Lipo-chitooligosaccharides: from specific rhizobial signals to nearly ubiquitous fungal quorum sensing molecules

By

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This dissertation is dedicated to

my father, Dr. M.C. "Chuck" Rush,

who was not able to see it completed but introduced me to the world of science and discovery,

which became my lifelong passion.

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Abstract

Microbial signaling is the driver to initiate microbe-host and microbe-microbe interactions. When the host perceives a signal, it will somehow decide whether to activate innate immunity or mutualistic cooperation between the organism(s). However, decoding and understanding any coded signal is an enigma, especially if we do not know what organisms produce the signal and why they use it. Lipo-chitooligosaccharides are an example of a coded signal which are recognized as "symbiotic" communication signals produced by rhizobia and by two genetically different mycorrhizal fungi, *Rhizophagus irregularis* and *Laccaria bicolor*.

Given the discovery of lipo-chitooligosaccharides being produced by such diverse fungi, here we tested the hypothesis that lipo-chitooligosaccharides are ubiquitous signal molecules among fungi. Using both biological assays (legume root hair branching and *ENOD11* expression), and high-performance liquid chromatography coupled to mass spectrometry, we analyzed fungal exudates of 61 fungi across five phyla in the fungal kingdom and three species of oomycetes. We were able to detect lipo-chitooligosaccharides in fungal exudates in 55 fungi across the Fungal Kingdom with various lifestyles and growth habits but not in non-dimorphic yeasts (*Saccharomyces cerevisiae* and *Candida glabrata*) and oomycetes. Moreover, chitooligosaccharides were detected in all fungi and oomycetes tested.

To further understand why non-symbiotic fungi would produce lipo-chitooligosaccharides, we tested the hypothesis that lipo-chitooligosaccharides is a quorum sensing molecule. We used specific chemically synthesized lipo-chitooligosaccharides (sulfated C16:0; non-sulfated C16:0; sulfated C18:1; and non-sulfated C18:1) and naturally produced chitooligosaccharides (CO4, CO5, and CO8). We were interested in answering the following questions: 1) Can exogenous lipo-

chitooligosaccharides influence growth and development of a fungus that produces lipo-chitooligosaccharides? 2) Can exogenous lipo-chitooligosaccharides influence growth and development of a fungus that does not produce lipo-chitooligosaccharides? 3) What is the extent of growth and development affected by exogenous lipo-chitooligosaccharides in the Fungal Kingdom? To answer these questions, we tested these synthesized lipo-chitooligosaccharides against two ascomycetes; *Aspergillus fumigatus*, which produces lipo-chitooligosaccharides, and *Candida glabrata*, which did not have lipo-chitooligosaccharides detection. Moreover, we tested *Rhodotorula mucilaginosa*, which is a basidiomycete yeast, to determine if the lipo-chitooligosaccharides effect on fungi can be across phyla. Our results determined that exogenous lipo-chitooligosaccharides influence the behavior, growth, and development, and gene expression in fungi, regardless of their ability to produce lipo-chitooligosaccharides or not, and their phylogenic placement in the Fungal Kingdom.

Finally, we wanted to test the hypothesis that the genes responsible for lipo-chitooligosaccharides production in fungi are homologous to the genes found in rhizobia. In the latter, the genes encoding for chitin synthase, chitin deacetylase, and acetyltransferase are well characterized and have been demonstrated to be responsible for lipo-chitooligosaccharides production based on gene knockout studies. We found two homologous chitin deacetylase genes in *Aspergillus fumigatus* Af293 strain that matched with rhizobia chitin deacetylase genes. We constructed a single and double chitin deacetylase knockout mutant to determine if chitin deacetylase encoding genes are indispensable for lipo-chitooligosaccharides production in fungi. Our results determined that the homologous chitin deacetylase genes are essential for lipo-chitooligosaccharides in fungi.

In conclusion, results from this dissertation have determined that: 1) lipo-chitooligosaccharides are ubiquitous signal molecules found throughout the Fungal Kingdom, 2) lipo-chitooligosaccharides influence behavior, growth and development, and gene expression in fungi, and 3) rhizobia and fungi share homologous genes that are responsible for the production of lipo-chitooligosaccharides.

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Chapter 1: Introduction

1.1 Microbial interactions are an intertwined network that microbes establish with each other and with their hosts.

The establishment of life is dependent on the intertwined network of microbial interactions. Numerous studies have demonstrated how microbes interact with humans, plants, minerals, soil, and other microbes which had shaped our environment and living conditions (Chisholm *et al.*, 2006; Badri *et al.*, 2009; Dong, 2010; Bermudez-Brito *et al.*, 2013; Zhu *et al.*, 2017). Based on microbial interaction studies, the central idea is that these complex relationships improve biological fitness, which means the ability to survive to reproductive age and produce an offspring to increase evolutionary genetics (Orr, 2009). Moreover, microbial interactions are critical for 1) the adaptability and establishment into new environments, 2) the co-evolution between two or more organisms, 3) the scavenging for nutrient supplies and resources; 4) the ability to acquire new molecular functions; and 5) the survivability and proliferation (Chisholm *et al.*, 2006; Orr, 2009; Badri *et al.*, 2009; Dong, 2010; Bermudez-Brito *et al.*, 2013; Zhu *et al.*, 2017).

Microbial interactions can be either positive or negative (Agrios, 2005; Mougi, 2016). Within these positive and negative interactions, microbes that have a mutualistic, commensals, predation, or pathogenic relationship with their hosts are called symbionts (Ebert, 2013). Symbiosis is the Greek word for "living together," and is defined as a close and prolonged association between two or more organisms of different species that may last for the lifetime of one or all partners and influences fitness (Dimijian, 2000; Orr, 2009; Gil & Latorre, 2019). Symbiont-host interactions are a complex system requiring the symbiont, the microbiome, and their environment (Brinker *et al.*, 2019). Microbiome influences symbiont-host interaction (Brinker *et al.*, 2019). Symbionts will use mixed-mode transmission which includes both, vertical

gene transmission, a transfer of genetic material from parent to descendants or horizontal gene transmission, which provides for sexual or environment transmission of genetic material (Ebert, 2013). However, not all symbionts use mixed-mode transmission.

Nonetheless, symbiont-host interactions shape the evolution of all organisms. The positive interactions are mutualism, and commensalism. Mutualism is when the fitness of both partners is increased, whereas commensalism is when the relationship is beneficial to one partner and neutral to the other. These positive interactions provide useful features such as an exchange of nutrients between organisms, a biological control activity against pathogens, and enhanced growth and development or sexual reproduction (Schirawski & Perlin, 2018). Symbiotic microbes such as rhizobia or mycorrhiza fungi are examples of microbes with positive interactions with their host. Negative interactions are ammensalism (antagonism), parasitism, predation, and competition. Ammensalism is when one partner harms the other partner without increased fitness, parasitism is when one partner hurts another partner for improved fitness, whereas predation is when an organism feeds on another organism(s) for substrates. Competition is when one or both partners harm each other searching for substrates which results in decreased fitness. These negative interactions are a well-studied category in science because it houses the studies of pathogens' interaction with animals, plants, and other microbes. **Figure 1** shows the continuum of symbiosis between a microbe and another organism.
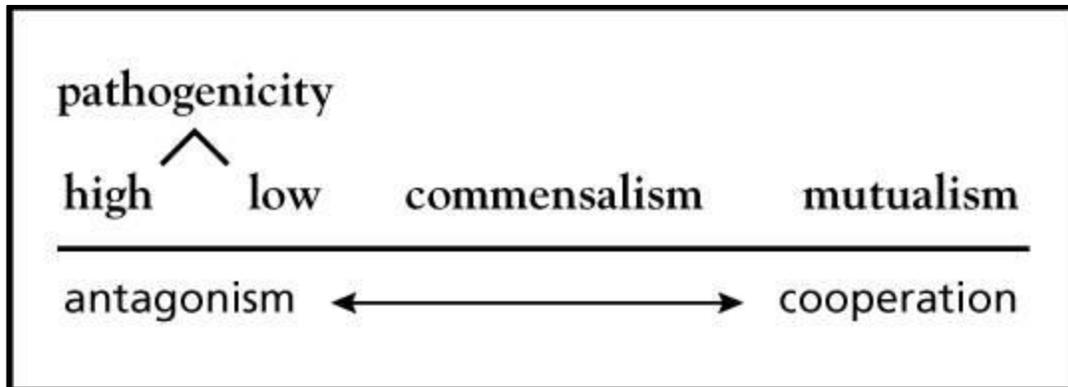
Figure 1 **Continuum from antagonism to cooperation (Dimijian, 2000).**

In the context of this dissertation, I will address microbe-host interactions, with a focus on pathogen-host interactions and mutualistic microbe-host interactions. Moreover, I will discuss microbe-microbe interaction. This dissertation displays the oddity and unique findings regarding a previously described "symbiotic" communication signal believed to be exclusively found in mutualistic symbiotic microbes (rhizobia and mycorrhizal fungi) but now is found in most fungi that are described as necrotrophic, hemi-biotrophic and biotrophic organisms. Moreover, I will present my results on how this communication signal is used between fungi and how it influences behavior, growth, and development.

1.1.1 Microbe-host interactions are critical for characterizing symbiotic or pathogenic microbes

For over a century, researchers have investigated the microbe-host interactions in a multitude of different ecosystems. Nowadays, our understanding of how microbes interact with their host leads to the development of modern agriculture and medicine (Casadevall & Pirofski, 2000). The concept of microbe-host communications often implied that a microorganism would infect/colonize a host, resulting in disease progression or improvement of the host (Casadevall & Pirofski, 2000). However, not all microbes should be viewed as harmful. Many microbes such as rhizobia and mycorrhizal fungi are known to improve plant development and establishment into new environments. Terminology is an issue when describing microbe-host interactions. Currently,

the term, "infect," referred to infection or colonization of an organism that causes disease, as shown in pathogenic microbes (Casadevall & Pirofski, 2000). The term "infect" is also used for symbiotic microbes. However, since symbiotic microbes, like rhizobia and mycorrhizal fungi do not cause disease, the term that should be used is "colonize."

Understanding how microbes interact with their host led to the development of Koch's postulates, where the microbe is isolated from a symptomatic host, grown, and then tested for its ability to recolonize the host and cause the same symptoms previously seen. However, many issues arose from performing Koch's postulates to understand microbe-host interactions such as: 1) how can you isolate an organism that cannot be grown without the host? 2) what if the environmental conditions are not conducive to cause disease in the host? 3) what if the microbes need the host to have a weakened immune system or specific receptor before causing disease or symptoms? 4) what if many microbes need to be present together to cause disease in the host? 5) what if there is an interaction between the microbe and host, but it does not conclude in disease symptoms?

1.1.1.1 Pathogen-Host Interactions

The host's ability to fight pathogens is called immunity, which includes different mechanisms in animals and plants but has the same common ends for a successful defense (Menezes & Jared, 2002). The definition of immunity has garner reexamination considering that all living organisms have preformed defenses and induced resistance against pathogens (Biella *et al.*, 2002; Nathan, 2006; Rowley & Powell, 2007; Abedon, 2012; Uehling *et al.*, 2017). Moreover, microbes have similar preformed defenses that are comparable to those of plants and vertebrates (**Figure 2**).

Plant preformed defenses	Vertebrate preformed defenses	Microbial preformed defenses
1) Physical Barriers 2) Chemical Barriers	1) Physical Barriers 2) Biochemical and physiological barriers	1) Physical Barriers 2) Chemical Barriers
Plant induced-resistance mechanisms	**Vertebrate induced resistance**	**Microbial induced resistance**

Figure 2 shows a diagram for the performed defenses and induced resistance for plants (green), animals (blue) and microbes (yellow). The figure is adapted and modified from (Menezes & Jared, 2002) with additional results from (Biella *et al.*, 2002; Nathan, 2006; Abedon, 2012; Uehling *et al.*, 2017).

In plants, there are preformed defenses such as physical and chemical barriers, like the cell wall, terpenoids, and phenolic compounds (Cassab & Varner, 1988; Fry, 2004). There are also the plant induced-resistance mechanisms where the plant's resistance (R) proteins recognize the pathogen's avirulence (AVR) proteins. This will initiate the signal transduction pathway, and produce salicylic acid and jasmonic acid, which results in a local response, like programmed cell death, cell wall lignification, superoxides, or phytoalexins synthesis and/or systemic signal that leads to systemic acquired resistance, like pathogensis-related proteins, inhibitor proteins, lipid-transfer proteins, and peroxidase, to name a few (Menezes & Jared, 2002).

In animals, there are vertebrate preformed defenses such as physical barriers like the skin and biochemical and physiological walls like saliva, mucus, lysozymes, vaginal, and gastric acidity (Menezes & Jared, 2002). Moreover, vertebrates have induced-resistance mechanisms such as, natural antibodies, T lymphocytes, and Toll family members, which will trigger the signal induction pathway, like cytokines and chemokines, and initiate innate immunity, like phagocytosis and inflammatory responses, then adaptive immunity (Menezes & Jared, 2002).

In microbes, the microbial innate immunity research is still in its infancy in comparison to plant and animal. Here are some examples of microbial innate immunity. In bacteria, the adaptive immunity associated with the discovery of the CRISPR/cas systems indicates that there is bacterial immunity against bacteriophages (Abedon, 2012; Barrangou & Marraffini, 2014). In fungi, programmed cell death will occur when the fungus is infected with a virus (Biella *et al.*, 2002). Moreover, fungi have NLRs like plants and animals, which raises the questions: 1) can fungi identify non-self; and 2) what is the extent of fungal innate immunity? (Uehling *et al.*, 2017).

Preformed defenses between plants, animals, and microbes, pathogen-host interaction allow us to understand the mechanisms of innate immunity. Pathogen associated molecular patterns (PAMPs) or microbe associated molecular patterns (MAMPs), will initiate PAMP-triggered immunity (PTI). Also, effector-triggered immunity (ETI), which are proteins expressed by pathogens to aid infection of specific species, can initiate innate immunity (Toruño *et al.*, 2016). Understanding PTIs and ETIs will allow the researcher to study how specific molecular components from the pathogen, like flagella or chitin, or chemical signals that are produced by pathogen can trigger innate immunity. However, one question that remains is how does a host know whether the microbe is a pathogen or a mutualistic organism?

1.1.1.2 Mutualistic Microbe-Host Interactions

Hosts can established their niche through mutualistic relationships (Margulis & Bermudes, 1985). There are multiple examples of mutualism between two or more organisms, to cite some: bees as flower pollinators, rumen bacteria in cow's digestion tract, flagellated protozoans and termites, rhizobia and legumes or mycorrhizae fungi and land plants (Brugerolle & Radek, 2006; Greenleaf & Kremen, 2006; Winfree *et al.*, 2007; Bonfante & Anca, 2009; Denison & Kiers, 2011; Oldroyd *et al.*, 2011; Venkateshwaran *et al.*, 2013b, 2015; Delaux *et al.*, 2013, 2015; Eisler *et al.*, 2014; Jayaraman *et al.*, 2014; Malmuthuge & Guan, 2017; Kamel *et al.*, 2017). Mutualistic relationships are often between members of different kingdoms, which account for major evolutionary innovations (Leigh, 2010). However, what maintains mutualistic relationship between two or more organisms is still unknown. In order to understand mutualistic relationships, a hypothesis has been developed based on Adam Smith's market economic theory. The hypothesis is that mutualistic relationships abide by the laws of supply and demand, suggesting that symbiosis is driven by a series of biological markets and reciprocal reward systems (Kiers *et al.*, 2011; Wyatt *et al.*, 2014; Noë & Kiers, 2018). Economic markets have checks and balances to prevent fraud; however, do microbes have the same standards? How do two or more organisms continue a long-term mutualistic relationship without one of the partners becoming a cheater? (Scannerini, 1991; Doebeli *et al.*, 1998). Cheaters are organisms that receive a benefit at the cost of another organism, although they were formally identified as being mutualistic partners (Genini *et al.*, 2010). There is a controversy on whether cheaters are ubiquitous in all mutualistic systems. In this regard, Genini et al. (2010) had demonstrated that cheaters in a previously known mutualistic relationship could influence the network topology. A review about cheaters (Kiers & Van Der Heijden, 2006) highlighted experiments in which a host is colonized by arbuscular mycorrhizal fungi, and there were strains not exchanging nutrients due potentially to the presence of multiple strains on one

host or because of the stamina of the host being compromised. Altogether, it is unknown how signals produced by mutualistic microbes are interpreted by their hosts. Perhaps could chemical signaling between a symbiont and their host play a role in the theory of a biological market? Moreover, could chemical signaling influence the microbe's ability to be a cheater?

1.1.2 Quorum sensing plays an influential role in microbe-microbe interactions

Mediators of communication between microbes are gaining interest where researchers are trying to "decode" microbial messages that can be useful in agricultural and medical fields (Scherlach & Hertweck, 2018). Microbial symbionts thrive on syntrophy (Scherlach & Hertweck, 2018). Whether microbes are using other microbes for substrates, competition for a host, piggybacking to gain entry to a host, or talking with each other to increase biological fitness, microbe-microbe interactions are the influencer of the microbiome of any environment. As previously mentioned in the microbial innate immunity section, there are pathogens of microbes that exist. The most well-known microbial pathogens are viruses called bacteriophages which attack bacteria or mycoviruses that attack fungi. There is evidence demonstrating that plant pathogenic viruses can be acquired and transmitted through plant pathogenic fungi, without causing symptoms to the fungal host, but be transmitted to the host plant (Andika *et al.*, 2017). To establish microbe-microbe interactions there should be chemical communications, like quorum sensing.

Quorum sensing (previously known as autoinduction) function through signals from small and diffusible molecules produced by a microbe to ensure cell to cell communication. These molecules can be perceived by the producing microbes, influence behavior, growth and development of these microbes, but also might be perceived by other microbes (Visick & Fuqua, 2005; Albuquerque & Casadevall, 2012; Mehmood *et al.*, 2019). Quorum sensing can influence

bioluminescence, biofilms, the production of bioactive compounds, virulence factors, and the production of metabolites (Nealson *et al.*, 1970; Hornby *et al.*, 2001; Turovskiy *et al.*, 2007; Albuquerque & Casadevall, 2012). The first time a mechanism was proposed for quorum sensing was in 1970. The bacterium, *Vibrio harveyi,* has been shown to display different intensity of bioluminescence in correlation with the density of the bacteria present (Nealson *et al.*, 1970). After that, multiple studies began investigating quorum sensing in bacteria, under the assumption that this phenomenon only occurred in bacteria.

Bacterial species belonging to the genera *Bacillus*, *Chromobacterium*, *Erwinia, Myxococcus, Pseudomonas*, *Staphylococcus*, *Vibrio,* and *Xanthomonas* are known to produce and perceive quorum sensing molecules (Visick & Fuqua, 2005). These quorum sensing molecules have been described as A-signaling amino acids, modified peptide chains, homoserine lactones, quinolone signals, or autoinducers-1 or -2. The bacteria's usage of quorum sensing molecules had improved biological functions such as the ability to uptake DNA, pigment production, virulence, biofilm formation, enzyme production, antibiotic production, and bioluminescence (Visick & Fuqua, 2005).

In 2001, a monumental shift occurred in the idea that quorum sensing is unique to bacteria. Hornby et al., (2001) had determined that the pathogenic yeast, *Candida albicans*, is able to produce and respond to a signaling chemical called farnesol, which controlled filamentation. Since then, several studies have shown that fungi produced and perceive quorum sensing molecules (Albuquerque & Casadevall, 2012). However, quorum sensing in fungi as compared to bacteria is still a less known mode of communication (Padder *et al.*, 2018). Quorum sensing is considered ubiquitous in fungi, although all examples are based on fungi found in Basidiomycota and Ascomycota (Hornby *et al.*, 2001; Albuquerque & Casadevall, 2012; Mehmood *et al.*, 2019). The

quorum sensing molecules that have been described from fungi are pheromones, farnesol, tyrosol, volatile organic compounds, lactone containing molecules, and oxylipins (Hornby *et al.*, 2001; Albuquerque & Casadevall, 2012; Gessler *et al.*, 2017; Mehmood *et al.*, 2019).

Given the evidence that quorum sensing molecules might be ubiquitous in all microbes, the next question is, what are the criteria to consider a molecule a quorum sensing molecule? There is no single consensus for the required features of a quorum sensing molecule. However, based on multiple reviews there are four key commonalities which are: 1) The organism ability to detect and respond to its own cell-like population resulting in an observable and repeatable phenotype(s); 2) the molecule alters its gene expression, allowing the organism to respond to its environment; 3) signal perception where the signal is recognized by a specific sensor protein; and 4) they are small diffusible molecules (Visick & Fuqua, 2005; Gonzalez & Keshavan, 2006; Deep *et al.*, 2011; Li & Tian, 2012; Rutherford & Bassler, 2012; Grandclément *et al.*, 2016; Verbeke *et al.*, 2017; Abisado *et al.*, 2018; Mehmood *et al.*, 2019).

1.2 Symbiotic microbes produce lipochitooligosaccharides as a communication signal with their hosts' symbionts.

Lipo-chitooligosaccharides (LCOs) are symbiotic signaling molecules produced by rhizobia, arbuscular mycorrhizal fungi, and ectomycorrhizal fungi to activate a common symbiotic pathway within the host plants (Oldroyd 2013; Venkateshwaran *et al.*, 2013; Cope *et al.*, 2019). There is further discussion about LCOs in section 1.3.

1.2.1 Rhizobia

The genus *Rhizobium* (Frank 1889) was firstly described in 1888, when the type species, *Rhizobium leguminosarum* was isolated and characterized. The name was derived from the Greek words, "*rhiza*" which means root and "bios" which means life. The term "rhizobia" is a collective

term that includes all bacteria that produce nodules (**Figure 3**) and fix nitrogen on leguminous hosts.

Figure 3 shows nodules on leguminous roots and caused by infection of rhizobia. Scale bar is 3 mm.

Multiple genera that belong to rhizobia were described, most notable are *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium*. There are β-proteobacteria in the genera, *Burkholderia* and *Ralstonia,* that are rhizobia (Chen *et al.*, 2003). Identifying the genes and their function in bacteria became possible in the 70s after a protocol was developed to make bacterial mutants using genetic recombination (Cohen *et al.*, 1973). This milestone research led to the development of methods to genetically engineer rhizobia. Altogether, these events lead to an increase in the number of publications on rhizobia-legume interaction and their ability to fix nitrogen. Thus the study of rhizobia-legume interaction was readily investigated throughout the world's laboratories because (i) both legumes and rhizobia can easily be handled and manipulated by molecular techniques, and (ii) the agronomical importance of the nitrogen-fixing symbiosis (Göttfert, 1993).

The beginning of LCOs in rhizobia-legume interaction history started in 1984 when Kondorosi *et al.* (1984) identified the genetic region that contains the symbiotic genes in *Sinorhizobium meliloti* (syn: *Rhizobium meliloti*) based on a mutant screen of rhizobia incapable of inducing nodule formation. The characterization of the physical, genetic map of the nodulation and nitrogen fixing genes (*nod-nif*) region allowed Faucher et al. (1988) to describe the function of the *nodABC* operon, which housed the *nod* genes. Then, the *nodABC* operon was identified in multiple species of rhizobia and was determined to be a common symbiotic genes (Faucher *et al.*, 1988). *Nod* genes are involved in the biosynthesis of *nod* factors.

Discovery of LCOs

Since the discovery of *nod* operon, rhizobia had become the model organism to investigate the assembly and function of *nod* genes. After identifying this genetic region, Lerouge *et al.,* (1990), characterized the genes responsible for LCO production. Subsequently, they were able to purify LCOs through a butanol extraction method. LCOs were determined to be butanol-soluble. When these molecules were applied to *Medicago sativa* (alfalfa)*,* they triggered root hair branching (Lerouge *et al.*, 1990). The N-acylated portion was on the non-reducing end with a fatty acid chain that makes the amphiphilic LCO molecule. The Lerouge et al. (1990) publication was the first one to determine that rhizobia have chitin assembly genes.

Now that an LCO (*nod* factor) purification method existed, different species of rhizobia were studied to determine what types of LCOs were present and if there is specificity. Rhizobia are restricted to leguminous hosts and the unrelated species of *Parasponia* (Dénarié *et al.*, 1996; Op Den Camp *et al.*, 2012). Therefore, rhizobia were extracted from several leguminous hosts during the 1990s, and their LCO production and structure was characterized.

Cumulatively, after two decades of research, 26 *nod* genes were identified from rhizobia. From this list, six genes are common in all rhizobia and have a specifically designated function. Here we cite: (i) *nodD* which regulates all the other *nod* genes (Mulligan & Long, 1985; Fisher & Long, 1992); (ii) *nodA* which codes for an acetyltransferase that adds the fatty acid to the CO backbone (Geremia *et al.*, 1994; Spaink *et al.*, 1994; Kamst *et al.*, 1995) (iii) *nodB* which codes for a chitin deacetylase that removes the acetyl group from the non-reducing end before the addition of a fatty acid (John *et al.*, 1993; Atkinson *et al.*, 1994; Spaink *et al.*, 1994); (iv) *nodC* which codes for a chitin synthase that is necessary to make the CO (Röhrig *et al.*, 1994; Ritsema *et al.*, 1996), and (v) *nodI* and *nodJ* which are involved in the secretion of the *nod* factors (Evans & Downie, 1986; Vázquez *et al.*, 1993; McKay & Djordjevic, 1993; Spaink *et al.*, 1995; Cardenas *et al.*, 1996).

With the identification and description of the function of common *nod* genes, the mechanisms on how rhizobia colonize their host, create an infection thread, and start the process of nodulation could be characterized. The process of colonization and nodule formation by rhizobia is as followed. The host plant will recruit rhizobia through hydrophilic amino acids, organic acids, sugars and sugar alcohols (Ma et al., 2005; Webb et al., 2015). Then, plant flavonoids will induce transcription of the genes for biosynthesis of *nod* factors in rhizobia through plant root chemical secretions (Liu & Murray, 2016). Flavonoids are a group of natural substances with variable phenolic structures. Flavones are plant pigments that fall within this group of flavonoids, and they are critical signaling molecules between legumes and rhizobia (Liu & Murray, 2016). Leguminous flavonoids, isoflavonoids and betaines, such as apigenin, naringenin, and luteolin, induce *nod* factors from rhizobia. The rhizobia perceives flavonoid, which is mediated by *nodD* (Mulligan & Long, 1985; Spaink *et al.*, 1987, 1989). Once rhizobia are next to the tip of the

root, *nodD* is activated and mediates *nodABC* genes. The final product of *nodABC* genes are three proteins, which allow the host plant to recognize rhizobia as a "friend." Then the root hair will begin to curl around the bacteria. The bacteria will form a micro colony. The curled root hair with the attached rhizobia will allow the bacteria to degrade the cell wall and form an infection thread (**Figure 4 a-c**). The infection thread is an intracellular tube through which the rhizobia travels to reach the root cortex (**Figure 4 d-e**). Simultaneously, nodule organogenesis will initiate, where cortical cells will divide and develop the nodule (**Figure 4 f**).

The **infection** process during **nodule organogenesis**
— occur simultaneously

(A) Plant: attractants / Rhizobia: Nod factors — Root hair, Rhizobia
(B) Curling growth
(C) Cell wall degradation / Infection thread formation — Golgi vesicle, Infection thread, Golgi body
(D) Fuse with the membrane of root cell — Infection thread membrane fuses with cell membrane
(E) Penetrate and new infection thread formation
(F) Branching and extending bacteria released into the cytosol — Vesicle containing rhizobia

Bacteroids: a nitrogen-fixing endosymbiotic organelles (p. 301R)

Figure 4 is a diagram of rhizobia and leguminous root hair interaction modified from Plant Physiology 3rd edition (Lazar 2003). The following steps are: (a) the gathering of rhizobia at the tip of the root hair, (b) the curling growth around the aggregated bacteria,

(c) the degradation of the plant cell wall and formation of the infection thread, (d) the rhizobia fusing with the membrane of the root cell, (e) the penetration of the bacteria through the cell wall, and (f) the beginning process of organogenesis.

Within the nodule, nitrogen gas from the atmosphere will be converted to ammonium, then assimilated into amino acids, nucleotides, and other all components. Nitrogen fixation in nodules is oxygen sensitive; therefore, leghemoglobin will be produced. In most cases, the production of LCOs from rhizobia is absolutely necessary for successful infection and nodule development (Polacco *et al.*, 2011)

1.2.2 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi are obligate biotrophic fungal symbionts that colonize 60% to 85% of land plants (Brundrett & Tedersoo, 2018). These fungi can concurrently colonize multiple hosts. They are one of four symbiont groups that colonize inside the cell, which makes them part of a collective group called endomycorrhiza (Bonfante & Genre, 2010a; Luginbuehl & Oldroyd, 2017). Arbuscular mycorrhizal fungi are monophyletic and genetically placed in the early divergent part of the Kingdom Fungi, within the phylum Mucoromycotina, and subphylum Glomeromycotina (Sanders & Croll, 2010; Spatafora *et al.*, 2017), which houses all described arbuscular mycorrhizal fungi. It is believed that arbuscular mycorrhizal fungi helped with the establishment of land plants 400 to 500 million years ago (Sanders & Croll, 2010). This group of symbiotic fungi is known to colonize their host plant without leaving any damage. Unlike other pathogenic fungi that produce plant cell wall degrading enzymes when they infect their host plant, arbuscular mycorrhizal fungi do not have the genes encoding for these enzymes (Tisserant *et al.*, 2013).

Similarly, to the plant-root chemical responses described earlier for rhizobia, the host plant will excrete phytochemicals (i.e. strigolactones) which will influence arbuscular mycorrhizal fungi growth and behavior. Strigolactones will cause increase germination and branching before the formation of the appressorium on arbuscular mycorrhizal fungi (Akiyama *et al.*, 2005) and will activate the mitochondria (Besserer *et al.*, 2006). Other plant exudates such as flavonoids, and more specifically, formononetin had been shown to increase during the colonization of roots by *Rhizophagus intraradices* (Volpin *et al.*, 1994, 1995). Moreover, high levels of transcripts encoding for five enzymes responsible to produce phenylpropanoid, flavonoid, isoflavonoids in *Medicago truncatula* roots were reported when they were colonized by *Rhizophagus versiforme* (Harrison & Dixon, 1994). Moreover, Harrison and Dixon (1994) determined that the plant hormones are related to arbuscule development within the root. Overall plant flavonoids and isoflavonoids will bind to the fungal estrogen receptors which will induce a defense response from the arbuscular mycorrhizal fungi (Harrison, 1999).

In connection to rhizobia, since plants produce chemical signals to attract symbiotic microbes, it was hypothesized that mycorrhizal fungi must be producing signaling molecules allowing the plant to recognize the microbe to start the colonization process. In 2011, Malliet *et al.* published the first evidence that *Rhizophagus intraradices* produces LCOs and this was confirmed by observing the root hair branching phenotype in *Medicago truncatula* and through high performance liquid chromatography. Furthermore, the structure of LCOs produced was identified by mass spectrometry. The identified LCO structure, fatty acids, and chemical substitutions were comparable to LCOs found in rhizobia. These results were of great significance as: (i) it was the first time showing that a fungus produces LCOs; (ii) it expands the correlation between LCOs and symbiosis; (iii) it supports the hypothesis that rhizobia have potentially

obtained the genes required to produce LCOs from arbuscular mycorrhizal fungi through horizontal gene transfer (Parniske, 2008); and (iv) it develops the narrative that LCOs are most likely a symbiotic signal merely found in symbiotic microbes. In the same paper, Malliet et al., (2011) have also tested the ability of the non-dimorphic yeast, *Saccharomyces cerevisiae* to produce LCOs. Findings from this study determined that *S. cerevisiae* did not have the presences of LCOs, thus concluding that these molecules are unique to symbiont microbes.

