically, the CYP86A and CYP94B monoxygenase members that can catalyze É-hydroxylation of fatty acids, respectively, from fatty acids in a NADPH dependent manner (Höfer et al., According to Serra et al. (2009), RNAi knockdown of a CYP tuberous sunheads to lower retention of water by the periderm (WT) because of a changed suberin composition. FAHs in soybean c

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

1.8 Soybean transformation

In order to begin to work on changing the expression o
(GmFAE1) genes, a method of plant transformation must be se
the insertion of suberin biosynthetic genes of interest into
analysis of their changed expression can be undertaken to
roots. Traditional transformation techniques use the soil
& Towns (=Rhizobium radiobacter [Beijerinck & van Delden] Young et a
bacterium that can be engineered to carry foreign genes.
transformation efficiency and long plant regeneration times
transformation is not always feasible (Cao et al., 2009; Ts
An alternative strategy using the bacterium (Rhizobium
Rhizobium rhizogenes [Riker et al.] Young et al., 2001) to
generation of transgenic hairy roots.

1.9 Agrobacterium rhizogenes and hairy roots

Agrobacterium rhizogenes, especially the members of the
family Rhizobiaceae, leads to extensive adventitious root growth
root syndrome at the site of infection (Riker et al., 1930). Hairy root forma
dependent upon the successful transformation and integration of plant cell
bacterial genes. Although the transformation mechanism is a

tumefaciens-mediated transformation, Agrobacterium genes lead to adventitious root for
rather than the formation of undifferentiated cell masses (Nilsson and w
and Olsson, 1997). Ri (root inducing) plasmid DNA is transferred a
of several hundred base pairs, and integrated into the host genome. T
T-DNA region may contain auxin biosynthesis genes as well as genes
genes that lead to increased sensitivity of the plant tissue
al., 1991). Expression of the particular is what causes increa
sensitivity to endogenous auxin, which leads to root formation.
Consequently, mutant strains of Agrobacterium lead to adventitious ro
formation (White et al., 1985). These oncogenes lead to n
adventitious root growth), and lead to opine production. The motivation for
this is to provide a growth substrate (opines) for bacteria
thus giving rhizogenes a competitive advantage over other species o
in Nilsson and Olsson, 1997). Ri plasmid DNA can be engineered to carry co
to knock down or over express native plant genes, or to int
Given that hairy root formation depends upon T-DNA, the successf
region and genes from bacteria, the relative proportion of trans
tissue is greater. Accurate and repeated transformation since tra
cells differentiate into adventitious roots. It is important
hairy roots are transformed because of local phytohormones
induce cell differentiation into root tissue.

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

deposition might be affected. Even though hairy root cultures have been
more than 100 species, their anatomy and has not been studied in cell wall ch
(Cano-Canche and Loyola-Vargas, 1999). Notably, California poppy (Eschscholzia
californica Cham.) and opium poppy (Papaver somniferum L.) (Park and Facchini,
2000), and tomato (Solanum lycopersicum L.) (Peres et al., 2001) differences in cell
arrangement and structure between WT and transgenic roots have sometimes
In particular, poppy hairy roots had a loose cortical arrang
whereas tomato hairy roots had a higher number of vascula
This suggests that hairy roots differ between species imperative to. Thu
study the anatomy and chemistry of hairy root cultures of t
to understand the impact those differences may have on fu

Soybean hairy roots can be induced and cultured (Cho et al., 2000
Induction of hairy roots involves wounding plant material,
hypocotyls, stems or young leaves, and simultaneously infe
engineered A. rhizogenes strain. Usually within a few weeks adventitious root grov
begins and small roots appear at the site of inoculation. O
roots can be cultured in solid or liquid nutrient-rich media.
stability and ease of regeneration is most appealing to plant biologists.
(2009) have found that Nicotiana benthamiana hairy roots retain morpholo
stability in culture medium and thus can be cultured relatively easily a
Thus, studying plant roots may be infected is simpler and more efficient
system that has a fast growth rate, is stable and can be viewed in a
flask

Hairy roots have been used to study a wide variety of plant biology top including specific gene expression (Diouf et al., 1995; Kouchi et al., 1999) and root nodule development (Doyle et al., 2003; Hayashi et al., 2008). They have also been us to study the interactions between plant roots and pathogens such as fungi. For instance, a similar approach has been used to study *Fusarium solani* (Mart.) Sacc soybean (Lozovaya et al., 2004), and *Fusarium oxysporum* Schlecht.-tomato (Mandal and Mitra, 2008) pathosystems, suggesting that hairy roots can be u study plant root and pathogen interactions. For instance, isoflavonoid accumu particular, glyceollin and coumestrol synthesis was measur liquid chromatography in two different cultivars of soybean af *F. oxysporum* inoculation and results were compared to uninoculated control (Lozovaya et al., 2004).

