# Characterization of a novel, ubiquitous fungal endophyte from the rhizosphere and root endosphere of *Populus* trees

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**Abstract**

We examined variation in growth rate, patterns of nitrogen utilization, and competitive interactions of *Atractiella* sp. isolated from the roots of *Populus* hosts. *Atractiella* grew significantly faster on media substituted with inorganic nitrogen sources and slower in the presence of another fungal genus. To determine plausible causal mechanisms we used metabolomics to explore competitive interactions between *Atractiella* strains and *Fusarium oxysporum* or *Leptosphaerulina chartarum*. Metabolomic screening of potential microbial inhibitors showed increased levels of glycosides produced *in vitro* by *Atractiella* when grown with a different fungal genus, relative to when grown alone. Cumulatively, these results suggest *Atractiella* is a poor competitor with other fungi via direct routes e.g. faster growth rates, but may utilize chemical interactions and possibly nitrogen sources to defend itself, and niche partition its way to abundance in the plant host root and rhizosphere.

**Key Words**

*Atractiella*, fungal endophytes, competition, metabolomics, organic and inorganic nitrogen, symbiosis, pathogen, mutualist

**Introduction**

The microbiome associated with the rhizosphere of any given plant species is highly diverse and includes at least some microbial species capable of spanning the symbiotic spectrum from antagonist to mutualist. Plant-fungal interactions are well-documented as complex, with many fungal species demonstrating the capability of shifting from plant mutualists to antagonists based on environmental context (Craven et al. 2001, Wicklow et al. 2005, Hartmann et al. 2008, Peay et al. 2008, Hamilton et al. 2009, Rodriguez et al. 2009, Hamilton et al. 2010, Kennedy et al. 2011, Wyrebek et al. 2011, Davidson et al. 2012, Hamilton and Bauerle 2012, Hamilton et al. 2012, Bonito et al. 2014). The ability of a fungal endophyte to regulate facets of the plant's microbiome, and therefore impact the plant host phenotype, has not been explored. *In vitro* competitive experiments between fungal endophytes are a means of determining plausible mechanisms by which interactions may occur and possibly impact plant-fungal symbiotic outcome. For example, a slow-growing fungal endophyte or one with reduced competitive capabilities may be

overwhelmed by a faster growing species when competing for space and nutrient availability within limited host niche space (Garrett 1951, Arora and Upadhyay 1978, Bennett and Lynch 1981, Whipps 2001, Bais et al. 2006, Jones et al. 2009, Taylor et al. 2014). The plant host is also capable of expressing selective preference by favoring specific fungal species, thus influencing the microbiome diversity present in the rhizosphere and plant roots (Grayston et al. 1998, Raynaud et al. 2008, Saunders and Kohn 2008, Gottel et al. 2011, Hafidh et al. 2011).

The order *Atractiellales* is housed in the Pucciniomycotina and consists of three families, ten genera, and 34 species identified to date (Oberwinkler and Bandoni 1982, Aime et al. 2006, Bauer et al. 2006, Kottke et al. 2010). Our recent studies have identified several *Atractiella* strains as prominent root endophytes of *Populus* and other woody plants sampled from across a broad geographic range, including from studies of Eastern Cottonwood (*P. deltoides*) in Tennessee and North Carolina, and Black Cottonwood (*P. trichocarpa*) in Oregon and California (Gottel et al. 2011, Shakya et al. 2013, Bonito et al. 2014). *Atractiella* species also appear to be enriched within the root endosphere compartment, as compared to the surrounding rhizosphere. Though the Atractiellomycetes have been placed phylogenetically within the Pucciniomycotina, an order containing many rust fungi (Kottke et al. 2010), little is currently known about their ecology, and the limited evidence to date suggests these fungi may have variable life history strategies (Oberwinkler and Bandoni 1982, Oberwinkler 1989, Kottke et al. 2010, Avila-Diaz et al. 2013). For example, species of *Atractiellales* were first isolated from decomposing matter such as decaying potatoes (Oberwinkler and Bandoni 1982), suggesting a saprophytic life-strategy. More recently, a potential mycorrhizal-like role has been observed between Atractiellomycetes and orchids (Kottke et al. 2010, Avila-Diaz et al. 2013). This supports research showing multiple fungal genera in fungal-plant interactions are variable and dynamic in terms of the life-strategy employed (Hamilton and Bauerle 2012, Hamilton et al. 2012). This is illustrated by organisms in the *Fusarium oxysporum* complex as well as the *Epichloë*/*Neotyphodium* complex (Craven et al. 2001, Clay and Schardl 2002). *Fusarium oxysporum* have been shown to reduce tomato plant damage caused by the pathogenic fungus *Meloidogyne incognita* (El-Fattah Adnan Dababat and Sikora 2007), but also

