Discovering signaling events in the actinorhizal symbiosis

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DISCOVERING SIGNALING EVENTS IN THE ACTINORHIZAL SYMBIOSIS

BY

NICHOLAS J. BEAUCHEMIN
B.S., University of New Hampshire, 2008

THESIS

Submitted to the University of New Hampshire
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PREFACE

The research in this thesis was completed in conjunction with research performed by our colleagues at the Institut de Recherche pour le Développement (IRD) in Montpellier, France. Dr. Tisa’s lab and the IRD have worked together in discovering communication events in the actinorhizal plant-microbe interactions. Their advice, friendship, and research have been crucial in completing the work presented in this thesis. Research on Frankia Ccl3 auxin biosynthesis completed by Dr. Tisa’s lab and research on plant auxin transporters in mature nodules completed by the IRD was recently published in Plant Physiology (2010) 154:1372-1380. Our collaboration is very similar to the actinorhizal mutualistic symbiosis; their expertise in actinorhizal plant genetics and physiology and our expertise in Frankia genomics and physiology allow for extraordinary research into the molecular interactions of the actinorhizal symbiosis.
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ABSTRACT

DISCOVERING SIGNALING EVENTS IN THE ACTINORHIZAL SYMBIOSIS

by

Nicholas Beauchemin

University of New Hampshire, May, 2011

Although the symbiosis between *Frankia* and actinorhizal host plants has been widely studied, very little is known about the initial molecular interactions. To address this issue, *Casuarina cunninghamiana* root exudates were collected and tested on *Frankia Ccl3*. *Frankia* growth yield was enhanced by root exudates but *Frankia* could not utilize them as a sole carbon and energy source. Exposure to root exudates caused *Frankia* hyphal curling and surface property changes in fatty acids and carbohydrates. Pre-exposure to root exudates also decreased the time required for nodule initiation. The results show that root exudates and *Frankia* physiological changes for symbiosis are involved in the actinorhizal symbiosis. *Frankia* auxin production *in planta* was also researched as a possible signaling molecule. Bioinformatics of auxin synthesis genes and expression analysis of putative *Frankia* genes revealed *Frankia* can likely produce auxins in mature nodules, suggesting auxins may be a signaling molecule in the actinorhizal symbiosis.
CHAPTER 1

INTRODUCTION

The Actinorhizal Symbiosis

The actinorhizal symbiosis is a mutualism that occurs between actinorhizal plants and the actinomycete Frankia (Wall 2000). The symbiosis is based upon the ability of the symbiont Frankia to fix a normally unusable form of nitrogen ($N_2$) into a biotic form. Because nitrogen is the most common limiting growth factor in the environment, nitrogen fixation is extremely important to both the host plant and the environment. This intimate symbiosis takes place in a specialized root structure called a nodule. Inside the nodule, Frankia provides nitrogen for the plant and the plant provides a carbon source for Frankia.

Actinorhizal plants consist of 8 different plant families, including over 200 different species of woody dicotyledonous trees and shrubs (Schwenke and Carú 2001; Zhong et al. 2010; Wall 2000). The globally distributed actinorhizal plants are found on every continent except Antarctica and are able to grow in a diverse set of natural habitats including arid lands, plains, tundra, and temperate forests. Actinorhizal plants are pioneer plant species that are able to grow in extremely nutrient-poor soil conditions and, with the aid of Frankia, are able to reclaim surrounding soil in disrupted environments. This pioneering ability accounts for their most environmentally important role as the initial step in forest succession.
In developing nations, actinorhizal plants have an important economic role for use as a fuel source and in soil stabilization. Due to their symbiotic relationship with *Frankia*, actinorhizal plants have extremely fast growth rates, but many retain a hardwood-like property with very high BTU contents that lose very little biomass during coal production. These properties, along with the ability to grow in many different soils, make actinorhizal plants extremely useful in economically poor areas. Other uses for actinorhizal plants include land reclamation (utilizing the natural pioneer species ability) and nurse cropping (inter-planting actinorhizal plants to provide a natural fertilizer for economic crops). Actinorhizal plants may soon also be used for bioremediation purposes and research is being conducted both into the plants and *Frankia* to determine their best uses (Sayed 2003; Richards et al. 2002). Bioremediation occurs when plants are strategically introduced to heavy metal or toxic compound contaminated sites to reclaim the land. While actinorhizal plants are not a large economic crop in developed nations, like the legume-*Rhizobium* system, they are extremely important in the environment and in developing nations.

**The Symbiont, *Frankia***

*Frankia* is a gram-positive actinomycete and exists in either a free-living state in the soil or in symbiosis with actinorhizal plants (Benson and Silvester 1993; Schwenke and Carú 2001; Chaia et al. 2010). As an actinomycete, *Frankia* maintains hyphal morphology, but also produces two distinct morphological structures: vesicles and sporangia. *Frankia* growth occurs only at the hyphal tips.
causing a slow growth rate (typically a 24-48 hour doubling time). Vesicles are specialized structures that are the site of nitrogen fixation. Spores, formed in the sporangia, are dormant resting structures that aid in the dispersal of *Frankia* in the environment.

As a nitrogen-fixing bacterium, *Frankia* produces the enzyme nitrogenase to drive the nitrogen fixation process. However, nitrogenase is extremely sensitive to oxygen and requires a strategy to protect the enzyme from oxygen damage. To protect the nitrogenase from oxygen inactivation, *Frankia* uses a compartmentalization strategy to separate the enzyme from oxygen and forms vesicles to serve that function. Vesicles are surrounded by layers of hopanoids that are built up around the exterior which serve to restrict oxygen from entering the center of the vesicle.

**Actinorhizal Symbiosis: Intracellular and Intercellular Infection Pathways**

While the actinorhizal symbiosis has been well studied at a morphological level since the 1970s, very little is known about the molecular interactions that occur between the plant host and *Frankia* during the establishment of the association. The establishment of the symbiosis has been termed the infection and nodulation processes (Wall 2000; Alloisio et al. 2010; Obertello et al. 2003; Mastronunzio and Benson 2010). In general, the bacterium needs to recognize a host plant and the host needs to identify the bacterium as a friend, not a foe. The bacteria enter the plant and establish the association after several steps. For the
actinorhizal symbiosis, there are two different infection and nodulation pathways: the intracellular pathway and the intercellular pathway (Figure 1).

For the intracellular pathway, the process probably initiates with rhizosphere communication between the plant and Frankia. Although other chemical signals may be involved in the initiation process, the first known step involves the release of Frankia root deformation factor(s) which cause host plant root hairs to deform (Van Ghelue et al. 1997). Frankia infects the deformed root hair, forming an infection thread in symbiosis with the plant (Berg 1999). The infection thread consists of the growing Frankia and a plant membrane that contains the Frankia in the plant cell. Signals are communicated into the root to cause cells in the cortex to divide, thus creating a prenodule (Laplaze et al. 2000). Lateral root formation starts in the pericycle of the root while Frankia is contained in the prenodule. When the lateral root has grown, Frankia infects plant cells in the lateral root. The lateral root becomes modified to form a mature nodule. The mature nodule enables compartmentalization of Frankia within individual plant cells allowing metabolic exchange.

For the intercellular infection pathway, Frankia invades the host by penetrating in-between plant cells and moves into the root without forming an infection thread. For this process, cellular communication is probably required especially to start lateral root formation. After lateral root formation, an infection thread is generated to move the bacteria into cells of the lateral root, creating a mature nodule.
Figure 1: Depiction of intracellular and intercellular infection and nodulation processes in the actinorhizal symbiosis. Image taken from Perrine-Walker et al. (2011) with permission from Bentham Science Publishers Ltd.
Rhizosphere Interactions

Through active transport, diffusion, and cell lysates, roots secrete plant compounds into the soil that are termed root exudates (Badri and Vivanco 2009). Root exudates often contain carbohydrates, flavonoids, amino acids, organic acids, proteins, and many other compounds and play a crucial role as a communication signal between host plants and both symbionts and pathogens (Bertin et al. 2003; Badri et al. 2009). A variety of complex interactions between plants, microbes, and other organisms that occur within the soil involve plant root exudates. These rhizospheric interactions may involve the recognition of beneficial microbes including interactions that take place in the initial step of the actinorhizal symbiosis or defense against pathogens. The interactions take place through exchanges with plant root exudates. In the legume-\textit{Rhizobium} symbiosis, the root exudates provide multiple functions (Bais et al. 2006; Hirsch et al. 2003). First, legume root exudates contain a chemo-attractant and their release generates a gradient in the rhizosphere that \textit{Rhizobium} can detect. \textit{Rhizobium} senses the gradient and travel towards host roots (Gaworzewska and Carlile 1982). Flavonoids are another compound within root exudates, and function to act as an initiator of the \textit{Rhizobium} infection process. Each specific host legume exudes a unique flavonoid that causes its specific \textit{Rhizobium} symbiont to produce another chemical signal (a Nod factor). Nod factors are a very specific lipochito-oligosaccharide that interacts with their specific host legumes to start an infection thread and control host specificity. Exposure of \textit{Rhizobium} to host plant root exudates induces physiological changes to these bacteria including
surface polysaccharides, secreted proteins, and other signaling compounds (Cooper 2007).