Afterwards, other investigations began to study the interactions between LCOs produced by arbuscular mycorrhizal fungi and economically important crops. To understand the activation of the common symbiosis pathway by COs and LCOs produced by *Rhizophagus intraradices*, Sun et al. (2015), extracted the signaling molecules and applied them to *Medicago truncatula* and *Oryza sativa* (rice) roots. Their results showed that the activation of the common symbiotic pathway occurred with the sole application of COs, but not LCOs in rice. Calcium spiking assays confirmed these results. However, the application of LCOs to *M. truncatula* activated the common symbiotic pathway. Interestingly, with mixed applications of LCOs and COs from *Rhizophagus intraradices,* the common symbiosis pathway (discussed in section 1.2.2-Recognition between symbiotic microbes and host partners) was activated in rice. The results indicate that there is a response by the host which was dependent on the signaling compound extracted from *R. intraradices*. However germinated spore exudates from *R. intraradices* induce signaling of the common symbiosis pathway in both *Medicago truncatula* and *Oryza sativa,* but it can be inhibited by ethylene production (Oláh *et al.*, 2005; Mukherjee & Ané, 2011). Moreover, regulation of the common symbiosis pathway by ethylene production in both monocots and eudicots is conserved (Mukherjee & Ané, 2011).

The discovery of LCOs in a symbiotic fungus created new theories about how the plant can discern these symbiotic signals and establish colonization. It was hypothesized that once the fungus is close to the root LCOs are produced, which allows the host plant to recognize the microbe as a "friend." Simultaneously, different responses occur. Plants will secrete flavonoids and isoflavoids that will influence hyphal growth and differentiation, and root colonization in arbuscular mycorrhizal fungi. Moreover, plants will secrete strigolactones that will stimulate hyphal branching, increase spore germination and increase mitochondrial density. Then, the arbuscular mycorrhizal fungi will start the process of colonization. It remains currently unknown whether LCOs are needed for the colonization to occur in arbuscular mycorrhizal fungi. **Figure 5** depicts the arbuscular mycorrhizal colonization of the root, which will start with the production of hyphopodium by the fungus, which is a specialized structure that will press against a root surface, build up pressure and then push the hyphae into the cell wall. Once inside the cell wall, the hyphae will vaginate into the cell and produce arbuscules, which are specialized structures involved in the exchange of nutrients with the host plant (Bücking & Shachar-Hill, 2005; Bücking & Kafle, 2015). The host plant will provide carbon to the fungus, and in return, the fungus will provide nitrogen and phosphate (Kiers *et al.*, 2011; Fellbaum *et al.*, 2012), which is dependent on the host genotype (Wang *et al.*, 2016). There is an exchange of water (Birhane *et al.*, 2012) and lipids (Keymer *et al.*, 2017) between host and fungus. After the exchange of nutrients, the fungus will leave the cell of the plant, without any damage.

Arbuscular mycorrhiza

Hypohopodium
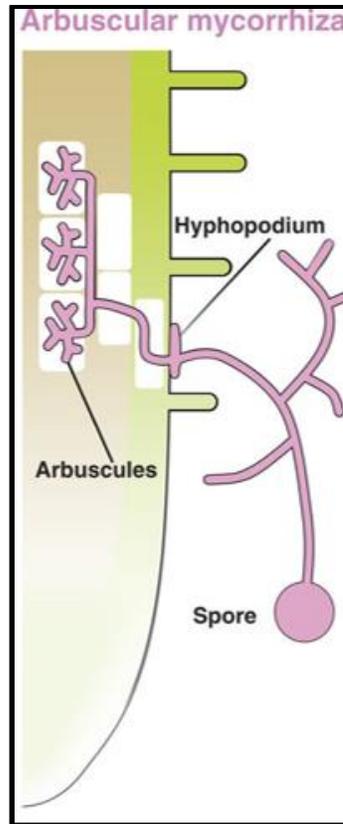
Arbuscules

Spore

Figure 5 Arbuscular mycorrhizal fungi will produce a spore that will germinate and produce mycelium. From the mycelium, a hyphopodium, a lobed outward growth from the mycelium, will attach and penetrate the root epidermis. Intraradical colonization will occur intra- and intercellularly which will produce arbuscules inside the inner cortical cells (Bonfante & Anca, 2009).

Now with the knowledge that arbuscular mycorrhizal fungi produce LCOs, this indicates that other fungi might also produce LCOs. It is hard, if not impossible, to make transformants of arbuscular mycorrhizal fungi, most likely due to its obligate biotroph lifestyle (Bonfante & Genre, 2010a; Luginbuehl & Oldroyd, 2017). However, there is one report of biolistic transformation that has been developed, but not extensively used (Forbes *et al.*, 1998). Lastly, no group has tested the influence of LCOs on growth and development on fungi. However, there are several reports about

LCOs influencing a fungus ability to colonize their host plant. Xie et al. (1995) reported for the first time that application of LCOs, from rhizobia to a root colonized by arbuscular mycorrhizal fungi and rhizobia resulted in a tripartite synergistic relationship that improved root health and colonization rates of both microbes. Marburger et al. (2018) showed that a foliar application of LCOs on soybeans did not affect disease symptoms of *Fusarium virguliforme*, but there was an increase in stem rot development caused by *Sclerotinia sclerotiorum*. However, it is unknown if the observed effect is due to the plant exposure to LCOs, and not dependent on the fungus.

1.2.3 Ectomycorrhizal fungi

Ectomycorrhizal fungi name is derived from the Greek words "ektos" meaning outside, "mykes" meaning fungi, and "rhiza" meaning root. Ectomycorrhizal fungi are known for their extensive soil-borne mycelial network which can span over hundred miles and for the longevity of a single specimen. These mycelial networks help to obtain, transport and supply nutrients between the fungus and their hosts. Ectomycorrhizal fungi are polyphyletic, which are found in Basidiomycota, Ascomycota, and Mucoromycota (Spatafora *et al.*, 2017). *Cenococcum geophilium* is an example of ectomycorrhizal fungi present in the class, Dothideomycetes, which is known as devastating plant pathogens (Peter *et al.*, 2016).

Ectomycorrhizal fungi colonize nearly 6,000 tree species (Martin *et al.*, 2016). Moreover, tree species require ectomycorrhizal fungi for their nutrient supply (Anderson & Cairney, 2007). Most ectomycorrhizal fungi and arbutoid mycorrhiza fungi are symbionts that do not penetrate the cell wall. As explained in Bonfante & Genre (2010a) (**Figure 6**) unlike endomycorrhiza fungi, the ectomycorrhizal fungi will grow a sheath of mycelium around the root of their host which is called a mantle. The mantle will increase in diameter between the cell wall and form a Hartig net. The

Hartig net is where nutrients, like phosphate, are exchanged with the host plant for carbon (Bücking & Heyser, 2003). An ectomycorrhizal symbiont will permanently colonize their host.
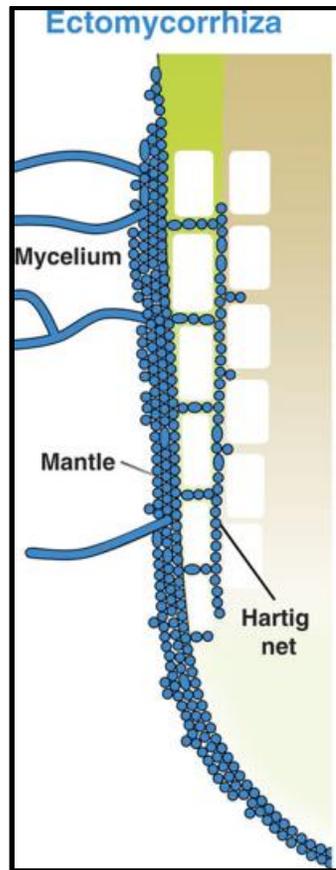
Figure 6 Ectomycorrhizal fungi will surround the root tip and create a thick hyphae structure around the root tip which is called the mantle. A Hartig net will grow around the epidermal cells where nutrient exchange between the plant and fungus will occur (Bonfante & Genre, 2010b).

There are three examples of basidiomycetes fungi-hardwood host interactions that are well-studied which are: 1) *Laccaria bicolor* communication with species of *Populus* (Vayssières *et al.*, 2015); 2) *Hebeloma cylindrosporum* with species of *Pinus* (Debaud and Gay 1987; Casieri et al. 2013); and 3) *Paxillus involutus* with both species of *Populus* and *Pinus* (Chalot *et al.*, 2000; Pestaña Nieto & Santolamazza Carbone, 2009; Jargeat *et al.*, 2014; Zhang *et al.*, 2017). These

three species of ectomycorrhizal fungi belong to Agaricomycetes in the division Basidiomycota. Moreover, their whole genomes have been sequenced (Martin *et al.*, 2008; Kohler *et al.*, 2015a; Doré *et al.*, 2015).

Similarly, to arbuscular mycorrhizal fungi, ectomycorrhizal fungi has none to few plant cell wall degrading enzymes, as shown in *L. bicolor* (Martin *et al.*, 2008) or have a reduced complement of genes as shown in *P. involtus* (Kohler *et al.*, 2015b). However, they do have the LCO assembly genes required for LCO production.

Moreover, *L. bicolor* has both sulfated and non-sulfated LCOs which cause root hair branching in *M. truncatula* and *V. sativa,* respectively. The fungal exudates induce calcium spiking in epidermal cells in lateral roots of poplar. This discovery is of great importance as it provides evidence, for the first time, that *L. bicolor* has all the genetic compounds to produce LCOs. Since both *L. bicolor* and *Rhizophagus intraradices* are not phylogenetically closed, yet both produce LCOs, it is unknown why distinctly different symbiotic fungi have the ability to produce LCOs.

1.3 Lipo-chitooligosaccharides bridge microbe-host and microbe-microbe interactions, expanding the role of this communication signal.

Communication is the key to sustainable life. There is a multitude of molecules or microorganisms that can initiate innate immunity between a microbe and their host or small diffusible signals that are used to communicate. What is unclear is how a host recognizes whether another organism is a friend or a foe. The current theory is that hosts can recognize specific signals from a microbe and then recognize this microbe as harmful or beneficial.

To address this theory, I'm investigating in this dissertation specific signal molecules that were briefly introduced in the previous sections which are LCOs. These signaling molecules are a

chitin-based oligomer with a fatty acid chain at the terminal end and can be decorated with various chemical substitutions. However, the presence or absence of a sulfate group at the non-reducing end is prominent. LCOs are produced by rhizobia and some mycorrhizal fungi. Rhizobia requires the production of LCOs to initiate colonization from their leguminous hosts. However, it remains unclear whether mycorrhizal fungi need to produce LCOs to colonize their host or not. Given that LCOs are only found in known symbiotic microbes; the signal is considered a symbiotic communication signal between host and microbe.

1.3.1 The lipochitooligosaccharides (LCOs) biological structure and functionality

Chitin is the second most abundant polysaccharide in nature, typically found in fungi, exoskeletons of arthropods, insects, and crustaceans (Tharanathan & Kittur, 2003; Knogge & Scheel, 2006). Chitin in fungi provides rigidity and structure support to thin cells of fungi (Tharanathan & Kittur, 2003). Moreover, chitin also elicits defense signaling pathways recognized by receptors in plants which trigger plant and animal immunity responses (Menezes & Jared, 2002; Knogge & Scheel, 2006; Wan *et al.*, 2008). Fatty acids are carbon chains ranging between four to 28 carbons (Das, 2006). They can be saturated, which means they do not have a carbon to carbon double bond, or unsaturated, contains more than one carbon to carbon double bond (Das, 2006; Kaur *et al.*, 2014). The most commonly found fatty acids are palmitic acid (C16:0); stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) (Srivastava & Srivastava, 2002; Das, 2006). N-acetyl glucosamine has been found in gram negative bacteria, although it is a rare occurrence. Both N-acetyl glucosamine and fatty acids have been found in gram-negative bacteria to create molecules used in signaling pathways as shown in lipopolysaccharides endotoxins produced only by pathogenic bacteria (Raetz & Whitfield, 2002)

and LCOs which are commonly referred as a "promiscuity" symbiotic signaling molecules not associated with pathogenic microbes (Perret *et al.*, 2000).

The basic structure of LCOs shown in **Figure 7** consists of the molecular joining of two structures which are: 1) chitooligosaccharides (CO), a chitin backbone of three to five residues of N-acetylglucosamine (GlcNAc), represented by (n) and 2) a fatty acid attached to the non-reducing end (Ritsema *et al.*, 1996; Debellé *et al.*, 1996), represented by R_2. The fatty acid attached after the R_2 position is usually deacetylated. Besides, various chemical decorations are found on the LCO structure, represented by R_3, R_4, R_5, R_7, R_8, R_9, and R_{10}. At the R_6 position, a chemical substitution that consists of a sulfate group or a hydrogen has been characterized. Based on the different possible combinations, there should be at least 1,000 possible combinations of unique LCOs which are usually determined by the: (i) length of the CO; (ii) type of the fatty acid; (iii) presence or absence of the sulfated group; (iv) different chemical substitutions which can vary on each structure, and lastly (v) presence, absence or both of hydrogen and methyl group at the R_1 position (Dénarié *et al.*, 1996; Maillet *et al.*, 2011) (**Figure 7**).
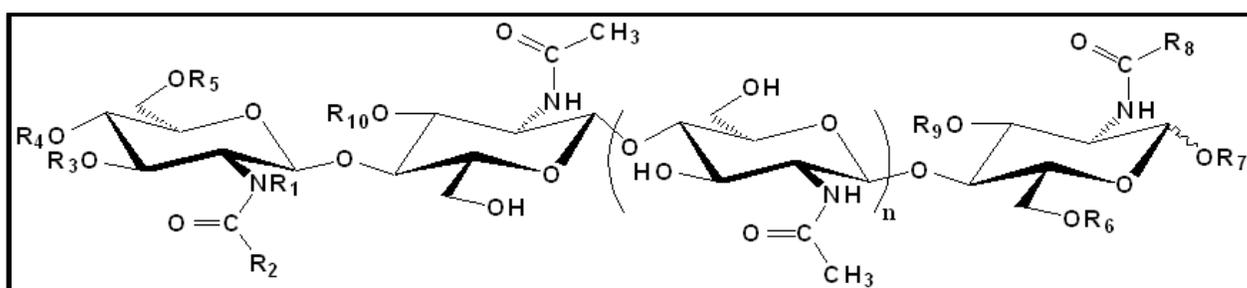
Figure 7 Diagram of the LCO structure. (n) is the number of chitin oligomers in the structure, (R_1), ($R_{3,4,5}$), and (R_6) are chemical substitutions and (R_2) is the type of lipid group. Lipid chains are identified as saturated fatty acids, palmitic acid (C16:0), stearic acid (C18:0), and arachidic acid (C20:0), and unsaturated fatty acid, oleic acid (C18:1). (Ac) is

acetylated, (Cb) is carbamylated, (Fuc) is fucosylated, (FucS) is fucosylated sulfate, (H) is hydrogen, (MeFuc) is methyl fucosylated, and (S) is sulfated.

The biological structure of chitooligosaccharides (COs)

The COs are water soluble molecules which are linked at the β 1-4 linked polymer of N-acetyl-D-glucosamine (GlcNAc). There can be a linkage of three to eight chitin monomers to form a CO. Moreover, COs with a degree of polymerization of six to eight chitin monomers are known as microbe-associated molecular patterns (MAMPs) and pathogen-associated molecule patterns (PAMPs) which will evoke a host plant immune responses (Knogge & Scheel, 2006; Eckardt, 2008; Wan *et al.*, 2008). However, most identified LCOs were found to have a fatty acid attached to a CO with three to five GlcNAc monomers. There are no reports of LCOs with a GlcNAc monomer of six to eight. It is also rare to find an LCO with three GlcNAc monomers (Poinsot *et al.*, 2016). The most abundant ones contain four or five GlcNAc monomers (Perret *et al.*, 2000; Maillet *et al.*, 2011; Poinsot *et al.*, 2016).

Types of fatty acids

Fatty acids are carboxylic acids consisting of a hydrocarbon chain and a terminal carboxyl group (Srivastava & Srivastava, 2002; Kaur *et al.*, 2014). The chitin deacetylase is required to deacetylate the acetylate group on the non-reducing end of the CO. Afterwards the acetyltransferase will attach a fatty acid group to the CO, thus creating an LCO. Oleic acid (C18:1) is the most abundant fatty acid substitute found in rhizobia (Spaink *et al.*, 1991, 1995; Carlson *et al.*, 1993; Orgambide *et al.*, 1995; Perret *et al.*, 2000; Poinsot *et al.*, 2016). Palmitic acid (C16:0) and palmitoleic acid (C16:1) have also been reported in most rhizobia (Dénarié *et al.*, 1996; Maillet *et al.*, 2011; Poinsot *et al.*, 2016).

Moreover, there have been reports about up to seven different types of fatty acid substitutes which are: hexadecadienoic acid (C16:2), stearic acid (C18:0), linoleic acid (C18:2), stearidonic acid (C18:4), arachidic acid (C20:0), and paullinic acid (C20:1) (Dénarié *et al.*, 1996; Perret *et al.*, 2000; Maillet *et al.*, 2011; Poinsot *et al.*, 2016).

Chemical substitutions

Since the first description of the LCO structure, there have been many reports characterizing the chemical decorations found. All studies involving LCOs comprised "LCOs cocktails," which are LCOs with various CO backbone lengths, fatty acid attachments, and chemical substitutions (**Figure 7**). However, these LCO cocktails are separated into two categories: sulfated and non-sulfated LCOs.

At the reducing end at the R_6 position, there is a hydrogen or a sulfate group; and this position is regulated by the *nodH* gene (Roche *et al.*, 1991; Truchet *et al.*, 1991; Freiberg *et al.*, 1997; Hanin *et al.*, 1997). Moreover, the presence of an hydrogen or a sulfate group is linked with root hair deformation on specific hosts, which is further discussed in section 1.2.1. In this regard, sulfated LCOs can elicit root hair branching, which is a lateral shank from a root hair, in *Medicago truncatula* and *M. sativa,* whereas non-sulfated LCOs elicit the same response in *Vicia sativa* (Lerouge *et al.*, 1990; Roche *et al.*, 1991; Heidstra *et al.*, 1994*).* However, there are other chemical substitutions in LCOs constituently found at the R_1, R_3, R_4, R_5, R_7, R_8, R_9, and R_{10} positions. At the R_1 location, the site of deacetylation can have hydrogen, methyl or both chemical groups. This site is under the control of the *nodS* gene that encodes for a methyl transferase responsible for the N-methylation of the acetyl group (Geelen *et al.*, 1995; Cakici *et al.*, 2010). R_3 and R_4 positions are for the addition of hydrogen or a carbamate group. The R_5 site is for the acquisition of a hydrogen or an acetylated group (Lerouge *et al.*, 1990; Carlson *et al.*, 1993; Mergaert *et al.*, 1997).

The R_6 position can commonly have an array of chemical substitutions such as a hydrogen, an acetylated-fucosylated group, a fucosylated group, a methyl fucosylated group, a N-methylated group, a N-methylated with a carbamoylated group, or a sulfated group (Jabbouri *et al.*, 1995; Dénarié *et al.*, 1996; Mergaert *et al.*, 1997; Etzler & Esko, 2009). Lastly, uncommon chemical substitutions were reported to occur at positions R_8, R_9, and R_{10}. The confirmation of the presence of LCOs and determination of the LCO structures is done with analytical assays such as high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS). The analytic assays can detect up to 10^{-10}M concentrations of LCOs. In addition to all the chemical decorations mentioned earlier, it has been hypothesized that there are LysM domains that can also bind to specific sulfated and non-sulfated LCOs (Knogge & Scheel, 2006; Mulder *et al.*, 2006; Limpens *et al.*, 2015; Rasmussen *et al.*, 2016; Gough *et al.*, 2018).

Biosynthesis of LCOs

Rhizobia can infect and trigger nodules on a legume. Moreover, rhizobia have a symbiotic relationship with leguminous hosts, which will lead to nitrogen fixation. In 1984, the genetic region that housed the genes responsible for signaling with the leguminous host was identified and called nodulation genes (*nod* genes) (Kondorosi *et al.*, 1984). These *nod* genes were only found in symbiotic rhizobia. Shortly after, Faucher *et al.*, (1988) characterized the physical and genetic map of the nodulation and nitrogen fixation genes (*nod-nif*). More importantly, the *nodABC* operon was identified and characterized. The *nodABC* operon was only found in symbiotic rhizobia and was believed to be responsible for the production of signaling molecules that will start the process of nitrogen fixation. The *nod* genes were responsible for producing Nod factors which were thought to be the symbiotic signaling molecule between rhizobia and legumes. Lerouge *et al.*, (1990) were able to isolate, purify, and characterize Nod factors through extraction methods and HPLC/MS.

The Nod factors are amphiphilic molecules of N-acetylglucosamine (GlcNAc) with a fatty acid chain on the non-reducing end and were retained in the butanol phase.

Moreover, Lerouge *et al.*, (1990) were able to demonstrate that there are different chitin backbone of three to five residues of GlcNAc which were designated as chitooligosaccharides: CO-III, CO-IV, or CO-V or lipochitooligosaccharides: LCO-III, LCO-IV, or LCO-V, and that various chemical substitutions can be found on the LCOs. In addition, Lerouge *et al.*, (1990) demonstrated that *nod* factors elicit a specific root hair deformation, which was called root hair branching (**Figure 8**).
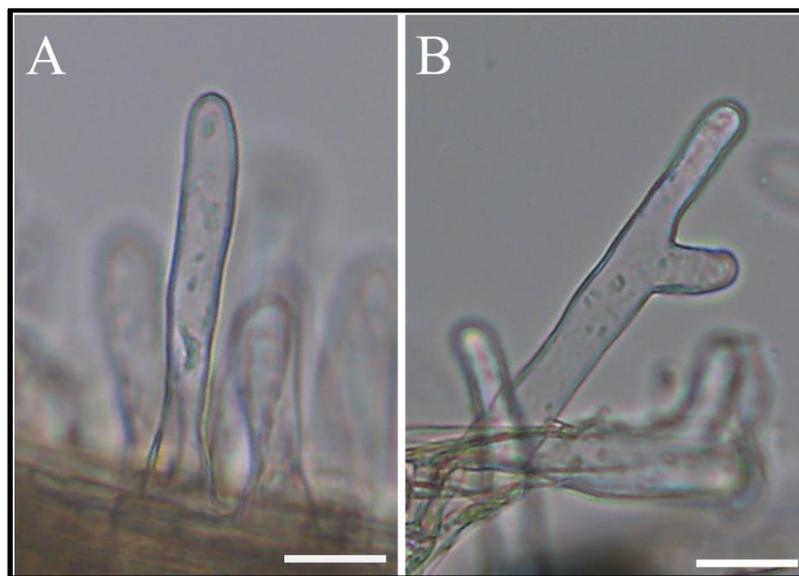
Figure 8 shows (a) a root hair without branching and (b) a root hair with branching. Scale bar is 1µm.

Since the *nodABC* operon was identified, genes responsible for *nod* factors were identified and characterized as listed in Table 1.

Table 1: Gene names and purpose for the assemble of LCOs in rhizobia

Gene Name	Name	Purpose

Nod A	Acyltransferase (N-acyltransferase)	Transfer fatty acids from an acyl carrier protein to the non-reducing terminal GlcNAc of LCOs
Nod B	Chitin deacetylase (chitin N-deacetylase)	removes the acetyl group from the non-reducing terminal GlcNAc
Nod C	Chitin synthase (N-acetylglucosaminyltransderase	Polymerize UDP-GlcNAc into chitin (usually CO-IV and CO-V)
Nod S	N-Methylation (SAM-dependent methyltransferase)	N-methylates the non-reducing terminal GlcNAc of Nod factors
Nod U	Carbamoylation (O-carbomoyltransferase)	O-carbamoyltransferase that carbamoylates C-6 of the non-reducing terminal GlcNAc of Nod factors
Nod Z	Fucosylation (α-1,6-fucosyltransferase)	Fucosylates C-6 of the reducing terminal GlcNAc of Nod factors
Noe P	Arabinosylation	Involved the D-arbinofuranosylation of C-3 of the reducing-end GlcNAc of Nod factors

Table 1 shows the names of the genes and their function for the assembly of LCOs. NodA, NodB, and NodC are responsible for the construct of the basic LCOs structure, whereas NodS, NodU, NodZ, and NoeP are responsible for the addition of chemical decorations. Table 1 is adapted from Poinsot *et al.*, (2016).

The current nomenclatures have changed for the term, *nod* factors, which are produced by *nod* genes. Nod factors are diffusible molecular signals, produced by rhizobia, and are necessary for the beginning of the nodulation process on their leguminous host. Nod factors are LCOs. However, in 2011, Malliet et al. found similar genes, in arbuscular mycorrhizal fungi, which does not produce nodules in legumes. Mycorrhizal (Myc) factors are diffusible molecules produced by arbuscular mycorrhizal fungi and are perceived by their plant hosts. Myc factors are a mixture of COs and LCOs. Recently, Myc factors found in ectomycorrhizal fungi, *Laccaria bicolor* (Garcia *et al.*, 2015; Cope *et al.,* 2019). It is unknown if the homologous genes found in arbuscular mycorrhizal fungi were functional homologous genes found in rhizobia.

Although the role of each *nod* gene was identified, the biosynthetic order to produce an LCO was unknown. *NodC* is provided in the inner membrane and catalyzes the chitin monomers

at the β1-4 glycoslidic linkage (Barny and Downie, 1993). *NodC* and *nodABC* deletion mutants were unable to produce COs or LCOs (Geremia *et al.*, 1994; Mergaert *et al.*, 1995; Poinsot *et al.*, 2016). *NodB* removes the acetyl group from the non-reducing end of the chitin oligomer, which is the site where the fatty acid is attached (Röhrig *et al.*, 1994). *NodB* deletion mutants were still able to produce COs, however LCOs were not detected (Poinsot *et al.*, 2016). *NodA* is responsible for transferring the fatty acid to the non-reducing end, thus creating an LCO (Poinsot *et al.*, 2016). Mutants lacking the *nodA* gene lost their ability to produce LCOs but were able to produce COs with the following chemical substitutions, carbamoyl, fucose, and N-methyl. Despite the alphabetical order of the nomenclature *nodABC,* the order to assemble LCOs was hypothesized to be: first, *nodC,* which made chitin backbone of four to five residues of GlcNAc; then *nodB*, which deacetylated the non-reducing end; then *nodA*, which added the fatty acid chain to the non-reducing end and finally the other *nod* genes (*nodS, nodU, nodZ,* and *noeP*), with no specific order, which will decorate the final LCO structure with chemical substitutions (**Schematics 1**). All Nod genes are under the regulation of the transcription factor, *nodD* (Peck *et al.*, 2006).
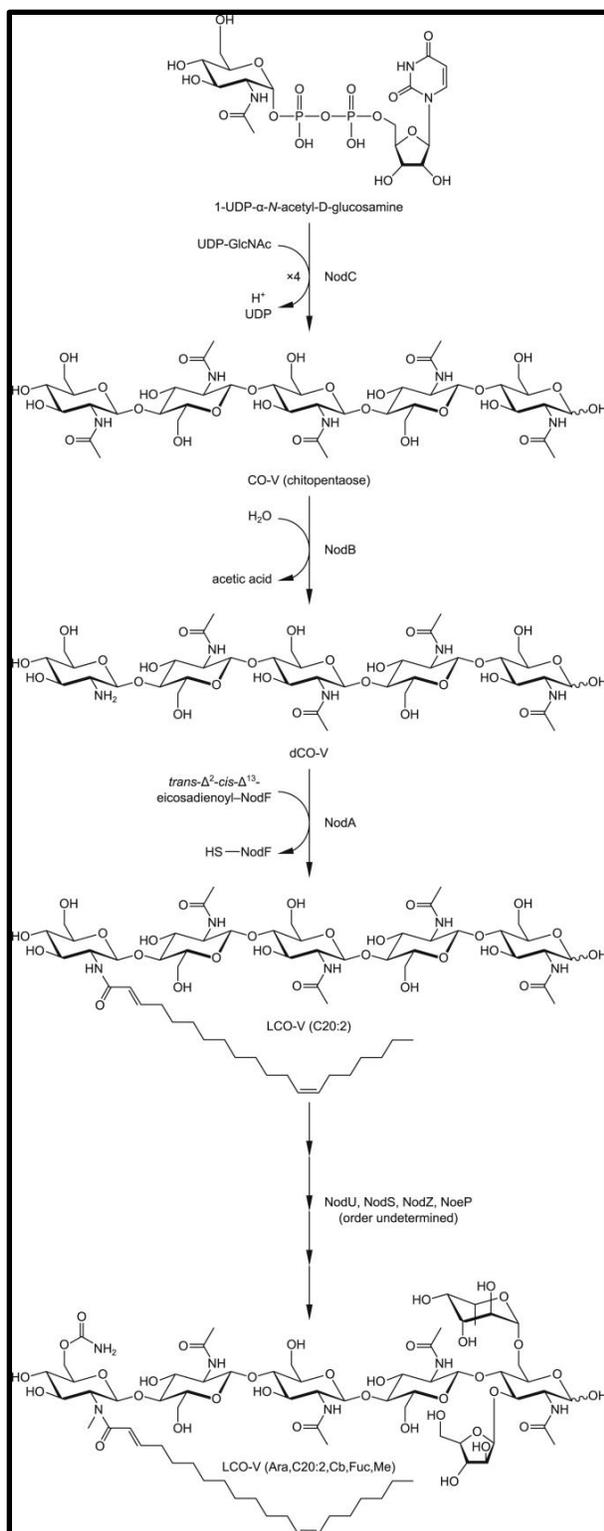
Schematic 1, which shows the previously proposed pathway to assemble LCOs in rhizobia (Poinsot *et al.*, 2016).