serve as mutualistic endophytes to banana plants (Mendoza and Sikora 2009). Other *F. oxysporum* are known to be pathogenic to members of numerous plant families, such as the Malvaceae, Solanaceae, and Fabaceae (Windels 1991, Kistler 1997). *Epichloë* foliar endophytes can range from mutualistic to pathogenic depending upon the clade (Craven et al. 2001) as well as in response to host species colonized. They can also change life-strategy in response to as yet unknown triggers, within the same symbiotum (Clay and Schardl 2002). Thus, there is a precedent in the literature supporting both mutualistic as well as pathogenic roles for a fungal species (Veldre et al. 2013), in this case *Atractiellales*, to be both closely related to a pathogenic order, Pucciniomycotina, and to show diverse symbiotic outcomes. The impact of competition between species within the rhizosphere must also be explored, as competitors may influence growth via metabolite secretions (Whipps 2001, Demirci et al. 2011). Two or more species may naturally segregate to occupy separate and distinct niches which they are adapted to, decreasing competitive interactions with each other (Elton 1946, Hutchinson 1957, Leibold 1995, Al-Naimi et al. 2005, Neubert et al. 2006).

Limited resource availability may drive speciation and in turn alter observable species-species interactions (Hutchinson 1959, Connell 1981, Leavitt et al. 2013, Winkelmann et al. 2014). The efficiency and type of nitrogen (N) used by fungi can be important for several reasons including: (1) agricultural production (Reynolds et al. 2005, Jones et al. 2009, Hamilton et al. 2016), (2) understanding how resource utilization impacts plant-microbial interactions (Bais et al. 2006, Harrison et al. 2007, Raynaud et al. 2008, Johnson et al. 2010, Kennedy et al. 2011), and (3) increasing our understanding of how microbial members of the plant microbiome interact via resource utilization (Porter and Carter 1938, Bais et al. 2006, Raynaud et al. 2008, Kennedy et al. 2011, Engelmoer et al. 2014). To better understand rhizosphere community dynamics, both the nitrogen used and the efficiency with which it is used by fungi can be important. For example, the effects of inorganic or organic N sources on the growth of various fungi have been repeatedly studied (Baar et al. 1997, Baar and Stanton 2000, Hodge et al. 2001, Digby et al. 2010, Whiteside et al. 2012, Taylor et al. 2014) as has the impact of organic versus inorganic N forms on fungal species biomass (Baar et al. 1997, Baar and Stanton 2000, Hodge et al. 2001, Reynolds et al.

2005, Digby et al. 2010). Hawkins et al. (2000) demonstrated both N transport and uptake by arbuscular fungi was positively correlated with the amount of N available in the soil environment and that organic N forms were preferred in *in vitro* studies.

Here we explore: (1) growth characteristics of a collection of *Atractiella* strains isolated from *Populus deltoides* and *P. trichocarpa*, (2) N source utilization patterns in a model isolate, *Atractiella* sp. (PMI 95), and (3) competitive potential of *Atractiella* against a panel of co-isolated *Populus* rhizosphere fungi. In addition, we explore the potential chemical basis for competition observed from these treatments via metabolomics.

## Materials and Methods

### *Fungal strain collection and characterization*

Twenty-three distinct strains of *Atractiella* species were isolated from trap-plants (Bonito et al. 2014) grown in soils originating from under *P. trichocarpa* in a common garden experiment in Placerville, California, USA (Evans et al. 2014), and wild populations of *P. deltoides* on the Yadkin River in North Carolina, USA (Shakya et al. 2013). We selected a subset of *Atractiella* isolates to grow with a panel of other potential competitor fungi isolated from the same *Populus* roots (Table 1). Fungal isolates were obtained and maintained on Modified Melin-Norkrans (MMN) media (Marx 1969) at 20$^{o}$C. The *Atractiella* sp., *F. oxysporum* and *L. chartarum* isolates were verified based on sequence identity of the ITS1 and ITS2 region and morphological characteristics (e.g. shape of conidia, colony color). The species description, systematics and population structure of the new *Atractiella* sp. are described in Bonito et al. (2016).