Although studies on the effects of root exudates on the actinorhizal symbiosis have been limited, research in other symbiotic systems suggests that root exudates may play a role in the *Frankia*-actinorhizal plant interactions. Currently, exposure of *Frankia* to root exudates causes noted physiological changes in a few observed areas and modifies nodulation patterns (Hammad et al. 2001; Lavenus 2008; Gabbrini and Wall 2008). The addition of *Alnus glutinosa* root exudates causes a slight modification of the protein profiles of *Frankia* sp. ACN14a grown in culture (Hammad et al. 2001). Dominant proteins identified were stress proteins, implying that the exudate response is a stress response to toxic components within actinorhizal root exudates. Although *Frankia* Arl3 initiates plant infection by causing root hair deformation, exposure to *Alnus* root exudates did not significantly alter the ability of *Frankia* Arl3 to cause root hair deformations (Van Ghelue et al. 1997). However, root hair deformation factor is not specific for infective *Frankia* strains, as non-compatible *Frankia* and non-*Frankia* bacteria will also induce root hair deformation (Perrine-Walker et al. 2011). Preliminary studies by Lavenus (2008) showed that methanol extracts of *Casuarina glauca* root exudates modified frankial polysaccharide levels and gene expression. Methanol extracts of root exudates up-regulated expression of two genes, an auxin efflux carrier and a WhiB transcriptional regulator. WhiB transcriptional regulator expression is of particular interest since these regulators
in *Mycobacterium* have been linked to many functions including infection and stress response (Raghunand and Bishai 2006).

While there is a limited database for *Frankia* on the physiological effects of root exudates, my hypothesis is that actinorhizal plants communicate with *Frankia* in the rhizosphere. Preliminary studies by Lavenus (2008) indicated that *Frankia* pre-exposed to extracts from nitrogen-deprived *C. glauca* root exudates formed root nodules on a greater number of plants than the control (cells without exposure to root exudate extracts). With another actinorhizal plant system *Discaria trivenis*, numbers of plants present affect the nodulation pattern (Gabbarini and Wall 2008). For the same inoculum size, the nodulation frequency and numbers increased as the number of plants per container increased. Thus, plant crowding had two effects. First, the time required to establish a mature nodule decreased with increased plant numbers. Second, the number of nodules per plant increased (Gabbarini and Wall 2008). The authors postulated that the larger number of plants caused an increase in root exudates, thus affecting the time required to establish a mature nodule. While these studies have indicated an effect of root exudates on the infection, the phenomena deserves further study to clarify the effects and overall process.

**Auxins and their Role in the Actinorhizal Symbiosis**

Auxins are one well-known family of molecular signals that are involved in pathogenic and symbiotic interactions. Auxins are plant phytohormones involved in a diverse set of plant developmental processes, especially gravitropism and
lateral root development (Aloni et al. 2006; Teale et al. 2006). Due to the importance of auxins in plants, many organisms have adapted ways to utilize auxins as signals, especially in symbiotic and pathogenic organisms. Auxins have been implicated in many different plant-microbe interactions and were shown to be particularly important in the legume-*Rhizobium* symbiosis (Spaepen et al. 2007). The reason that auxins play such a large role in these interactions is due to the ability of symbiotic and pathogenic microorganisms to produce auxins identical to those synthesized by the host plant. By increasing the auxin concentrations at specific steps in the infection process, an organism can influence plant growth and development, hence using auxins as a signal to the plant (Spaepen et al. 2007).

Auxins have been suspected of being involved in the actinorhizal symbiosis (Peret et al. 2007). Exposure of *A. glutinosa* to phenylacetic acid (PAA), an auxin, causes formation of pseudo-nodules, suggesting an early role for auxins in the actinorhizal symbiosis (Hammad et al. 2003). Treatment of *C. glauca* with auxin influx transporter inhibitors causes a delay in the nodulation process and an overall decrease in nodule size (Peret et al. 2007). Actinorhizal plants possess multiple auxin transporters and their expression pattern within mature nodules is different from normal root cells (Figure 2) (Peret et al. 2008). While the work described above suggests modified concentration and expression of auxin and auxin transporters, (Peret et al. 2007; Peret et al. 2008; Hammad et al. 2003) research still needs to be completed on auxin production and flow inside the mature nodule.
Normal Root Cells

Infected Nodule Cell

Figure 2: Representation of regulated auxin transport. (Left) Depiction of normal root cell auxin flow. (Right) Currently known auxin flow in a *Frankia* infected nodule cell.
Currently, five *Frankia* genomes have been completely sequenced and several more are in the pipeline. Analysis of the first three genomes (ACN14a, Ccl3, and EAN1pec) resolved many questions about *Frankia* (Normand et al. 2007b). One major discovery is that the *Frankia* genomes do not contain any of the common *nod* genes similar to the *Rhizobium* genomes. This result suggests that actinorhizal symbiosis uses novel signal compounds during the infection process than those used in the legume-*Rhizobium* association. One major difference between the *Rhizobium*-legume symbiosis and the actinorhizal symbiosis is plant host specificity. *Rhizobium* strains are highly specific and have an extremely narrow host range, while *Frankia* strains have a much broader plant host range. *Rhizobia* species are limited to specific legume species host. *Frankia* strains vary from a very narrow host range (one plant genus) to a very broad host range (multiple genera in different plant families). Plant host range specificity appears to correlate with *Frankia* genome size (Normand et al. 2007a). *Frankia* Ccl3 has the smallest genome (5.4 Mb) and the narrowest host range, while *Frankia strains* EAN1pec, Eu11c, and EUN1f have the largest genomes (about 9 Mb) and broadest plant host ranges. *Frankia* strains ACN14a and QA3 which have a medium plant host range possess a 7.5 and 8.0 Mb genome, respectively.

The availability of genome databases has opened the door to other powerful genome-enabled approaches including transcriptomic, proteomic, and data-mining techniques. Transcriptome analysis of *A. glutinosa* nodules and free-
living *Frankia* ACN14a identified several groups of genes that are up- or down-regulated in mature nodules providing insight on nodule physiology (Alloisio et al. 2010). Further insight in nodule physiology comes from proteome profiles for wild root nodules of *Casuarina americanus*, *A. glustinosa*, and *Elaeagnus angustifolia* (Mastronunzio and Benson 2010). These proteomic studies revealed a large increase in proteins involved in nitrogen fixation, nitrogen metabolism, and energy metabolism. Besides these expected proteins, several proteins for ABC transporters and multiple drug transporters were elevated within the nodule, thus suggesting a role in nutrient or signal transport to the host plant. These abundant proteins present inside the actinorhizal nodules provide a guide to the components involved in the functioning mature symbiosis and serve a guide for genome-enabled approaches.

**Research Objectives**

The overall objective of this study was to identify events involved in the molecular signaling for the actinorhizal symbiosis. There is a paucity of information on molecular interactions occurring between actinorhizal host plants and their symbiont, *Frankia*. My working hypothesis is that actinorhizal host plants excrete a chemical signal in the rhizosphere to *Frankia* that alters the bacteria for the infection process. The goal of this study was to test this hypothesis through the use of *Casuarina cunninghamiana* root exudates and *Frankia* Ccl3. To achieve this goal, two different approaches were used.
The first approach was to collect *C. cunninghamiana* root exudates in aqueous solutions that were not extracted by the use of a solvent to mimic the effects found in the soil environment. Root exudates from plants of various ages and under different conditions were tested in order to determine environmental effects on host root exudates.

The second approach centered on modification(s) of *Frankia* Ccl3 physiology including plant infectivity in response to *C. cunninghamiana* host root exudates. For this study, *C. cunninghamiana* and *Frankia* Ccl3 were chosen for several reasons. The *Frankia* Ccl3 genome was completed and represents a narrow host range symbiont. The relationship between the plant host and symbiont including the nodulation process is well-studied (Zhang et al. 1984; Torrey 1976; Laplaze et al. 1999; Laplaze et al. 2000) and genetic tools are available for the plant host including GUS fusions in developmentally repressed genes (Svistoonoff et al. 2010).

Lastly, a bioinformatics approach was used to identify potential signaling events for nodule development. Our study concentrated on data mining the *Frankia* Ccl3 genome to identify the *Frankia* genes responsible for auxin biosynthesis. Gene expression studies were performed to confirm the identity of these *Frankia* Ccl3 auxin biosynthesis genes and their role in nodule development.
CHAPTER 2

METHODS AND MATERIALS

Growth Media and Culture Conditions

Frankia strain Ccl3 was grown and maintained in basal MP growth medium with 5.0 mM NH\textsubscript{4}Cl as a nitrogen source and 5.0 mM propionate as a carbon source at 28°C, as described previously (Tisa et al. 1999).