1.10 Rationale: why hairy roots?

Since *Phytophthora sojae* is a root pathogen and *Agrobacterium rhizogenes* transformation leads to the hairy root phenotype, it seems that using soyb of suberin in the *P. sojae*-soybean pathosystem is an excellent option. transformation system, suberin levels could be manipulated by u appropriate constructs to alter the expression of GmFAE genes (for exam further allow testing the hypothesis that suberin plays a vital role in the pathogen growth within soybean roots. Generating a whole cells and cells that may or may not be transformed can tak However, transformed hairy roots can be regenerated within a few weeks

pathogen preferentially attacks roots, a whole plant need r
as mentioned earlier, the aliphatic domain of suberin is clo
partial resistance in soybean roots. Therefore, it becomes imperat
whether or not the deposition of suberin in hairy roots mim
reason, hairy roots must be investigated more carefully be
model to study the role of suberin in soybean. Transpiration and these issues
into my thesis objectives.

1.11 Objectives

1. Establish soybean hairy root cultures from soybean cu
   6.
2. Confirm that established *A. rhizogenes* hairy roots are ideal for investigating
   the expression of *RSH* genes using PCR.
3. Study the anatomy and suberin deposition pattern in es
   cultures using histochemical techniques.
4. Characterize the aliphatic suberin (quantitatively and qualitatively) in soybean
   hairy roots.
5. Investigate the infection process of *Phytophthora sojae* on hairy
   roots and compare to WT roots.

By fulfilling these objectives, I hope to gain a clear pictur
and functional similarity of hairy roots to WT roots, thus esta
tool to investigate the role of suberin in the soybean-*Phytophthora sojae* system.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant materials

Seeds of *Glycine max* cultivar Conrad and line OX760-6 from field grown plant (year of cultivation 2006) were obtained from Agriculture and Agri-Food Canada (London, Ontario). Seeds were surface sterilized in 20% (v/v) commercial bleach, Clorox Co. Ontario, Brampton, Canada) for 20 minutes and rins with sterile water. Surface sterilized seeds were then plac with sterile water in petri dishes. Petri dishes were sealed using p 25°C incubator at 16:8 (light:dark) photoperiod. Once the cotyledons e were excised and used to generate hairy roots as described below.

2.2 *Agrobacterium rhizogenes*

Agrobacterium rhizogenes strain LBA9402, a gift from Dr. Patrick C (National Reseach Council Canada) was used to generate h Stock cultures were maintained on yeast mannitol agar medium (YMA (4 mM K_2HPO_4, 0.8 mM $MgSO_4$, 1.7 mM NaCl, 5 mM mannitol, 0.4 g yeast extract, and 15 pH 7 with 1 M NaOH) at 4°C in the dark.

2.3 Generation of hairy roots

Hairy roots were generated using a protocol developed by Cho et a slight modifications. Briefly, soybean cotyledons were ino *A. rhizogenes* the abaxial side by wounding the major vein using a scalpe

coated with *A. rhizogenes* from culture that had been grown overnight on medium at 30 °C. Inoculated explants were placed on solid (0.8% w/v) agar medium containing MS (Murashige and Skoog) salts (4.4 g L^{-1}), sucrose (3% w/v) and cefotaxime (500 mg L^{-1}), adjusted to pH 5.7-5.8 with 1 M NaOH. The antibiotic is used to p the growth of *A. rhizogenes* anywhere but the site of inoculation. The inoculated explants were placed in an incubator (dark, 16:8 h light photoperiod, 25 °C).