### *Radial growth screening media preparation and experimental design*

Two kinds of culture media were employed to compare fungal growth rates: PDA and P5 (Kottke et al. 2010, Avila-Diaz et al. 2013) agar. The P5 agar is composed of 0.5 g $l^{-1}$ di-ammonium tartrate, 1 g $l^{-1}$ potassium dihydrogen phosphate, 1 g $l^{-1}$ magnesium sulfate heptahydrate, 5 g $l^{-1}$ D(+)-maltose, 20 g $l^{-1}$ D(+)-glucose, 1000 µL $l^{-1}$ thiamine-HCl solution at 100 mg $l^{-1}$, 1000 µL $l^{-1}$ Kanieltra stock solution, and 20 g $l^{-1}$ of agar. HIMEDIA© brand PDA mix composed of 200 g infusion from potatoes, 20 g dextrose

and 15 g agar was used to prepare media by suspending 30 g in one liter of deionized water. Cultures were grown in the dark at room temperature (~25$^{o}$C). A flame-sterilized, metal palm inoculator was used to pull a five mm diameter fungal disc-shaped plug, centered in a 25 mL Petri dish for single colony growth rates.

*Qualitative competition screening of Atractiella with a panel of Populus rhizosphere isolates*

Pairwise interaction experiments were conducted between three *Atractiella* strains and a panel of 30 fungi co-isolated from the endosphere and rhizosphere of the same greenhouse trap-plant studies. Isolates were chosen due to the high frequency at which they were co-isolated with *Atractiella*. Those chosen were inoculated onto MMN media with a 0.5 cm diameter agar plug extracted with a cork borer from pure colonies of each fungal genus or *Atractiella* strain. Agar plugs of distinct genera were placed one to two cm apart. Interactions were then classified as negative, positive or none between *Atractiella* and each of the isolates assayed.

*Quantitative competition studies between Atractiella and Fusarium or Leptosphaerulina*

To identify potential competitive interactions, each petri dish was inoculated separately with *Atractiella* alone or in combination with either *F. oxysporum* or *L. chartarum*. A flame-sterilized, metal palm inoculator was used to pull a five mm diameter fungal disc-shaped plug, centered in a 25 ml Petri dish for single colony growth rates. In order to explore fungal growth in paired fungal interactions, the five mm plugs were placed equidistant from each other and the petri dish wall. To accommodate the relatively faster radial growth rates of pathogenic fungi, mycelial plugs were added after approximately one week of *Atractiella* growth. Fungal colonies were measured every two to three days using an ordinal grid system to quantify rate of growth by recording growth distance from the plug's perimeter.

*Metabolomic profiling of interactions with Fusarium and Leptosphaerulina*

To explore differences in metabolic profiles of fungi growing alone or with a putative competitor, sample plugs of agar media were taken following the final growth measurements of *Atractiella* and the competitor species. Agar fungal plugs were frozen and stored at -80°C prior to processing. Frozen samples were weighed into vials containing the extraction solvent, 2 ml of 80% ethanol and 30 µl of

sorbitol (1 mg $ml^{-1}$ aqueous solution), and the sample weight recorded. Using internal standard differences in extraction efficiency and derivatization efficiency we corrected accordingly, and changes in sample volume during heating were included in data analysis. Samples were vortexed for several minutes until finely dispersed. Samples were allowed to extract for two hours at room temperature, after which they were syringe filtered through 0.2 µm nylon filters. One ml was dried under a stream of $N_2$ and then dissolved in 500 µl of silylation–grade acetonitrile, followed by the addition of 500 ml N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) (Restek, Bellefonte, PA), and then heated for one hour at 70°C to generate trimethylsilyl (TMS) derivatives (Li et al. 2012, Tschaplinski et al. 2012). After two days, 1 µl aliquots were injected into an Agilent Technologies Inc. (Santa Clara, CA) 5975C inert XL gas chromatograph-mass spectrometer, fitted with a Restek Rtx-5MS with Integra-guard (5% diphenyl/95% dimethyl polysiloxane) 30 m x 250 µm x 0.25 µm film thickness capillary column. The standard quadrupole GC-MS was operated in the electron impact (70 eV) ionization mode with gas (helium) flow set at 1 ml per min and the injection port configured in the splitless mode. The injection port, MS Source, and MS Quad temperatures were set to 250°C, 230°C, and 150°C, respectively. The initial oven temperature was held at 50°C for 2 min and was programmed to increase at 20ºC per min to 325ºC and held for another 11 min, before cycling back to the initial conditions. The GC-MS total ion current outputs for plugs taken from the same plate were overlapped to identify putative inhibitors produced by either of the fungi. Metabolite peak extraction, identification, and quantification were as described previously (Tschaplinski et al. 2012), and unidentified metabolites were denoted by their retention time as well as key mass-to-charge (m/z) ratios. Ratios of metabolites were determined with the data from the plug of the closest fungus used as the numerator and the more distant fungus used as the denominator, assuming that if a metabolite is higher on the side of the plate closest to one fungus, it was likely produced by that fungus.