For experimental conditions, Frankia cultures were grown in MP (Tisa et al. 1983; Tisa et al. 1999) or BAP growth media (Peret et al. 2007) as described previously. Basal MP growth medium consisted of MOPS-phosphate buffer (50 mM MOPS, 10 mM K\textsubscript{2}HPO\textsubscript{4}, pH 6.8) supplemented with metals mix containing (Final Conc.) 1 mM Na\textsubscript{2}MoO\textsubscript{4}, 2 mM MgSO\textsubscript{4}, 20 \textmu M FeCl\textsubscript{3} with 100 \textmu M nitrilotriacetic acid (NTA), and modified trace salts solution (Tisa et al. 1983). Propionate (5mM) was added as a carbon and energy source. Basal BAP growth medium was composed of the following components: 0.01 M KH\textsubscript{2}PO\textsubscript{4}, 0.01 M K\textsubscript{2}HPO\textsubscript{4}, 0.135 mM CaCl\textsubscript{2}, 5 mM propionate, 0.045 mM MgSO\textsubscript{4}*7H\textsubscript{2}O, 0.1% (v/v) Fe-EDTA stock, 0.1% (v/v) Oligoelements, 0.1% (v/v) Wolf’s vitamins, pH 6.7 supplemented with 2% (v/v) Mes-Tris (0.5 M 2[N-Morpholino] ethan sulfonic acid, adjusted to pH 6.8 with Tris). Fe-EDTA stock contains 195 mM FeNa\textsubscript{2}EDTA. Oligoelements contains 0.115 mM H\textsubscript{3}BO\textsubscript{3}, 0.08 \textmu M CuSO\textsubscript{4}*5H\textsubscript{2}O, 2.3 \textmu M MnCl\textsubscript{2}*4H\textsubscript{2}O, 0.19 \textmu M ZnSO\textsubscript{4}, 0.026 \textmu M Na\textsubscript{2}MoO\textsubscript{4}*2H\textsubscript{2}O. Wolff’s vitamin stock contains 0.059 mM pyridoxine HCl, 0.036 mM \textit{p}-aminobenzoic acid, 0.024
mM lipoic acid, 0.04 mM nicotinic acid, 0.013 mM riboflavin, 0.016 mM thiamine HCl, 0.01 mM calcium DL-pantothenate, 0.008 mM biotin, 0.004 mM folic acid, and 0.0737 μM vitamin B12.

For growth under nitrogen-sufficient conditions, 5 mM NH₄Cl was added to either MP or BAP medium. For growth under nitrogen-deficient conditions, N₂ was the sole nitrogen source.

For plant growth and root exudate studies, plants were grown in commercially available ¼ strength Hoagland’s Modified Basal Salt Solution (1/4 HS; MP Biomedicals, Solon, Ohio) containing 0.25 mM (NH₄)₃PO₄, 115 μM H₃BO₃, 1 mM Ca(NO₃)₂, 0.08 μM CuSO₄*5H₂O, 22.53 μM Na₂EDTA, 22.5 μM FeSO₄*7H₂O, 0.5 mM MgSO₄, 2.3 μM MnCl₂*4H₂O, 0.0275 μM MoO₃, 1.5 mM KNO₃, 0.19 μM ZnSO₄*7H₂O, pH 5.5. Plants without a source of nitrogen were grown in ¼ strength Hoagland’s solution (1/4 HS(N₂)) which contains 22.5 μM Na₂EDTA, 22.5 μM FeSO₄*7H₂O, 0.5 mM MgSO₄*7H₂O, 0.5 mM K₂SO₄, 0.625 mM CaSO₄*2H₂O and Hoagland’s Micronutrients at a final pH 5.5. Hoagland’s Micronutrients contained 0.115 mM H₃BO₃, 0.08 μM CuSO₄*5H₂O, 2.3 μM MnCl₂*4H₂O, 0.19 μM ZnSO₄, and 0.026 μM Na₂MoO₄*2H₂O.

**Seed Sterilization and Plant Growth Conditions**

Prior to use, *C. cunninghamiana* seeds (Sheffield’s Seed Company, Locke, New York, or F. W. Schumacher Company, Sandwich, Massachusetts) were incubated overnight in sterile tap water at room temperature (RT). Seeds were surface sterilized by treatment with 15 mL of 30% hydrogen peroxide
containing two drops of Tween 20. The suspension was incubated for 5 min under agitation at RT. The sterilized-seeds were washed 4 times with 10 mL sterilized deionized H2O (sdH2O). Sterilized seeds were aseptically sown into a Magenta GA-7 box (Magenta Corp., Chicago, Illinois) containing 100 mL perlite (Scotts Company, Marysville, Ohio) and 45 mL ¼ HS medium. Seeds were germinated at 28°C with a 16 h light period and 8 h dark period.

**Conditions for Production and Collection of Root Exudates**

Figure 3 outlines the process for production of plant root exudates. At 7 to 12 days after seed germination, 5 *C. cunninghamiana* seedlings were aseptically transplanted from perlite (Scotts Company, Marysville, Ohio) and ¼ HS medium to a Brite-Kote aluminum screen (Phifer, Tuscaloosa, Alabama) in a magenta box suspended over 50 mL of ¼ HS medium. The plants were incubated at 28°C with a 16 h light period. For each magenta box, spent growth medium was replaced weekly with fresh sterile ¼ HS medium. Root exudates (spent plant growth medium) were collected each month and filter-sterilized by the use of a 150-mL 0.2 μm filter unit (Nalgene, Rochester, New York). The filter-sterilized root exudates were portioned out (45 mL each) and stored at −20°C until use. The same procedure was repeated with seedlings incubated in 50 ml ¼ HS(N2) medium. Table 1 summarizes all of the *C. cunninghamiana* root exudates collected in this study.
Figure 3: Diagram of workflow to grow aseptic seedlings for root exudates collection and nodulation studies.
Table 1: Samples of root exudates collected from *C. cunninghamiana* used in this study.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Age of Plants</th>
<th>Seed Company</th>
<th>Nitrogen Condition</th>
<th># of Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil</td>
<td>2 months</td>
<td>Sheffield’s Seed Co.</td>
<td>Nitrogen-deficient</td>
<td>20</td>
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<tr>
<td>Soil</td>
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<td>Sheffield’s Seed Co.</td>
<td>Nitrogen-sufficient</td>
<td>20</td>
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<td>Axenic</td>
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<td>Sheffield’s Seed Co.</td>
<td>Nitrogen-deficient</td>
<td>20</td>
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<tr>
<td>Axenic</td>
<td>3 weeks</td>
<td>Sheffield’s Seed Co.</td>
<td>Nitrogen-sufficient</td>
<td>20</td>
</tr>
<tr>
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<td>F. W. Schumacher Co.</td>
<td>Nitrogen-deficient</td>
<td>25*</td>
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<tr>
<td>Axenic</td>
<td>3 weeks</td>
<td>F. W. Schumacher Co.</td>
<td>Nitrogen-sufficient</td>
<td>25*</td>
</tr>
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<td>F. W. Schumacher Co.</td>
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<td>F. W. Schumacher Co.</td>
<td>Nitrogen-sufficient</td>
<td>25*</td>
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<td>Axenic</td>
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<tr>
<td>Axenic</td>
<td>2 months</td>
<td>F. W. Schumacher Co.</td>
<td>Nitrogen-sufficient</td>
<td>25*</td>
</tr>
</tbody>
</table>

*Root exudates collected at different ages from the same plants*
Plant Growth Conditions in Soil and Procedures for Root Exudates

Collection

*C. cunninghamiana* seeds were incubated overnight in tap water at RT. Seeds were sown into Miracle Grow Seed Starter Mix (Scotts Co., Marysville, Ohio) and incubated at 25°C on 16 h light cycle for 2 months. Two-month-old seedlings were washed in tap water and sdH$_2$O. The seedlings were aseptically transferred to 15 mL culture tubes containing 14 mL of ¼ HS medium. Seedlings were allowed to acclimate to ¼ HS medium for one week. After the acclimation period, the plant medium was replaced and the plants were incubated at 25°C on 16 h light cycle. After one-week incubation, root exudates or spent growth media were collected, filtered through a 150 mL 0.25 μm filter (Nalgene, Rochester, New York), and stored at -20°C. The process was repeated for plants under nitrogen-deficient conditions with ¼ HS(N$_2$) medium. Information about the soil root exudates collected are presented in Table 1.

Production and Collection of *Elaeagnus angustifolia* Root Exudates

*E. angustifolia* seeds (F. W. Schumacher Company, Sandwich, Massachusetts) were sterilized by a 1 h soak in 1 L 30% bleach at RT. The treated seeds were washed 4 times with 1 L sdH$_2$O. Washed seeds were soaked in 10 mL 30% H$_2$O$_2$ for 10 min and washed 4 times with 40 mL sdH$_2$O. Seeds were sown and germinated in Magenta GA-7 boxes (Magenta Corp., Chicago, Illinois) containing 100 g of sterilized pebbles and 25 mL ¼ HS medium. After germination, seedlings were transferred to hydroponic solutions, as described
above, containing ¼ HS or ¼ HS(N\textsubscript{2}) medium. Root exudates were collected under aseptic conditions, as described above.

**Phenolic Quantification**

The total phenolic content of root exudates was measured by a modification to the Waterhouse method (2002). Root exudates (25 µL) were added to 125 µL Folin-Ciocalteu reagent and samples were mixed by a Vortex-Genie 2 (VWR Scientific, Radnor, Pennsylvania). After the mixture was incubated for 5 min, 375 µL of 20% sodium carbonate and 475 µL of H\textsubscript{2}O were added to each sample. The samples were mixed by a Vortex-Genie 2 (VWR Scientific, Radnor, Pennsylvania) and incubated at RT for 2 h. Phenolic content was determined by measuring the A\textsubscript{760} on a UV-2401 PC spectrophotometer (Shimadzu, Norwell, Massachusetts) using a Gallic acid standard (10-200 µg/mL).