Recently, Poinsot *et al.*, (2016) determined the role of the other genes that attached the chemical substitutions and proposed a new order and function of the enzymes encoded by these genes. At the time they were certain unknown information that need clarification, mainly those related to the order of the genes in the biosynthetic pathway for CO and LCO assembly, and the role of the chemical substitutions. Based on forward and reverse genetics with *Rhizobium* sp. IRBG 74, the proposed schematic to assemble LCOs was the following: NodC, NodZ, NodB, NodS, NodU, NodA, and NoeP (Poinsot *et al.*, 2016). It should be noted that roughly 46% of CO-IV and CO-V was detected with fucose before the NodB step; which means that fucosylation could play a role in determining the number of chitin monomers present in COs or LCOs (Poinsot *et al.*, 2016). Other than fucosylation, mono-deacetylation (Nod S) is the sole primary driver for the addition of

chitin monomers to the chitin backbones of COs and LCOs. This indicates that the chemical decoration plays a larger role in the biosynthesis of LCOs in rhizobia. The latest proposed biosynthetic process of assembling LCOs is shown in **Schematic 2**.
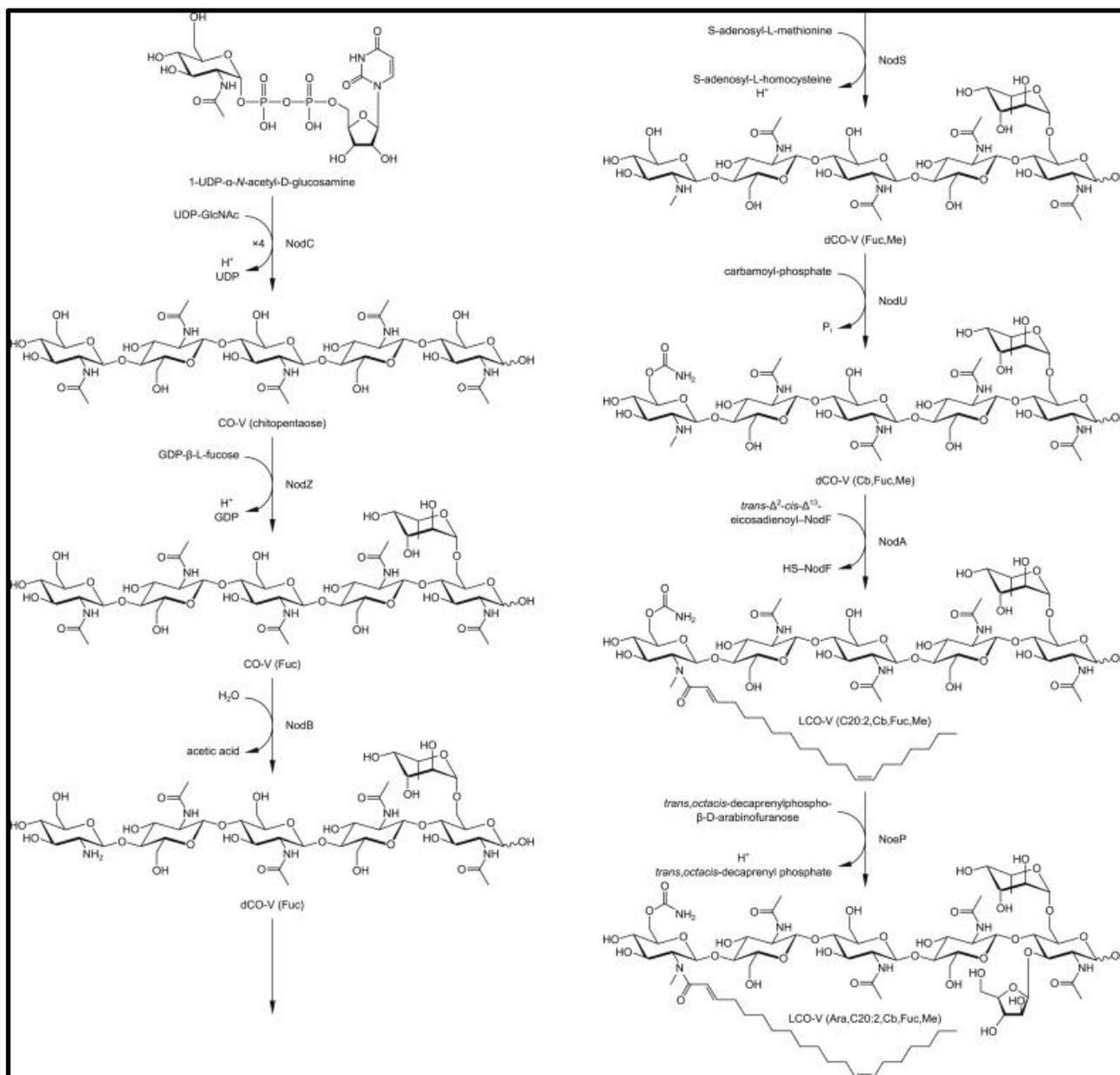
Schematic 2 shows the LCO biosynthetic pathway proposed in *Rhizobium* sp. IRBG74.

Maillet *et al.*, (2011) determined that *Rhizophagus intraradices* produces LCOs as shown through root hair branching assays, *pENOD11:GUS* assays, and confirmation by HPLC analysis.

They also learn the structure of the LCOs through mass spectrometry. Moreover, Cope *et al.,* (2019) have shown that the ectomycorrhizal fungus, *Laccaria bicolor*, also produces LCOs. Two vastly genetically different fungi, arbuscular mycorrhizal fungi, and ectomycorrhizal fungi (*L. bicolor*) which are respectively in Mucoromycota and Basidiomycota phyla share the same symbiotic signal as rhizobia. What is the link between these symbiotic microbes producing the same signal? Garcia *et al.*, (2015) investigated the commonalities between arbuscular mycorrhizal fungi and ectomycorrhizal fungi. They examined the symbiosis behavior, conserved genes, and symbiosis specific genes found in the common symbiosis pathway between the hosts of these two group of fungi. They determined that angiosperms, *Populus trichocarpa, Malus domestica, Prunus persica,* and *Eucalyptus grandii* all shared the same symbiosis behavior from arbuscular mycorrhizal fungi and ectomycorrhizal fungi; the same conserved genes (CCD7 and CCD8); and the same symbiosis specific genes (Garcia *et al.*, 2015).

Moreover, arbuscular mycorrhizal fungi and rhizobia both produce COs and LCOs and trigger the common symbiosis pathway in *Medicago truncatula*. Cope *et al.,* (2019) showed that *Laccaria bicolor* also produced COs and LCOs, which triggered the common symbiosis pathway in *Populus trichocarpa*. This raises the question: Are the same *nod* genes that are found in rhizobia are responsible for LCOs production in fungi?

Several arbuscular mycorrhizal fungi have their genome sequences such as: *Gigaspora rosea*, *Rhizophagus irregularis, R. diaphus*, and *R. cerebriforme* to name a few (Chen *et al.*, 2018; Morin *et al.*, 2019). These fungi contain putative *nod A/B/C* -like genes (Chen *et al.*, 2018; Morin *et al.*, 2019). However, to conduct knockout studies in arbuscular mycorrhizal fungi are notoriously difficult because they are obligate symbionts, which means it is difficult to get protoplasts because they will die without a host (Forbes *et al.*, 1998). Although particle

bombardment has been used to transform arbuscular mycorrhizal fungi (Forbes *et al.*, 1998). Several ectomycorrhizal fungi have their genome sequenced such as: *Laccaria bicolor, Hebeloma cylindrosporum, Paxillus involutus,* and *Tuber melanosporum* (Martin *et al.*, 2008; Kohler *et al.*, 2015a). *Laccaria bicolor* and *Tuber melanosporum* has the putative *nod A/B/C* -like genes (Garcia *et al.*, 2015). However, they determined that these two ectomycorrhizal fungi several copies of *nod* genes (over ten copies each) present in the genome. Without knowing the role of these genes, or if they are clustered together in the genome, it is challenging to make transformants of these ectomycorrhizal fungi. Recently, the previously accepted hypothesis that LCOs are only produced in symbiotic microbes was proven wrong by Rush *et al.,* (unpublished - Chapter 2). 56 fungi of different lifestyle were able to produce LCOs, including the fungal genetic model, *Aspergillus fumigatus* Rush *et al.,* (unpublished - Chapter 2).

 1.3.2 The recognition of LCOs, activation of the common symbiosis pathway and root hair deformations.

Recognition between symbiotic microbes and host partners

 Plant receptor-like kinases (RLK) genes, found in plant roots, are involved in symbiosis with rhizobia and mycorrhizal fungi. LysM-receptor-like kinases (part of the RLKs) are required for successful nodulation in legumes. It is unknown if LysM-receptor-like kinases are necessary for mycorrhization. The first LysM-type receptors were described by Radutoiu *et al.*, (2003) and Madsen *et al.*, (2011), which were *NFR1* and *NFR5* found in *Lotus japonicus.* These receptors were able to recognize LCOs from the bacterium, *Mesorhizobium loti.* Furthermore, the authors concluded that *NFR1* and *NFR5* are required for physiological and cellular responses. Thereafter, other LysM domain receptors kinases, *LYK3* and *NFP* were identified in *Medicago* (Mulder *et al.*, 2006; Smit *et al.*, 2007). *NFP* and *NFR5* are orthologous genes. *LYK3* and *NFR1* are orthologous

genes with different phenotypes. These receptors are entry receptors that perceives LCOs, which initiates a cascade of signal transduction. The order of signal transductions in *Medicago truncatula* are: 1) nodulation receptor-like kinases (NORK) or doesn't make infections (DMI2), 2) cation channels *DMI1*, 3) nucleoporins NUP85/NUP133/NENA, 4) *DMI3* or calcium and calmodulin-dependent kinases (CCaMK), and lastly 5) interacting partner of the calcium/calmodulin-dependent protein (IPD3) (Ané *et al.*, 2002; Ane, 2004; Lévy *et al.*, 2004; Mitra *et al.*, 2004; Imaizumi-Anraku *et al.*, 2005; Kanamori *et al.*, 2006; Zhu *et al.*, 2006; RIELY *et al.*, 2006; Riely *et al.*, 2007; Messinese *et al.*, 2007; Smit *et al.*, 2007; Kevei *et al.*, 2007; Chen *et al.*, 2008; Groth *et al.*, 2010; Horváth *et al.*, 2011; Venkateshwaran *et al.*, 2013a; Singh *et al.*, 2014; Genre & Russo, 2016b).

Our current understanding of the mechanisms involved in the common symbiosis pathway is limited. Previously, LysM-like receptors kinases were found in *Arabidopsis thaliana* (Cao *et al.*, 2014). The *LYK5* found in the leaves of *Arabidopsis* is a receptor closely related to *LYK3* and was shown to be the primary chitin receptor in *Arabidopsis* (Cao *et al.*, 2014). Moreover, it is unknown if LysM-like receptor kinases can be non-specific binding targets for LCOs (Dworkin, 2018). Therefore, when these common symbiosis pathway signal transductors encounter LCOs, early stage read outs will activate gene regulation that leads to root hair deformation phenotypes and activation of the Early Nodulation 11 gene (*ENOD11*) (Genre & Russo, 2016a). Later stages lead to symbiont accommodation.

Host responses to exogenous COs and LCOs

Root hair deformations

Root hairs are tip growing extensions of the epidermal cell that grow straight (Ryan *et al.*, 2001; Wais *et al.*, 2002). When legume root hairs encounter LCOs from compatible rhizobia,

deformations occurs (Wood & Newcomb, 1989; Lerouge *et al.*, 1990; Heidstra *et al.*, 1994). There are two types of well-characterized root hair deformations which are: 1) root hair curling and 2) root hair branching (Lerouge *et al.*, 1990; Roche *et al.*, 1991; Heidstra *et al.*, 1994; Esseling *et al.*, 2003; Patriarca *et al.*, 2004; Maillet *et al.*, 2011). As shown in **Figure 9**, there are other types of root hair deformations such as intertwined, adjoined, spatulated (sometimes called "bulbed"), deformed and waves (Patriarca *et al.*, 2004).

Moreover, intertwined, adjoined, spatulated, deformed and wavy root hair deformations are the beginning stages of a plant response in the presence of LCOs. When a root hair perceives LCOs, the root will stop growing and root deformations will occur. However, to elicit root hair branching response, a lateral shank is formed from the root hair. Thereafter, the root hair will continue to grow in an upward direction. Plant parasitic root knot nematode can elicit a wavy root deformation yet root hair curling or root hair branching were not observed in any of the treatments (Weerasinghe *et al.*, 2005).
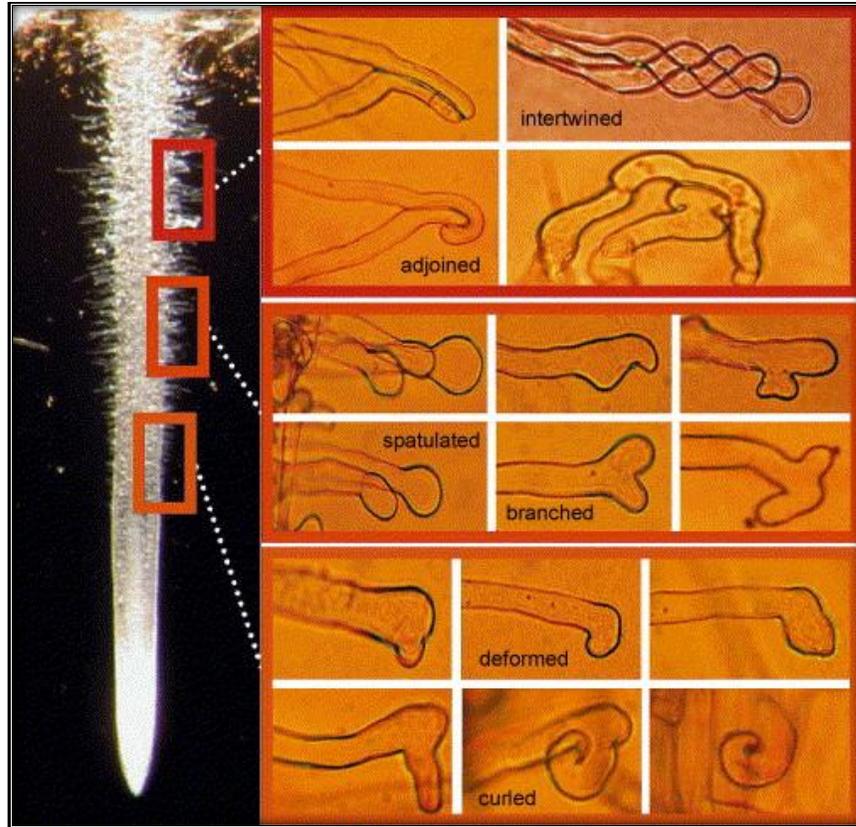
Figure 9 show root hair deformations on *Phaseolus vulgaris* (common bean) inoculated with *Rhizobium etli* (Patriarca *et al.*, 2004).

Root hair branching assays are a sensitive and specific way to determine if LCOs are present. COs do not trigger root hair branching (Cope *et al.* 2019). Sulfated-LCOs will elicit a root hair branching response in *M. truncatula* whereas the same phenotype is obtained in *V. sativa* and *L. japonicus* caused by the detection of non-sulfated LCOs. The root hair branching is the most sensitive assay to detect LCOs as both leguminous hosts will show root hair branching phenotypes with the addition of 10^{-13} M concentrations of purified LCOs (Lerouge *et al.*, 1990; Spaink *et al.*, 1991; Heidstra *et al.*, 1994; Geurts & Bisseling, 2002; this study).

Nuclear Calcium Spiking

Nuclear calcium spiking is a signal transduction event characterized by oscillations in the concentration of ionic calcium in the nucleoplasm and perinuclear region (Meyer & Stryer 1991; Charpentier *et al.* 2008; Capoen *et al.* 2011; Dupont *et al.* 2011; Cope *et al.,* 2019). Nuclear calcium spiking is perceived by a calcium and calmodulin-dependent protein kinase (CCaMK), located in the nucleoplasm and is released from the inter-nuclear-membrane space and endoplasmic reticulum lumen (Capoen *et al.*, 2011). Then *CASTOR* and *POLLUX,* originally described as potassium channels, and *DMI1* are the calcium gated calcium channels and will allow calcium influx to flow through the nuclear membrane (Ané *et al.*, 2004; Charpentier *et al.*, 2008). Once this influx of calcium occurs MCA8, a calcium ATPase will provide a downstroke of the oscillation, returning the calcium to the inter-nuclear-membrane space of the endoplasmic reticulum lumen (Capoen *et al.*, 2011). The activation of the common symbiosis pathway, as observed through calcium spiking, has been induced by COs and LCOs in *Medicago truncatula, Lotus japonicus, Oryza sativa* (rice), and *Populus* (Sun *et al.*, 2015; Cope *et al.,* 2019). Calcium spiking can detect CO4 or non-sulfated LCOs up to nanomolar concentrations and sulfated LCOs to picomolar concentrations (Kosuta *et al.*, 2008; Chabaud *et al.*, 2011; Genre *et al.*, 2013b; Sun *et al.,* 2015). Altogether, calcium spiking is an indicator that the common symbiotic pathway perceives COs and LCOs in various host plants at different molar concentrations.

pENOD11: GUS staining

Another biological assay for the detection of LCOs is the promoter early nodulation 11: β-glucuronidase reporter gene staining (*pENOD11: GUS*) (Journet *et al.*, 2001). ENOD11 is an early activator gene in the rhizobial and arbuscular mycorrhizal legume symbiosis transcriptionally up-regulated when LCOs are applied to roots. *M. truncatula* A17 *pENOD11: GUS* is a genetically

modified organism originated from the *M. truncatula* Jemalong A17 line (Journet *et al.*, 2001; Marsh *et al.*, 2007; Horváth *et al.*, 2011).

The *M. truncatula* Jemalong A17 line has a transgene that is the promoter of *ENOD11* fused to the *GUS* coding sequence (Journet *et al.*, 2001; Marsh *et al.*, 2007; Horváth *et al.*, 2011). Therefore, whenever LCOs are applied to these genetically modified roots, the promoter is activated and the β-glucuronidase enzyme is expressed indicating the presence of LCOs (Marsh *et al.*, 2007; Svistoonoff *et al.*, 2010; Horváth *et al.*, 2011; Maillet *et al.*, 2011). COs activates *pENOD11:GUS* in *M. truncatula* at a concentration of 10^{-7} M (Sun *et al.,* 2015) The *pENOD11: GUS* assays have been thoroughly applied as another biological assay to confirm root hair branching assays and can detect LCOs at picomolar concentrations (Journet *et al.*, 2001; Maillet *et al.*, 2011).

1.4 Justification and Goals

As discussed in previous sections, LCOs are signal molecules that will activate the common symbiosis pathway and elicit root hair deformations. These molecules were reported to be merely produced by symbiotic microbes such as 1) rhizobia, 2) arbuscular mycorrhizal fungi and 3) ectomycorrhizal fungi. Rhizobia require the production of LCOs to colonize their host and start nodulation. The role of LCOs for colonization in mycorrhizal fungi is unclear.

The discovery of LCOs from two vastly distant fungal relatives, arbuscular mycorrhizal fungi and ectomycorrhizal fungi, call into question the extent of LCOs' production in fungi. The questions that arise from this discovery are: 1) Is LCO production restricted to symbiotic microbes? 2) What are the genes responsible for LCO production in fungi? 3) Does LCOs influence fungal growth and development?

The goal of my Ph.D. was to find answers to these questions to fill some critical knowledge gaps. Therefore, Chapter 2 of my Ph.D. dissertation will be devoted to determining the extent of LCOs production in the Fungal Kingdom and to establish the impacts that LCOs have on growth and development. Chapter 3 will be dedicated to the identification of genes responsible to produce LCOs in fungi and to the characterization of LCOs as quorum sensing molecules. Lastly, Chapter 4 will discuss how findings from this Ph.D. study impact the current knowledge on the common symbiosis pathway and how they influence future directions of the research on LCOs. The previous research on LCOs were restricted to plant symbiosis. Now, LCOs could have an impact on fungal pathogens that infect animals, insects, plants and other microbes. In addition, LCOs can be used for identifying new therapeutic medicines by controlling the behavior of certain pathogens. Lastly, if LCOs are a major communication signal for all pathogens, then inhibiting the receptor that perceives it could be the next step in resolving pathogenicity in agricultural crops and humans.

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Chapter 2: Lipo-chitooligosaccharides are a wide-spread fungal quorum sensing molecule that regulates fungal growth and development.

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ONE SENTENCE SUMMARY

Signals thought to be produced exclusively by plant symbionts were found in most fungi, and control fungal growth and development.

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2.1 Abstract

Lipo-chitooligosaccharides (LCOs) are short chitin oligomers substituted by an acyl chain at the non-reducing end. Such molecules were first discovered as the primary signaling molecules produced by rhizobial bacteria and perceived by their legume hosts. Recently, LCOs were identified in arbuscular and ecto-mycorrhizal fungi that associate with plants. Here we tested if other fungi can produce LCOs. Using biological assays and mass spectrometry, we were able to detect LCOs in 55 fungi across the five phyla in the fungal kingdom. Notably, we did not detect LCOs in the yeasts *Saccharomyces cerevisiae* and *Candida glabrata*. Treatment of *Aspergillus fumigatus*, and *Candida glabrata* with LCOs affected fungal growth and development in a dose-dependent manner, indicating that LCOs are a common signal between fungal species.

2.2 Introduction

Few symbiotic associations between bacteria and their eukaryotic hosts have been studied as the nitrogen-fixing symbiosis between rhizobia and legumes that leads to the development of root nodules. Rhizobia often exhibit a high level of host specificity and the development of genetic tools in rhizobia led to the identification of lipo-chitooligosaccharides (LCOs) (*1*). These molecules, commonly known as Nod factors, are the primary signals produced by rhizobia to be recognized by their legume host and initiate the symbiosis. LCOs are short chitin chains, polymers of three to five β-1,4-linked N-acetyl-D-glucosamine residues, substituted at the non-reducing end by an acyl group (*2*). They are required in most rhizobia for colonization (infection) of the legume host and nodule development. The type of lipid chain and the presence of various substitutions on the chitin backbone are the primary determinants of host specificity for rhizobia. These diffusible and amphiphilic signals are recognized by LysM-receptor-like kinases in legumes at nanomolar to picomolar concentrations, and they elicit responses such as root hair branching, calcium spiking and the expression of genes such as *Early Nodulin 11* (*ENOD11*) (*3*). Genetic studies in model legumes such as *Medicago truncatula* (Medicago) and *Lotus japonicus* showed that many mutants in the early Nod factor signaling pathway are not only affected in the rhizobium-legume symbiosis but also associations with arbuscular mycorrhizal fungi indicating commonalities in the molecular mechanisms of the two symbioses and the existence of a "common symbiosis pathway"(*3*). Recently, LCOs as well as short chitin oligomers (COs) were described in the exudates of an ectomycorrhizal fungus, *Laccaria bicolor*, and were shown to trigger symbiotic responses in a CSP-dependent manner (*4–6*), thus, raising questions about the production of LCOs by other fungi.

2.3 Results

We tested the presence of LCOs in 61 different fungi covering all the phyla and almost all the subphyla of the fungal kingdom (**Fig. 1**).
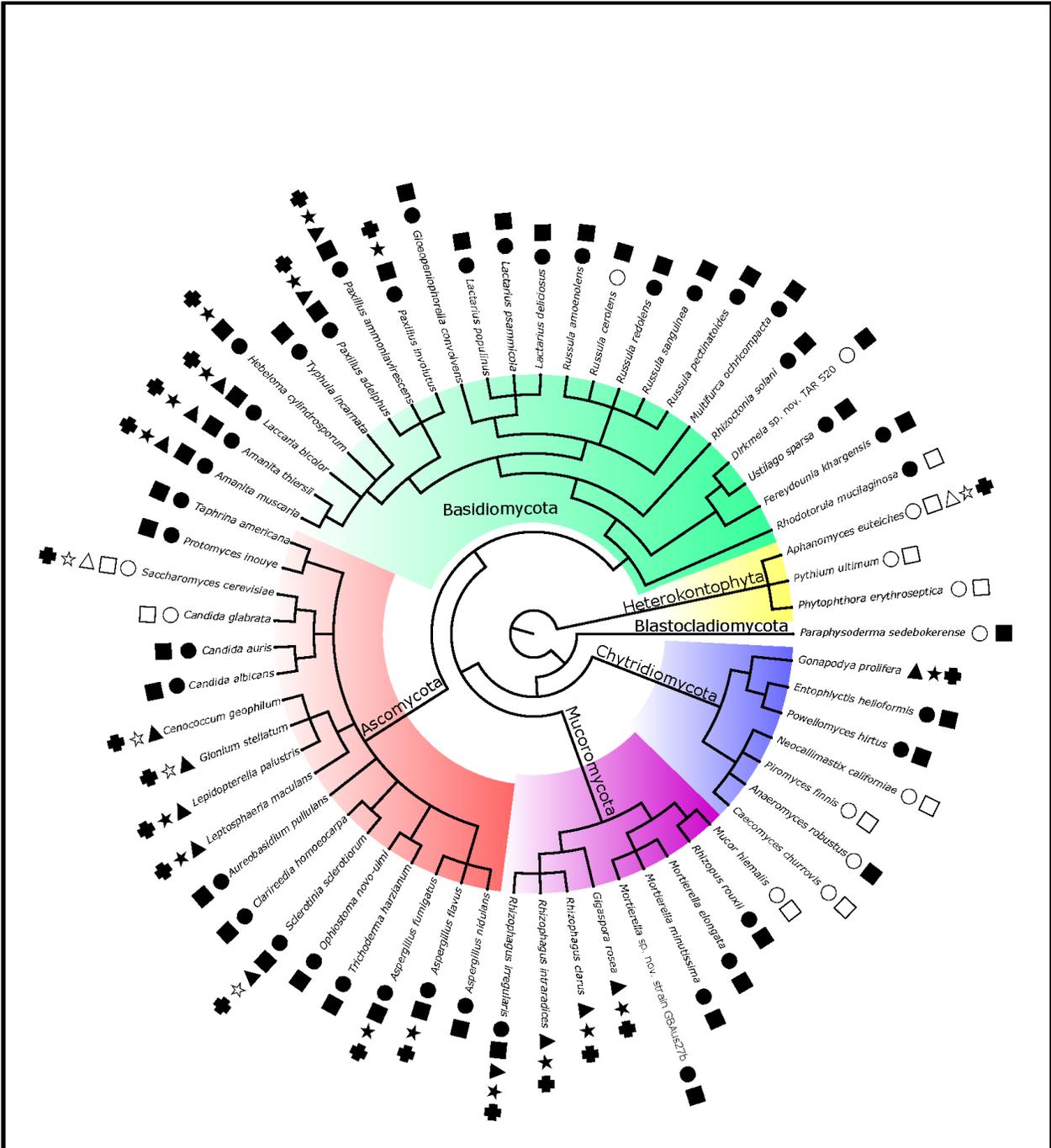
Figure 1: Widespread production of chitooligosaccharides (COs) and lipo-chitooligosaccharides (LCOs) throughout the Fungal Kingdom. 61 fungi from six of the eight fungal phyla and 3 oomycetes were tested for the presence of COs and LCOs in their culture supernatants. (●) Detection of (s)LCOs-using the root hair branching assay with *M. truncatula*. (■) Detection of (ns)LCOs using the root hair branching assay with vetch. (▲) Detection of (s)LCOs using *M. truncatula ENOD11* expression assay with butanol extracts. (★) Detection of LCOs by HPLC/MS from butanol extracts. (✚) Detection of COs by HPLC/MS from water extracts. Clear symbols indicate no detection.

To screen many fungal exudates, we took advantage of root hair deformations triggered by LCOs in Medicago and vetch (*Vicia sativa*) (*1*). However, some of these root hair deformations such as waving, or bulbs are not specific to LCOs. Therefore, we only used root hair branching with a distinct branch coming out of the shank as our bioassay (**Supplementary Fig. 1-5**). As expected, Medicago responded only to sulfated LCOs, whereas vetch responded to non-sulfated ones. LCOs can be detected down to picomolar concentrations using these assays. None of the short or long COs tested or the media used to grow these fungi triggered root hair branching confirming the specificity of this bioassay (**Supplemental Fig. 6-7**). Besides, we confirmed that our fungal samples were not contaminated through microscopy and amplifying the DNA with fungal-specific and bacterial-specific primers (**Supplemental Fig. 8**). We observed root hair branching in Medicago or vetch in 55 fungi out of 61, with 51 of them triggering branching in both legumes which suggested the presence of sulfated and non-sulfated LCOs in most fungi across the fungal kingdom, which represent different ecological lifestyles (**Fig. 1**). We confirmed the presence of sulfated LCOs in some fungal exudates using the ability of sulfated LCOs to trigger *ENOD11* expression in Medicago (**Fig. 1**). We did not observe root hair branching or *ENOD11*

expression with exudates from several *Saccharomyces cerevisiae* isolates (**Fig. 1** and **Supplemental Fig. 2**). We investigated further in the Saccharomycotina clade and tested *Candida albicans, C. auris,* and *C. glabrata,* which are often found infecting immunocompromised patients, yet they are genetically and phenotypically different (*7, 8*). We detected LCOs in *Candida albicans* and *C. auris* that can have both yeast and filamentous forms but not in exudates from *Candida glabrata* which is related to *S. cerevisiae* and is reported to have only a yeast form (**Supplemental Fig. 2**) (*7*).

We used mass spectrometry to confirm the bioassay results and to determine the structure of LCOs produced by these fungi. The culture media were fractionated with a butanol/water phase separation. The water phases were analyzed directly for the presence of COs, and we detected the presence of short COs exuded in the culture medium of all the fungi tested (**Fig. 1, 2**). The butanol phases in which LCOs were expected to be solubilized, were directly analyzed or were further purified by chromatography on different columns to minimize matrix effect. Using Multiple Reaction Monitoring, we found at expected retention times, mass signals in the culture media of four fungal phyla. Fungal LCOs identified were highly decorated with sulfate, methyl, carbamoyl, fucose and methylfucose residues (**Fig. 2**).

Given that oomycetes like fungi can produce COs (*9*), we also tested the presence of LCOs in exudates of three oomycetes (*Aphanomyces euteiches*, *Pythium ultimum*, and *Phytophthora erythroseptica*) using root hair branching assay and mass spectrometry (**Fig.1, Supplemental Fig. 5**). We confirmed the presence of short COs by mass spectrometry but did not detect LCOs in our two assays suggesting that the ability to produce LCOs may be restricted to the fungal kingdom and rhizobia (**Fig. 1, 2**).
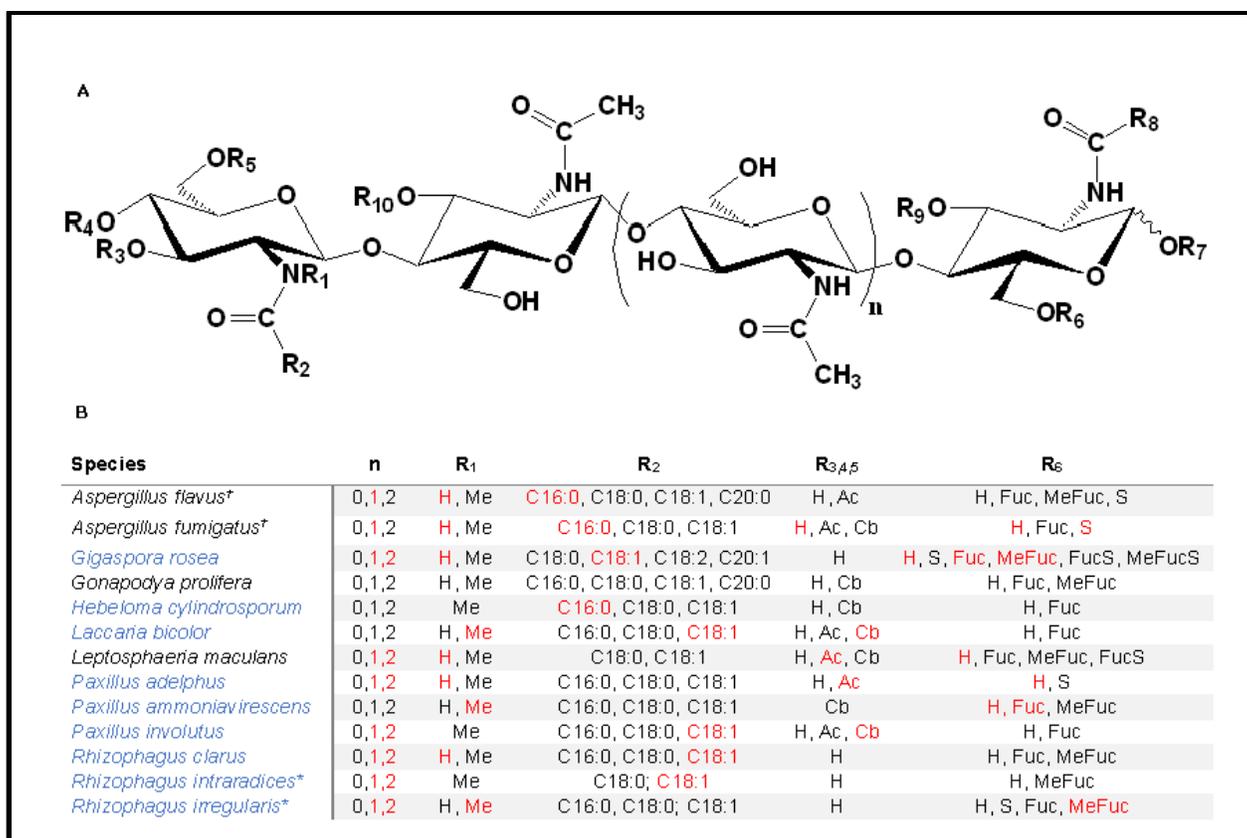
Figure 2: Structures of LCOs in symbiotic and non-symbiotic fungi. (**A**) Structure of lipo-chitooligosaccharides molecule. (**B**) Symbiotic fungi (shown in blue) and non-symbiotic fungi (shown in black) were examined for the abundance (shown in red) and various LCOs structures found in butanol extraction phase through MS/HPLC analysis. (*) indicates more than one strain was considered and (†) indicates samples were tested at 25°C or 37°C after 24 hours growth or 96 growth in liquid broth or water. (n) is the number of chitin oligomers in the structure, (R_1), ($R_{3,4,5}$), and (R_6) are chemical substitutions and (R_2) is the type of lipid group. Lipid chains are identified as saturated fatty acids, palmitic acid (C16:0), stearic acid (C18:0), and arachidic acid (C20:0), and unsaturated fatty acid, oleic acid (C18:1). (Ac) is acetylated, (Cb) is carbamylated, (Fuc) is fucosylated, (FucS) is fucosylated sulfate, (H) is hydrogen, (MeFuc) is methyl fucosylated, and (S) is sulfated.