2.4 Maintenance of hairy roots

Once hairy roots emerged (approximately 2-4 weeks), when 5 cm in length they were excised from the parent explants and placed on MS medium (same as above with same concentration of cefotaxime). Once free of bacterial growth, they were plated on MS medium without antibiotic. Plates with hair placed in an incubator at 25 °C (dark, 16:8 h light photoperiod). The first 4-5 cm of root was subcultured every few weeks onto a fresh plate as described above to propagate and maintain the hairy roo

2.5 PCR and *rolB* gene

In order to confirm the origin of adventitious root grow *rolB* gene was amplified from genomic DNA obtained from established hair polymerase chain reaction. Genomic DNA was extracted fro both Conrad and OX760-6 using modified (CTAB) Cetyl Trimethyl Ammonium Bromide) protocol of Kang and Yang (2004). Briefly, hairy roots were ground

in liquid N_2 using a sterile mortar and pestle and approximately … was re-suspended in 600 µL of pre-heated (65°C) CTAB buffer (1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 2% CTAB) along with 0.2% v/… mercaptoethanol and 2 µL of RNase A and incubated at 65°C for 1 hour. To t… suspension, 600 µL of Chloroform:Isoamylalcohol (24:1) was add… centrifugation at 13,000 rpm for 12 min. The resulting supernatant was tran… to a fresh microcentrifuge tube and 0.7 volumes of isopropanol were a… was mixed by inverting the tubes. The sample was cooled at 4°C for 30 minu… centrifuged again at 13,000 rpm for 20 minutes at 4°C. The supernatant obtained after centrifugation was discarded and the pellet was washed wi… again at 13,000 rpm to collect the pellet. Ethanol was removed completely using a… aspirator and the pellet was re-suspended in 30-50 µL of Milli-Q® H_2O.

The *rolB* gene was amplified using primers described by Zebarjadi et al. (2011)… F: 5'-GCTCTTGCAGTGCTAGATTT-3' and R: 5'-GAAGGTGCAGCTACCTCTC-3'… 430 bp fragment was amplified. Genomic DNA from hairy and WT roots from both cultivars was used (WT roots = negative control). PCR was also run using whole bacterial preparation as a positive control. The 25 µL PCR… µL $MgCl_2$ (50 mM), 2.5 µL 10 X PCR buffer, 1 µL dNTP mixture (100 mM), 0.3… (5 U), 100 ng of genomic DNA or 5 µL of whole bacterial preparation and… to volume with Milli-Q® H_2O. The enzyme and primers were obtained… The PCR reaction was conducted with an initial denaturation temperature of 94°C for 4 minutes followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, 72°C… annealing temperature was 55°C and final extension was do…

The PCR products obtained were run on a 1.0% agarose gel (applying a voltage of 10 and stained with ethidium bromide. Gels were visualized on Bio-Rad Gel Doc (Universal Hood II, S76S/01511).

A relatively easy method of determining whether or not transformed was also employed, which allowed visual confirmation. In vitro grown cotyledons (obtained as described above) we groups. One group was mock inoculated as described abov whereas, the second group was inoculated with A. rhizogenes. After 10 days the presence or absence of hairy root formation was noted.

2.6 Histochemical analysis

In order to view the pattern of suberization in hairy roots, fluorescent and non-fluorescent dyes were employed. Free-hand sections of hairy roots cultivar S-14 (1-2 cm in length) were made at intervals 1.5 along the length root. Sections were viewed using a Zeiss Axioimager Z1 Microscope. The C band in the endodermis was detected using Neutral Red (NR, Sigma) and Toluidine Blue (TBO, Sigma-Aldrich) according to Lulai and Morgan (1992 free hand cross sections were stained with fresh TBO (0.05 4.4) for 1 minute followed by rinsing with neutral red (0.1% in 0.01 M phospha pH 6.5) for 30 s. Stained sections were then rinsed several times in water on slides. Sections were viewed with blue light (-490 nm), filter set dichrotic mirror 510 nm, barrier filter 515-565 nm). Suberin and Casparian band fluoresce yellow. Images were taken with AxioCam MR3. Suberin lamellae w

viewed using fluorol yellow (FY) (Brundrett et al., 1991). Root cross sections were placed in 0.01% (w/v) FY in polyethylene glycol-90% glycerol (1:1) for 1 hour. These sections were then rinsed several times in fresh water and mounted in 75% glycerol and viewed (filter set: excitation 540-560 nm, dichroic mirror 585 nm, barrier images were taken with an AxioCam MR3. Under these conditions, cell walls containing suberin lamellae fluoresce bright yellow-white. Finally, suberin was detected using Sudan Red VIIB (fat red). Root cross sections were placed in 0.1% (w/v (in 1:1 polyethylene glycol:glycerol) for 2 hours (Brundrett et al., 1991). rinsed with clean water, mounted in 75% glycerol and viewed with brig were taken with an AxioCam ICc1. Suberized cell walls stain pink to red.