**Flavonoid Quantification**

Flavonoid content of root exudates was measured by a modification of the Chang et al. (2002) method. Root exudates (300 µL) were added to 900 µL 95% ethanol, 60 µL 10% aluminum trichloride, and 60 µL of 1 M potassium acetate. The reaction mixtures were mixed by a Vortex-Genie 2 (VWR Scientific, Radnor, Pennsylvania) and incubated at RT for 30 min. Samples were measured by reading the A\textsubscript{415} on a UV-2401 PC spectrophotometer (Shimadzu, Norwell, Massachusetts) using a Quercetin standard (10-200 µg/mL).
Gas Chromatography (GC)

Volatile organic acid (C2-C5 acids) content of root exudates was measured by gas chromatography and the use of an 80/120 carbopack column (Kappell et al. 2005). Root exudates (1 μL) were injected into the Gas Chromatograph HP6890 (Agilent, Santa Clara, California) fitted with an 80/120 carbopack B-DA Carbowax 20 M packed column (Sigma Aldrich (Supelco 11889), Saint Louis, Missouri) and flame ionization detector. The following parameters were used: injector port temperature (200°C), column temperature (175°C), column N₂ carrier flow rate (24 mL/min), and flame ionization detector temperature (200°C). The retention time and peak height of the samples were analyzed and compared to volatile acids standard (Retention Time [min]/Area, mM): Formic Acid (0.654/106.5, 10mM), Acetic Acid (1.722/1562, 10mM), Propionic Acid (3.377/2490, 10mM), Isobutyric Acid (5.706/2414, 10 mM), Butyric Acid (7.4/2738, 10 mM), Isovaleric Acid (13.9/2410, 10mM), and Valeric Acid (17.3/2481, 10 mM) (Supelco Inc, Bellefonte, Pennsylvania).

Ammonium Quantification

Ammonium content in root exudates was determined using the indolephenol blue assay as described in Daniels et al. (1996). Root exudates (10 mL) were combined with 50 μL 0.003 M MnSO₄. The solution was mixed and 500 μL 1% sodium hypochlorite (pH 7.3) and 600 μL Phenol Reagent (0.625 N NaOH and 1.06 M Phenol) were added and mixed continuously during addition. The
reaction was incubated at RT for 10 min. Ammonium content was determined by measuring the $A_{630}$ on a UV-2401 PC spectrophotometer (Shimadzu, Norwell, Massachusetts) using a NH$_4$Cl standard (0.01-0.5 µg/mL).

**Nitrate Quantification**

Nitrate content in root exudates was determined as previously described by Karlsson et al. (1995). Root exudate (1 mL) nitrate content was determined by measuring the $A_{220}$ and $A_{270}$ on a UV-2401 PC spectrophotometer (Shimadzu, Norwell, Massachusetts) using a KNO$_3$ standard (0.01-0.2 mM). The $A_{270}$ was a reference reading and was subtracted from the $A_{220}$.

**Plant Nodulation Studies**

After seeds were germinated and seedlings were placed in Magenta boxes containing 5 seedlings as described above, plants were incubated at 28°C with a 16 h light period for two weeks in ¼ HS(N$_2$) medium. As inocula, 14 day-old *Frankia* sp. Ccl3 cultures grown in MP medium with 5.0 mM NH$_4$Cl and 5.0 mM propionate were harvested and washed twice with 10 mL ¼ HS(N$_2$) medium to remove all traces of nitrogen. The washed cells were resuspended in a 25 mL solution of aqueous root exudates or ¼ HS(N$_2$) medium (the control) and incubated at 28°C for 6 days. The treated *Frankia* suspensions were adjusted to an equivalent final protein concentration (60 µg/ml) in ¼ HS(N$_2$) medium. Spent plant growth medium was decanted from each Magenta box and replaced with 50 mL of the *Frankia* suspension. Control boxes contained 50 mL fresh ¼ HS(N$_2$)
medium. After inoculation, the plants were incubated at 25°C with 16 h light period and plant growth medium was replaced weekly for the duration of the experiment. The plant roots were monitored daily for nodule formation. Both the number of plants with nodules and the total number of nodules formed were recorded. Statistical analysis was completed using ANOVA on JMP software (JMP, Cary, North Carolina).

Experiments on the Effect of Root Exudates Exposure on *Frankia*

**Physiology**

For these experiments, 14-day-old cultures grown on MP medium with 5.0 mM NH₄Cl and 5.0 mM propionate were used as the inocula. *Frankia* strain Cc13 cultures were harvested by centrifugation at 10,000 x g for 15 min at RT and washed twice with 10 mL MP buffer. The washed hyphae were resuspended in 10 mL MP buffer and fragmented by glass tissue homogenizer. The homogenate was adjusted to an OD$_{600}$ of 0.08 in 25 mL of 2X MP growth medium with or without 1 mM propionate. The diluted suspension (0.5 mL) was added to wells of 24-well microtiter plate (Corning, Corning, New York). To each well, 0.5 mL of varying concentrations of root exudates were also added to make a final volume of 1 mL 1X MP growth medium. The plates were sealed with parafilm and incubated for 14 days at 28°C. After incubation, the cultures were analyzed for several physiological properties described below.
Total Cellular Protein Determination

Protein content was measured by the BCA method (Smith et al. 1985). *Frankia* mycelia were harvested by centrifugation at 13,000 x g for 30 min at RT and washed once with sdH$_2$O. The cell pellet was resuspended in 200 uL of 0.5 N NaOH and frozen at -20°C. Protein was extracted by boiling the suspension at 95°C for 10 min. Solubilized protein and cell mass were separated by centrifugation at 13,000 x g for 10 min. For each sample, 10 uL was used in the BCA assay according the manufacturer’s instructions (Pierce, Rockford, Illinois). BCA assay was incubated at 37°C for two hours. Protein concentrations were quantified on a Tecan plate reader (Tecan, Durham, North Carolina) at A$_{562}$ using a bovine serum albumin (BSA) standard (0-800 µg/mL). Growth yield was determined by subtracting the protein content of the inocula. Statistical analysis was completed using the Dunnett’s test and Student’s T-test on JMP software (JMP, Cary, North Carolina).

Dry Weight Determination

Total cellular dry weight was determined as described previously (Tisa et al. 1983). Samples (5 mL) were collected on tared membrane filters (0.45 µm pore size, polycarbonate, Millipore, Billerica, Massachusetts). The filters were then placed in a Petri dish over desiccant and dried overnight at 80°C to constant weight. The filters were weighed three times. Averages of the three weights were subtracted from the initial tared reading of the filter paper. Dry weight growth
yield was determined by subtracting inoculum dry weight from sample dry weight. Statistical analysis was completed as described above.

**Microscopy of Frankia Cultures**

Photomicroscopy of *Frankia* cultures was performed by the agar method of Pfenning and Wagner (1986). Culture samples (10 µL) were placed onto the surface of solid 0.8% Ion Agar that was thinly spread on a glass microscope slide. *Frankia* was observed at 400X magnification on an Olympus BH2 phase contrast microscope (Olympus, Center Valley, Pennsylvania) and the image was captured by a Retiga 1300 camera and QCapture software (Qimaging, Surrey, British Columbia, Canada).

**Congo Red Binding Assay for Surface Property Changes**

Bacterial surface property changes were measured by a modification of the Congo red dye-binding assay (Etienne et al. 2002). *Frankia* cultures were incubated in MP growth medium with 5 mM propionate at 28°C for 11 days. At day 11, 10 µL of Congo red (10 mg/mL) was added to the 1 mL culture. After 3 more days incubation at 28°C, *Frankia* mycelia were collected by centrifugation at 13,000 x g for 10 min and washed 3 times with sdH2O. The pelleted washed cells were resuspended in 200 µL acetone and incubated with shaking for 2 h at RT. Acetone was separated from the debris by centrifugation at 13,000 x g for 10 min and transferred to a flat transparent 96-well plate (Nunc, Rochester, New York). The Congo red concentration was determined by measuring the A₄₈₈ on a
Fourier Transforming Infrared Spectroscopy (FTIR)

For these experiments, 5-day-old cultures were harvested by centrifugation at 13,000 x g for 10 min and washed 3 times in sdH₂O. The washed cells were frozen at –80°C and lyophilized for 48 h in a Freezezone 6Lplus freeze dryer (Labconco, Kansas City, Missouri). FTIR analysis of the lyophilized samples were performed according to a modified Turick et al. (2003) method on a diamond attenuated total reflectance (ATR) Nicolet iS10 (Thermo Scientific, Waltham, Massachusetts). ATR is a recent advance in FTIR that allows direct infrared-spectroscopy instead of the KBr pellet used in Turick et al. (2003). The average FTIR spectrum for 3 replicates and spectrum variation of replicates were determined by the use of the Omnic software package (Thermo Scientific, Waltham, Massachusetts).

Bioinformatics Studies of Auxin Synthesis Genes

The FASTA amino acid sequences of the three finished Frankia genomes (Ccl3, NCBI RefSeq: NC_007777; ACN14a, NCBI RefSeq: NC_00827; EAN1pec, NCBI RefSeq: NC_009921) and the three Frankia draft genomes (Eul1c, NCBI RefSeq: NC_014666; EUN1f, http://genome.ornl.gov/microbial/fran_eun1f/; the uncultured Frankia symbiont of Datisca glomerata http://genome.ornl.gov/microbial/fran_sym/) were obtained.
from Genbank or Oak Ridge National Laboratory Genome Channel (http://compbio.ornl.gov/channel/). Functionally analyzed genes for the IAA and PAA biosynthesis pathways were identified by the use of Kyoto Encyclopedia of Genes and Genomes and published literature (Kanehisa and Goto 2000; Spaepen et al. 2007; Patten and Glick 1996). Genes in the Indole-3-acetamide (IAM), Indole-3-pyruvate (IPyA), tryptamine (TAM), Indole-3-acetonitrile (IAN), and phenylacetic acid pathways were used in this study (Table 2). BLASTP analyses were performed on each Frankia genome database using the identified proteins in the IAA and PAA biosynthesis pathways as a query subject (Altschul et al. 1997).
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene</th>
<th>Organism</th>
<th>Accession #</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>IAM</td>
<td>Tryptophan monooxygenase</td>
<td><em>Agrobacterium tumefaciens</em></td>
<td>AAD30489</td>
<td>(Otten et al. 1999)</td>
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<tr>
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<td>(Arias et al. 2008)</td>
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</tbody>
</table>
**Frankia Growth Condition for Gene Expression Experiments**

For these experiments, *Frankia* Ccl3 inoculum was washed twice in 10 mL BAP medium. The washed mycelium was fragmented in a tissue homogenizer and inoculated into BAP medium with and without 5.0 mM NH₄Cl. *Frankia* cultures were incubated with stirring for 5 days at 28°C.