The fact that LCOs were found in fungi with various lifestyles and, in particular, fungi that are associated with animals and not plants suggested that LCOs could play a role in fungal biology independent of their role in plant symbioses. To explore this hypothesis, we applied chemically synthesized sulfated or non-sulfated LCOs with different chains of lipids (C16:0 or C18:1), short (CO4 and CO5), and long (CO8) COs to *Aspergillus fumigatus.* A 41% decrease in the number of secondary branches per micrometer of apical branch was observed after treatment with 10^{-8} M sulfated LCOs with a C16:0 chain compared to other treatments (**Fig. 3B**). There was no difference in the length of apical branches with any of the treatments (**Supplemental Fig. 9**). The "hypo-branching" effect of sulfated LCOs with C16:0 was dose dependent (**Fig. 3C**). C16:0 sulfated LCO and CO4 also increased the germination of spores of *A. fumigatus* (**Supplemental Figures 10**). The application of C16:0 sulfated LCO regulated the expression of several genes compared to the control even after 30 or 120 minutes (**Fig. 3, D and E, Supplemental Fig. 11**).
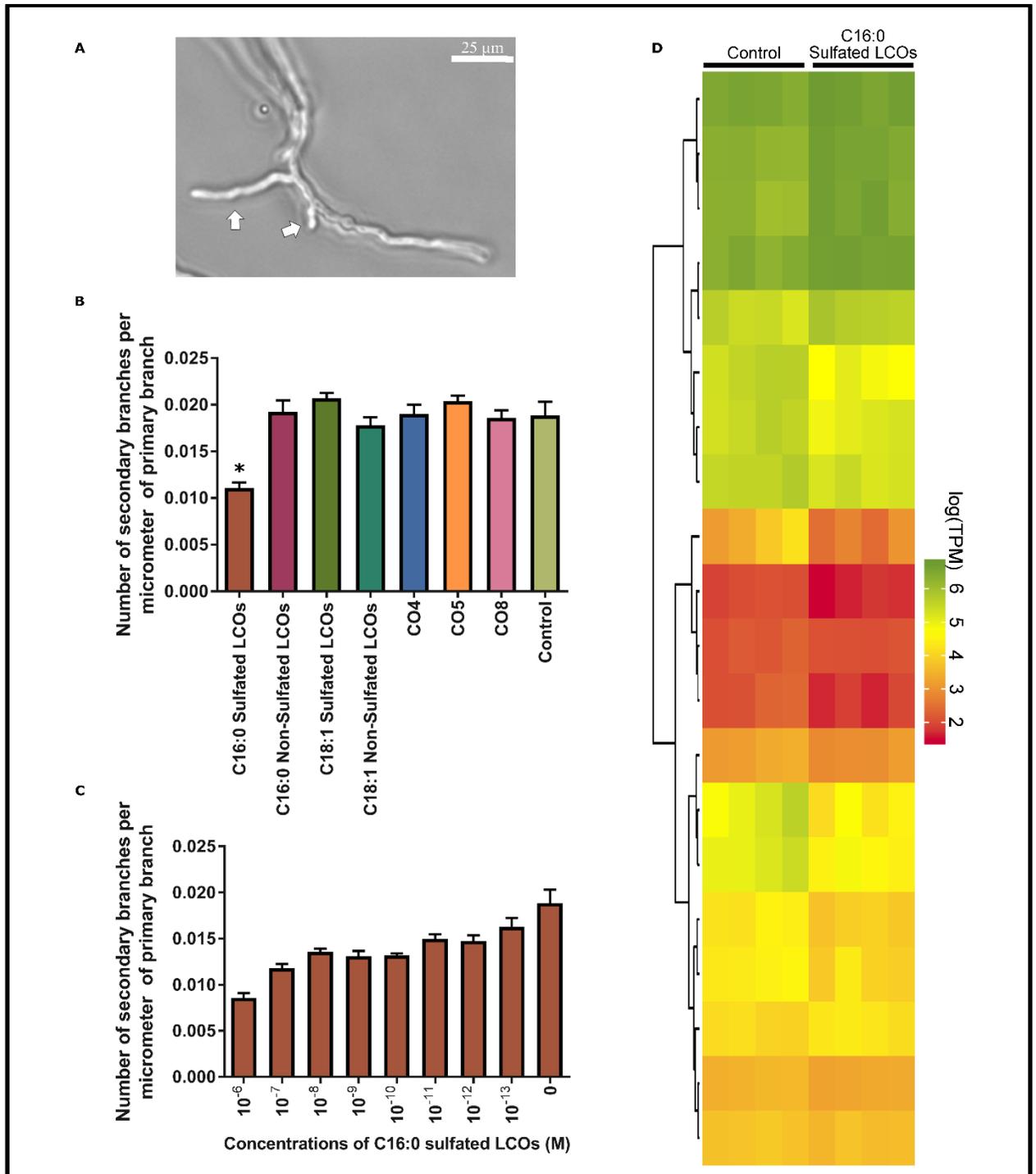
Figure 3: LCOs influence the growth, development, and gene expression in *Aspergillus fumigatus*. **(A)** Regular branching are germinated spores observed in control with an apical branch that has two to three secondary branches. White arrows indicate secondary branches. Scale bar is

25 µm **(B)** Ratio of secondary branches from the apical branch in *A. fumigatus* Af293 in response to various LCOs and COs at 10^{-8} M. * indicates significant difference from the control with a P-value <0.001 **(C)** The ratio of secondary branches from the apical branch is dependent on the concentrations of sulfated C16:0 LCOs. **(D)** Transcriptomic analyses revealed gene expression changes at 30 minutes following treatment with LCOs. Heatmaps show the top 20 differentially expressed genes, with the highest expression levels indicated in red and the lowest in green.

We also tested the effect of LCOs on the growth of *C. glabrata*, a yeast in which we did not detect LCOs using our bioassays. Application of C16:0 sulfated LCOs at 10^{-8} M led to a significant increase in the number of pseudo-hyphae observed (**Fig. 4B; Supplemental Videos 1, 2, and 3**) and this effect was dose-dependent too (**Fig. 4C**). To test if fungi outside the Ascomycota can respond to LCOs, we used *Rhodotorula mucilaginosa*, a basidiomycete causing allergies in humans. C16:0 sulfated LCOs at 10^{-8} M led to a significant growth increase of *R. mucilaginosa* compared to the control (**Supplemental Fig. 12**).
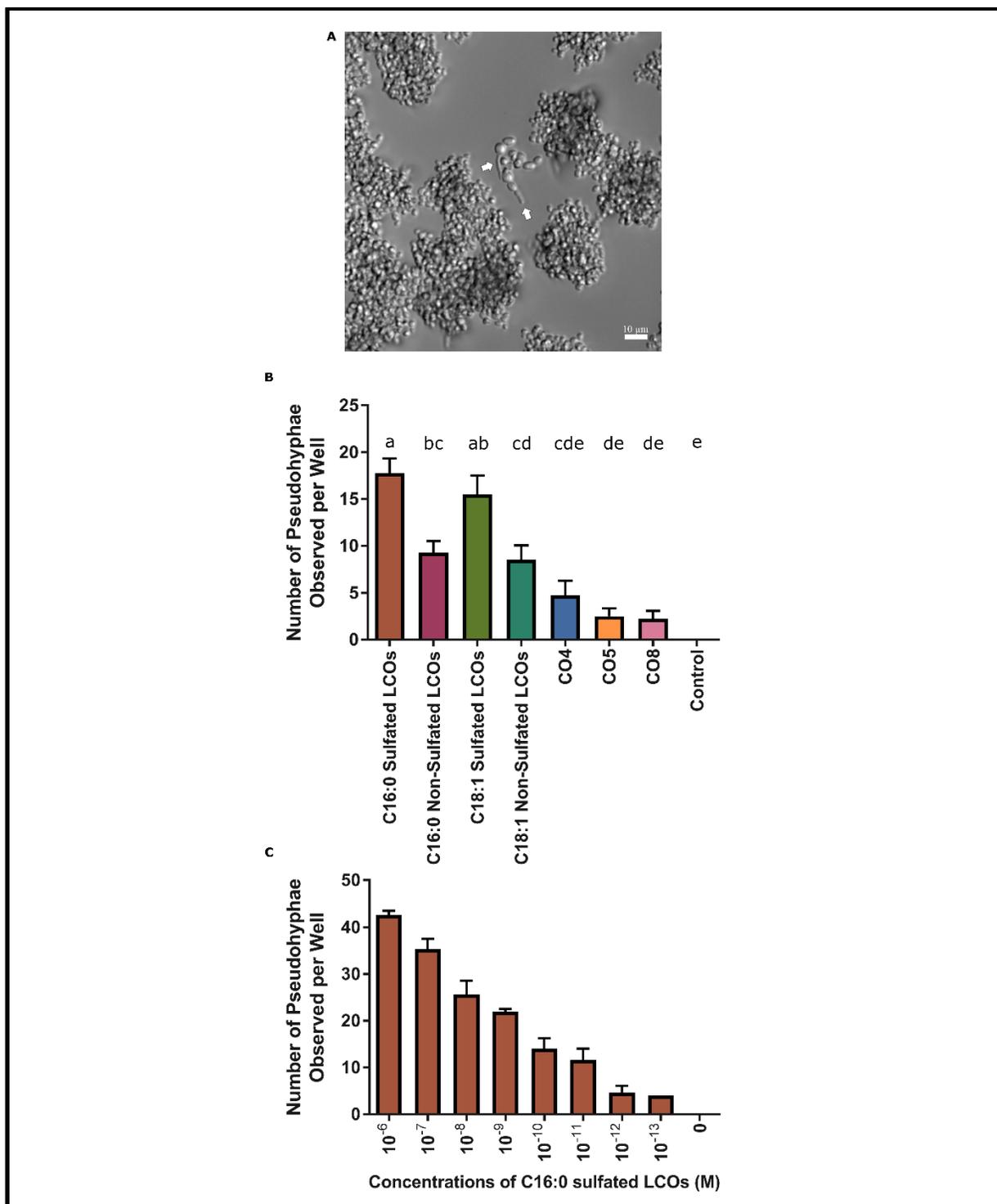
Figure 4: LCOs induce the formation of pseudohyphae in *Candida glabrata* under standard environmental conditions. **(A)** White arrows show pseudohyphae of *C. glabrata* (see **Supplemental Videos 1,2, and 3**). Scale bar is 10 µm. **(B)** Number of pseudohyphae observed

per well in response to various LCOs and COs at 10^{-8} M. ANOVA was significant (P-value <0.01). Tukey's single-step multiple comparison procedures were conducted where different letter groupings indicate significant differences. **(C)** Pseudohyphae formation was dependent on the concentrations of sulfated C16:0 LCOs.

To test if fungi outside the Ascomycota can respond to LCOs, we used *Rhodotorula mucilaginosa*, a basidiomycete causing allergies in humans. C16:0 sulfated LCOs at 10^{-8} M led to a significant growth increase of *R. mucilaginosa* compared to the control (**Supplemental Fig. 12**).

2.4 Discussion

For over twenty years, LCOs have been studied mostly in the context of the rhizobium-legume symbiosis. Interest in these molecules widened when arbuscular and ecto-mycorrhizal fungi were found to produce similar molecules (*4*, *6*). The results presented here represent a paradigm shift by revealing that fungi with no interaction with plants produce LCOs whose structures are very close to Nod factors. This discovery raises questions on how legumes can distinguish rhizobia from fungi in the soil or, how land plants can distinguish mycorrhizal fungi from all the other fungi producing LCOs. It seems likely that signals other than LCOs must provide this specificity. It is also tempting to speculate that the ability of plants to recognize LCOs emerged to recognize fungi by eavesdropping on a widespread fungal signal. Plant roots have LysM receptor-like kinases that perceive LCOs and activate the "common symbiosis pathway" that regulates plant defenses and allows endosymbiosis with rhizobia or arbuscular mycorrhizal fungi (*10*). It is currently unknown if LCOs from other fungi can be recognized the same type of LysM receptor and pathway. Application of LCOs to leaves of several plants also leads to a suppression of microbe-associated molecular pattern (MAMP)–triggered immunity (*11*) and increase fungal disease (*12*). Considering that many fungi produce LCOs, it is tempting to speculate that plants

may decrease their defenses to accommodate leaf fungal endophytes. C16:0 sulfated LCOs were detected through HPLC/MS in 84% of fungi tested and had the most substantial impact on fungal growth and development. Many questions remain such as how and where LCOs are synthesized in fungi. It will be interesting to determine if they are produced intracellularly as in rhizobia or form the degradation and modification of longer chitin molecules on the fugal wall. We were able to detect LCOs in culture using a sensitive bioassay, but it is unknown if specific environmental signals can effect LCO production by fungi. Given that the LCO structures produced by different fungi seem similar, it is tempting to speculate that they could be not only autocrine but also paracrine signals. The fact that only specific LCO structures and not COs affect fungal growth and development suggest that receptors may perceive them as in plants. Given the production and influence that LCOs have on *Aspergillus fumigatus*, we used this fungus as our main model to study LCOs in fungi. Before this study, pseudohyphae had only been observed previously under harsh conditions in *C. glabrata* (*13*, *14*), but infections with *C. glabrata* have often been reported in the presence of *Candida albicans* (*7*, *8*). We propose that the pathogenicity of *C. glabrata* may be regulated by the perception of LCOs produced by other fungi. Also, the lack of LCO production may be linked to the ability to develop hyphae, and that non-dimorphic yeasts in the Saccharomycotina may lack the ability to produce these molecules. Lastly, we propose that this signal may be perceived across phyla within the Fungal Kingdom, as shown in the *Rhodotorula mucilaginosa* experiments. Altogether, given that fungi produce and perceive LCOs and that fungal responses are dependent on the LCO concentration, our data indicate that LCOs are a new type of fungal quorum sensing signal. A page is now turning in the history of LCOs from being symbiotic signals for plants to a quorum sensing signal controlling growth and development across the Fungal Kingdom.

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2.6 Materials and Methods

2.6.1 Protocols

<u>**Detection and confirmation of COs and LCOs from fungal exudates.**</u>

Preparation and usage of 125ml Erlenmeyer flasks. Brand new Sylon CT (Sigma-Aldrich) coated 125ml flasks were used for all experiments. The interior of each flask was completely coated once with Sylon CT for 10 minutes with shaking. After coating, toluen (Sigma-Aldrich) and methanol (Fischer-Scientific) were added to the flasks for washing. The flasks were dried overnight under the fume hood. To prevent cross-contamination between experiments, sylon-covered flasks were washed, twice with liquid soap for an hour, then autoclave at 121 °C for 30 minutes.

Strains and cultures. The list and source of fungal and oomycete strains and their authorities used in these experiments are presented in **Supplemental Table 1**. The 61 fungal species are representative of five divisions, with each sub-division represented within the fungal kingdom. The following fungal divisions are listed from most divergent to least divergent fungal clades based on (*15*, *16*): Basidiomycota (Agaricomycotina, Pucciniomycotina, and Ustilaginomycotina), Ascomycota (Pezizomycotina, Saccharomycotina, and Taphrinomycotina), Mucormycota (Glomeromycotina, Mortierellomycotina, and Mucoromycotina), Chytridiomycota (Chytridiomycetes, Monoblepharidomycetes, and Neocallimastigomycetes), and Blastocladiomycota (Blastocladiomycetes). These fungal species were grown on media as listed in **Supplemental Table 2** with the medium recipes presented in **Supplemental Material and Methods 1**. There were three different methods used in extraction of fungal exudates: i) using a

concentration of 1X filtered fungal exudates after optimal growth conditions and period; ii) using raw liquid exudates from cellophane cultures that were concentrated to 10X, and iii) using liquid exudates from cellophane cultures that were then separated into LCOs and COs by butanol extraction. *Amanita muscaria, A. thiersii, Aphanomyces euteiches, Hebeloma cylindrosporum, Laccaria bicolor, Paxillus ammoniavirescens, P. involutus, Saccharomyces cerevisiae,* and *Sclerotinia sclerotiorum* were selected to be examined by all three methods to determine congruency between different methods.

  Extraction of filtered fungal exudates. Brand new, sylon coated, 125ml glass Erlenmeyer flasks, with no previous contact with any rhizobia, were washed, dried and autoclaved with 50ml of the optimal liquid broth media. The growth period, temperature and dark conditions are specified in **Supplemental Table 2** for each of the following fungal species : *Amanita muscaria, A. thiersii, Anaeromyces robustus, Aphanomyces euteiches, Aspergillus flavus, A. fumigatus, A. nidulans, Aureobasidium pullulans, Caecomyces churrovis, Candida albicans, C. auris C. glabrata, Clarireedia homoeocarpa, Dirkmeia* sp. nov. TAR 520, *Entophlyctis luteolus, Fereydounia khargensis, Globisporangium ultimum, Gloeocystidiellum convolvens, Hebeloma cylindrosporum, Laccaria bicolor, Lactarius deliciosus, L. populinus, L. psammicola, Mortierella elongata, M. minutissima, Mortierella* sp. nov. strain GBAus27b, *Mucor hiemalis, Multifurca ochricompacta, Neocallimastix californiae, Piromyces finnis, Ophiostoma novo-ulmi, Paraphysoderma sedeokerense, Paxillus adelphus, P. ammoniavirescens, P. involutus, Phytophthora erythroseptica, Powellomyces hirtus, Protomyces inouye, Rhizoctonia solani, Rhizopus ruoxii, Rhodotorula mucilaginosa, Russula amonenolens, R. cerolens, R. praetervisa, R. redolens, R. sanguinaria, Saccharomyces cerevisiae, Sclerotinia sclerotiorum, Taphrina americana, Trichoderma harizanum, Typhula incarnata* and *Ustilago sparsa.* Each species had

five replications. After the optimal growth conditions and period, samples were filtered through a 0.22µm Millipore Express® PES Membrane (Millipore Sigma, Darmstadt, Germany). The 0.22µm Millipore Express® PES Membrane was attached to a sterile glass reagent bottle (Wheaton, Millville, NJ, USA) with a screw cap. Liquid exudate were examined for bacterial or fungal contamination microscopically and through observing the broth in the glass containers for bacterial or fungal growth. The final concentration was 1X.

Extraction of liquid fungal exudates from cellophane on solid medium. Aphanomyces euteiches, Hebeloma cylindrosporum, Laccaria bicolor, Paxillus adelphus, P. ammoniavirescens and *P. involutus, Saccharomyces cerevisiae,* and *Sclerotinia sclerotiorum* were floated on cellophane with sterile water **(Cope *et al.,* 2019)** These fungal exudates were concentrated to a 10X concentration and filtered through a sterile 0.22µm polyethersulfone pore membrane (Sigma-Aldrich, St. Louis, MO, USA). *Aphanomyces euteiches, Cenococcum geophilum, Glonium stellatum, Laccaria bicolor, Hebeloma cylindrosporum, Lepidopterella palustris, Leptosphaeria maculans, Paxillus adelphus, P. ammoniavirescens, P. involutus, Saccharomyces cerevisiae,* and *Sclerotinia sclerotiorum* cultures were initiated on solid medium overlaid with cellophane membrane, with one implant per 55 mm Petri dish or three implants per 90 mm Petri dish. One exception was for *Gonapodya prolifera* that was cultivated without cellophane because it can digest it. After two to three weeks, mycelia were transferred to liquid medium in Petri dishes as described in **Supplemental Table 2.** The mycelium growing on solid medium was crushed and transferred to liquid medium. The cultures were maintained at 22 °C, in the dark, without agitation. At least 10 Petri dishes (90 mm) were used for each culture series and several independent series were carried out for each strain (3 to 20).

Extraction and separation of lipo-chitooligosaccharides. The culture media for *Aphanomyces euteiches, Cenococcum geophilum, Glonium stellatum, Hebeloma cylindrosporum, Hydnomerulius pinastris, Lepidopterella palustris, Leptosphaeria maculans, Paxillus adelphus, P. ammoniavirescens,* and *P. involutus* (100 to 400 mL depending on the series) was extracted twice with butanol (1:1 v/v). The pooled butanol phases were washed with distilled water and evaporated under vacuum. The dry extract was re-dissolved in 4 ml water:acetonitrile (1:1 v/v) and dried under nitrogen. This crude extract was re-suspended in 1 ml of 20% acetonitrile in water and separated on Hypersep C18 (500 mg, 3 ml, Thermo scientific) using 3 ml elutions: 20%, 50% and 100% acetonitrile in water. Then the samples were dried under nitrogen. Occasionally the 50% sample was re-suspended in 75% acetonitrile in water and separated on Chromabond HILIC (500 mg, 3ml REF) using 3 ml elution: 100%, 80%, 75% acetonitrile in water. Then the samples were dried under nitrogen.

Bioassays. We used three bioassays to screen 1X and 10X concentrations of filtered fungal exudates and butanol-extracted media from each fungal or oomycete species for the presence of LCOs. These included: (i) root hair branching in *Vicia sativa* (vetch), which is induced by non-sulfated (ns)LCOs (*17*), (ii) root hair branching in *Medicago truncatula* accession Jemalong A17 which is induced by sulfated (s)LCOs, and (iii) the expression of the *MtENOD11::GUS* construct in *M. truncatula*, which is also induced by (s)LCOs (*18*). The root hair branching assay in vetch were conducted using two methods: i) vetch growth conditions and methods[1] and ii) germinated seedlings were grown for three days in Petri dishes (five plants per plate), on Fahräeus agar, in a vertical position in a growth chamber (22°C, 100 µmol m^{-2} s^{-1}, 16h day/8h night).

Each root was treated with either 1 ml of 1x or 10x filtered fungal exudates or 40 µl of sample and plates were put back into the growth chamber for 30 hours. Root hair formation were

observed using two methods: i) through a light inverted microscope (Leica DMi1, Leica Microsystems, Buffalo Grove, IL, USA) on a wet slide, or ii) performed in a 0.02% methylene blue solution under a light microscope. Five to ten seedlings were tested per sample and compared to a mock treatment (0.005% EtOH in water or 5% acetonitrile in water). Plants treated with Nod factors purified from *Rhizobium leguminosarum* biovar *viciae* supernatant were used as a positive control. *Medicago truncatula* accession Jemalong A17 were prepared as in **Cope *et al.,* (2019)** with 8 to 10 seeds per plate with sterile growth paper on Fahräeus agar surface. One milliliter was applied to each 1-week old *M. truncatula* accession Jemalong A17 root and stored at 25 °C for 48 hours. Three centimeters from the root tip was used to count for root hair branches that were present in each assay.

The *MtENOD11* gene induction assay was performed in square (120 x 120 mm) Petri plates, with sterile growth paper on the Fahräeus agar surface. Forty microliters treatment were used per seedling root. Ten plants were tested by sample and compared to a mock treatment (0.005% EtOH in water or 5% acetonitrile in water). Plants treated with Nod factors purified from *Sinorhizobium meliloti* supernatant were used as a positive control for both *M. truncatula* accession Jemalong A17 and *MtENOD11* gene induction assays.

For both tests, two kinds of samples were used: butanol extracts were diluted 100 times in water, Hilic column fractions were diluted 10 times. Arbitrary scales were used to quantify the frequency of root hair branching or GUS-staining.

Mass spectrometry analyses. Synthetic lipochitinic standards (LCO IV-C16:0, LCO IV-C16:0 S, LCO IV-C18:1, LCO IV-C18:1 S) obtained from Hugues Driguez (CERMAV, Grenoble, France) were used to identify retention times and optimize HPLC/Q-TRAP tandem mass spectrometry detection by MRM, at 10^{-5} M, in acetonitrile (ACN):water (1/1, v/v). (*4*)The HPLC

3000 (Dionex) was equipped with a C18 reverse-phase column C18 Acquity (2.1 x 100 mm, 1.7 µm, Waters). The separation was done with a gradient of ACN/water:acetic acid (1000:1, v:v), started at 30% ACN in water for 1 min, followed by a gradient to 100% ACN for 8 min, followed by an isocratic step at 100% ACN for 2 min, at a constant flow rate of 450 µL.min^{-1}. Samples of 10 µL were injected. The mass spectrometer was a 4500 Q Trap mass spectrometer (Applied Biosystems, Foster City, USA) with an electro-spray ionization in the positive ion mode. The samples were analyzed using the multiple reaction monitoring (MRM) mode and by the Enhanced Mass Spectrometry-Enhanced Product Ion (EMS-EPI) mode for the most concentrated ones. For the MRM mode, a list of predicted transitions was established based on known Nod factor structures. It consisted, for each potential precursor proton adduct ion $[M + H]^+$, of three fragment ions corresponding to the loss of one, two or three N-Ac Glucosamines at the reducing end (bearing or not additional groups). It led to a total of 3000 possible MRM transitions sensitive, this targeted analytical approach was suitable for samples with very few molecules. However, it could not provide a comprehensive analysis of all the molecules present, because only 16 MRM transitions could be searched per injection. For samples with higher content of molecules, full scan EMS-EPI analyses were performed. Using this mode, precursor ions are automatically detected, selected and after collision, further analyzed with their product ions accumulating in the trapping module. This more comprehensive, non-targeted, mode could only be used with two *Paxillus* LCO-rich samples. The capillary voltage was fixed at 4500 V, source temperature at 400 °C. Fragmentation was performed by collision-induced dissociation (CID) with nitrogen at a collision energy between 22 and 54 V; declustering potential was between 90 and 130 V, optimized for each synthetic available molecule.

Routine cultivation of Neocallimastigomycetes fungi. Anaerobic fungal strains *Anaeromyces robustus*, *Caecomyces churrovis*, and *Neocallimastix californiae* were isolated via reed canary grass enrichment from the feces of goat or sheep collected from the Santa Barbara Zoo, as described previously. (*19–21*) *Piromyces finnis* was enriched and isolated from the feces of a horse collected from Verrill Farm Stables in Concord, MA, USA. (*19–21*) The strains were grown in anaerobic serum bottles or Hungate tubes containing liquid medium at 39˚C with CO_2 in the headspace. Fungi were routinely transferred via sterile syringe-needle technique every 3-4 days into fresh anaerobic medium with 0.1 g of 4-mm milled reed canary grass as growth substrate. *P. finnis* was cultivated in Medium C, whereas all other strains were cultivated in a minimal formulation of Medium C containing 0.25 g of yeast extract (Thermo Fisher Scientific), 0.5 g Bacto™ Casitone, and 7.5 vol% clarified rumen fluid per liter of medium. Growth proliferation was measured using the pressure transducer technique due to invasive growth of the fungi into fermentation substrate. (*22*, *23*)

Preparation of Neocallimastigomycetes fungi for LCO analysis. Minimal Medium C was prepared as described above omitting yeast extract or Bacto™ Casitone. Twenty milliliters of modified minimal Medium C were aliquoted while sparging with CO_2 into 60 mL borosilicate serum bottles containing 0.2 g switch grass to support growth of anaerobic fungi. After autoclaving serum bottles, 0.2 mL of sterile filtered 100x vitamin solution was added to each bottle (*24*). About 1 mL of fungal zoospores from each of the four strains was inoculated in separate, serum bottles (preheated to 39 °C) with at least two replicates per strain. Fungal cultures were incubated with substrate anaerobically for 144 hours at 39 °C. Cultures were then transferred into 50 mL Falcon tubes and centrifuged at 4000 g using a fixed angle rotor (Eppendorf F-34-6-38) at 4°C. Sample

supernatants and cell pellets were shipped on dry ice to the University of Wisconsin-Madison for LCO analysis.

   Preparation of Russulales fungi for LCO analysis. Pure culture of the following strain, *Russula sanguinea* PMI158, *Lactarius populinus* PMI44, *Russula pectinatoides* PMI72, *Russula cerolens* PMI56, *Russula redolens* BPL29, *Gloeopeniophorella convolvens* OM19405, *Russula amoenolens* BPL10, *Multifurca ochricompacta* BPL690, *Lactarius psammicola* BPL869 and *Lactarius deliciosus* BPL912 were maintained in solid Modified Melin-Norkrans (MMN) medium and kept at 4 °C as stock cultures. This Russulaceae culture collection is deposited at the Oak Ridge National Laboratory in the PMI microbial library. They are available by request at the https://pmiweb.ornl.gov/ for public accessibility. Three liquid cultures of each of these fungi were initiated from three 0.5-cm diameter plugs in 12-ml autoclaved MMN medium (pH=5), in glass culture tubes (https://phytotechlab.com/culture-tube-phytotech-reg-brand-25-x-95-mm-flat-bottom-glass.html). These cultures were grown for 2 weeks at 23˚C in dark without agitation. For LCO extraction, each culture was emptied into a Nalgene 0.45-µm bottle top filter with a 150 mL receiver after application of a sterile cheesecloth on top of the filter. Fifteen milliliters of sterile water were added to the top of the filter prior to each filtration. Cultures were later filtered by plugging the filtering system to a vacuum pump. The exudates were transferred from the receiver to 15-ml falcon tubes and stored at 4 °C until LCO detection analyses.

Procedures used for *Aspergillus fumigatus* experiments.

   Organism and inoculum. *Aspergillus fumigatus,* strain Af293, was used in this study. The growth media used is glucose minimal media (GMM). Af293 strain was previously described.(*25*)

Evaluation of germination rates. The germination rates of *A. fumigatus* strain Af293 were monitored under microscopy in GMM broth supplemented with various treatments of COs and LCOs at a final concentration of 10^{-8} M in the media. The LCO treatments used were sulfated C16:0 LCO(s); non-sulfated C16:0 LCO(s); sulfated C18:1 LCO(s); non-sulfated C18:1 LCO(s). The CO treatments were: CO4, CO5, and CO8. The negative control for these analyses consisted of 0.005% EtOH, the solvent in which all the treatments were prepared. The spore concentration was adjusted to 10^6 spores/mL of medium. Briefly, one milliliter of each mixture was distributed into 2 replicate wells of a 24-sterile well plate. Time-course microscopy was carried out over 24 hours at 37 °C using a Nikon Ti inverted microscope. Two replications with ten pictures were taken for each well every hour, beginning three hours post-incubation. Hundred spore germlings were counted for each replication per treatment and recorded. The percentage of germinated spores was plotted against time, and the germination rates were determined. Four separate trials were performed for this same experiment. Dose response experiments were carried out as previously mention except the treatments were of sulfated C16:0 LCO(s) at concentrations between 10^{-6} M and 10^{-13} M.

Evaluation of branching hyphae. Hyphae branches were evaluated from the germinated spores mentioned in ***Evaluation of germination rates*** section. Time-course microscopy was carried out over 24 hours at 37 °C using a Nikon Ti inverted microscope. Two replications with ten pictures were taken for each well every hour, beginning three hours post-incubation. At 12 hours post inoculation, the length of the apical branch, number of secondary branches per apical branch and number of total secondary branches were counted for 100 germinated spores. Branches were counted for each replication per treatment and recorded. The ratio of secondary branches per apical branch were determined. Four separate trials were performed for this same experiment.

Dose response experiments were carried out as previously mentioned except for the treatments with sulfated C16:0 LCO(s) for which concentrations between 10^{-6} M and 10^{-13} M were used.