2.7 GC-MS analysis

Suberin is a cell wall polymeric network and in order to quanti it in plant tissue, it has to be depolymerized into monomers. Both whole roots and isolated epidermal and endodermal tissues were analyz

For whole root, hairy roots from both cultivars (1-12 cm) were frozen in liquid N_2 and ground to a powder using a mortar and pestle.

To isolate epidermal and endodermal tissues from three separate root inoculations (three roots per replicate, total of were used from both cultivars. Roots 1-14 cm in length were separated into 3 seg 0-4, 4-8 and 8-12 cm. Any lateral branching was removed. Root cross the epidermis was removed; three separate diameter measu

for any root sections that were not cylindrical. This was done so that the surface area of the roots could be calculated and suberin amount per mm^2 value. The epidermis was then removed by pressing. Roots were pulled through the wire, which stripped the epidermis and few cortical left behind the endodermis, stele and a few cortical cells. The epidermis removed, it was frozen in liquid N_2 and ground using a pre-cooled mortar. Ground tissue was placed in microcentrifuge tubes and kept frozen at -80°C until extraction. Root cross-sections were made in the endodermal tissue to measurements were made at three different locations. The endodermal tissue was also ground from the epidermis and placed in a separate microcentrifuge tube.

In order to quantify the amount of suberin monomers found in root tissue was first extracted in chloroform as described by Meyer et al., 2011). Briefly, frozen hairy root tissue from both tissue layers and cellulose thimbles and dried overnight. The dried tissue was extracted in chloroform:methanol (2:1) for 3.5 hours. The solution turned a dark green color and was removed and clear around bottom and flask. The remaining tissue was washed for with 3.5 hours of fresh chloroform:methanol (2:1). This solution was also collected and the tissue was extracted in 100% chloroform overnight. The third aliquot was combined with the previous extracts and dried, as was the removed from the thimbles, weighed and transferred to vials in 5 mL solution. Components were obtained by drying down in chloroform:methanol mixtures using a rotary evaporator. When approximately 1

bottom flasks, the solutions were removed using a glass pi weighed clean 5 mL glass screw cap vials. Remaining chloroform:methanol was evaporated using a steady stream of nitrogen and the mass weighing the vials again.

Suberin monomers were obtained by using a methanolic-HCl transesterification protocol according to Meyer et al. (2011). Samples (3 mg for separated hairy roo approximately 6 mg for intact hairy roots) were incubated w HCl (Supelco-Sigma-Aldrich; 3 N diluted to 1 M with dry methanol) at 80°C for Once samples were brought to room temperature, 1 mL saturated salt water w stop the reaction after which 10 µL of triacontane (1 mg mL^{-1}) was added as an intern standard. Samples were extracted three times with hexane and the vial was sealed and shaken vigorously, the upper layer was then re clean screw cap glass vial and extraction with hexane was hexane solubles were dried under a steady stream of nitrog µL of pyridine and 50 µL BSTFA + 1% TMCS (N,O-bis(trimethilsilyl)trifluoroacetamide; Supelco-Sigma-Aldrich) were added and incubat at 70°C for 40 minutes to derivatize remaining hydroxyl an groups. The final sample was transferred to 2 mL glass screw cap vials with inserts (Agilent). Concentrations of esterified monomers and any TMS analyzed on Varian CP-3800 Gas Chromatograph equipped with a flam detector (GC/FID) and an ion trap mass spectrometer (GC/MS). The FID and mass spectrometer were connected through CP-Sil 5 CB low bleed MS column (WCOT silica 30 m X 0.25 m). One µL samples were inject

injector temperature set at 250°C. Samples were eluted fr following temperature settings: 70°C for 2 min, 40°C/min to to 300°C, hold 9.42 min, total 50 min. High purity helium w flow rate of 1 mL/min (Yang and Bernards, 2006). Compou ified using EI-mass spectra and quantified with GC/FID using intern and external calibration curves established from standards

2.8 Hairy root-pathogen interactions

To follow the infection process in hairy WT roots and comp *Phytophthora sojae* race 2 (strain P6497) cultures were obtained as Gijzen (Agriculture and Agri-Food Canada, London, Ontario). The oom maintained on 20% V8-juice agar (8.4 g agar, 1.6 g $CaCO_3$, 150 mL V8 juice [Campbell Soup Company, Toronto, Ontario, Canada], and 440 mL of H_2O) for 5-7 days after which zoospore production was induced. To induce zoospores, plate cultures were flooded overnight with sterile distilled water at room temperature. The next day the water was replaced with sterile distilled water every half hour for 5 hours. Zoospore produ viewed using a microscope at 40X magnification and when zoospores were detec water was decanted to a sterile beaker and zoospore concentration using conce 0.1% w/v aniline blue in lactophenol (1:1:1 85% lactic acid was added to 1 mL of the zoospore suspension and concent hemocytometer. The zoospore concentration was adjusted t 10^{4} zoospores mL^{-1} with sterile distilled water.