**RNA Extraction**

For these experiments, all solutions and material were DEPC-treated to prevent RNA degradation. RNA extractions were performed by the Triton X-100 method as previously described (Niemann and Tisa 2008). *Frankia* mycelia were harvested by centrifugation at 6,000 x g for 15 min at RT and washed with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The washed pelleted cells were resuspended in 500 µL TE buffer containing 0.2% Triton X-100 and incubated at 95°C for 10 min. Samples were transferred to an ice bath and cooled for 1 min. An equal volume of cold chloroform was added to the lysed samples and mixed by inversion 100 times. Cell lysates were removed by centrifugation at 13,000 x g at 4°C for 15 min. The aqueous layer was transferred to a fresh tube and the chloroform treatment was repeated. A 1/10th volume of 3M sodium acetate (pH 5.2) was mixed with the aqueous layer in a fresh tube and 2 volumes cold absolute ethanol were added to the mixture. Samples were incubated overnight at -60°C. The RNA was recovered by centrifugation at 13,000 x g for 60 min at 4°C and the pellet washed with 1 mL 80% cold ethanol. The RNA pellet was air-dried and resuspended in 44 µL RNAse-free H₂O.
**DNAse Treatment and RNA Quantification**

RNA samples were treated with DNAsel (New England Biolabs, Ipswich, Massachusetts) according to the manufacturer’s instructions. Samples were incubated at 37°C for 30 min with 1 U of DNAse I in 1X DNAse I buffer. RNA samples were quantified with a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware) and stored at -60°C until use.

**cDNA synthesis**

The cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, California) according to the manufacturer’s instructions. The reactions were performed in 20μL reaction volumes with 400 ng RNA template. To each sample, 1 μL of 10 mM dNTPs, 0.1 μL of [300 ng] random primers, and 1 μL sdH₂O were added and the mixture was incubated at 65°C for 5 min. The tubes were chilled on ice for 1 min and 4 μL 5x SuperScript III buffer, 1 μL DTT, 1 μL RNAseOUT (Invitrogen, Carlsbad, California), and 1 μL (200 U/μL) SuperScript III (Invitrogen, Carlsbad, California) was added to each sample. Tubes were incubated 25°C for 5 min, 50°C for 50 min, and 70°C for 15 min. Samples were treated to remove RNA by adding 1 μL (2 U) Ribonuclease H (Invitrogen, Carlsbad, California) and 29 μL H₂O to the cDNA sample. The cDNA was incubated at 37°C for 30 min and quantified by Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware) and diluted to 10 ng/μl working stock in RNAse-free H₂O.
**Reverse Transcription (RT)-PCR**

The RT-PCR was performed in 26 µL total volume with [100 ng] of cDNA as template, 1 µL [0.4 µM] primer Forward, 1 µL [0.4 µM] primer Reverse, 13 µL AmpliTaq Gold 360 (Applied Biosystems, Carlsbad, California), and water up to 26 µL following the manufacturer’s instructions (Table 3). The thermocycler program parameters were the following: 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 10 min. Amplicons were resolved by 2% agarose LE (Lonza, Rockland, Maine) in 1X Tris-acetate EDTA buffer (TAE, 40mM Tris-acetate, 1 mM EDTA, pH 8.1-8.5) on a submarine electrophoresis unit (Hoefer Scientific Instruments Inc., Holliston, Massachusetts).

**Quantitative Real Time (qRT) PCR**

The mRNA expression levels were quantified using 2X SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, California) following the manufacturer’s instructions. The primers used are listed in Table (Table 3). Each 26 µL reaction contained 10 µL [100 ng] template cDNA, 3 µL [400 nM] Forward and Reverse primer mix, and 13 µL [1X] SYBR® Green PCR Master Mix. Parameters for the Applied Biosystems 7300 (Applied Biosystems, Carlsbad, California) were as follows: (1) 95°C 10 min, (2) 40 cycles of 95°C for 15 s and 60°C for 30 s, (3) 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. Reactions were performed in triplicates and relative gene expression was analyzed by the ΔΔCt
method. Primer efficiencies were determined using a *Frankia* CcI3 gDNA standard curve. The primer efficiency for each primer set was determined by plotting the log change in gDNA concentration vs. cycle threshold (Ct).
<table>
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<th>Primer</th>
<th>Primer set (5' to 3')</th>
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<td>This Study</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase 2</td>
<td>F GCG GCA TTC GGC GGA TAC</td>
<td>Francci3_2944</td>
<td>This Study</td>
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<tr>
<td></td>
<td>R TGT TCT TGG TCT GGC TGT AGT G</td>
<td></td>
<td>This Study</td>
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Physiological Responses of Frankia to Root Exudates

Production of Root Exudates and General Properties

Although previous studies on root exudates have used solvent extracted samples, aqueous root exudates were chosen for use in this study (Lavenus 2008, Benoit and Berry 1997, Popovici et al. 2010, Hughes et al. 1999). Our rationale for this approach centers on the hypothesis that root exudates released under these conditions would be similar to those released in the soil. Root exudates from several different conditions were collected and analyzed for their chemical properties (Table 4). Two-month-old soil and three-week-old axenic grown C. cunninghamiana root exudates contained phenolic and flavonoid compounds (Table 4). Three-week-old axenic root exudates were also tested for C2-C8 organic acids using gas chromatography (GC) analysis (Table 4 and Figure 4). Acetic and propionic acid were found in the root exudates at very low concentrations. A small amount of formic acid was identified in three-week-old axenic root exudates collected under nitrogen-deficient conditions. Isobutyric, butyric, isovaleric, and valeric acids were not found in the three-week-old root exudates (data not shown). GC analysis also revealed unidentified organic acids including a peak at 1.2 min retention time. This peak had the largest area observed for root exudates grown in nitrogen-sufficient and -deficient conditions.
The chemical composition of this peak was not determined. All nitrogen-deficient root exudates contained ammonium concentrations below detectable levels and between 0.04-0.07 mM nitrate, except three-week-old axenic root exudates which contained 0.3 μg/mL ammonium and 0.23 mM nitrate. Nitrogen-sufficient root exudates contained 0.41-0.45 μg/mL of ammonium and nitrate concentrations above detectable levels, except two-month-old soil grown root exudates which contained 0.14 μg/mL ammonium.
Table 4: Analysis of *C. cunninghamiana* root exudates from nitrogen-sufficient (NH$_4$Cl) and deficient (N$_2$) growth conditions.

<table>
<thead>
<tr>
<th>Root Exudates</th>
<th>Flavonoid</th>
<th>Phenolic</th>
<th>Acetic Acid</th>
<th>Propionic Acid</th>
<th>Formic Acid</th>
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<tr>
<td>Three-week-old axenic (N$_2$)</td>
<td>2 µg/mL</td>
<td>15.4 µg/mL</td>
<td>0.92 mM</td>
<td>0.17 mM</td>
<td>5.5 mM</td>
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<tr>
<td>Three-week-old axenic (NH$_4$Cl)</td>
<td>2.7 µg/mL</td>
<td>8.9 µg/mL</td>
<td>0.25 mM</td>
<td>0.03 mM</td>
<td>BD$^1$</td>
</tr>
<tr>
<td>Two-month-old soil (N$_2$)</td>
<td>4.9 µg/mL</td>
<td>ND$^2$</td>
<td>ND$^2$</td>
<td>ND$^2$</td>
<td>ND$^2$</td>
</tr>
<tr>
<td>Two-month-old soil (NH$_4$Cl)</td>
<td>ND$^2$</td>
<td>18.6 µg/mL</td>
<td>ND$^2$</td>
<td>ND$^2$</td>
<td>ND$^2$</td>
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</tbody>
</table>

$^1$BD = below detection limit  
$^2$ND = not determined
Figure 4: Gas chromatograph analysis of volatile organic acids in root exudates. (A) Volatile organic acid standards, (B) three-week-old *C. cunninghamiana* root exudates (nitrogen-deficient), and (C) three-week-old *C. cunninghamiana* root exudates (nitrogen-sufficient) were analyzed by a gas chromatograph as described in Methods. All spectra were shortened to 5 min from the original 40 min spectral run.
Effect of Root Exudates on *Frankia* Ccl3 Growth

The effect of *C. cunninghamiana* root exudates on cell growth was tested and growth yield was determined by total cellular protein after 14 days (Figures 5 and 6). In growth medium devoid of a carbon source, root exudates were unable to support the growth of *Frankia* Ccl3. Under these conditions, the protein content was lower and significantly different than the inoculum (data not shown). This protein reduction was proportional to the concentration of root exudates. In the presence of an additional carbon source (propionate), the addition of root exudates caused an increase in growth yield. For these experiments, cells exposed to root exudates were more difficult to pellet than control cells, suggesting a change in their surface properties. Soil-grown two-month-old *C. cunninghamiana* root exudates caused a 1.8-fold increase in protein yield, while axenic three-week-old root exudates caused a 3.7-fold increase in protein yield (Figures 5 and 6). Nitrogen added to *Frankia* supplemented with root exudates did not cause a reduction in the response (Figure 7). This suggests that the nitrogen content of the root exudates was not a significant factor in the enhanced growth. These results show that *Frankia* Ccl3 was unable to utilize *C. cunninghamiana* root exudates as a sole carbon and energy source for growth, but growth in the presence of an exogenous carbon source was enhanced when supplemented with root exudates.