RNA isolation, library preparation and RNA sequencing

Growth Conditions: *Aspergillus fumigatus* strain Af293 was grown in GMM broth supplemented with either sulfated C16:0 LCO(s) at concentration of 10^{-8} M or the control which is 0.005% EtOH, the solvent used to prepare sulfated C16:0 LCO(s). The spore concentration was adjusted to 10^6 spores/mL of medium, and the cultures were maintained at 37 °C and 250 rpm. Spores were collected at two time points: 30 minutes post-inoculation (mpi) and 120 mpi. Four replications were done per treatment.

RNA extraction: The spores were collected and ground to a fine powder in liquid nitrogen and transferred into 50-ml centrifuge tubes. Total RNA was extracted using QIAzol Lysis Reagent (Qiagen) according to the manufacturer's instructions with additional phenol:chloroform:isoamylacohol (24:1:1) extraction step before RNA precipitation. For the preparation of RNAs suitable for RNA-sequencing, total RNAs were further cleaned up using RNeasy Mini Kit. RNA samples digested with DNase and kept at -80 °C until further use. NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA.) was used to quantify and assess purity of RNA. Nanodrop readings for samples were between 112.24 ng/μL to 491.44 ng/μL.

Library preparation and RNA sequencing: Sixteen libraries of RNAseq single-end reads were prepared using Truseq library preparation protocol and sequenced with illumina Hiseq2500 machine. The raw reads of 16 libraries, four biological replicates for each of the four treatments (control: 30 mpi; sulfated C16:0 LCO(s): 30 mpi; control: 120mpi; and sulfated C16:0 LCO(s)). Read quality was assessed with FastQC. Read quality was good and adapter sequences were

minimal, so read were not trimmed. Paired-end reads were pseudoaligned and quantified using

Kallisto 0.42.3 against the reference transcriptome of *Aspergillus fumigatus* Af293 from

downloaded from Genome database of Joint Genome Institute,

(https://genome.jgi.doe.gov/portal/Aspfu1/Aspfu1.download.html) genome released on

December 21, 2012 by project P.I. Katherine McMahon and downloaded from Genome database

of Joint Genome Institute,

(https://genome.jgi.doe.gov/portal/Aspfu1/Aspfu1.download.html).(*26*, *27*) Bootstrap values

were 100. Pair-wise transcriptomic comparisons were completed in Sleuth (*28*). We defined

transcripts as differentially expressed if they had a false discovery rate (FDR, q-value) <0.05, p-

value <0.01, and beta values less than -0.4 or greater than 0.4. GO-enrichment analysis for the

Aspergillus fumigatus genome was carried our using the Gene ID, gene ontology enrichment,

from http://fungidb.org.

Preparation and procedures used for *Candida glabrata* experiments

  Organism and inoculum. *Candida glabrata* isolate, CG 006 was used in this study. CG006

strain was previously described.(*29*) Yeast was stored in 15% (vol/vol) glycerol stock at -80°C and

maintained on yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, 2%

dextrose), supplemented with uridine (80 µg/ml) prior to experiments. For overnight culture, single

colonies were propagated in 3mL YPD supplemented with uridine at 30°C on an orbital shaker at

200 rpm. Ten microliters of the overnight culture were diluted 1:1,000 in Dulbecco'c phosphate

buffered saline (-calcium, -magnesium) DPBS (Hyclone Laboratories Inc., Logan, UT), and

enumerated using a hemocytometer.

Evaluation of pseudohyphae formation. The growth and development of *C. glabrata* cells were monitored under microscopy in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fischer Scientific, Co., Waltham, MA.), supplemented with various treatments of COs and LCOs at a final concentration of 10^{-8} M in the media. The LCO treatments used were sulfated C16:0 LCO(s); non-sulfated C16:0 LCO(s); sulfated C18:1 LCO(s); non-sulfated C18:1 LCO(s). The CO treatments were: CO4, CO5, and CO8. The negative control for these analyses consisted of 0.005% EtOH, the solvent in which all the treatments were prepared. The cell concentration was adjusted to 10^6 spores/mL of medium. Briefly, 300 µl of each mixture, cells and treatments, were distributed into wells of a µ-Slide 8 well sterile plate (ibidi USA, Inc., Fitchburg, WI.). Time-course microscopy was carried out over 12 hours at 37 °C using a Nikon Ti inverted microscope. Each well represents one treatment and five pictures were taken for each well every 10 minutes. After 12 hours, the entire well was scanned to look for pseudohyphal formation. The total number of pseudohyphae observed per well was counted. Four separate trials were performed for this same experiment. Dose response experiments were carried out as previously mentioned except for the sulfated C16:0 LCO(s) treatments that were done at concentrations ranging between 10^{-6} M and 10^{-13} M.

Preparation and procedures used for *Rhodotorula mucilaginosa* experiments

Organism and inoculum. *Rhodotorula mucilaginosa,* strain AR 1356-LV16, was used in this study. This strain was stored using the same procedure described earlier for the *C. glabrata* isolate. For overnight culture, single colonies were propagated in 50 mL potato dextrose broth (PDB) in 125ml flasks at 25 °C on an orbital shaker at 250 rpm. About 10^6 cells were determined using the Countless[TM] cell counting chamber slides and Countless II program (Invitrogen, Inc., Carlsbad, CA.).

Evaluation of OD$_{600}$ reading. The OD$_{600}$ of *R. mucilaginosa* cells were determined by Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Co., Winooski, VT.). PD broth supplemented with various treatments of COs and LCOs at a final concentration of 10^{-8} M in the media. The LCO treatments used were sulfated C16:0 LCO(s), non-sulfated C16:0 LCO(s), sulfated C18:1 LCO(s), and non-sulfated C18:1 LCO(s). The CO treatments were: CO4, CO5, and CO8. The negative control for these analyses consisted of 0.005% EtOH, the solvent in which all the treatments were prepared. The cell concentration was adjusted to 10^6 spores/mL of medium. Briefly, 200 µl of each mixture, cells and treatments, was distributed into wells of a 96 Costar well sterile, flat-bottom, assay plate (Corning Inc., Corning, NY.). Time-course OD readings were carried out over 24 hours at 25 °C with 0.5 rpm. Outer wells were filled with sterile MiliQ water to prevent evaporation. After 24 hours, the maximum V was analyzed to determine the final OD$_{600}$ reading per treatment. Six replications were done per treatment and three separate trials were performed for this same experiment.

Statistical Analysis

Statistical analysis was done using R Studio (RStudio Team 2015, RStudio, Inc., Boston, MA.) and GraphPad Prism software (La Jolle, CA.). Analysis of variances for all results that were significant had a p-value <0.05. For the Aspergillus fumigatus and Rhodotorula mucilaginosa experiments, the post-hoc analysis and Dunnett pairwise test were used to compare the treatments to the control. Statistical differences were based on p-values <0.05. For the Candida glabrata experiments, Tukey's post hoc analysis was used to compare all treatments to each other, since the control had no pseudohyphae formation. The error bars in all figures indicate the standard error of the mean.

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2.6.2 Media recipes used

1. **0.2g switchgrass in modified MC' medium (without yeast extract or bacto casitone)**
    Adapted from Teunissen et al., Journal of General Microbiology (199 1), 137, 140 1 – 1408, and Archives of Microbiology September 1991, Volume 156, Issue 4, pp 290-296

Step 1, combine ingredients

For 1 liter, add to 700 ml Milli Q water in a Fernbach flask:

Chemical or solution	Volume or weight	Final concentration
• Solution A	150 ml	15%
• Solution B	150 ml	15%
• NaHCO3 (sodium bicarbonate)	12 g	143 mM
• Trace elements solution (100x)	10 ml	1%
• Hemin solution (100x)	10 ml	1%
• Resazurin solution (0.1 %, w/v)	1ml	0.0001%

Optional: for monoculture of fungi or for co-culture, add:

o Yeast extract	2.5 g	0.25%
o Bacto-Casitone	10 g	1%

Step 2, boil off oxygen and cool

*   Heat flask with medium in microwave, ~ 10 minutes, to boil off oxygen. Watch carefully during the last 3 minutes and make sure it doesn't boil over. Expect to lose ~200 ml during this process.
*   Cool. ~ 1h. Sparge medium with gas to prevent dissolution of oxygen into the cooling liquid. If you are making media for fungi or for co-culture, you can use CO2.
*   Transfer cooled media to a 1L bottle containing
    *   o L-cysteine·HCl   1 g   8.25 mM
*   Bubble with CO2 and cap.

Step 3, aliquot media for 10 ml cultures
*   Prepare Hungate tubes (16 x 125 mm, Chemglass CLS-4208-01).

- With a partner, dispense 9 ml media into Hungate tubes – one person will dispense media, taking care to keep CO2 or CO2 flowing into the head space of the open bottle of media, the other will displace air from Hungate tubes by filling with CO2 or CO2 prior to media addition, then quickly capping tubes after media has been added.

Step 4, sterilize
- Autoclave 25 minutes at 121° C
- Cool to room temperature and store at 4° C until ready to use

Step 5, add final components
- Warm tubes to incubation temperature
- Add Modified vitamin solution (1000x)   1 ml   0.1%
  Add:
  Soluble C source (cellobiose, 20%)   0.25ml   0.5%

RECIPES

- Solution A, 1 liter (6.66x)
- Dissolve in 800 ml Milli Q water:

Chemical or solution	Volume or weight	Final concentration	Final conc. in M2
• KH_2PO_4	3.0 g	22 mM	3.3 mM
• $(NH_4)_2SO_4$	3.0* g		
• NaCl	6.0 g		
• $MgSO_4 \cdot 7\ H_2O$	0.6 g		

Dissolve in 100 ml Milli Q water:
- $CaCl_2 \cdot 2\ H_2O$ 0.6 g
- Combine and bring volume to 1 L
- Store at 4° C

NOTE, Solns A and B are identical to those used in anaerobic fungal Medium C, with the exception that Soln A $(NH_4)_2SO_4$ is present at 6 g/L in Medium C recipe.

Solution B, 1 liter (6.66x)

Chemical or solution	Volume or weight	Final concentration	Final conc. in M2
• K_2HPO_4	3.0	0.45	
• Vitamin solution 2 (mg/L) = 1000x			Final µg/L
• Thiamin·HCl	5		5
• Riboflavin	5		5
• Calcium D-pantothenate	5		5
• Nicotinic acid	5		5
• Folic acid	2		2
• Cyanocobalamin	1		1
• Biotin	1		1
• Pyridoxin HCl	10		10
• p-aminobenzoic acid	5		5

Trace element solution (g/L) = 100x   Final mg/ml
(from Lowe et al., 1985, J. Gen Microbiol, 131:2225-2229)

- - prepared in 0.2 M-HCl
        2mM

$MnCl_2 \cdot 4H_2O$	0.25	2.5
$NiCl_2 \cdot 6H_2O$	0.25	2.5
$NaMoO_4 \cdot 2H_2O$	0.25	2.5
H_3BO_3	0.25	2.5
$FeSO_4 \cdot 7H_2O$	0.20	2.0
$CoCl_2 \cdot 6H_2O$	0.05	0.5
SeO_2	0.05	0.5
$NaVO_3 \cdot 4H_2O$	0.05	0.5
$ZnCl_2$	0.025	0.25
$CuCl_2 \cdot 2H_2O$	0.025	0.25

Hemin solution = 100x
(from Lowe et al., 1985, J. Gen Microbiol, 131:2225-2229)

Hemin	0.1 g	1 mg/L
• dissolve in	10 ml ethanol	0.01%
• adjust volume to 1L with	0.05 M NaOH	0.5 mM

2. **2% Malt Extract Broth**

   For 1 L solution:

   - 17 g Malt Extract (VWR Life Science, Solon, Ohio, USA)
   - 3 g Peptone (Difco^{TM}, Sparks, Maryland, USA)
   - pH 5.4±0.2 at 25 ºC

3. **CG Medium**

   For 1 L solution:

   - 50 mg $CaCl_2$
   - 1 g KH_2PO_4
   - 300 mg $(NH_4)_2HPO_4$
   - 500 mg $MgSO_4$ $7H_2O$
   - 1 mg $FeCl_3$ $6H_2O$
   - 2 g casein peptone (ThermoFisher, Illkirch,France)
   - 20 g glucose (Merck, Darmstadt, Germany)
   - 0.1 mg thiamin (ACROS-ThermoFisher, Illkirch, France)
   - 0.1 mg $ZnSO_4 \cdot 7H_2O$
   - 0.1 mg H_3BO_3
   - 10 mg $MnSO_4 \cdot 7H_2O$, 3 mg $CuSO_4 \cdot 7H_2O$
   - 3 mg $AlCl_3$
   - 3 mg $NiCl_2$ $6H_2O$
   - 3 mg $MoNa_2$
   - 1 mg KI
   - solid media were supplemented with 10 to 15 g agar (Merck, Darmstadt,Germany)

4. **Corn Meal Broth (**Sigma-Aldrich, St. Louis, Missouri, USA)
   a. Follow company's instructions

5. **Floated on Cellophane with Sterile MilliQ Water**
6. **Modified Melin-Norkran's Medium** (PhytoTechnology Laboratories, Shawnee Mission, Kansas, USA)
   a. Follow company's instructions
7. **Infected Wheat was mixed with Sterile MilliQ Water in 50ml Falcon Tube**
   a. Wheat with *Puccinia striiformis f. sp. tritici* was collected from Arlington Agricultural Research Station, Arlington, WI, USA in 2016.
   b. A single blade of infected wheat was placed in five sterile 50ml Falcon tubes (Thermo Fisher Scientific, Waltham, MA, USA) with sterile MiliQ water for 5 days at 25 $^{\circ}$C in the dark.
8. **MP Medium**

   For 1 L solution:
   a. 50 mg $CaCl_2$
   b. 25 mg NaCl
   c. 500 mg KH_2PO_4
   d. 250 mg $(NH_4)_2HPO_4$
   e. 150 mg $MgSO_4 \bullet 7\ H_2O$, 1 mg $FeCl_3 \bullet 6\ H_2O$
   f. 1 g casein peptone (ThermoFisher, Illkirch,France)
   g. 5 g malt extract (Merck, Darmstadt, Germany)
   h. 5 g glucose (Merck, Darmstadt, Germany)
   i. 0.1 mg thiamin (ACROS-ThermoFisher, Illkirch, France)
   j. 0.1 mg $ZnSO_4 \bullet 7\ H_2O$, 0.1 mg H_3BO_3, 10 mg $MnSO_4 \bullet 7\ H_2O$
   k. 3 mg $CuSO_4 \bullet 7\ H_2O$, 3 mg $AlCl_3$, 3 mg $NiCl_2 \bullet 6\ H_2O$
   l. 3 mg $MoNa_2$
   m. 1 mg KI
   n. solid media were supplemented with 10 to 15 g agar (Merck, Darmstadt, Germany)

9. **MP modified** (MPm): MPm; same as MP medium except that it contained only 2.5 g L^{-1} glucose as carbon source)
10. **Peptonized milk, tryptone, glucose broth (PmTG)**
    a. 1.0g Peptonized milk (Himedia, Mumbai, India)
    b. 1.0g Tryptone (BD 211705, Sigma-Aldrich, St. Louis, Missouri, USA)
    c. 5.0g glucose (Fischer Scientific, Fair Lawn, New Jersey).
       solid media were supplemented with 10 to 15 g agar (Merck, Darmstadt, Germany
11. **Potato Dextrose Broth** (Difco^{TM}, Sparks, Maryland, USA).
    a. Follow company's instructions

2.7 Acknowledgments

sedebokerensis, and *Powellomyces hirtus;* and Dr. Anne Pringle for cultures and samples of *Amanita muscaria* and *A. thiersii.* We also like to thank Mr. Jacob Golan and Mrs. Katie Morey Gold for statistical advice.

2.8 Supplemental Tables

Supplemental Table 1: Fungal culture collection information and authority.

Phyla	Species	Culture Collection
Ascomycota	*Aspergillus flavus*	NRRL 3377
Ascomycota	*Aspergillus fumigatus*	Af293
Ascomycota	*Aspergillus nidulans* strain RDIT 9.32 WT	FGSC A1252
Ascomycota	*Aureobasidium pullulans* strain 222	Cultures obtained from Ms. Karen Vanderwolf, the Department of Pathobiological Sciences, University of Wisconsin-Madison, available upon request
Ascomycota	*Candida albicans* strain SN250	Noble et al. 2010; Cultures obtained from Dr. David Andes, the Department of Medicine, University of Wisconsin-Madison, available upon request
Ascomycota	*Candida auris*	Noble et al. 2010; Cultures obtained from Dr. David Andes, the Department of Medicine, University of Wisconsin-Madison, available upon request
Ascomycota	*Candida glabrata* strain CG006	Cultures obtained from Dr. David Andes, the Department of Medicine, University of Wisconsin-Madison, available upon request

Ascomycota	*Cenococcum geophilum* strain WSL 1.58	Culture obtained from Swiss Federal Institute for Forest, Snow and Landscape Research (WSL) and from Dr. Frances Martin, Laboratoire d'Excellence Advanced Research on the Biology of Tree and Forest Ecosystems, Unité Mixte de Recherche Institut National de la Recherche Agronomique–Université de Lorraine, available upon request
Ascomycota	*Clarireedia homoeocarpa* (Syn: *Sclerotinia homoeocarpa*) strain NL 715	Cultures obtained from Dr. Paul Koch, the Department of Plant Pathology, University of Wisconsin-Madison, available upon request
Ascomycota	*Glonium stellatum* strain CBS207.34	Cultures obtained from Dr. Frances Martin, Laboratoire d'Excellence Advanced Research on the Biology of Tree and Forest Ecosystems, Unité Mixte de Recherche Institut National de la Recherche Agronomique–Université de Lorraine
Ascomycota	*Lepidopterella palustris*	Cultures obtained from Dr. Frances Martin, Laboratoire d'Excellence Advanced Research on the Biology of Tree and Forest Ecosystems, Unité Mixte de Recherche Institut National de la Recherche Agronomique–Université de Lorraine
Ascomycota	*Leptosphaeria maculans* strain V23 1.3 (JN3)	?
Ascomycota	*Ophiostoma novo-ulmi*	Cultures obtained from Dr. Brian Hudelson and Armila Francis, the Department of

		Plant Pathology, University of Wisconsin-Madison, available upon request
Ascomycota	*Protomyces inouye* strain 1321	Cultures obtained from Ms. Karen Vanderwolf, the Department of Pathobiological Sciences, University of Wisconsin-Madison, available upon request
Ascomycota	*Saccharomyces cerevisiae* strain INV Sc1	Thermo Scientific: C8100
Ascomycota	*Saccharomyces cerevisiae* strain yHD0554 (S288c)	Cultures obtained from Dr. Chris Hittinger, the Department of Genetics, University of Wisconsin-Madison, available upon request
Ascomycota	*Taphrina americana* strain 1380	Cultures obtained from Ms. Karen Vanderwolf, the Department of Pathobiological Sciences, University of Wisconsin-Madison, available upon request
Ascomycota	*Trichoderma harizanum*	Cultures obtained from Dr. Brian Hudelson and Armila Francis, the Department of Plant Pathology, University of Wisconsin-Madison, available upon request
Basidiomycota	*Amanita muscaria* strain Koide BX008	Cultures obtained from Dr. Anne Pringle, the Department of Botany and Bacteriology, University of Wisconsin-Madison, available upon request
Basidiomycota	*Amanita thiersii* strain Skay 4041	Cultures obtained from Dr. Anne Pringle, the Department of Botany and Bacteriology, University of Wisconsin-Madison, available upon request

Basidiomycota	*Amanita thiersii* strain Skay 4041 HET	Cultures obtained from Dr. Anne Pringle, the Department of Botany and Bacteriology, University of Wisconsin-Madison, available upon request
Basidiomycota	*Dirkmeia* sp. nov. strain TAR 520	CBS 12832
Basidiomycota	*Fereydounia khargensis* strain TAR 509	CBS 135682
Basidiomycota	*Gloeocystidiellum convolvens* (syn: *Gloeopeniophorella convolvens)*	Fungal exudates obtained from Dr. Jessy Labbé, Fungal Systems Genetics and Biology Laboratory, Oak Ridge National Laboratory, Oak Ridge, Tennessee; available upon request
Basidiomycota	*Hebeloma cylindrosporum* strain h7	Cultures obtained from Université de Lyon, France
Basidiomycota	*Lactarius deliciosus* strain BPL912	Fungal exudates obtained from Dr. Jessy Labbé, Fungal Systems Genetics and Biology Laboratory, Oak Ridge National Laboratory, Oak Ridge, Tennessee
Basidiomycota	*Lactarius populinus* strain PMI44	Fungal exudates obtained from Dr. Jessy Labbé, Fungal Systems Genetics and Biology Laboratory, Oak Ridge National Laboratory, Oak Ridge, Tennessee
Basidiomycota	*Lactarius psammicola* strain BPL869	Fungal exudates obtained from Dr. Jessy Labbé, Fungal Systems Genetics and Biology Laboratory, Oak Ridge National Laboratory, Oak Ridge, Tennessee
Basidiomycota	*Multifurca ochricompacta* strain BPL690	Fungal exudates obtained from Dr. Jessy Labbé, Fungal Systems Genetics and Biology Laboratory, Oak

		Ridge National Laboratory, Oak Ridge, Tennessee
Basidiomycota	*Paxillus ammoniavirescens* strain Pou09.2	Cultures obtained from Laboratorie Evolution and Diversité Biologique, Toulouse, France
Basidiomycota	*Paxillus involutus* strain Bel09	Cultures obtained from Laboratorie Evolution and Diversité Biologique, Toulouse, France
Basidiomycota	*Rhizcotonia solani* strain LP2	Cultures obtained from Dr. Paul Koch, the Department of Plant Pathology, University of Wisconsin-Madison, available upon request
Basidiomycota	*Rhodotorula mucilaginosa* strain AR 1356-LV16	Cultures obtained from Ms. Karen Vanderwolf, the Department of Pathobiological Sciences, University of Wisconsin-Madison, available upon request
Basidiomycota	*Russula amonenolens* strain BPL10	Fungal exudates obtained from Dr. Jessy Labbé, Fungal Systems Genetics and Biology Laboratory, Oak Ridge National Laboratory, Oak Ridge, Tennessee
Basidiomycota	*Russula cerolens* strain PMI56	Fungal exudates obtained from Dr. Jessy Labbé, Fungal Systems Genetics and Biology Laboratory, Oak Ridge National Laboratory, Oak Ridge, Tennessee
Basidiomycota	*Russula praetervisa* (syn: *Russula pectinoides)* strain PMI72	Fungal exudates obtained from Dr. Jessy Labbé, Fungal Systems Genetics and Biology Laboratory, Oak Ridge National Laboratory, Oak Ridge, Tennessee
Basidiomycota	*Russula redolens* strain BPL29	Fungal exudates obtained from Dr. Jessy Labbé, Fungal Systems Genetics and Biology Laboratory, Oak

		Ridge National Laboratory, Oak Ridge, Tennessee
Basidiomycota	*Russula sanguinaria* (syn: *Russula sanguinea)* strain PMI158	Fungal exudates obtained from Dr. Jessy Labbé, Fungal Systems Genetics and Biology Laboratory, Oak Ridge National Laboratory, Oak Ridge, Tennessee
Basidiomycota	*Sclerotinia sclerotiorum* strain 1980	ATCC 18683D2
Basidiomycota	*Typhula incarnata* strain OJN	Cultures obtained from Dr. Paul Koch, the Department of Plant Pathology, University of Wisconsin-Madison, available upon request
Basidiomycota	*Ustilago sparsa* strain TAR 523	CBS 12833
Mucoromycota	*Gigaspora rosea*	Cultures obtained from Laboratorie Evolution and Diversité Biologique, Toulouse, France
Mucoromycota	*Mortierella elongata* strain NVPG4	Cultures obtained from Dr. Gregory Bonito, Department of Plant, Soil and Microbial Sciences, Michigan State University, available upon request
Mucoromycota	*Mortierella minutissima* strain ADO51	Cultures obtained from Dr. Gregory Bonito, Department of Plant, Soil and Microbial Sciences, Michigan State University, available upon request
Mucoromycota	*Mortierella* sp. nov. strain GBAus27b	Cultures obtained from Dr. Gregory Bonito, Department of Plant, Soil and Microbial Sciences, Michigan State University, available upon request

Mucoromycota	*Mucor hiemalis* strain TAR 706	Cultures obtained from Dr. Brian Hudelson and Armila Francis, the Department of Plant Pathology, University of Wisconsin-Madison, available upon request
Mucoromycota	*Rhizophagus clarus*	Cultures obtained from Laboratorie Evolution and Diversité Biologique, Toulouse, France
Mucoromycota	*Rhizophagus intraradices*	Cultures obtained from Laboratorie Evolution and Diversité Biologique, Toulouse, France
Mucoromycota	*Rhizophagus irregularis*	Mycorise® ASP
Mucoromycota	*Rhizopus ruoxii* (syn: *Mucor ruoxii*)	Cultures obtained from Dr. Brian Hudelson and Armila Francis, the Department of Plant Pathology, University of Wisconsin-Madison, available upon request
Chytridiomycota	*Anaeromyces robustus* strain S4	Cultures obtained from Dr. Michelle O'Malley, Department of Chemical Engineering, University of California, Santa Barbara, available upon request
Chytridiomycota	*Caecomyces churrovis* strain Ceco	Cultures obtained from Dr. Michelle O'Malley, Department of Chemical Engineering, University of California, Santa Barbara, available upon request
Chytridiomycota	*Entophlyctis luteolus* strain JEL129	James et al. (2006)
Chytridiomycota	*Gonapodya prolifera* strain JEL 478	Cultures obtained from Dr. Joyce Langcore, School of Biology and Ecology, University of Maine; available upon request.

Chytridiomycota	*Neocallimastix californiae* strain G1	Cultures obtained from Dr. Michelle O'Malley, Department of Chemical Engineering, University of California, Santa Barbara, available upon request
Chytridiomycota	*Piromyces finnis* strain Finn	Cultures obtained from Dr. Michelle O'Malley, Department of Chemical Engineering, University of California, Santa Barbara, available upon request
Chytridiomycota	*Powellomyces hirtus* strain BR81	James et al. (2006)
Blastocladiomycota	*Paraphysoderma sedebokerense* strain JEL821	James et al. (2006)
Hetereokontophyta	*Aphanomyces euteiches* strain P22	ATCC 201684 and from Laboratorie de Recherche en Sciences Végétales, Castanet-Tolosan, France
Hetereokontophyta	*Pythium ultimum* strain Grav	Cultures obtained from Dr. Brian Hudelson and Armila Francis, the Department of Plant Pathology, University of Wisconsin-Madison, available upon request
Hetereokontophyta	*Phytophthora erythroseptica*	Cultures obtained from Dr. Brian Hudelson and Armila Francis, the Department of Plant Pathology, University of Wisconsin-Madison, available upon request

Supplemental Table 2: Conditions and methods used for the extraction of fungal exudates.

Species	Media	Growth Time Periods	Growth Temperature
Amanita muscaria **strain Koide BX008**	Floated on Cellophane with Sterile MilliQ Water and Grown in Rich Medium	5 days/ 1 month	25C

Amanita thiersii strain Skay 4041	Floated on Cellophane with Sterile MilliQ Water and Grown in Rich Medium	5 days/ 1 month	25C
Amanita thiersii strain Skay 4041 HET	Floated on Cellophane with Sterile MilliQ Water and Grown in Rich Medium	5 days/ 1 month	25C
Dirkmeia sp. nov. strain TAR 520	Potato Dextrose Broth	5 days	25C
Fereydounia khargensis strain TAR 509	Potato Dextrose Broth	5 days	25C
Gloeocystidiellum convolvens (syn: *Gloeopeniophorella convolvens*)	Potato Dextrose Broth		
Hebeloma cylindrosporum strain h7	Floated on Cellophane with Sterile MilliQ Water and Grown in Modified Melin-Norkran's Medium	5 days/ 1 month	25C
Laccaria bicolor strain S238N	Floated on Cellophane with Sterile MilliQ Water and Grown in Modified Melin-Norkran's Medium	5 days/ 1 month	25C
Lactarius deliciosus	Potato Dextrose Broth		
Lactarius populinus	Potato Dextrose Broth		
Lactarius psammicola	Potato Dextrose Broth		
Multifurca ochricompacta	Potato Dextrose Broth		
Paxillus adelphus strain Ve08.2h10	Floated on Cellophane with Sterile MilliQ Water	5 days	25C
Paxillus ammoniavirescens strain Pou09.2	Floated on Cellophane with Sterile MilliQ Water and Grown in Modified Melin-Norkran's Medium	5 days/ 1 month	25C
Paxillus involutus strain Bel09	Floated on Cellophane with Sterile MilliQ Water and Grown in Modified Melin-Norkran's Medium	5 days/ 1 month	25C
Rhizcotonia solani strain LP2	Potato Dextrose Broth	5 days	25C

Rhodotorula mucilaginosa **strain AR 1356-LV16**	Potato Dextrose Broth	5 days	25C
Russula amonenolens	Potato Dextrose Broth		
Russula cerolens	Potato Dextrose Broth		
Russula praetervisa (syn: *Russula pectinoides)*	Potato Dextrose Broth		
Russula redolens	Potato Dextrose Broth		
Russula sanguinaria (syn: *Russula sanguinea)*	Potato Dextrose Broth		
Typhula incarnata **strain OJN**	Potato Dextrose Broth	1 month	4C
Ustilago sparsa **strain TAR 523**	Potato Dextrose Broth	5 days	25C
Aspergillus flavus **strain NRRL 3377**	Glucose Minimal Medium	24 hours or 96 hours	37C
Aspergillus fumigatus **strain AF293**	Glucose Minimal Medium	24 hours or 96 hours	37C
Aspergillus nidulans **strain RDIT 9.32**	Glucose Minimal Medium	24 hours or 96 hours	30C
Aureobasidium pullulans **strain 222**	Potato Dextrose Broth	5 days	25C
Candida albicans **strain SN250**	Potato Dextrose Broth	5 days	25C
Candida auris	Potato Dextrose Broth	5 days	25C
Candida glabrata **strain C60G**	Potato Dextrose Broth	5 days	25C
Cenococcum geophilum **strain WSL 1.58**	Floated on Cellophane with Sterile MilliQ Water on soild CG Medium	2 to 3 weeks on solid medium then transferred liquid exudates were incubated for and additional 4 weeks	24C
Clarireedia homoeocarpa (**Syn:** *Sclerotinia homoeocarpa*) **strain NL 715**	Potato Dextrose Broth	5 days	25C
Glonium stellatum **strain CBS207.34**	Floated on Cellophane with Sterile MilliQ Water on soild MP/MPm Medium	2 to 3 weeks on solid medium then transfered liquid exudates were incubated for and additional 4 weeks	24C

Lepidopterella palustris	Floated on Cellophane with Sterile MilliQ Water on soild MP/MPm Medium	2 to 3 weeks on solid medium then transferred liquid exudates were incubated for and additional 4 weeks	24C
Leptosphaeria maculans **strain V23 1.3 (JN3)**	Floated on Cellophane with Sterile MilliQ Water on soild MP/MPm Medium	2 to 3 weeks on solid medium then transferred liquid exudates were incubated for and additional 4 weeks	24C
Ophiostoma novo-ulmi	Potato Dextrose Broth	5 days	25C
Protomyces inouye **strain 1321**	Potato Dextrose Broth	5 days	25C
Saccharomyces cerevisiae **strain INV Sc1**	Potato Dextrose Broth	5 days	25C
Saccharomyces cerevisiae **strain yHD0554 (S288c)**	Potato Dextrose Broth	5 days	25C
Sclerotinia sclerotiorum **strain 1980**	Potato Dextrose Broth	5 days	25C
Taphrina americana **strain 1380**	Potato Dextrose Broth	5 days	25C
Trichoderma harizanum	Potato Dextrose Broth	5 days	25C
Gigaspora rosea	Floated on Cellophane with Sterile MilliQ Water	5 days	25C
Mortierella elongata **strain NVPG4**	2% Malt Extract Broth	5 days	25C
Mortierella minutissima **strain ADO51**	2% Malt Extract Broth	5 days	25C
Mortierella **sp. nov. strain GBAus27b**	2% Malt Extract Broth	5 days	25C
Mucor hiemalis **strain TAR 706**	Potato Dextrose Broth	5 days	25C
Rhizophagus clarus	Floated on Cellophane with Sterile MilliQ Water	5 days	25C
Rhizophagus intraradices	Floated on Cellophane with Sterile MilliQ Water	5 days	25C

Rhizophagus irregularis	Floated on Cellophane with Sterile MilliQ Water	5 days	25C
Rhizopus ruoxii (**syn: Mucor ruoxii**)	Potato Dextrose Broth	5 days	25C
Anaeromyces robustus **strain S4**	0.2g switchgrass in modified MC' medium (without yeast extract or bacto casitone)	144 hours	39C
Caecomyces churrovis **strain Ceco**	0.2g switchgrass in modified MC' medium (without yeast extract or bacto casitone)	144 hours	39C
Entophlyctis luteolus **strain JEL129**	0.2g switchgrass in modified MC' medium (without yeast extract or bacto casitone)	3 weeks	25C
Gonapodya prolifera **strain JEL 478**	Peptonized milk, tryptone, glucose broth	2 to 3 weeks on solid medium then transferred liquid exudates were incubated for and additional 4 weeks	22C
Neocallimastix californiae **strain G1**	0.2g switchgrass in modified MC' medium (without yeast extract or bacto casitone)	144 hours	39C
Piromyces finnis **strain Finn**	0.2g switchgrass in modified MC' medium (without yeast extract or bacto casitone)	144 hours	39C
Powellomyces hirtus **strain BR81**	Peptonized milk, tryptone, glucose broth	3 weeks	25C
Paraphysoderma sedebokerense **strain JEL821**	Peptonized milk, tryptone, glucose broth	3 weeks	25C
Aphanomyces euteiches **strain P22**	Corn Meal Broth	5 days	25C
Pythium ultimum **strain Grav**	Corn Meal Broth	5 days	25C
Phytophthora erythroseptica	Corn Meal Broth	5 days	25C

Supplemental Table 3: Differentially expressed genes for 30mpi and 120mpi.