Hairy roots were incubated directly in zoospore suspension for 24 and 72 hours. Hand cross sections were made 1 cm from the root tip for microscopy using a protocol described by Ranathunge et al., 2003. Roots were cleared using 10% KOH for 30 min and stained with 2% trypan blue in lactoglycerol (1:1:1 of water, glycerol and 85% lactic acid) for 5 min. Cross sections were mounted in 75% glycerol and viewed under bright light using the microscope described above. Images were taken using CamICc1.

2.9 Statistical analysis

Student-t test was used to compare mean amount of poly(aliphatic) suberin in intact hairy roots from both cultivars. Analysis of Variance (ANOVA) was used to compare mean amount of poly(aliphatic) suberin of isolated exodermis from both cultivars. ANOVA was also done to compare mean amount of suberin monomers distributed by chain length in intact hairy roots of both cultivars and waxes of isolated exodermis from both cultivars. In the case of significance 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 soybean cultivars, OX760-6 and Conrad, were generated in the lab using a modified protocol of Cho et al. (2000). When cotyledons were inoculat with *Agrobacterium rhizogenes* strain LBA9402, tissues surrounding the point of inoculation became necrotic within a couple of days, and approximately one week inoculation (pi), callus growth appeared (Figure 3 A, B). Hairy root growth from the callus region appeared at approximately 10-14 days pi (Figure 3 C). Previous studies have reported a similar timeline for hairy root generation in different al., 2009; Fernandez et al., 2009; Zhang et al., 2010). Although many roots were ge per cotyledon within 10-14 days pi, root growth was also noticed before a time range. This could be attributed to T-DNA insertion, copy number of dif which in turn influences the amount of endogenous hormone produced, levels in the tissue. This in turn affects the time when hairy root growth is abundant (T 1997). Furthermore, the site of insertion can also affect the amount and frame of hairy root generation (Sekine et al., 2005). Hairy roots cultured on solid medium had growth rates of approximately 2-5 mm per week.

Figure 3. Time course of hairy root induction and emergence from explants inoculated with *Agrobacterium rhizogenes* A, Necrosis on wounded hypocotyl 3 days after inoculation. B, Callus growth 7 days pi. C, roots emerging from callus 14 days pi. D, Two weeks old hairy roots in nutrient rich agar medium. Bar = 1 cm (A-C), 5 cm (D).

Cotyledons obtained from in vitro germinated soybean for hairy root production, which proved to be a more efficient system rather cotyledons from soybean plants germinated in the greenhou cotyledons from greenhouse-grown soybean followed by inoculation with bact necrosis and eventually embryonic leaf death before any ha

Once putative hairy root cultures were established it w whether or not they are a result of *A. rhizogenes* mediated transformation. For t analysis was done using genomic DNA isolated from hairy r along with WT roots. The presence of the *rolB* (430 bp) PCR amplicon fo gel (Figure 4) confirmed the *A. rhizogenes* origin of hairy root genotype. This important because in order to manipulate suberin levels in system is required that will alter the soybean genome, and not only hormonal changes. However, successful generation of hair visually. That is, cotyledons wounded with a scalpel but n *A. rhizogenes*, did not produce any roots whereas those inoculated formation at the site of inoculation (data not shown). It is necessity of conducting PCR analysis on hairy roots, becau genetically transformed. *Agrobacterium rhizogenes* not only has the ability to transform plant tissue with a native plasmid, but it can also transform a secondary plasmid, thus is efficient as a binary vector sy through the use of a binary vector system, explants transformed soy as well as a gene encoding a protein product which fluoresces u any generated hairy root which fluoresces red is truly tran

reported that 34% of roots generated from reverse transformed were initiated due to hormonal effects.