Growth yield was also determined by measuring cellular dry weight (Figure 8). In the presence of a carbon source, three-week-old axenic *C. cunninghamiana* root exudates caused an 8-fold increase in the total dry weight
yield. One-month-old axenic root exudates produced a 6-fold increase in growth yield. The overall patterns of the dry weight results were similar to total protein results: *Frankia* growth was enhanced by the addition of *C. cunninghamiana* root exudates. However, the growth yields for total mass (dry weight yield) showed a larger fold increase than those measured for protein yield. These results would suggest that the root exudates influence *Frankia* physiology by affecting cellular components beyond total protein levels.
Figure 5. The effect of two-month-old soil grown *C. cunninghamiana* root exudates (nitrogen-deficient) on *Frankia* CcI3 growth yield. *Frankia* was grown with propionate-MP medium supplemented with root exudates. Control conditions contained 1/4 HS(N$_2$) medium. After 14 days, cellular protein content was determined as described in Methods and corrected for the inoculum value. Control Dunnett’s test was used to determine statistical significance. *p*-value of less than 0.05
Figure 6: The effect of three-week-old axenic *C. cunninghamiana* root exudates (nitrogen-deficient) on *Frankia* Cc13 growth yield. *Frankia* was grown with NH$_4$Cl and propionate-MP medium supplemented with root exudates. Control conditions contained 1/4 HS(N$_2$) medium. After 14 days, cellular protein content was determined as described in Methods and corrected for inoculum value. Control Dunnett’s test was used to determine statistical significance. *p-value of less than 0.05
Figure 7: Effect of supplemented nitrogen on *Frankia* response to root exudates. *Frankia* was grown with propionate–MP medium supplemented with or without 5 mM NH$_4$Cl and (dark gray) $\frac{1}{2}$ concentration axenic (nitrogen-sufficient) *C. cunninghamiana* root exudates. (light gray) Control conditions contained $\frac{1}{4}$ HS medium. After 14 days, cellular protein content was determined as described in Methods and corrected for the inoculum value. Students T-test was used to determine statistical significance. *p*-value of less than 0.05.
Figure 8: The effect of axenic *C. cunninghamiana* root exudates on *Frankia* Ccl3 growth yield as determined by total cellular dry weight. *Frankia* was grown with propionate-MP medium supplemented with root exudates. Control conditions contained 1/4 HS(N₂) medium. After 14 days, cellular dry weight was determined as described in Methods and corrected for inoculum value. Control Dunnett's test was used to determine statistical significance. *p*-value less than 0.05
Root Exudates Causes Hyphal Curling

Since *C. cunninghamiana* root exudates affected growth of *Frankia* Ccl3, their effects on cell morphology was investigated. *Frankia* Ccl3 cultures were exposed to different root exudates preparations and the effects on cell-morphology are shown in Figure 9. *Frankia* cultures exposed to two-month-old soil root exudates (Figure 9B) or one-month-old axenic root exudates (Figure 9C) exhibited a hyphal curling response. The tips of the hyphae were bent or curled. Control cells did not exhibit the hyphal curling effect (Figure 9A). Hyphal curling was induced by root exudates collected under nitrogen-deficient and -sufficient conditions, but curling occurred to a greater degree in nitrogen-deficient root exudates. Root exudates collected from the same plants at different ages also induced the response, but did not show an age-dependent response (data not shown). The curling response by *Frankia* Ccl3 was specific for host plant root exudates. *Frankia* Ccl3 did not respond upon exposure to *E. angustifolia* (non-host actinorhizal plant) root exudates (data not shown). Taken together, these results suggest two possible hypotheses: (1) hyphal curling is a potential chemotrophic response to a chemical plant signal and/or (2) curling is an indication of surface property changes. Because of technical difficulties in investigating chemotrophic responses, the effect of root exudates on *Frankia* Ccl3 surface properties was further examined.
Figure 9: Hyphal curling response of Frankia Ccl3 upon exposure to host root exudates. Frankia Ccl3 incubated for 14 days in propionate-MP medium containing (A) ¼ HS(N₂) medium (Control), (B) soil-grown two-month-old (nitrogen-sufficient) C. cunninghamiana root exudates, or (C) axenic one-month-old (nitrogen-deficient) C. cunninghamiana root exudates. Cultures were observed under phase contrast microscopy at 400X. Size bar represents 10 μm. Arrows point to regions showing curling effects.
Frankia Surface Properties

Dye absorption changes have been used extensively to investigate bacterial surface property changes (Etienne et al. 2002; Leigh et al. 1985; Payne and Finkelstein 1977). Mycobacteium cell surface changes are detected by Congo red dye binding, which binds to lipids and lipoproteins (Cangelosi et al. 1999). Congo red binding was used to identify any broad changes to Frankia surface properties in response to host root exudates. Exposure of Frankia Ccl3 to axenic C. cunninghamiana root exudates reduced Congo red binding compared to the control cells (Figure 10). These results imply that a change in the lipid or lipoprotein content of Frankia Ccl3 cell surface occurred in response to host root exudates.

To further examine surface property changes, Fourier Transform Infrared Spectroscopy (FTIR) analysis was used to characterize the general types of molecules present in microbes based on specific wavelength areas (Naumann 2006; Wang et al. 2010). FTIR spectra were collected for Frankia Ccl3 exposed to host root exudates and control cells (Figure 11). Exposure to root exudates caused several changes in the spectral pattern. Specific changes in the wavelength regions of 2850-2960 cm\(^{-1}\), 1370-1400 cm\(^{-1}\), and 906-1170 cm\(^{-1}\) indicate alterations in fatty acids, fatty acids and proteins, and cell wall carbohydrates, respectively. The bacterial fingerprint region (600-900 cm\(^{-1}\)) was also changed between root exudates exposed cells and control cells. The protein peaks (1600 and 1500 cm\(^{-1}\)) and the H\(_2\)O peak (3200 cm\(^{-1}\)) had no difference between the conditions. These spectral changes were observed for nitrogen-
deficient root exudates collected from soil and axenic grown plants. The averaged spectral changes were conserved in root exudates collected in nitrogen-sufficient conditions, but the variance was too large to determine if they were true changes. The fatty acid changes confirm the Congo red results, and FTIR also revealed changes to the cell wall carbohydrates.
Figure 10 The effect of exposure to host root exudates on *Frankia* Ccl3 Congo red binding. Cultures were incubated for 14 days in propionate-MP medium supplemented with axenic collected (nitrogen-deficient) *C. cunninghamiana* root exudates or ¼ HS(N₂) medium (Control). Congo red dye was added during the last three days of incubation and treated as described in the Methods. Control Dunnett’s test was used to determine statistical significance. *p-value less than 0.05
Figure 11: Effect of host root exudates on the FTIR spectra of *Frankia* Cc13. Cultures were incubated for 5 days in propionate-MP medium containing 2-month-old soil *C. cunninghamiana* root exudates (light gray) or ¼ HS(N₂) medium (Control) (black). After incubation, the cultures were collected and treated as described in the Methods. FTIR scans of lyophilized cells were taken and the averaged scans are presented (n=3). Numbers in figure represent areas of change and correspond to specific chemical signatures: (1) fatty acids, (2) fatty acids and proteins, and (3) cell wall carbohydrates. Areas of Change were determined by the variance of individual conditions.
Root Exudates Exposure Influences Plant Nodulation

Although surface property changes suggest major modifications in the bacterial surface, these alterations need to be correlated to their effects on plant interactions. The effect of host root exudates on plant infectivity was investigated. *Frankia* cultures were pre-exposed to axenic *C. cunninghamiana* root exudates (nitrogen-deficient) or ¼ HS(N2) medium for 6 days without an additional carbon source. Although *Frankia* Ccl3 did not grow under these conditions, inoculum levels were standardized to minimize possible growth differences between inocula. After inoculation, *C. cunninghamiana* roots were monitored for nodulation as described in the Methods. Nodule structures appeared after 14 days and are shown in Figure 12. Figure 13 shows the time course for nodulation. The data presented are the average percentage of plants with nodules for three independent experiments. At 15 days, *Frankia* Ccl3 pretreated with host root exudates first produced root nodules, while control cells formed nodules at day 19.6 (Figure 14). The frequency of nodulation increased over time, reaching 60% and 55% for treated and control cells, respectively. In one experiment the percent nodulated roots with *Frankia* Ccl3 pretreated with host root exudates reached almost 100% (data not shown). There was no difference in the number of nodules produced per plant between the pre-treated cultures and the control (data not shown). These results indicate that *Frankia* exposed to host root exudates are predisposed to infect their host plants and initiate the infection process earlier than control cells.
Figure 12: Photographs of *C. cunninghamiana* roots. (A) Control plants (uninoculated), (B) plants inoculated with *Frankia* Ccl3 pretreated with ¼ HS(N₂) medium for 6 days, and (C) plants inoculated with *Frankia* Ccl3 pretreated by 6 day exposure to *C. cunninghamiana* nitrogen-deficient root exudates. Arrows identify root nodules.
Figure 13: The effect of host root exudates pretreatment on plant nodulation frequency. Prior to plant inoculation, *Frankia* Ccl3 was pre-treated with *C. cunninghamiana* root exudates from nitrogen-deficient conditions (open-circles) or ¼ HS(N₂) medium (closed circles) for 6 days. The percentage of plants with nodules was determined as described in the Methods. Data presented are the average percentage for three independent experiments. The average number of plants per condition in the three experiments was n=10.
Figure 14: The average number of days required to initiate plant nodulation (n = 3). *Frankia* Ccl3 was pre-treated with *C. cunninghamiana* root exudates from nitrogen-deficient conditions or ¼ HS(N₂) medium (Control) for 6 days. ANOVA analysis showed this to be a significant difference (p-value < 0.05).
**Auxin Biosynthesis by Frankia**