Target ID	Function	Time Point	pval	qval	beta value	Regulation
Afu1g01350	Unknown	30 min	5.93E-11	2.84E-08	0.780818569	up
Afu1g04310	Hypothetical protein	30 min	2.13E-12	1.76E-09	0.865142263	up
Afu1g13550	Conserved hypothetical protein	30 min	5.80E-14	6.97E-11	0.606252692	up
Afu2g13890	Annexin ANXC3.2	30 min	6.57E-11	2.99E-08	0.967639169	up
Afu3g13440	Stomain family protein	30 min	6.13E-14	6.97E-11	0.575964158	up
Afu4g06920	DNAJ domain protein	30 min	4.07E-12	3.09E-09	0.461825957	up
Afu5g02500	Conserved hypothetical protein	30 min	1.15E-19	1.05E-15	1.31309033	up
Afu5g02800	C6 transcription factor	30 min	2.04E-18	9.29E-15	-0.578649006	down
Afu6g06590	DNAJ chaperone (Caj1)	30 min	3.19E-13	3.22E-10	0.508189388	up
Afu6g08270	Hypothetical protein	30 min	5.81E-12	4.07E-09	0.443772783	up
Afu6g10940	Conserved hypothetical protein	30 min	7.62E-16	1.74E-12	0.999595932	up
Afu8g05720	DUF567 domain protein	30 min	1.54E-15	2.34E-12	0.642549549	up
Afu1g01600	Deoxyribodipyrimidine photo-lyase Phr1	120 min	1.87E-13	1.53E-10	0.437511769	up
Afu1g02590	RNA polymerase I specific transcription initiation factor RRN3 superfamily	120 min	3.19E-13	2.22E-10	-0.481228188	down
Afu1g05690	Hypothetical protein	120 min	3.74E-16	5.63E-13	0.519936048	up
Afu1g13510	C6 transcription factor FacB/Cat8	120 min	2.44E-12	1.16E-09	0.402012445	up
Afu1g14640	Conserved hypothetical protein	120 min	2.87E-08	2.65E-06	-0.421029944	down

Afu2g01980	Nuclear protein (Sgd1)	120 min	1.48E-10	3.27E-08	-0.463036528	down
Afu2g02480	Conserved hypothetical protein	120 min	4.95E-12	2.03E-09	0.40131908	up
Afu2g03490	Calcium/calmodulin-dependent protein kinase	120 min	2.33E-16	5.05E-13	0.412415253	up
Afu2g06000	NAD+ dependent glutamate dehydrogenase	120 min	2.99E-38	2.70E-34	0.969996574	up
Afu2g10240	NAD binding Rossmann fold oxidoreductase	120 min	2.80E-16	5.05E-13	0.424406347	up
Afu2g17060	60S ribosome biogenesis protein Nip7	120 min	8.74E-15	9.87E-12	-0.472276622	down
Afu4g07500	Small nuclear ribonucleoprotein complex subunit	120 min	4.18E-15	5.39E-12	-0.43728531	down
Afu5g11110	Thiamin pyrophosphokinase-related protein	120 min	1.12E-18	3.36E-15	0.786919105	up
Afu6g12160	C6 transcription factor	120 min	1.78E-26	8.03E-23	0.57621974	up

2.9 Supplemental Figures
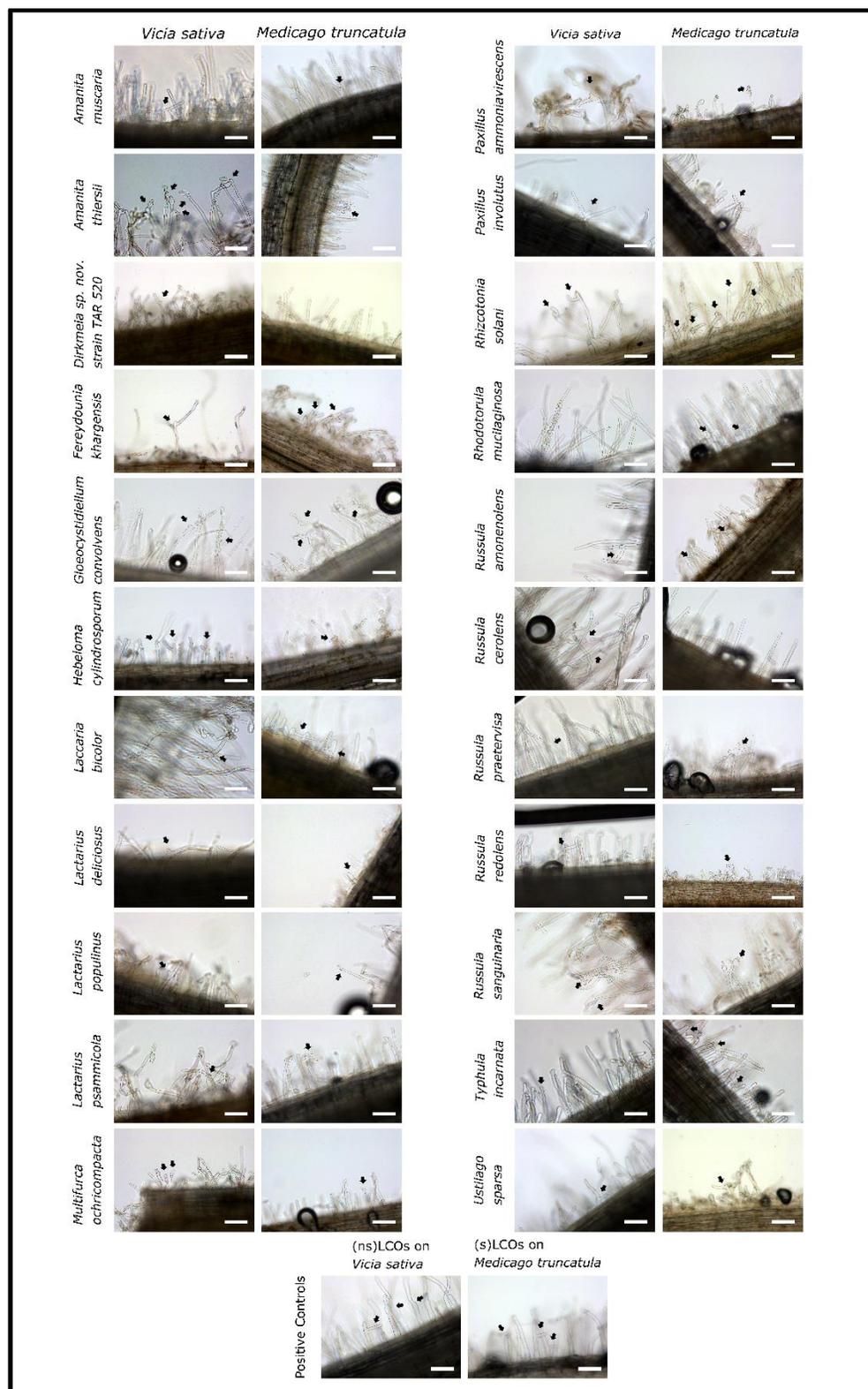
Supplemental Figure 1 **Bioassay results in Basidiomycota**

Filtered exudates from 22 species were applied to 1-week old *Vicia sativa* and *Medicago truncatula* accession Jemalong A17. After 48 hours at 25°C, roots were checked for root hair branches. Black arrows indicate root hair branch(es) were observed, and the lack of black arrows means no root hair branches were found. Scale bar is 1 µm. Positive controls are an application of a concentration of 10^{-8} M of (ns)LCOs on *V. sativa* and (s)LCOs on *M. truncatula*.

Vicia sativa   *Medicago truncatula*   *Vicia sativa*   *Medicago truncatula*

Aspergillus flavus

Aspergillus fumigatus

Aspergillus nidualns

Aureobasidium pullulans

Candida albicans

Candida auris

Candida glabrata

Clarireedia homoeocarpa

Ophiostoma novo-ulmi

Protomyces inouye

Saccharomyces cerevisiae strain INV Sc1

Saccharomyces cerevisiae strain yHD0554 (S288c)

Sclerotinia sclerotiorum

Taphrina americana

Trichoderma harizanum

Positive Controls

(ns)LCOs on *Vicia sativa*

(s)LCOs on *Medicago truncatula*
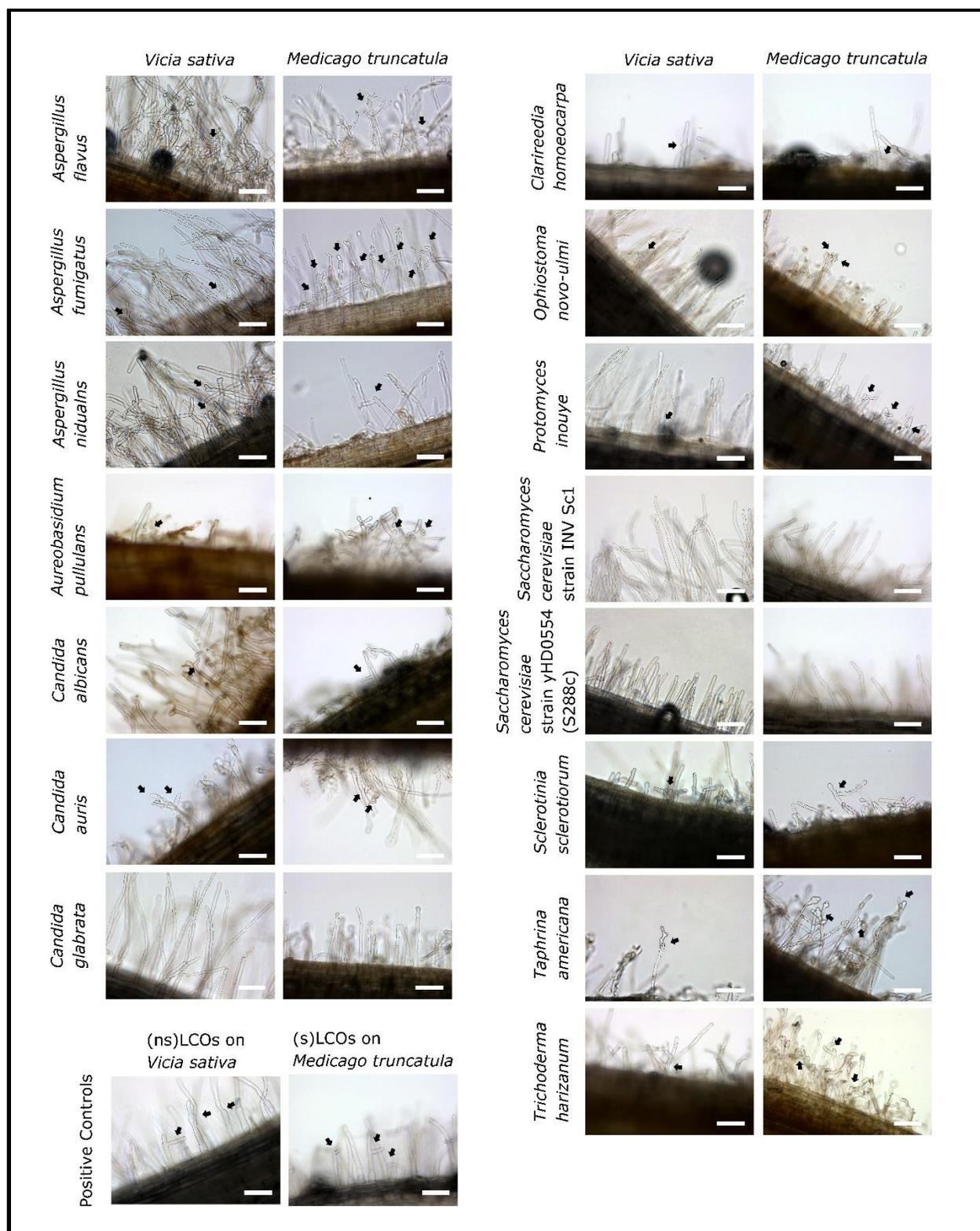
Supplemental Figure 2 **Bioassay results in Ascomycota**

Filtered exudates from 15 species were applied to 1-week old *Vicia sativa* and *Medicago truncatula* accession Jemalong A17. Two different strains (yHD0554 and INV Sc1) were examined for *Saccharomyces cerevisiae.* After 48 hours at 25°C, roots were checked for root hair branches. Black arrows indicate root hair branch(es) were observed, and the lack of black arrows means no root hair branches were seen. Scale bar is 1 µm. Positive controls are an application of a concentration of 10^{-8} M of (ns)LCOs on *V. sativa* and (s)LCOs on *M. truncatula*.

Vicia sativa *Medicago truncatula*

Mortierella elongata

Mortierella minutissima

Mortierella sp. nov. strain GBAus27b

Mucor hiemalis

Rhizophagus irregularis

Rhizopus ruoxii

Positive Controls

(ns)LCOs on *Vicia sativa*

(s)LCOs on *Medicago truncatula*
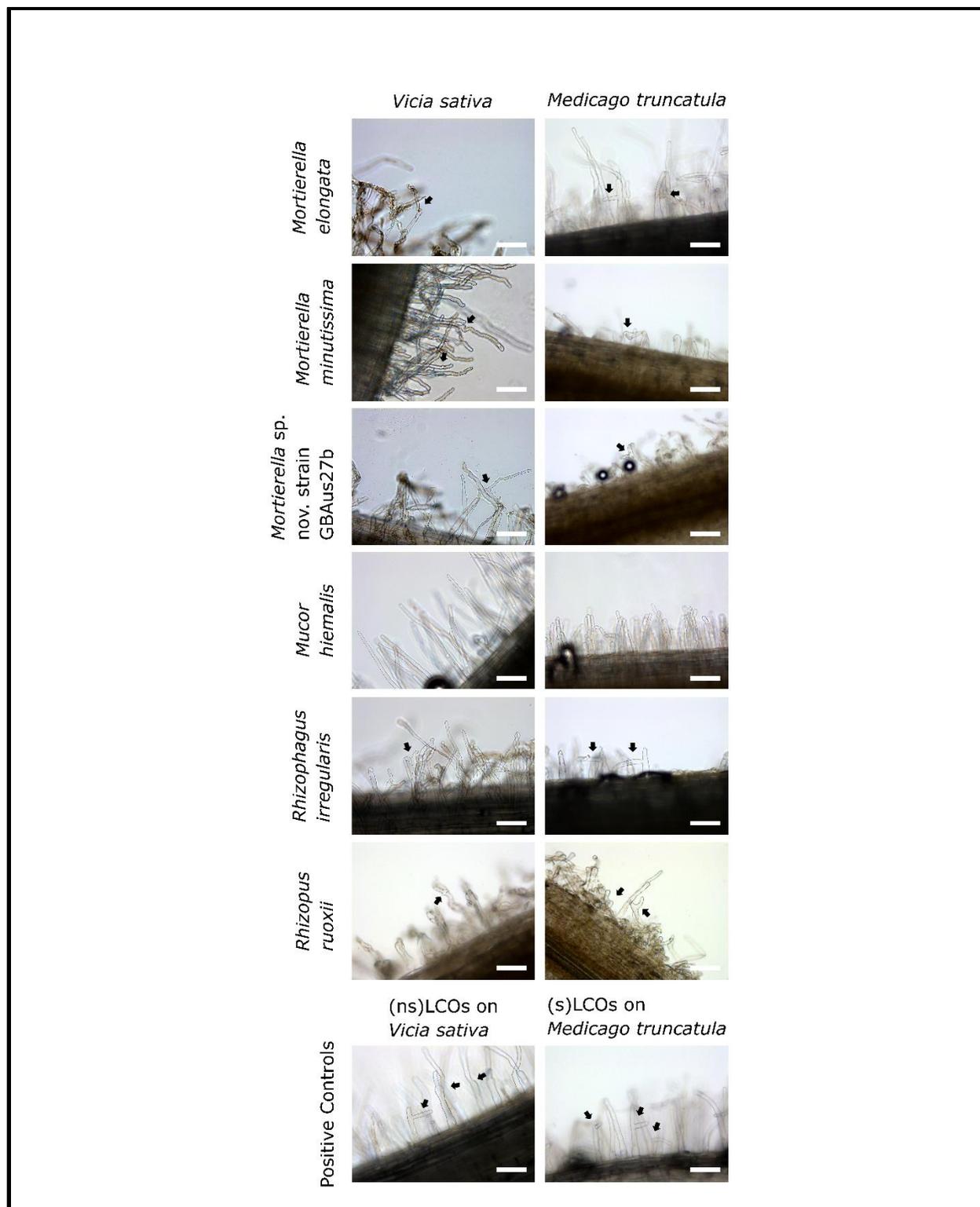
<u>Supplemental Figure 3</u> Bioassay results in Mucoromycota

Filtered exudates from six species were applied to 1-week old *Vicia sativa* and *Medicago truncatula* accession Jemalong A17. After 48 hours at 25°C, roots were checked for root hair branches. Black arrows indicate root hair branch(es) were observed, and the lack of black arrows means no root hair branches were found. Scale bar is 1 µm. Positive controls are an application of a concentration of 10^{-8} M of (ns)LCOs on *V. sativa* and (s)LCOs on *M. truncatula*.

Blastocladiomycota

Paraphysoderma sedebokerense

Vicia sativa   *Medicago truncatula*

Chytridiomycota

Anaeromyces robustus

Caecomyces churrovis

Entophlyctis luteolus

Neocallimastix californiae

Piromyces finnis

Powellomyces hirtus

Vicia sativa   *Medicago truncatula*

Positive Controls

(ns)LCOs on *Vicia sativa*   (s)LCOs on *Medicago truncatula*
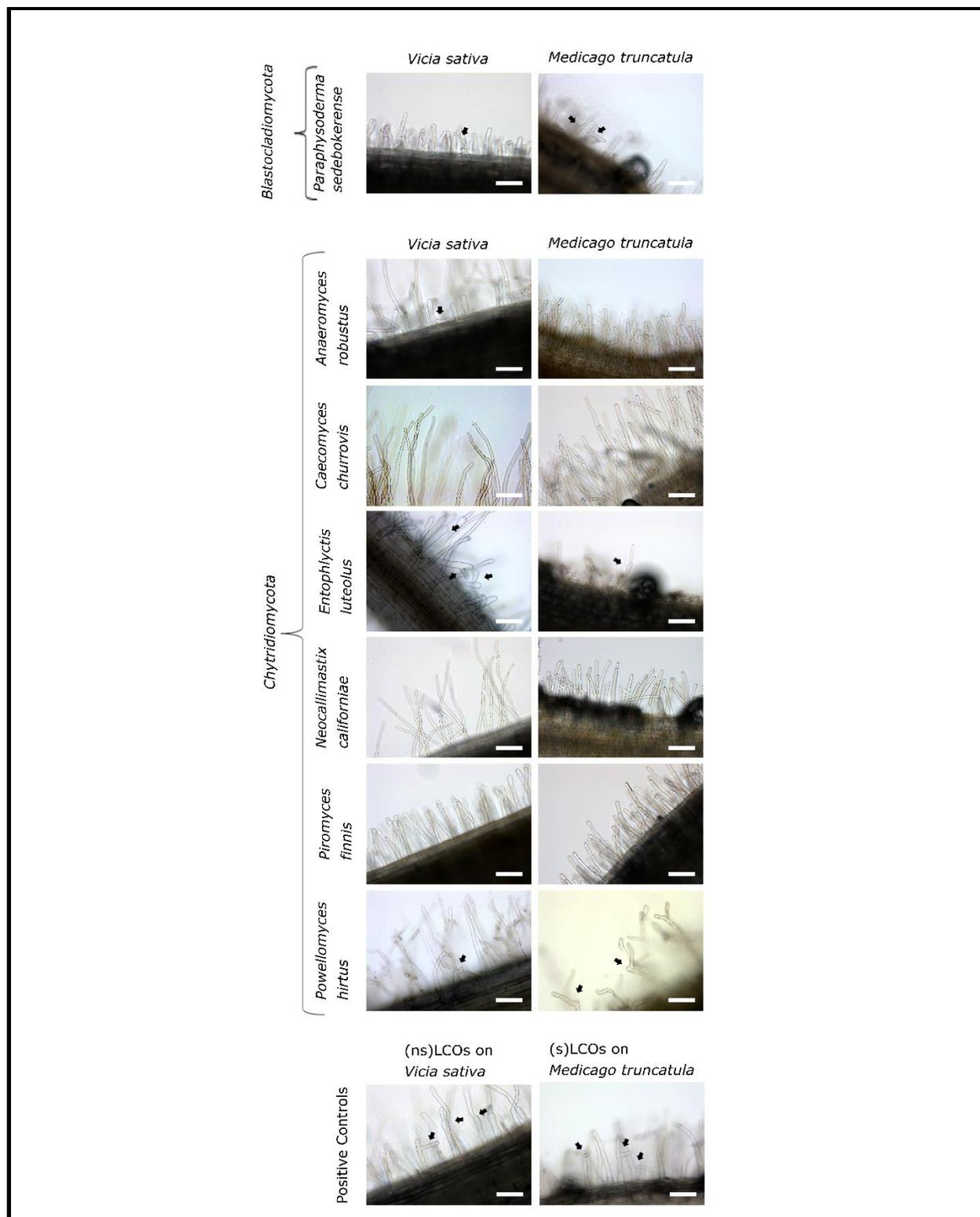
<u>Supplemental Figure 4</u> Bioassay results in Blastocladiomycota and Chytridiomycota

Filtered exudates from one species of Blastocladiomycota and six species of Chytridiomycota were applied to 1-week old *Vicia sativa* and *Medicago truncatula* accession Jemalong A17. After 48 hours at 25°C, roots were checked for root hair branches. Black arrows indicate root hair branch(es) were observed, and the lack of black arrows means no root hair branches were found. Scale bar is 1 µm. Positive controls are an application of a concentration of 10^{-8} M of (ns)LCOs on *V. sativa* and (s)LCOs on *M. truncatula*.

Vicia sativa　　*Medicago truncatula*

Aphanomyces euteiches

Pythium ultimum

Phytophthora erythroseptica

Positive Controls

(ns)LCOs on *Vicia sativa*　　(s)LCOs on *Medicago truncatula*
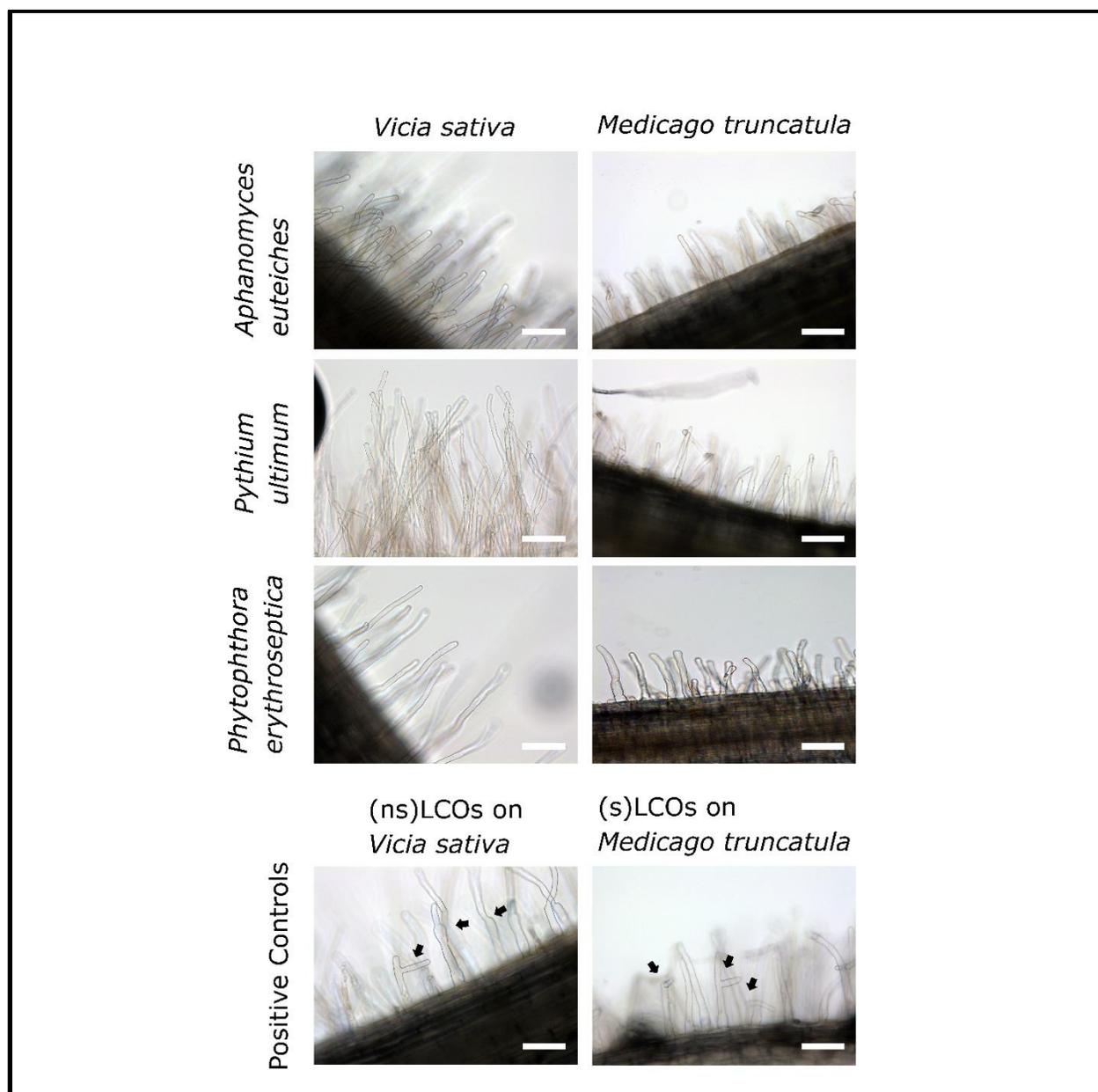
Supplemental Figure 5 Bioassay results in Oomycetes

Filtered exudates from three species were applied to 1-week old *Vicia sativa* and *Medicago truncatula* accession Jemalong A17. After 48 hours at 25°C, roots were checked for root hair branches. Black arrows indicate root hair branch(es) were observed, and the lack of black arrows means no root hair branches were seen. Scale bar is 1 µm. Positive controls are an application of a concentration of 10^{-8} M of (ns)LCOs on *V. sativa* and (s)LCOs on *M. truncatula*.

Vicia sativa

Medicago truncatula

CO4

CO5

CO8

(ns)LCOs on
Vicia sativa

(s)LCOs on
Medicago truncatula

Positive Controls
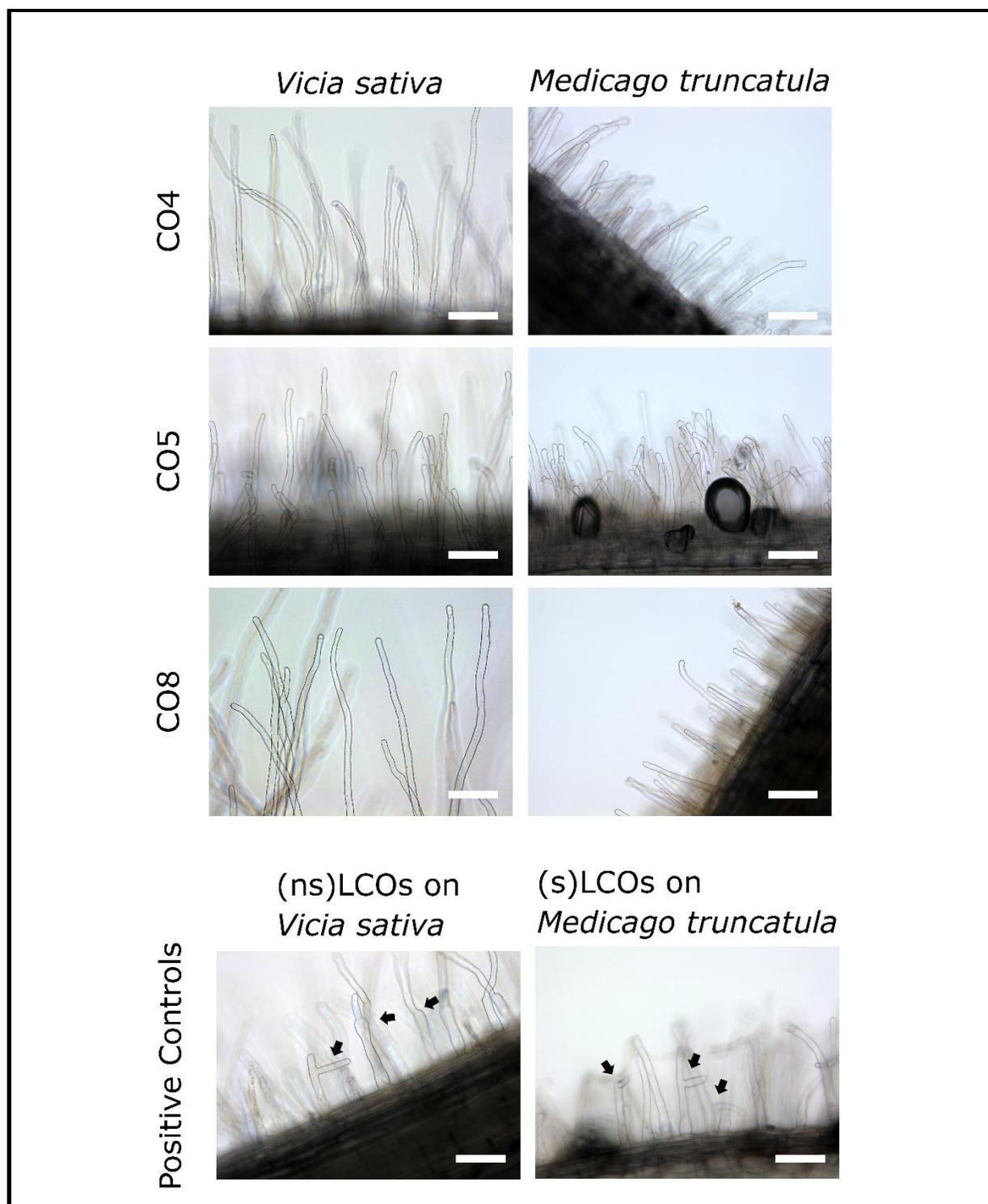
<u>Supplemental Figure 6</u> Bioassay results with chitooligosaccharides application

Purified 10^{-8} M concentrations of different types of chitooligosaccharides were applied to 1-week old *Vicia sativa* and *Medicago truncatula* accession Jemalong A17. After 48 hours at 25°C, roots were checked for root hair branches. Black arrows indicate root hair branch(es) were observed, and the lack of black arrows means no root hair branches were seen. Scale bar is 1 µm. Positive controls are an application of a concentration of 10^{-8} M of (ns)LCOs on *V. sativa* and (s)LCOs on *M. truncatula*.