Figure 4. PCR analysis of genomic DNA using primers designed for the *A. rhizogenes* rolB gene. Lane 1 = whole bacterial DNA preparation (positive control); Lane 2 = Conrad WT = genomic DNA isolated from uninoculated soybean (negative control) and Lane 3 = rolB-HR = genomic DNA isolated from soybean hairy root cultures. PCR products were run on 1% agarose gel. Select ladder (bp) are labeled on the left hand side of the gel. The 430 bp.

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

It was observed that hairy roots grow mainly aerial, i.e. roots emer callus and typically display negative geotropism (Figure 3E). suggested that fewer amyloplasts are found in hairy roots possibly leading to a weak gravitational response (Kim et a Furthermore, large air spaces were commonly present in the a (Figure 5). Teakle et al. (2011) reported that roots growin areas, such as agar, develop an aerenchyma to overcome the root tissue limiting O_2 conditions. These features were found in both Co OX7660.

3.2 Hairy root anatomy

Hairy roots are anatomically similar to WT roots as ca However, in addition to the air spaces found in the cortex, the vasculat hairy roots seems different than WT. In particular, in som

three xylem poles whereas, WT roots have four (Figure 5). (1957), auxin levels can influence the number of xylem poles formed in roots re from decapitated roots placed in auxin medium. Thus, sinc higher levels of endogenous auxin (or are more sensitive t that the number of xylem poles may be influenced in hairy roots.

I also noted that hairy roots did not appear to have the WT roots. This was especially evidenced during sectioning prone to breaking or collapsing. This may be characteristic of the soybean h air spaces present in the cortex (Figure 5) may also contri integrity. Additionally, it could be due to the influence of the moist m roots are first generated and then propagated. High moisture conditions can wea epidermal cell walls making them more prone to damage. H widely grown in liquid cultures and used as tools and mode questions involving plant roots, it seems that while media may have robustness it does not preclude their use as an experiment

Figure 5. Cross section of an OX760 root tip (top image) and an OX760 with root hair 4 cm from the root tip. Magnification = 55. Section stained with toluidine blue. Left hand side is epidermis, cortex and endodermis, and central stele.

3.3 Hairy root suberin histochemical analysis

In order to use hairy roots to test the hypothesis that *Phytophthora sojae* soybean pathosystem, it is imperative that suberin deposition in hairy roots resembles that of WT roots. Through the use of fluorescent dyes, fluorescence microscopy showed that cell wall suberin histochemistry of hairy roots is similar to WT roots (Figure 6). Previous research using WT roots documented that clover root has a state I endodermis, in other words, suberin deposition occurs only in the anticlinal walls and solute transport between the cortex and the vascular stele in the mature zone (Thomas et al., 2007). In cross section, the Casparian band is evident as two distinct points of fluorescence in endodermal cell walls staining with a fluorescent dye, and this same pattern can be seen in hairy roots (Figure 6C). Near the root tip, endodermal cell walls of hairy roots were more suberized resulting in lamellae. However, not every cell wall was suberized resulting in a characteristic suberin deposition pattern where regions of suberin deposition alternate with regions of no lamellae (Figure 6C). This may be to allow the passage of water and solutes into the stele even at these more distal parts of the root (Clarkson, 1975; Clarkson et al., 1978; Peterson and Enstone, 1996; Waduwara et al., 2008). Importantly, hairy roots also still had a Casparian band. Normally as roots mature, suberin deposition also increases (Peterson and Enstone, 1996). In hairy roots we found increased suberin deposition in epidermal cell walls distal to the tip along the root (Figure 6E; this was evident in greater staining of epidermal cell walls close to the root tip at about 10 cm and this was also observed in WT roots (Thomas et al., 2007).

Figure 6: Suberin deposition patterns in soybean hairy roots (A, C, E, F and G = soybean hairy roots and B, D and H = soybean wild type roots (cv. Raymond Thomas), free hand cross sections. Bar = 50 µm. A, Section stained with toluidine blue O and neutral red, 2 cm from the root tip. C present in the endodermis. B, Section stained with toluidi 8 cm from the root tip. C, Section stained with fluorol yellow (FY), 8 cm from the root Patches of cells with suberin lamellae (arrows) are present stained with FY, > 8 cm from the root tip. E to H, Sections lamellar suberin is present in the epidermal cell walls (arrows). E 2-4 cm from root tip, F 4-6 cm, G 6-8 cm and H 2-4 cm.