Nodule development by actinorhizal plants has been postulated to be driven by plant hormones including auxin (Peret et al. 2007). These studies were directed toward investigating auxin formation and its role in the actinorhizal symbiosis in collaboration with our colleagues at Institut de Recherche pour le Développement (IRD) in Montpellier, France and were recently published in Plant Physiology (2010) 154:1372-1380.

**Frankia Auxin Production**

Research performed by the IRD showed *Frankia* Ccl3 produced IAA and PAA when analyzed by LC-MS of culture supernatant. *Frankia* Ccl3 growth in BAP medium without a nitrogen source caused a significant increase in IAA and PAA concentrations compared to nitrogen-sufficient conditions. IAA levels only increased by a small amount, but there was a 3-fold increase in PAA production. These results indicate that *Frankia* Ccl3 auxin production is dependent on the nitrogen condition and that PAA is the major auxin form secreted.

**Auxin Genes Present in Frankia Ccl3**

The availability of several *Frankia* genome databases including *Frankia* Ccl3 allowed for a genome mining approach to identify genes potentially involved in auxin biosynthesis and transport. The *Frankia* Ccl3 genome contained predicted genes for the complete indole-3-pyruvate and phenyl pyruvate pathways for IAA and PAA biosynthesis, respectively (Table 5 and Figure 15).
With *Frankia* Ccl3, genes were putatively identified for other IAA biosynthesis pathways but were missing putative genes in at least one key enzyme (Table 5). All of the *Frankia* genomes contained the predicted genes for the indole-3-pyruvate and phenyl pyruvate pathways (data not shown). Since many beneficial plant-associated bacteria preferentially use the indole-3-pyruvate pathway for IAA biosynthesis, this result is not surprising (Spaepen 2007). A putative microbial auxin efflux carrier (Francci3_1249) was also identified from the automatic annotation of *Frankia* Ccl3 and orthologs were found in all *Frankia* genomes.
<table>
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<tr>
<th>Pathway</th>
<th>Enzyme</th>
<th>Accession #</th>
<th>Frankia Ccl3 Locus ID</th>
<th>% Identity</th>
<th>E-value</th>
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<td>Francci3_2944</td>
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</tr>
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<td></td>
<td>Francci3_3777</td>
<td>36</td>
<td>9e^{-76}</td>
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</table>
Figure 15: Putative IAA and PAA complete biosynthetic pathways found in *Frankia* Ccl3. Each enzyme is listed with the name of the enzyme, Accession #, *Frankia* Ccl3 locus tag, and BLASTP percent identity.
Expression Analysis of Putative Auxin Biosynthesis Genes

cDNA synthesis and RT-PCR experiments showed that *C. glauca* nodules and *Frankia* Ccl3 grown in BAP medium in nitrogen-sufficient and -deficient growth conditions expressed all of the genes necessary for the putative indole-3-pyruvate and phenyl pyruvate pathways (Figure 16). The putative auxin efflux carrier (Francci3_1249) was expressed in all conditions. *C. glauca* roots and nodule cDNA was provided by Laurent Laplaze at the IRD. This suggests that *Frankia* IAA and PAA biosynthetic pathways are active in planta. Further qRT-PCR analysis revealed that most of the genes were upregulated when *Frankia* Ccl3 was grown in BAP medium without a nitrogen source (Figure 17). Putative phenyl pyruvate decarboxylase (Francci3_2495) and phenylalanine aminotransferase (Francci3_0566) increased by 4-fold and 3.4-fold in nitrogen-deficient conditions, respectively. Putative tryptophan aminotransferase (Francci3_4054) and aldehyde dehydrogenase (Francci3_2944) increased by 2-fold and 1.1-fold in nitrogen deficient conditions, respectively. All of the auxin biosynthesis genes were down regulated in the nodule compared to *Frankia* Ccl3 grown in nitrogen-sufficient conditions, except a 4-fold increase in the putative tryptophan aminotransferase (Francci3_4054) (data not shown). The expression results show that putative auxin genes are overexpressed in the same conditions where there is a significant increase in auxin and are expressed in the mature nodule.
Figure 16: Expression of *Frankia* putative auxin synthesis genes. RT-PCR of cDNA from (1) *C. glauca* roots, (2) nodules, (3) negative control, (4) *Frankia* Ccl3 growth in BAP medium without a nitrogen source, and (5) with a nitrogen source. There was no expression found in *C. glauca* roots or negative control for all genes tested. cDNA and RT-PCR was performed as described in Methods.
Figure 17: Relative expression of putative auxin biosynthesis genes. RNA was collected after 4 days of Frankia Ccl3 growth in BAP medium without a nitrogen source (light-gray), and with a nitrogen source (dark gray). Relative expression was established using \( \Delta \Delta Ct \) as described in Methods. RpsA was used as the housekeeping gene and the nitrogen-sufficient culture was used as the calibrator.
CHAPTER 4

DISCUSSION

Aqueous Root Exudates

This study focuses on physiological changes in *Frankia* after exposure to *C. cunninghamiana* root exudates to understand the rhizospheric interaction between actinorhizal plants and *Frankia* in the environment. To study rhizospheric interactions similar to environmental conditions aqueous root exudates were collected from soil-grown *C. cunninghamiana*. Aqueous root exudates were used because they will contain any water soluble nutrient or signaling molecule secreted by the plant root. Soil growth conditions will be similar to environmental conditions because microorganisms released from the seed and the environment may metabolize the secreted compounds, resembling what takes place in the rhizosphere. However, microorganisms may also deplete the root exudates of possible signaling molecules. To ensure signaling molecules are present, axenic *C. cunninghamiana* root exudates were also collected. The axenic root exudates will contain any soluble molecules excreted by the roots that may contribute to the actinorhizal symbiosis without modification by microorganisms. Our results showed that *Frankia* responded to both soil- and axenic-grown *C. cunninghamiana* root exudates. This suggests that in the soil-grown root exudates contain signaling molecules or nutrients that affect *Frankia* physiology.
To better understand the types of compounds that may affect *Frankia* we compared our results with root exudates to previously published works. Based on specific flavonoids in the legume-*Rhizobium* symbiosis playing a key role in initiating Nod factor synthesis, most studies on actinorhizal rhizospheric interactions have focused on flavonoids instead of crude (aqueous) root exudates (Lavenus 2008; Benoit and Berry 1997; Hughes et al. 1999; Popovici et al. 2010; Vogel and Dawson 1985; Perradin et al. 1982; Cooper 2007). Flavonoid compounds have been identified from *A. glutinosa, Myrica gale*, and *C. glauca* (Benoit and Berry 1997; Lavenus 2008; Popovici et al. 2010). *C. cunninghamiana* root exudates also contained flavonoids that are likely to be most closely related to those in *C. glauca*. The concentration of flavonoids in *C. cunninghamiana* aqueous root exudates were above the minimum concentration (1 mg/L) of rare flavonoids extracted from *M. gale* fruit that influence *Frankia* growth (Popovici et al. 2010). Another known signaling molecule family that could be influencing the actinorhizal symbiosis are fatty acids. Plant fatty acids are signaling molecules in many plant-microorganism interactions (Farmer 1994). The fatty acid dipterocarpol aids *Frankia* isolation by influencing hyphae to grow out of *A. glutinosa* nodules (Quispel et al. 1989). Actinorhizal root exudates also contain compounds that may not be involved in the symbiosis like sugars and proteins (Vergnaud et al. 1987), but are important to the microbial communities in the rhizosphere. Our results confirmed the presence of organic acids in the aqueous root exudates and suggest that *C. cunninghamiana* aqueous root exudates contain types of compounds that could be used as signaling molecules.
Enhancement of *Frankia* Growth

The ability of *Frankia* Ccl3 to grow in the rhizosphere of *C. cunninghamiana* (Mirza et al. 2009) supports our hypothesis that *Frankia* has chemotrophic responses to its host plant. However, *Frankia* was unable to utilize the aqueous root exudates as an external carbon source. While this result does not disprove our hypothesis it suggests that the chemoattractant is not a growth factor. *Frankia* cannot grow solely on phenolic compounds, also contained in the root exudates, as a sole carbon source (Vogel and Dawson 1985). Since only a small quantity of volatile organic acids were identified in the aqueous root exudates the inability of *Frankia* to grow solely on the aqueous root exudates collected in this study is not surprising. One explanation for the contradiction between our results with aqueous root exudates and those of Mirza et al. (2009), with collected plant rhizosphere, is that usable carbon compounds may have accumulated in the soil over time. Aqueous root exudates were collected quickly (one week) compared to plants in soil that have continuously excreting carbon compounds.