Vicia sativa *Medicago truncatula* *Vicia sativa* *Medicago truncatula*

Modified MC Medium

Modeified Melin-Norkran Medium

2% Malt Extract Broth

PmTG Broth

Corn Meal Broth

Potato Dextrose Broth

Glucose Minimal Medium

0.005% EtOH (Control)

Positive Controls

(ns)LCOs on *Vicia sativa*

(s)LCOs on *Medicago truncatula*
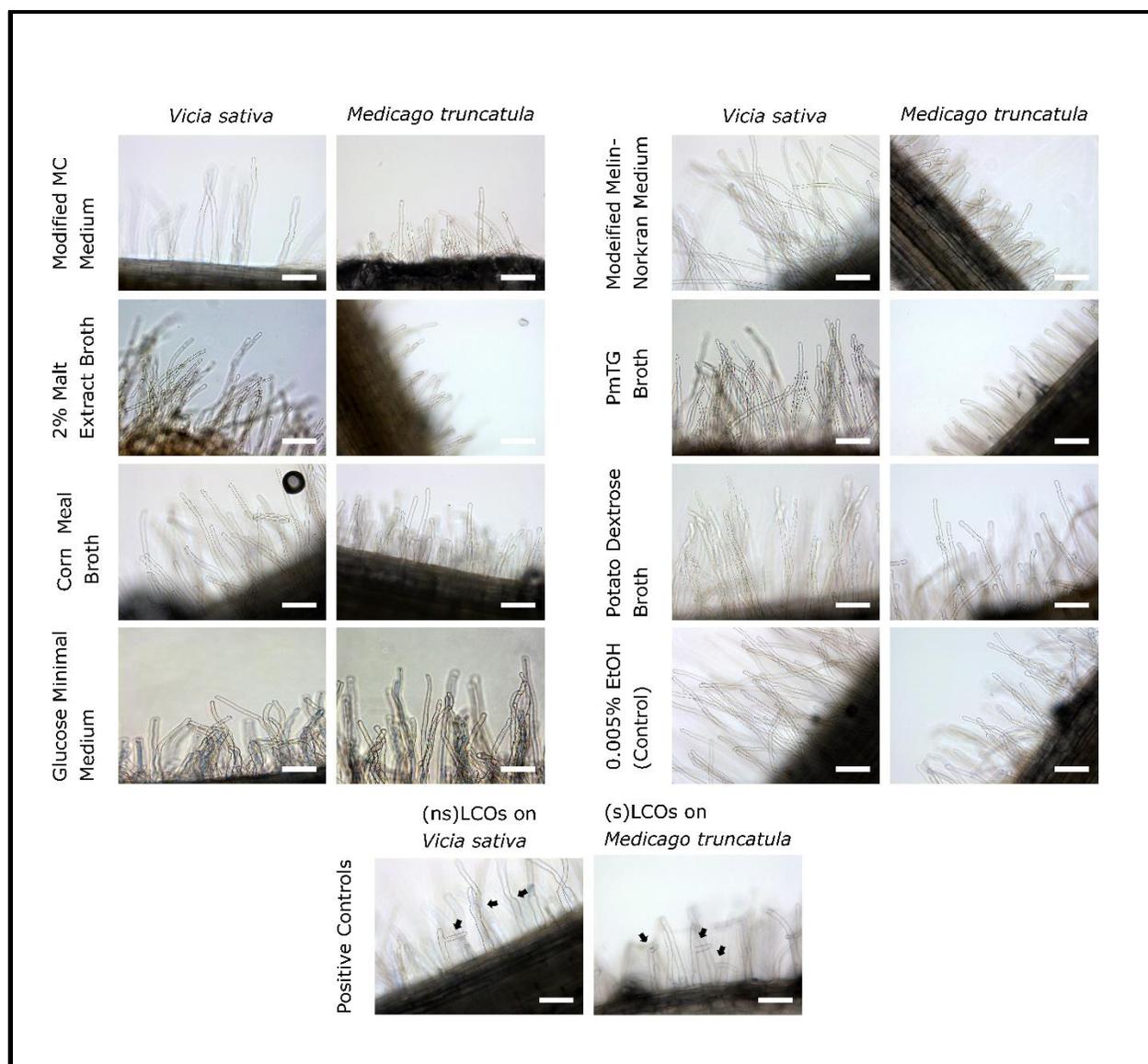
<u>Supplemental Figure 7</u> Bioassay results with liquid broth medium and negative control applications

Filtered liquid broth medium used for all species tested in these experiments and 0.005% of EtOH in sterile MilliQ water were applied to 1-week old *Vicia sativa* and *Medicago truncatula* accession Jemalong A17. After 48 hours at 25°C, roots were checked for root hair branches. Black arrows indicate root hair branch(es) were observed, and the lack of black arrows means no root hair branches were seen. Scale bar is 1 µm. Positive controls are an application of a concentration of 10^{-8} M of (ns)LCOs on *V. sativa* and (s)LCOs on *M. truncatula*.

A Basidiomycota

B Ascomycota

C Mucoromycota

D Blastocladiomycota and Chytridiomycota

E Oomycetes

F Fungi used for COs and LCOs confirmation through HPLC/MS

Supplemental Figure 8 Confirmation of fungal samples are not contaminated with bacteria.

DNA extraction of all fungal (**A** to **D**) and oomycete **(E)** samples was amplified with fungal-specific ITS primers (ITS1F and ITS4) as described in (Gardes and Bruns *et al.,* 1993; White *et al.,* 1990) and amplified with bacterial specific 16S primers (fD1/rP2) as described in (Weisburg *et al*., 1991). *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* were used the positive bacterial controls since we were mostly concerned about contaminations with rhizobia. Sterile MilliQ water which was used to make the master mix for PCR amplification was used a negative control. The ladder is 1kb. **(F)** Fungi used in the HPLC/MS was also confirmed using fungal-specific primers (ITS1 and ITS4) and bacterial-specific primers (Fd1/Rp2) with *Providencia rettgeri* and *Vicia sativa* nodules inoculated with rhizobia bacteria as positive controls.

A

Apical Branch

B

C

D

Secondary Branches
from Apical Branch

E

F
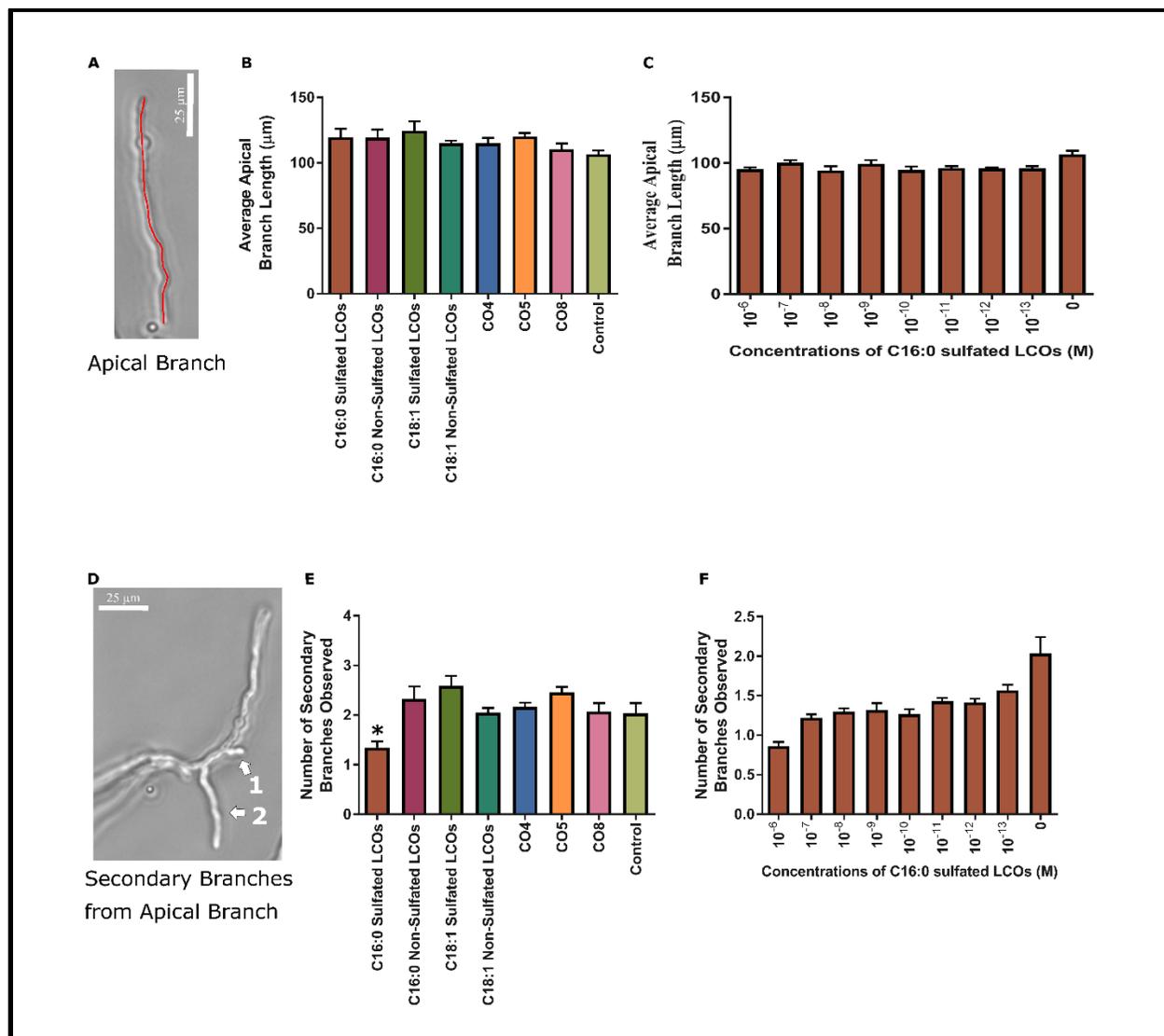
Supplemental Figure 9 **Apical branch length and number of secondary branches assays for**

Aspergillus fumigatus

(A) The apical branch is the primary hyphae germinated on one or both side of a spore (as shown in red). Scale bar is 25 µm. (B) Length of apical branches of *Aspergillus fumigatus* hyphae in response to treatments with COs or LCOs at 10^{-8} M. All treatments had eight replications where each replication had 100 branches observed. (C) Length of apical branches of *Aspergillus fumigatus* hyphae in response to a treatment with various concentrations of sulfated C16:0 LCOs. All levels had six replications where each replication had 100 branches observed. (D) Secondary branches are branches that stem from the apical branch as denoted by the white arrows with numbers. Scale bar is 25 µm. (E) Number of secondary branches per spore in response to treatments with COs or LCOs at 10^{-8} M. ANOVA P-value is <0.0001 and Dunnett's multiple comparison procedures had a P-value <0.0001. (*) denotes significant difference from the control group. (F) Number of secondary branches per spore in response to treatments with various concentrations of sulfated C16:0 LCOs. All levels had six replications where each replication had 100 branches observed.
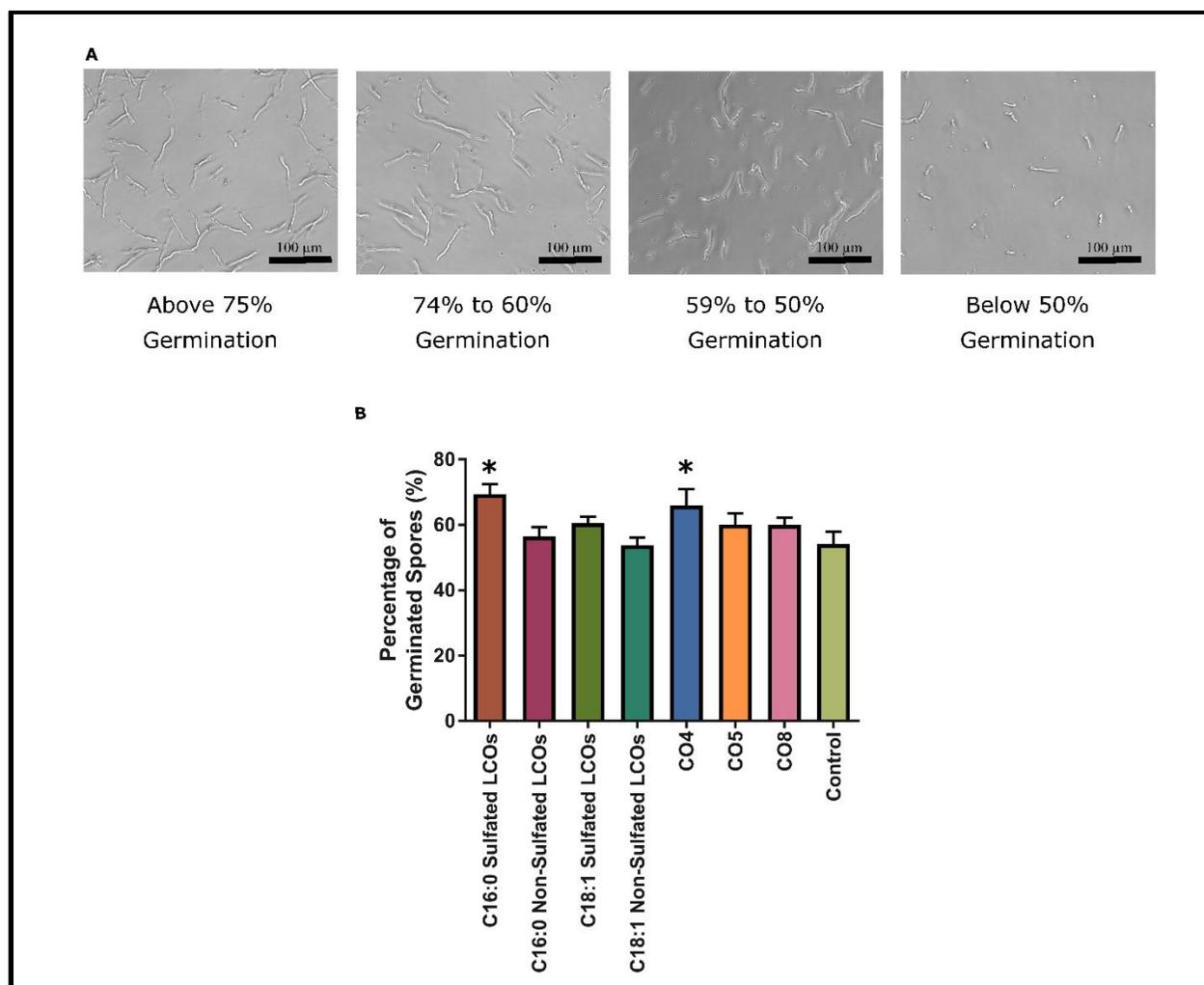
Supplemental Figure 10 Germination assays for *Aspergillus fumigatus*

(**A**) Examples of germination rates for *A. fumigatus* spores after 10 hours of treatments with COs and LCOs. Scale bar is 100 µm. (**B**) Percentage of germinated spores after treatment with COs and LCOs at 10^{-8} M. Over 3,000 spores were observed per treatment. ANOVA P-value is <0.0001 and Dunnett's multiple comparison procedures had a P-value <0.0001. (*) denotes significant difference from the control group.
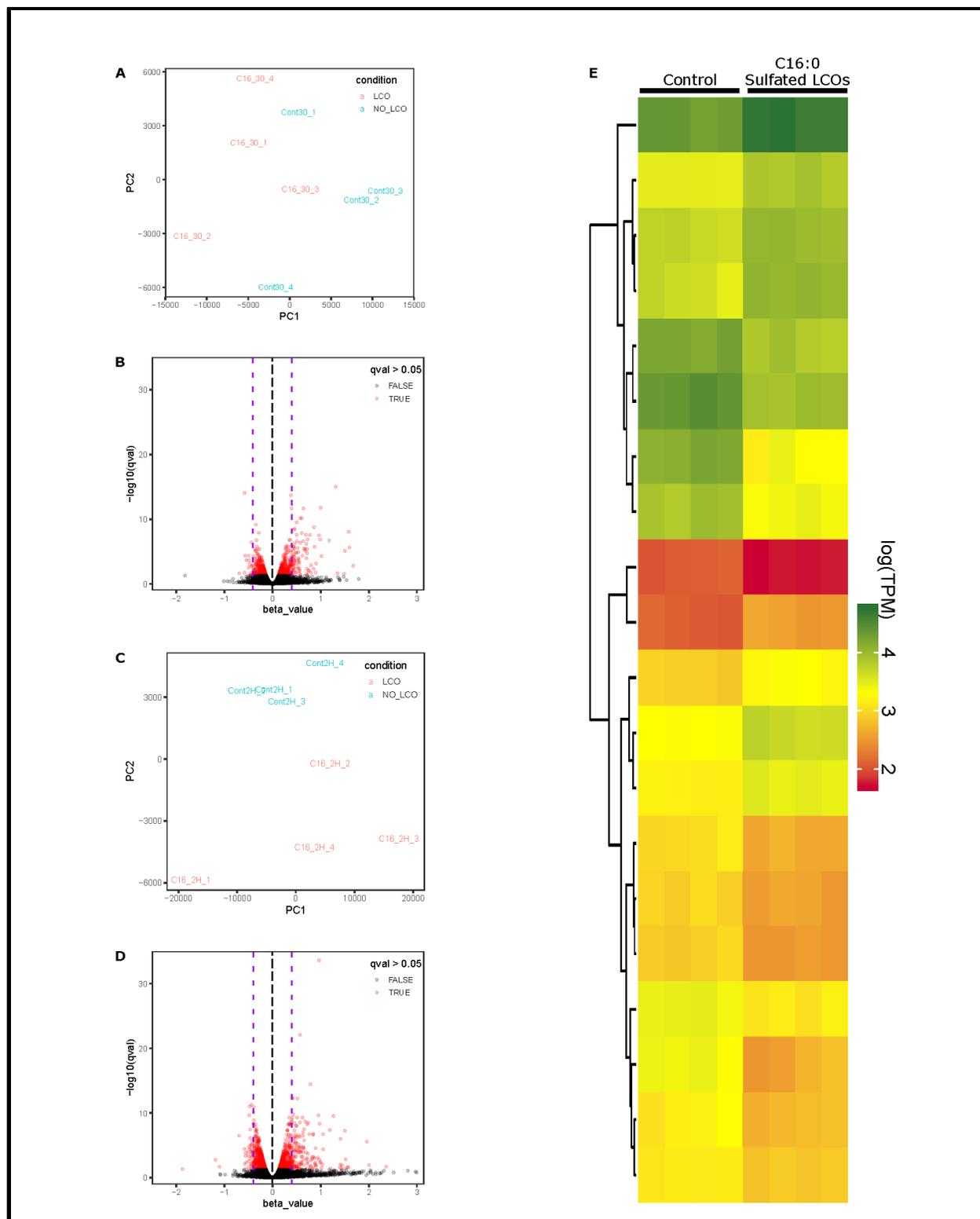
Supplemental Figure 11: **Differentially expressed genes (DEGs) when treated with 10^{-8} M sulfated C16:0 LCOs *vs.* control treatments.**

(**A**) Principal component analysis for the four replications used at 30 minutes post-treatment. PC1 represents 50% variation and PC2 represents 20% variation. (**B**) Significant DEGs with the -log10(q-value) and beta value for genes expressed below -0.4 or higher than 0.4 after 30 minutes. (**C**) Principal component analysis for the four replications used at 120 minutes. PC1 represents 75% variation and PC2 represents 15% variation (**D**) Significant DEGs with the -log10(q-value) and beta value for genes expressed below -0.4 or higher than 0.4 after 120 minutes. (**E**) Transcriptomic analyses revealed gene expression changes at 120 minutes following treatment with LCOs. Heatmaps show the top 20 differentially expressed genes, with the highest expression levels indicated in red and the lowest in green.
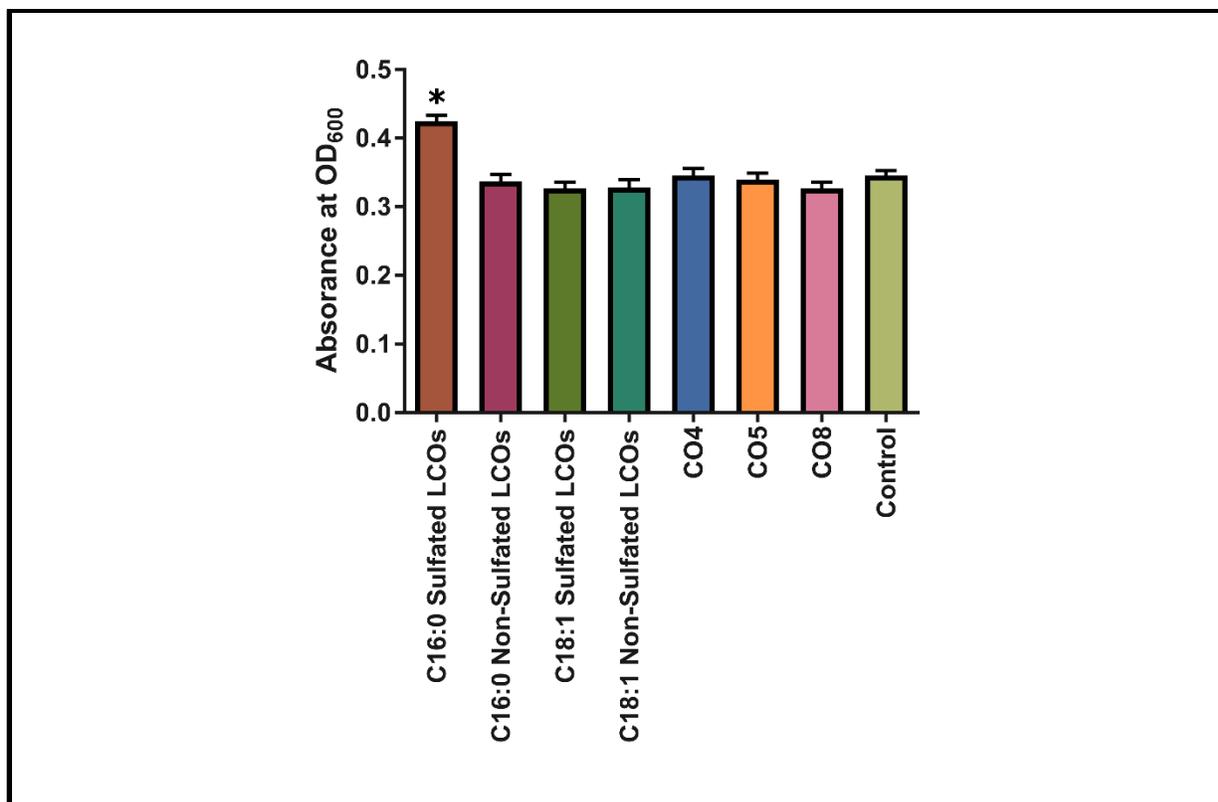
Supplemental Figure 12: LCOs influences on _Rhodotorula mucilaginosa_.

OD_{600} reading after 24 hours growth for three trials with six replications per treatments. Liquid broth medium was potato dextrose. ANOVA P-value is <0.0001 and Dunnett's multiple comparison procedures had a P-value <0.0001. (*) denotes significant difference from the control group.

2.10 Supplemental Movie Legends

<u>**Supplemental Video 1: Pseudohyphal formation and swollen cells in *Candida glabrata***</u>

Swollen cells and pseudohyphal formation are shown after 10 hours post inoculation treated with C18:1 sulfated LCOs with a concentration of 10^{-8} M. Scale bar is 50 µm.

<u>**Supplemental Video 2: A closer view of pseudohyphae formation in *Candida glabrata***</u>

The pseudohyphal formation is shown after 10 hours post inoculation treated with C16:0 sulfated LCOs with a concentration of 10^{-9} M. Scale bar is 10 µm.

<u>**Supplemental Video 3: Z-Stack of extensive pseudohyphae formation in *Candida glabrata***</u>

The 3-D image showing extensive pseudohyphae formation using Z-Stack imaging which takes several photos at different layers of the stage.

2.11 Additional Results

<u>**Additional Results Materials and Methods**</u>

Dry biomass analysis. Fungal growth was also evaluated regarding dry biomass. For each treatment, five 125 mL flasks were prepared with 50 mL of sterile GMM broth. There were two trials with five replication per treatment. Media were inoculated with 10^6 spores of the *A. fumigatus* spore suspension. The cultures were incubated at 25 °C, 250 rpm for 6 days or at 37 °C, 250 rpm for 4 days. After the appropriate incubation period, the fungal balls developed in the broth media were collected in 1.5 µl eppi tubes for samples at 25 °C and in 50 mL Falcon tubes for samples at 37 °C, lyophilized until a constant weight, corresponding to the dry biomass weight, was obtained.

Metabolite profiling using <u>UHPLC-MS</u>. The effect of COs and LCOs treatments on secondary metabolite production by *A. fumigatus* strain Af293 was assessed by UHPLC-MS analysis. About 10^6 fresh spores were grown in 125 mL flasks containing 50 mL of GMM broth

supplemented with the same treatments mentioned earlier. Two different growth conditions were assessed; the first one consisted on an incubation under 25 ℃ and 250 rpm for 6 days, whereas the second consisted on an incubation under 37 ℃ and 250 rpm for 4 days. After the incubation periods, fungal balls were collected and lyophilized to estimate the dry biomass. For secondary metabolite analysis, three milliliters of supernatant were homogenized with 3 mL of chloroform. Organic and aqueous layers were separated by centrifugation at 3000 rpm for 5 min and the organic layer was collected and dried down. Samples were later resuspended in Acetonitrile: Water (50:50) % (v/v) and filtered through an Acrodisc syringe filter with nylon membrane (0.45 µm, Pall Corporation) into 1 mL HPLC vials. Samples were subjected to High-resolution UHPLC-MS analysis.(*30*) Data acquisition and processing for the UHPLC-MS was done using the Thermo Scientific Xcalibur software. Files were converted to .mzXML using MassMatrix MS Data File Conversion grouped by condition, and run in the XCMS open-source package (https://xcmsonline.scripps.edu/).

Additional Results Figure 1: **Metabolite production at 25°C.**

Shows the regulation of metabolite production with significant p-value 0.05 and log2 fold

change. The legend indicates putative secondary metabolites.
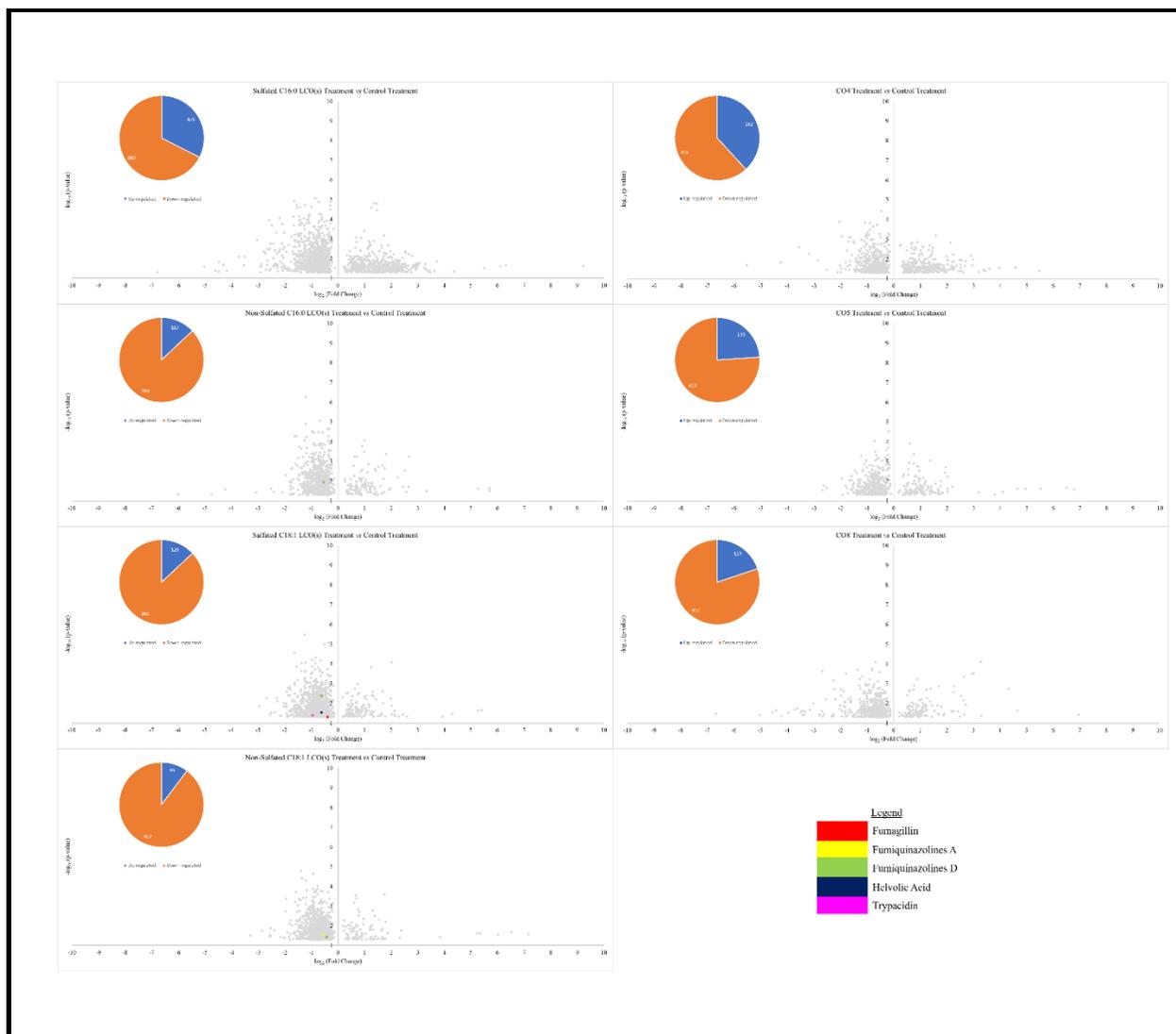
Additional Results Figure 2: Metabolite production at 37ºC.

Shows the regulation of metabolite production with significant p-value 0.05 and log2 fold change. The legend indicates putative secondary metabolites.