Hairy roots from both cultivars displayed similar suberin d
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 GC-MS analysis

After establishing suberization patterns of hairy roots,
poly(aliphatic) suberized tissues was measured (1-2 long,
see materials and methods for details) were analyzed for a
and amount.

3.4.1 Quantitative analysis

Quantitative analysis revealed that OX 7 hairy roots had more suberin
compared to Conrad (Figure 7). Although this difference is
is in contrast to published work where Conrad had approximately
amount of aliphatic suberin than OX 7 (Thomas et al., 2007), this difference
to both cultivars responding differently to either the transf
and/or the medium in which they are grown. Like many genetic traits,
suberin deposition is influenced by the environment. Previous work has shown that
has greater partial resistance to higher amounts of suberin in
suberizing tissues (Dorrance et al., 2003; Thomas et al., 2
al., 2008; Ranathunge et al., 2008). Anderson (1982) found tha
a 2% mortality rate when soybean seedlings were exposed to susceptible cultivar An

had 40% mortality rates. However, interestingly, the perce[-6]
fields changes between cultivars meyen Vadi ing to the fact that a chang
can influence level of suberin deposition and thus resistan
other words, CX-760 hairy roots may be more plastic to a changin
thus have higher (polysaccharide) suberin levels compared to Conrad ha
medium as opposed to growing in soil may contribute to thi
insertion may also influence expression of native genes (B... et al., 2003; Zia et al.,
2010) and thus cause hairy roots to have a different phenotype

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

Quantitative differences in aliphatic suberin were found in C-6 Conrad and OX760 hairy roots when the endodermis and epidermis were compared, although analy differences were not always statistically significant (Figure 8). In particular, sub was highest farthest away from the endodermis in C-6 hairy root lines of OX760. Two important aspects of suberin deposition in hairy root derma poly(aliphatic) suberin is higher in the epidermis compared to the endodermis in b cultivars, and 2) poly(aliphatic) suberin amounts increased root axis. Furthermore, there are quantitative differences between WT publ al., 2007) and hairy roots, where the aliphatic suberin in Conrad hairy roots approximately 8 times lower and in C-6 OX760 hairy roots 1.5 times lower than WT. In other words, amounts of aliphatic suberin on a per m^2 basis were more comparable to WT roots in the case of C-6 OX760. This is important because when developing a root model system it is necessary to understa model may have from the original system. With this data i histochemical data it seems C-6 OX760 hairy root system is an appropriate in vitr model compared to Conrad.

Figure 8. Quantification of insoluble poly(aliphatic) suberin in root tissues. Epidermal and endodermal tissues were isolated separated into 3 segments (0-4, 4-8 and 8-12 cm. from tip). Isolated tissues were subjected to MeOH-HCl transesterification to release poly(aliphatic) suberin. Suberin was measured using GC-MS and GC are expressed on basis of root endodermal surface area. Data represent average ± SE of three replicates with different letters are significantly different (P = 0.001)

3.4.2 Qualitative analysis

Next, it was also important to assess the monomeric co poly(aliphatic) suberin in hairy roots compared to WT. Qualitatively, hairy root aliphatic suberin contained fatty acids, ω-hydroxy fatty acids, fatty alcohols, and dioic acids as in WT roots (Thomas et al. 2007). Since the amount of tissue availab isolated dermal tissues from hairy roots was so low, some suberin componen accurate detection levels and thus component analysis was so, it was found that although all components are present, fatty acids, alcohols, dioi acids and ω-hydroxy fatty acids, not all chain lengths that are normally were found (Table 1). Generally 16 and 18 carbon chain le found, whereas longer chain lengths of fatty acids and alco more of suberized tissues, (reviewed in Bernards, 2002) were not d important feature to take note of is that ω-hydroxy fatty acids in both cultivars are of greatest abundance, which is similar to WT roots where they make up 65-70% of total aliphatic suberin (Thomas et al., 2007). In fact, it is this component that has been show to have a high-correlation with resistance to P. sojae (Thomas et al., 2007). Again OX760-6 not only has greater suberin composition, amounts a roots. Interestingly, Conrad had higher amounts than OX760-6 although this difference was not significant.

Table 1. Chain length distribution of hairy root aliphatics by subs
intact hairy roots of both Conrad and OX760. Data expressed as mg/mg $X\ 10^{-5}$ of h
root tissue subjected to $NaOCH_3$-HCl transesterification and measured using -MS;
averages of three replicates ± SE. Means followed by different
different (P = 0.006).