While carbon sources in the aqueous root exudates alone did not support *Frankia* growth, plant compounds in the root exudates enhanced the growth of *Frankia*. If a supplied carbon source was present *Frankia* growth was positively influenced by aqueous root exudates, suggesting that *C. cunninghamiana* aqueous root exudates provide a growth nutrient or plant-signaling compounds influencing *Frankia* physiology. Our results with aqueous root exudates confirm
previous studies on flavonoid effects on the growth of *Frankia* with methanol extracts or purchased phenolics, (Lavenus 2008; Perradin et al. 1982; Vogel and Dawson 1985; Popovici et al. 2010). In the presence of a carbon source, *Frankia* Ccl3 growth was positively influenced by *C. cunninghamiana* root and plant extracts, while *C. glauca* methanol extracts, containing flavonoids, have no effect on *Frankia* Ccl3 growth (Zimpher et al. 2004; Lavenus 2008). Interestingly, root exudates collected under nitrogen-sufficient and -deficient conditions cause the same positive influence in *Frankia* growth. These results suggest that flavonoids may not be the component in *C. cunninghamiana* aqueous root exudates causing the physiological changes in *Frankia*.

**Frankia Hyphal Curling**

The most spectacular physiological change caused by plant signaling-compounds was a hyphal curling response. No other studies have observed hyphal curling, but phenolic compounds increased hyphal branching (ramification) and vanillic acid caused the formation of morphological structures similar to vesicles (Perradin et al. 1982; Vogel and Dawson 1985). The hyphal curling was a host-specific response suggesting that these changes were caused by compounds excreted by actinorhizal plants that are hosts for *Frankia* Ccl3. It is currently unknown how *Frankia* and the host plant come into contact in the soil and what surface compounds *Frankia* may use to interact with host roots upon contact. The hyphal curling could be an *in vitro* observation of a chemotrophic response and/or changes to *Frankia* surface properties. Root exudates from
legumes are a chemoattractant for motile *Rhizobium* bacterium, but *Frankia* is a non-motile bacterium (Gaworzewska and Carlile 1982; Schwenke and Carú 2001). *Frankia* Ccl3 grows in the rhizosphere of *C. cunninghamiana* which suggests that a chemotrophic response is possible and has been suggested but not studied (Mirza et al. 2009; Vessey et al. 2005).

**Carbohydrates and Fatty Acid Surface Changes**

There were several lines of evidence to indicate a surface property change in response to aqueous root exudates. First, treated *Frankia* cells were more difficult to pellet. The surface property changes observed during pelleting could be caused by hydrophobicity changes on the cell surface. Second, Congo red dye binding was reduced for treated cells. Lastly, FTIR spectra of treated cells were different from untreated cells. Surface property changes were expected in *Frankia* in response to infection and nodulation because of the intimate intracellular infection pathway between *Frankia* Ccl3 and *C. cunninghamiana*. The lipid and carbohydrate surface property changes seen in this study are also necessary for many pathogenic and symbiotic infection pathways (Kucho et al. 2010; Fraysse et al. 2003; Schorey and Sweet 2008). In the legume-*Rhizobium* symbiosis polysaccharide changes are important to the intracellular infection (Fraysse et al. 2003). *Mycobacterium*, a closely related actinomycete to *Frankia*, has an array of complex lipids necessary for pathogenesis (Gago et al. 2011; Schorey and Sweet 2008). We do not know the specific type of molecules being produced but these chemical characteristics will
be extremely helpful in identifying the compound(s) structure. Further studies need to be done to establish what specific compounds are changing and how they benefit the actinorhizal symbiosis.

Physiological Changes to Root Exudates are Beneficial to Actinorhizal Symbiosis

The main hypothesis for this study was that root exudates will change the physiology of *Frankia* to allow for infection and nodulation. Here, we report that pre-exposure to root exudates allowed *Frankia* to nodulate host plants earlier than untreated cells suggesting that physiological changes to *Frankia* from host root exudates exposure were beneficial to the infection and nodulation process. This result is in agreement with the reduction in nodulation time by *Frankia* BCU110501 under plant crowding conditions, perhaps due to an increase of root exudates (Gabbarini and Wall 2008). We still do not know what changes in *Frankia* physiology allow for symbiosis with the plant, but it is clear that the observed physiological changes benefit the infection and nodulation process. Future studies will establish what changes are the most beneficial.

To predict what aqueous root exudates compound(s) are involved in affecting *Frankia* physiology to benefit the symbiosis our nodulation results were compared to previous studies. Fractionated extracts and root exudates focused on testing the effects of flavonoids on nodulation caused an increase in the number of nodules per plant, but do not affect the initiation of nodulation (Lavenus 2008; Perradin et al. 1982; Benoit and Berry 1997). *Frankia* CcI3 pre-
incubated with methanol extracts from *C. glauca* root exudates (the closest actinorhizal system to the one used in this study) do not reduce the time for the initiation of nodulation (Lavenus 2008). Since methanol extracts contain flavonoids, the above responses suggest that flavonoids are not the causative agent for our observed alteration in the nodulation timeline.

**Frankia Cci3 Auxin Production In Planta**

The results for *in vivo* and *in planta* expression of auxin synthesis genes show that *Frankia* has the potential to produce PAA and IAA inside the nodule as a possible signaling molecule. The expression of most auxin biosynthesis genes *in planta* were down regulated in comparison to *in vitro* expression. These results could be explained by tight regulation of auxin production inside the mature nodule. Alternatively, auxin expression could be time-dependent and may only be up-regulated at a specific nodule development stage(s). The results indicate that frankial PAA and IAA biosynthetic machinery is produced inside the nodule.

The increased expression of auxin biosynthesis genes and production of IAA and PAA in *in vitro* conditions needs to be further investigated. The increase may suggest a use of auxins in the soil as a possible signaling molecule to the host plant or other organisms. The increase in auxin production under nitrogen-deficient conditions support this hypothesis. Under nitrogen-sufficient conditions plant nodulation is inhibited and will only occurs under nitrogen-deficient conditions. It is known that PAA is located on the hopanoids in vesicles, which may explain the increase under nitrogen-deficient conditions (Hammad et al.
2003). Since *Frankia* Ccl3 does not produce vesicles in the nodule this may explain the difference in auxin biosynthesis gene expression in *in vitro* and *in planta* conditions (Benson and Silvester 1993).

The use of auxin as a signaling molecule fits well with the physiological changes seen during the infection and nodulation process and the known physiological changes caused by auxins. Auxin accumulation would also help explain hypertrophy in *Frankia* infected plant cells (Perrine-Walker et al. 2010). Currently, Dr. Louis Tisa’s lab is working to create targeted mutagenesis mechanisms in *Frankia* (unpublished). If this work succeeds, *Frankia* strains mutated in identified auxin biosynthesis pathways can be created to observe the effect of knocked out PAA and IAA production.

**Significance and Future Directions**

The effect of aqueous exudates on *Frankia* physiology and nodulation have shown that rhizospheric interactions between *Frankia* and actinorhizal plants do exist and that flavonoids may play a smaller role in the rhizospheric interactions of the actinorhizal symbiosis than in the legume-*Rhizobium* symbiosis. Based on FTIR and Congo red binding results, fatty acids are predicted to play a role in the actinorhizal symbiosis. *Frankia* possesses multiple predicted novel fatty acids and lipids that could be involved in symbiosis (Udwary et al. 2011). Future studies will focus on fractionating root exudates to discover which compound(s) can reproduce the physiological changes observed when *Frankia* Ccl3 is exposed to aqueous root exudates.
Frankia Ccl3 hyphal curling after aqueous root exudates exposure was host specific which suggests a very intimate interaction between C. cunninghamiana and Frankia Ccl3, which has an intracellular nodulation pathway and very narrow host range. Rhizospheric interactions are involved in the D. trivervis-Frankia system but the physiological changes caused in the intercellular infection and promiscuity of these broad host range strains needs to be further studied.

Our results illustrate that exposure to aqueous root exudates changes Frankia physiology and is beneficial to nodulation. Future studies will separate the different cellular components of Frankia and test them on transgenic C. glauca to detect changes in expression of known plant symbiosis genes (Svistoonoff et al. 2010). The different fractions will help better determine the type(s) of molecules that are involved in the actinorhizal symbiosis and use of the putative secretome of Frankia Ccl3 to help in deciding which molecules may cause the largest effect (Udwary et al. 2011). A bioinformatic approach will be used to discover genes up-regulated by root exudates using naturally occurring non-infective strains of Frankia, Frankia sp. CN3 and Frankia sp. DC12, and microarray analysis of Frankia Ccl3 exposed to root exudates. The molecular approaches along with the putative secretome will help in discovering which biosynthetic clusters are being expressed when Frankia is preparing to form a symbiosis with host plants and help in identifying what compound(s) are necessary for the actinorhizal symbiosis. Work will be completed to better elucidate if a chemotrophic response is involved in the actinorhizal symbiosis. This research has identified a rhizospheric interaction in the actinorhizal
symbiosis and putative *Frankia* production of auxin in the mature nodule. Hopefully future research will elucidate the specific molecular signals involved in the rhizospheric interactions and what auxin’s role is as a possible *Frankia* signaling molecule in the actinorhizal symbiosis.
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