A

Aspergillus fumigatus Dry Biomass Weight at 25 C
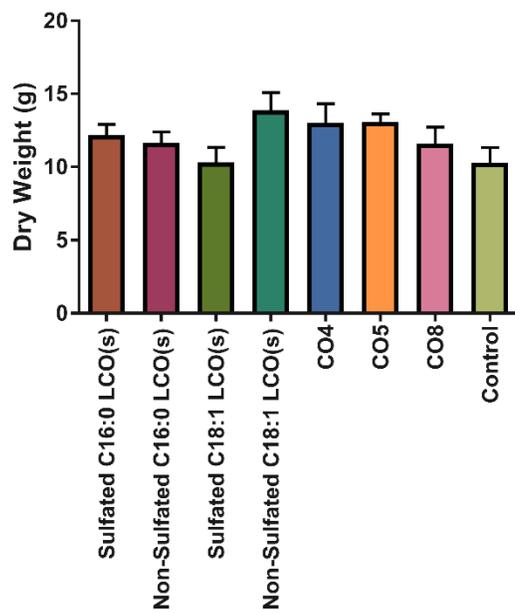
B

Aspergillus fumigatus Dry Biomass Weight at 37 C
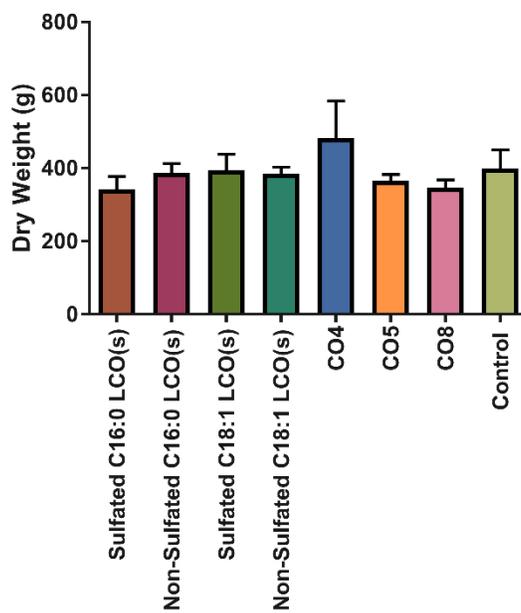
Additional Results Figure 3: Dry biomass weight at 25ºC and 37ºC.

Shows no difference in dry biomass weight between treatments at (**A**) 25ºC or at (**B**) 37ºC.

Additional Results Conclusion:

Several genes associated with primary metabolism were differentially expressed in response to LCOs which probably corresponds to the observed effects on fungal growth and development. Therefore, we investigated further the influence COs and LCOs have in metabolite production. We observed that the application COs or LCOs at 10^{-8} M overall stimulated metabolite production at 25ºC, yet overall reduced metabolite production at 37ºC but there was no effect on total biomass production at both temperatures.

Chapter 3: Homologous rhizobia chitin deactylase genes necessary for lipo-chitooligosaccharides production in fungi.

3.1 Abstract

The biosynthesis of lipo-chitooligosaccharides is well characterized in rhizobia. The nodulation genes (*nod* genes) functions are defined. With the recent discovery that lipo-chitooligosaccharides are produced by most fungi, it is unknown if the *nod* genes from rhizobia are homologous of the putative genes found in fungi. Chitin deacetylase catalyzes a critical step in the biosynthesis of lipo-chitooligosaccharides in rhizobia. Fungi also have genes encoding for chitin deacetylase. We hypothesized that the chitin deactylase in fungi are essential to the production of LCOs in fungi. Through protoplast transformations of the double auxotroph strain of *Aspergillus fumigatus* Af293.6, we knocked out the only two chitin deacetylase genes found in the genome of this species. There was an absence of LCOs detection in the double chitin deactylase mutants based on root hair branching assays in *Medicago truncatula* and *Vicia sativa*. Findings from this study demonstrate the implication of the chitin deacetylase gene in the biosynthesis of LCOs in fungi.

3.2 Introduction

Chitin deactylase had been hypothesized to be involved in the biological attack and defense systems in fungi (Zhao *et al.*, 2010). The presence of chitin deacetylase has been well documented throughout the Fungal Kingdom and was first identified in Mucoromycotina fungus, *Mucor ruoxii* (Araki and Ito, 1975; Zhao *et al.*, 2010). There are two types of chitin deacetylase in fungi, the first one is the chitin deacetylase secreted into the periplasm, which is called intracellular chitin deacetylase; and the second is the chitin deacetylase secreted into the culture medium which is called extracellular chitin deacetylase (Zhao *et al.*, 2010). Fungi that have intracellular chitin

deacetylases are the mucoromycetes, *Absidia coerulea* and *Mucor ruoxii*, whereas fungi with extracellular chitin deacetylase are the ascomycetes, *Colletotrichum lindemuthianum* and *Aspergillus nidulans* (Araki and Ito, 1975; Kauss *et al.*, 1983; Alfonso *et al.*, 1995; Gao *et al.*, 1995). Moreover, chitin deacetylase has been found to be highly expressed in fungi during certain stages of the fungus lifecycle for example during sporulation of *Saccharomyces cerevisiae*, during the vegetative growth of *Cryptococcus neoformans*, and during the development of the fruiting body in *Flammulina velutipes* (Christodoulidou *et al.*, 1999; Baker *et al.*, 2007; Yamada *et al.*, 2008). Experiments for the characterization of the mechanisms for the formation of chitin deacetylase were conducted in *Mucor ruoxii* and *Colletotrichum lindemuthianum,* through which they conclude that only a CO-III and CO-IV could be deacetylated (Tokuyasu *et al.*, 1997, 2000; Zhao *et al.*, 2010). The role of chitin deacetylase has been studied in *Saccharomyces cerevisiae* that harbor two chitin deacetylase genes (*cda1* and *cda2*). In this yeast strain, it has been speculated that chitin deacetylases are important for the rigidity and structure integrity of the cell wall (Mishra *et al.*, 1997; Zhao *et al.*, 2010). Moreover, *Candida neoformans* has four putative chitin deacetylase genes which are *cda1, cda2, cda3,* and *fpd1*. In this species, it has been determined that *cda1* is required for fungal pathogenesis (Upadhya *et al.*, 2018). In the rice pathogen, *Magnaporthe oryzae,* there are ten chitin deacetylase genes (*cda1-10*), and it was determined that *cda1* is necessary for chitin deacetylation in the septa and lateral cell walls of mature hyphae and for resistance to cell wall hydrolysis (Geoghegan & Gurr, 2017). In this same study, the *cda1-4* deletion mutant did not show differences in growth and development or pathogenicity compared to the wild type strain (Geoghegan & Gurr, 2017). In a separate study, chitin deacetylase was confirmed to be necessary for *Cbp1* activation*,* which is a gene required for appressorium formation (Kuroki *et al.*, 2017).

Despite the number of publications about the role of chitin deacetylase in fungi, none of them investigated the role of these genes in *Aspergillus fumigatus*. Based on our previous study by Rush *et al.,* (unpublished), the widespread presence of LCOs in the Fungal Kingdom, and the impact of these molecules on *A. fumigatus* growth and development, we propose this species as the model organism to understand the mechanisms responsible for LCO production and identify the genes implicated in their biosynthetic pathway.

Aspergillus fumigatus is a saprotroph widespread in nature, typically found in soil on decaying material or is airborne (Latgé, 1999). However, it is well studied because it can be an opportunistic human pathogen in immunocompromised patients, causing aspergillosis (Latgé, 1999). Aspergillosis can cause illnesses like asthma, cystic fibrosis, sarcoidosis, and chronic obstructive pulmonary disease. *Aspergillus fumigatus* is also known to produce lethal mycotoxins (Latgé, 1999). Most importantly, *A. fumigatus* is not a symbiotic fungus with plants, yet it produces LCOs as shown through biological assays and HPLC/MS (Rush *et al.,* unpublished). Moreover, *A. fumigatus* changes its behavior, growth, and development when exposed to exogenous synthesized LCOs. *Aspergillus fumigatus* displayed hypobranching, increased germination, different regulation of metabolite production, and differences in gene expression when exposed to 10^{-8} M of sulfated C16:0 LCOs (Rush *et al.,* unpublished). The complete genome of *Aspergillus fumigatus* strain Af293 has been sequenced and contains over 9,000 genes. Among these genes, the putative fungal *nod*A/B/C-like genes are present. There are eight *nodC* genes, two *nodB* genes, and 119 *nodA* genes identified. The two NodB proteins showed 76% and 61% homology with proteins from *Sinorhizobium meliloti*. Since *Aspergillus fumigatus* produces and response to LCOs, contain the putative *nod* genes, we were interested in testing the hypothesis that *nodB* is essential for LCO production in fungi.

Herein, we present evidence that chitin deactylase is essential to produce LCOs in fungi through a double knockout chitin deacetylase mutant in *A. fumigatus* strain Af293 and confirmation through biological assays.

3.3 Materials and Methods

3.3.1 Strain construction and confirmation

Construction of CDA1 and CDA2 deletion cassettes: The predicted sequences for the *A. fumigatus* CDA1 and CDA2 orthologs were obtained from AspGD (gene accession numbers *Afu5g11410* (XP_753508) and *Afu4g09940* (XP_751832), respectively) by conducting a protein BLAST search with a query coverage of 76% with the *Sinorhizobium meliloti* (WP_127634383) CDA1 and a query coverage of 61% with *Sinorhizobium meliloti* (WP_100674671) CDA2 amino acid sequences against the *A. fumigatus* genome scaffolds. The *A. fumigatus* CDA1 ortholog displayed 27.78% identity to the *Sinorhizobium meliloti* protein, whereas the CDA2 ortholog displayed 32.72% identity to the protein sequence from the same bacterial strain. This strain of *Sinorhizobium meliloti* was shown to be involved in the signal synthesis of LCOs and has been reported to be chitin deacetylase (John *et al.*, 1993). Deletion of both *cda1* and *cda2* genes was obtained by replacement of the ORFs with the *argB* and *pyrG* genes, respectively. To construct the deletion cassette, around 1000 bp of the 5′ and 3'flanking regions of the *cda1 ORF* were amplified using the primer pairs KO_cda1_5'F/R and KO_cda1_3'F/R. Both marker genes were amplified from the WT *A. fumigatus strain* Af293 using the primer sets argB_F/ argB_R and pyrG_F/ pyrG_R. Gene disruption cassette was constructed via a double joint PCR strategy as described previously (Lim *et al.*, 2012). The 3.7 kb and 4.1 kb linear *cda1* and *cda2* deletion cassettes (5'flank cda1-*argB*-3'flank cda1, and 5'flank cda2-*pyrG*-3'flank cda2) were amplified with primers cda1_nestedF/R and cda2_nestedF/R, respectively, using the Long Template Expand

PCR System (Roche, Indianapolis, IN, USA) and PCR conditions according to the manufacturer's instructions. The third round PCR product was used to transform protoplasts of the double auxotroph strain Af293.6. The schematic representations of both constructs are shown in Figures 3A and 3C and all the primers used are listed in Table 2. To create the double mutant strain (Δ*cda1*Δ*cda2*), the same deletion cassettes were used to complement the single auxotrophic mutants constructed in this study. The schematic representation of the *cda* double mutant strain construction is shown in Figure 3E.

Fungal transformation and mutant confirmation: To create the single mutants Δ*cda1* and Δ*cda2*, protoplasts from *A. fumigatus* double auxotroph strain (Af293.6) were generated using a well-established transformation protocol described by Palmer et al., (2008) with slight modifications. Transformants were first selected on Sorbitol Minimal Medium (SMM) supplemented with uracil and uridine for the Δ*cda1,* or arginine for the Δ*cda2*. After selection, mycelia from transformant strains were grown in GMM broth amended with the adequate supplements, gDNA was extracted (Lee *et al.*, 2017), and PCR screening reactions were run to confirm the correct integration of the cassette and concurrent deletion of the target sequence. For this purpose, primers which bind outside from the deletion construct in the flanking regions of the gene were used along with primers designed on the marker gene (KO_cda1_confF/R and KO_cda2_confF/R).

Primer Name	Sequence (5' to 3')
CDA1_5'F	CCAATTCCTTCCACGGCATCGG
CDA1_5'F Nested	CTCGATGTCTGCGTCTTCGACG
CDA1_5'R	TCTACGCCTCTCTTGGTCCGTCGTGATGACGG TGTTGTGTCACTTGTGGGAGG
argB_F	GTCATCACGACGGACCAAGAGAG
argB_R	GCTTGAGTGAGTGGATAGAAGG

CDA1_3'F	TCTGTATTCCTTCTATCCACTCACTCAAGCCGA GACTACAGGCTACAGAGGC
CDA1_3'R Nested	GCCTGGATGCAGACCATCAAGAAG
CDA1_3'R	GTTTACCGGTTCCACGTGGTCAG
CDA1 F confirmation	GCGTTTATGAAGTCCGTTCGTGAGTC
CDA1 R confirmation	TCGTTCTATTGCCACGTTCGGC
CDA2_5'F	CTTCCAGAATCGAGGAGACTACAC
CDA2_5'F Nested	GCGCATTCATTTTCTATCACTCTCCCC
CDA2_5'R	TTCGATATCAAGCTATCGATACCTCGACTCGA ACGAGTAAGAATGGACAACGAGTGTG
pyrG_F	GAGTCGAGGTATCGATAGCTTGATATC
pyrG_R	ATTCGACAATCGGAGAGGCTGCAG
CDA2_3'F	CTGTCGCTGCAGCCTCTCCGATTGTCGAATGA GTCCTTGTACGCAGTTAGTTTGTGC
CDA2_3'R Nested	CAATGCACTGGCCACACTTGACG
CDA2_3'R	CACTACTACATTAGATGCGATTCAAGAGC
CDA2 F confirmation	GCCCGTTGACAAGCCCTTGAAG
CDA2 R confirmation	CCATAATAATFTCAATGCCAGCAGCG

Table 2 shows the primers name and sequences used for constructs.

Positive transformants confirmed by PCR were later subjected to southern blot analysis to show that the deletion cassette was integrated one time at the targeted locus. Probes were prepared from *cda1* and *cda2* gene 5' and 3' flanking sequences, those used in the construction of the deletion cassette. For *cda1* southern blot analysis, genomic DNA from the *A. fumigatus* parental and transformant strains were digested with *Bam*HI (for the 5' side confirmation) and *Xho*I (for the 3'side confirmation). For *cda2* southern blot analysis, genomic DNA from the *A. fumigatus* parental and transformant strains were digested with *Apo*I (for the 5' side confirmation) and *Xho*I (for the 3'side confirmation). Digested DNAs were separated on a 0.8% agarose gel and blotted onto Hybond N+ nylon membranes (GE Healthcare). Probe labeling for detection was performed using [a-32P] dCTP with the Random Primers DNA Labeling System (Life Technologies). Labeled membranes were exposed to X-ray films, which were scanned for image processing.

3.3.2 Screening for LCOs production using bioassays.

Sulfated LCOs and non-sulfated LCOs were screened by applying fungal exudates to 1-week old *Medicago truncatula* or *Vicia sativa* roots. Root hair branches indicate a positive detection of the LCOs signal. Fungi were grown in glucose minimal medium (GMM) broth for 24 hours or 96 hours, in dark conditions at 37 C at 250 rpm. *Aspergillus fumigatus* strain Af293 wild type and purified LCOs were used a positive control. 0.005% and GMM broth were used a negative control. Protocols and procedures were followed as reported in Cope *et al.,* (2019) and Rush *et al.,* (unpublished). Double *cda* deletion prototroph mutants were examined, whereas single *cda* deletion prototroph mutants were not examined.

3.4 Results

3.4.1 Construction and confirmation of *Aspergillus fumigatus* single and double *cda* mutants

The *A. fumigatus* double auxotroph strain Af293.6 was used as the DNA recipient strain for the deletion of the *cda1* and *cda2* genes one at a time. Transformation with the *cda1* and *cda2* deletion cassette yielded several transformants on SMM containing uracil/uridine and arginine, respectively. One hundred transformants were randomly selected for each transformation and seventy-five were screened for insertion at the targeted locus by PCR using primers which bind outside from the deletion construct in the flanking regions of the gene along with primers designed on the marker gene. Seventeen out of the seventy-five isolates for cda1 deletion and seventeen out of the seventy-five isolates for *cda2* deletion showed a positive replacement event (data not shown). These positive transformants were further subjected to southern blot analysis which confirmed that six of these mutants display only one insertion of the deletion cassette at the *cda1* and five of these mutants display only one insertion of the deletion cassette at the *cda2* locus (Figures 3B, 3D). The confirmed strains for the *cda1* and *cda2* single mutations were labelled

TTAR1.1, TTAR1.2, TTAR1.3, TTAR1.4, TTAR1.5, TTAR1.6, and TTAR2.1, TTAR2.2., TTAR2.3, TTAR2.4, and TTAR2.5, respectively. The TTAR2.5 (Δ*cda2*) strain was later used as the DNA recipient strain for the construction of the double mutant Δ*cda1, Δcda2* using *argB* as selective marker. Transformation of the *Δcda2* strain with the *cda1* deletion cassette yielded eight transformants on SMM medium without supplements. The prototroph strains obtained were screened for the single insertion of the deletion cassette by southern blot analysis using the same restriction enzymes used for the confirmation of the single *cda1* deletion. Out of the eight strains, only two showed a single insertion of the deletion cassette and yielded the expected 4.5 Kb and 1.5 kb expected bands with the 5' and 3' probes, respectively (Figure 3 F). The two confirmed double mutant strains were labelled TTAR3.1 and TTAR3.2.
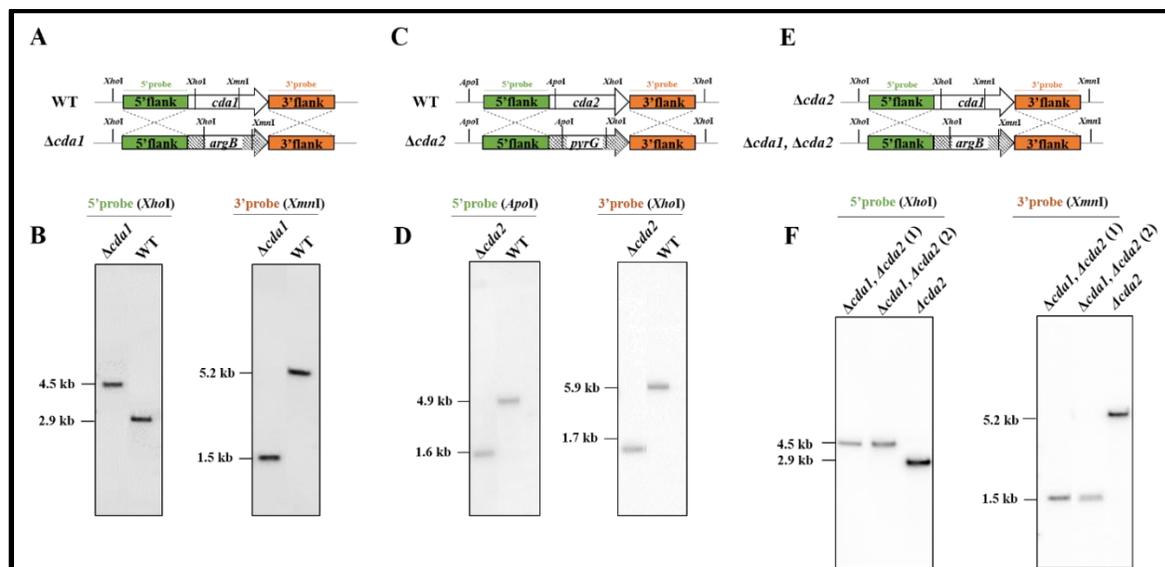
Figure 3 shows the deletion and confirmation of *cda* genes in *Aspergillus fumigatus*.
 (A) Schematic representation of the genetic construct for *cda1* deletion in *A. fumigatus* strain

AF293.6. The construct is constituted of the *argB* gene. (B) Southern blot analyzes of genomic

DNA from the WT and the Δ*cda1* strains. (C) Schematic representation of the genetic

construct for *cda2* deletion in *Aspergillus fumigatus* strain AF293.6. The construct is constituted of the *pyrG* gene. (D) Southern blot analyzes of genomic DNA from the WT and the Δ*cda2* strains. (E) Schematic representation of the genetic construct for *cda1* deletion in *Aspergillus fumigatus* strain TTAR2.1 (Δ*cda2*) constructed in the present study. The construct is constituted of the *argB* gene. (F) Southern blot analyzes of genomic DNA from the single (parental strain) and the two double mutant strains. For all southern blot analyses, ten micrograms of total DNA from each strain was digested with the appropriate enzymes and subjected to southern blot analysis using respectively the 5' flank fragment (green) and the 3'fragment (orange) as probes. The 1 kb DNA ladder from New England Biolabs was used to determine the size of the expected bands. The positions of the restriction enzyme cutting sites are shown on the A, C and E maps.

3.4.2 Screening for LCO production in single and double *cda* mutants

Two double mutant strains were constructed and confirmed by southern blot as shown in Figure 3F. Both strains were screened for LCO production. The first double mutant strain (TTAR3.1) was notable for its production of a pinkish pigment in the fungal exudates and resulted in an average of 2.4 root hair branches observed in 3 cm of root within 5 replications for both *Medicago truncatula* or *Vicia sativa.* The second double mutant strain (TTAR3.2) did not produce any visible pigment and resulted in no root hair branching phenotypes across five replications in 3 cm of roots for both *Medicago truncatula* or *Vicia sativa.*
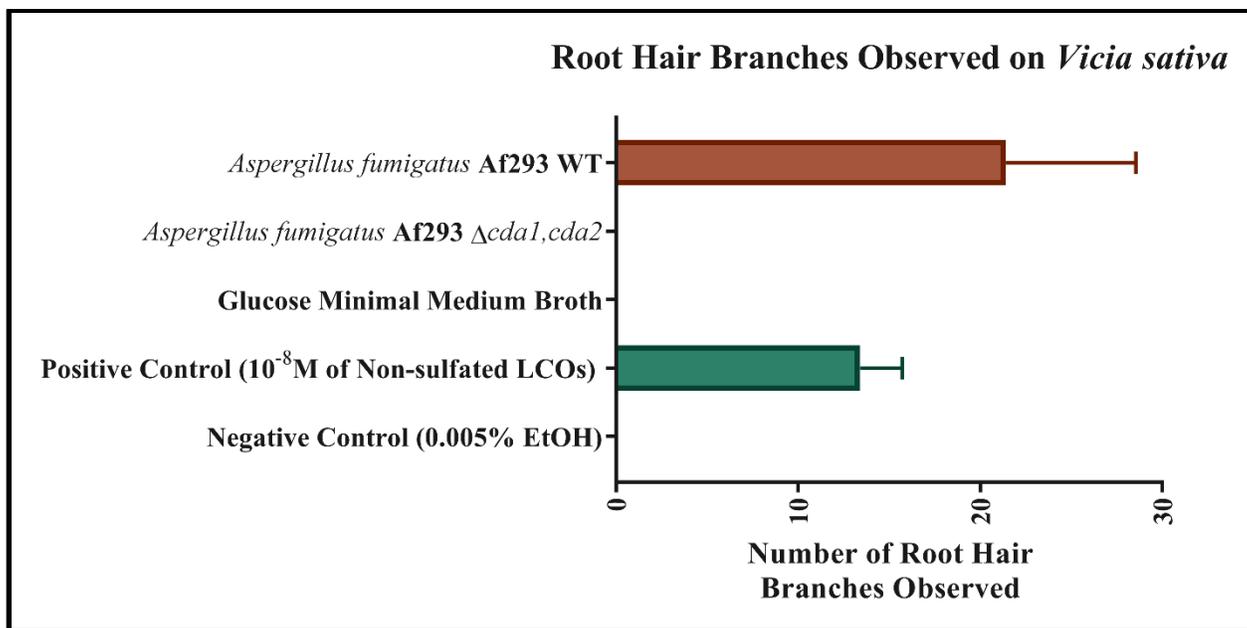
Figure 2 shows the root hair branches observed in 3cm of root for 5 replications.

Since the two double mutant strains showed different LCO production results, more double mutant strains are currently in the making to get more conclusive results as for the role of *nodB* in LCO biosynthesis.

3.5 Discussion

In this paper we were able to characterize one step of the biosynthetic pathway leading to LCO production in *A. fumigatus*. Further efforts are required in the future in order to characterize additional steps and identify the functions of other genes. To date it remains unknown if the fungal LCO biosynthesis is comparable to that in rhizobia. This paper reports for the first time the implication of the chitin deactylase encoding gene (*nodB*) in the biosynthesis of LCOs in a fungus on the basis of targeted mutation experiments. However, several questions remain unresolved to cite some: why are they more putative chitin deacetylase genes found in mycorrhizal fungi compared to non-mycorrhizal fungi? Are chitin deacetylase genes in rhizobia and fungi functional homologous? Lastly, why does *Saccharomyces cerevisiae* and *Candida glabrata* have putative

nodA/B/C-like genes, yet tested negative for the detection of LCOs? To answer all these questions further investigations are required in order to achieve a full understanding of LCOs biosynthesis in fungi.

Besides the role that chitin deacetylase has in LCO production in fungi, it is unknown what role it has in the pathogenicity of *Aspergillus fumigatus*. In this regard, there are conflicting results in the literature on whether chitin deacetylase is important for fungal pathogenicity. As shown in the filamentous pathogen, *Magnaporthe oryzae,* chitin deacetylase does not impact pathogenicity (Geoghegan & Gurr, 2017). Also, it has been reported that is unknown if chitin deacetylase is involved in pathogenicity for *Pochina chlamydosporia* (Aranda-Martinez *et al.*, 2018)*.* However, for the pathogenic yeast, *Cryptococcus neoformans*, chitin deacetylase was shown to have an impact on pathogenicity (Upadhya *et al.*, 2018). Perhaps, the role that chitin deacetylase has on pathogenicity depends on the filamentous or yeast growth stages. The double *cda Aspergillus fumigatus* mutants constructed in this study could be used to provide clarity and test whether chitin deacetylase plays a role in pathogenicity.

Another step in understanding the role of chitin deacetylase is classifying the different types of chitin deacetylase. There is limited information of how many types of chitin deacetylase are present in fungi (Aranda-Martinez *et al.*, 2018). Based on a protein BLAST search with the terms "chitin deacetylase and fungi" on the National Center for Biotechnology Information (NCBI), there are 6,381 results for fungi with chitin deacetylase. More specifically, there are chitin deacetylase protein sequences for 4,297 ascomycetes (with 364 sequences found in budding yeasts), 1,848 for basidiomycetes (with 160 sequences found in smut fungi), 110 for chytrids, 72 for glomeromycetes, 38 for microsporidians, and 16 for Blastocladiomycetes. Lastly, chitin deacetylase is not limited to fungi. Based on the BLAST search for "chitin deacetylase and

bacteria" on NCBI, there were 58,593 protein sequences. Comparing the chitin deacetylase sequences between bacteria and fungi and characterizing the types of chitin deacetylase that are known will be critical to understand the origin of chitin deacetylase and reveal the roles of chitin deacetylase in fungi and bacteria. To conclude, chitin deacetylase is an important protein which was confirmed to be indispensable for the production of LCOs in fungi and could have potential impact on the pathogenicity of the fungus.

3.6 References

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Chapter 4: Conclusions and Future Directions

4.1 Summary

Since the purification, identification, and determination of the role of LCOs nearly 30 years ago, there has been an extensive research for the better understanding of the mechanisms involved in LCO production. Moreover, the biosynthesis of LCOs in rhizobia has been well characterized. Given the recent discovery of LCO production in genetically different mycorrhizal fungi (Maillet *et al.*, 2011; Cope *et al.,* 2019), understanding the role that LCOs play in the Kingdom Fungi was critical. Prior to the findings from this dissertation, LCOs were thought to be a symbiotic signal that triggers the common symbiosis pathway in corresponding hosts. The conclusions of this dissertation have determined that: 1) LCOs are a widespread signal found throughout the Fungal Kingdom 2) LCOs are not unique symbiotic signal molecules and has alternative roles other than triggering the common symbiosis pathway, 3) LCOs can be defined as a quorum sensing molecule that influences behavior, growth, development and the transcriptome in fungi, 4) chitin deacetylase plays a role in LCO biosynthesis in fungi, and 5) *Aspergillus fumigatus* should be used as the model organism to characterize the biosynthesis of LCOs in fungi.

4.2 Discussion of the major findings

How does a plant differentiate between an LCO produced by a symbiotic microbe and a pathogenic microbe?

Rhizobia produce LCOs in order to colonize their leguminous hosts. It is unknown if mycorrhizal fungi produce LCOs for the same purpose. However it is known that LysM-RLKs are involved with arbuscular mycorrhizal associations. However, if most fungi produce LCOs, how does the plant distinguish between an LCO signal that is from a friendly or a nemesis microbe?

The use of specific LCO structure that is well defined and characterized through chemical synthetization, can be critical to answer this question.

Moreover, the recognition of specific types of LCOs might involve a check and balance, where you need a threshold of certain type of LCOs produced for the plant to have a response. To determine a possible limit based on the concentration of LCOs is problematic since there are no valid tools available to quantify LCO concentrations from raw exudates.

<u>How do fungi perceive LCOs?</u>

Similar to the thought that there might be unknown receptors in plants to recognize specific types of LCOs, fungi might also have receptors that have been identified already and can perceive LCOs or unknown receptors waiting to be characterized. Plant hormones are known to influence the growth and development of fungi (Besserer *et al.*, 2006; Chatterjee *et al.*, 2009; Degani *et al.*, 2015; Chanclud & Morel, 2016). Therefore, it should not be surprising that a microbe that produces a signal that is perceived by a host plant, perhaps could also respond to that same signal. This is the first study to determine specific types of LCOs influencing on the growth and behavior of fungi that may or may not produce the same signal. Preliminary data from this project have shown that a cocktail of LCOs can influence the growth and development in species of *Aspergillus, Laccaria, Hebeloma,* and *Mortierella* (unpublished data). However, from these experiments, *Aspergillus fumigatus* had the fewest phenotypes observed from exposure to exogenous cocktail of LCOs. Yet, when you parsed out the different types of LCOs, through synthetic chemistry, there was a significant influence by sulfated C16:0 LCOs that was not observed in the other specific types of LCOs. These data suggest that there could be interactions occurring between the different types of LCOs in these cocktails. Moreover, there might be receptors in the fungus that recognize specific types of LCOs and not the others.

Since LCOs are perceived by the LysM domain in plants, the LysM domain in fungi should be investigated. There is a large molecular diversity of LysM throughout the Fungal Kingdom based on whole genome sequencing (Akcapinar *et al.*, 2015). To test the hypothesis that a LysM receptor perceives LCOs in fungi, differentially gene expression data from our RNAseq analysis can be used and further investigated.

What is the biosynthetic pathway responsible for LCO production in fungi?

The role and order of the *nod* genes that made the chemical decorations were well characterized for rhizobia in the study by Poinsot *et al.*, (2016). However further investigations should determine what role these chemical decorations play in the biosynthesis of LCOs in fungi. Findings from this dissertation have raised an interesting question, can the homologous chitin deacetylase found between rhizobia and fungi be swapped and remained functional? This question can be answered through cross species genetic recombination of the *nod* genes between rhizobia and *Aspergillus fumigatus* strain Af293. I hypothesize that these *nod* genes are not functional homologs; they originated from the universal common ancestor and evolved.

Do other microbes produce LCOs and if so, why do they produce them?

Based on preliminary data, other microbes have putative *nodA/B/C* -like genes. However, they have not been examined for LCO production. If other microbes produce LCOs, what is the extent of LCO production throughout the tree of life? What is the origin of these *nod* genes? Are other microbes generating and perceiving LCOs as a communication signal? I hypothesize that LCOs are a universal microbial communication signal. These LCO signals might have divergent evolution, but the receptors that perceive them are still present in organisms. The evidence to support this hypothesis is that a rhizobia bacterium produces LCOs, which is perceived by their

host plants (Oldroyd *et al.*, 2011). Mycorrhizal fungi produce LCOs, which is also distinguished by their host plants (Maillet *et al.*, 2011; Cope *et al.,* 2019). A wide range of fungi produce LCOs and are recognized by plants and by fungi (this study). Lastly, LCOs modulate and are recognized by mammalian angiogenesis, which are blood vessels and tumor formations (Djordjevic *et al.*, 2014). Perhaps not all organisms can produce LCOs, but they might have the receptors to perceive it. With the ever-growing interest in microbiome studies, I predict that LCOs will be significant influencer on how microbes interact, which would affect microbiomes, thus making an impact on all scientific fields and requiring collaborations between diverse multidisciplinary mindsets.

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