3.4.3 Soluble wax analysis

With respect to soluble wax components, the pattern o
published data of WT roots (Thomas et al., 2007) for 6-DPI (Figure 9)
soluble waxes increased, however the increases were not stati
significant. Regardless of the lack of significance, soluble wax contains un
polymerized alkanes, and typically the actual amount of which in th
hairy roots may vary between distances from the root tip.
earlier, soybean WT roots with higher amounts of insoluble
more resistant to pathogen infection, therefore, it can be expected
growing in an artificial medium may not have the same amo
may be considered more plastic to a changing environment

Figure 9. Quantification of soluble and insoluble waxes in isolated soybean hai
Epidermal and endodermal tissues were isolated from soyb
3 segments, 4 and 12 cm. Soluble waxes were isolated by solv
Total suberin was the sum of soluble and insoluble waxes. Data are expressed on
root or endodermal suface area. Bars represent average ±
significant differences were found (P = 0.235).

3.5 Hairy root-pathogen interactions

As a final step in investigating the appropriateness of model for soybean WT roots, analyzed the interaction between root-and path 6 hairy roots had higher levels of suberin comparable to WT roots, and h more structurally impaired compared to Conrad, only OX760-6 hairy roots were used for this study. Hairy roots were exposed to incubated zoospore sus different times. found that at 2 h zoospores had attached to the epidermal c in preparation for germination. This is in contrast to experiments, where zoospore attachment was observed at the 1 h time point (Ranathunge et al., 2008); at 4 h however, zoospores had attached and showed evidence of germination (Figure Finally, within 22 h zoospores had penetrated the epidermi growth was seen between cortical cells (Figure 10B, C). T wherein 24 h post inoculation hyphal growth has reached the endodermis (Ra al., 2008). This difference could be due to the fact that ha between cortical cells (Figure 5), which may impede the pr despite this it was clear that P. sojae infection of hairy roots followed a si to that in WT roots, albeit it was slower. This is important effect of the change in suberin levels obtained through ger require a pathogenicity test, which in this case will involve P. sojae infection. With a baseline infection progress we can assess any change in in manipulated.

Figure 10. Development of *Phytophthora sojae* on soybean. Free-hand cross sections of 1-2 cm long hairy roots taken 0.5-6 cm from the root tip. A, after incubation zoospores attached and began germinating. B, 2 hr after incubation zoospore germinated and hyphal germ tube penetrated epidermis. C, 12 hr after incubation zoospore germinated and hyphae progressing between cortical cells (arrows). D, Appressoria on cortical cells (arrows). Bars = 50 µm.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Through this experimental work I have found that hairy roots are sim roots where root anatomy, suberin histochemistry, suberin cl response to pathogen attack are concerned. Even though c cultivars Conrad and OX760-6 are opposite to those found in WT roots, hairy roots can still be used as a system mimicking WT roots since the anatomy, pathogen response has been documented experimentally. V system, suberin levels can now be manipulated, in particular SPAD levels can be changed. For this, RNA interference (RNAi) knockdown experi which can down-regulate *GmFAÉH* transcription and activity, which limits the production of the SPAD. For example, Yi et al. (2010) hav *GmMYB176* in soybean hairy roots via RNAi leading to decreased production. Conversely, SPAD levels could potentially be enhan regulation of *GmFAÉH* genes.

Lin et al. (2011) have developed a new plant transformation ve from *A. rhizogenes*, pHairyRed, which can be used on soybean. It offers a greater detecti capability of *A. rhizogenes* transformed roots since transformed roots appear red ultraviolet light due to the expression of DsRed2 gene. Essentially, pHairyRed has been engineered with the capacity to carry foreign genes separa This can potentially be used in conjunction with RNAi to down-regulate *GmFAÉH* or a strong promoter, such as Cauliflower mosaic virus promoter to up-regulate it.

Additionally, a different pathogenicity test could be em
test of the effect of changed suberin levels on hairy root r
that relies on sectioning and a microscope. Fortunately, a method
hairy roots with zoospore suspensions and monitoring roots
caused by *P. sojae* can be an alternate method of testing the effect
manipulation. Such work has proved to be useful in this pa
expression of other root specific genes have been manipula

In essence, if manipulated levels of suberin affect *P. sojae* pathogenicity
soybean hairy roots, then this will further support the hypo
to soybean partial resistance against this pathogen.

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APPENDICES

APPENDIX A: Permission for copyrighted material.

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