*Screening to determine the impact of organic or inorganic N sources*

To determine N content and source impacts on *Atractiella* 95 growth rate, the C:N ratio of P5 media was adjusted using five separate N source stock solutions to 100:1 in order to mimic standard P5 media C:N

ratio, and 10:1 in order to mimic standard PDA media C:N ratio. Three inorganic and two organic N source stock solutions were used to adjust the C:N ratio of P5 media to either 100:1 or 10:1 for a total of two C:N ratios per N source. Inorganic N sources used included ammonium chloride, sodium nitrate, and ammonium nitrate. Organic N sources used included glycine and glutamate. For a 100:1 and 10:1 ratio, a total of 0.006 mol $L^{-1}$ and 0.06 mol $L^{-1}$ of ammonium chloride, sodium nitrate, glycine and glutamate were added to P5 media. For the same ratios with ammonium nitrate, a total of 0.003 mol $L^{-1}$ and 0.03 mol $L^{-1}$ were added to P5 media.

*DNA extraction and PCR*

To verify the identity of the *Atractiella* and competitors, isolates were grown on standard PDA topped with sterilized Millipore© 0.45 µm membranes, allowing removal of pure fungus without agar contamination. Inoculation of plates and fungal growth on the filters was as above described. The Powerplant Pro DNA Isolation Kit© was used to extract DNA samples as per kit instructions, and DNA samples were stored at -20$^{o}$C. Ribosomal DNA (rDNA) was amplified using fungal-specific internal transcribed spacer (ITS) primers ITS1 and ITS4 using the ProMega GoTaq © Master Mix kit, and cleaned using the Affymetrix, USB ExoSAP-IT © kit. Samples were Sanger sequenced on an ABI3730 Genetic Analyzer at the University of Tennessee at Knoxville (UTK), and sequences generated were analyzed against the NCBI database using BLASTN to verify identity.

*Data analysis of each experimental design*

All fungal growth rate analyses were completed using SAS© software. Data were tested for normality and assumptions of heteroscedasticity both within and between treatments. When necessary, data were transformed to achieve assumptions of normality and variance distributions. A repeated-measures, generalized linear model (GLM) was used with all effects being fixed, i.e., fungal identity (Strain), date measured (Time), and media substrate (Media). Effects were evaluated individually and in all possible combinations.

**Results and Discussion**

*Growth rate comparisons between Atractiella strains*

Radial growth rates among the nine *Atractiella* strains varied significantly from 1.58 to 3.18 mm day$^{-1}$ ($F_{6,10} = 4.63$, $p = 0.0166$; Table 2) . All fixed effects and some interactions between them were significant; Media and Strain ($F_{6,10} = 4.2$, $p = 0.0226$; Table 2), Media ($F_{1,10} = 228.08$, $p < 0.0001$; Table 2). On average, all *Atractiella* strains displayed a faster radial extension rate on PDA compared to growth on P5 media (Table 2). Time and Strain did not produce significant interaction effects ($F_{30,50} = 1.35$, $p = 0.1733$; Table 2).

The slower growth rate on P5 media suggests nutrient limitation or auxotrophy. Interestingly, a visual assessment of *Atractiella* suggested comparatively faster growth rates on a nitrate-rich cellulose membrane versus a C-rich cellulose membrane, a pattern similar to that observed by Reeslev and Kjöller (1995). This suggests either *Atractiella* requires nitrate inputs or other chemical(s) present in the nitrate-rich membrane, or C-rich cellophane membranes are inhibitory. This led us to test impacts of N forms available to *Atractiella* grown on P5 media. More work needs to be done in field experiments, but this supports the hypothesis that *Atractiella* is, in general, a poor competitor with other fungi, yet is found in relatively high percentages in plant host roots (Bonito et al. 2014) possibly through mechanisms such as resource partitioning (Rajala et al. 2011).

Quantitative and qualitative assessments of rate of diameter change (proxy for growth rate) in *Atractiella* grown solo or in the presence of a putative competitor produced interesting results. For example, both *Mortierella* and *Fusarium* grew faster than the *Atractiella* strains they were paired with (Table 3), eventually overgrowing *Atractiella* colonies. Many pairings led to growth stalemates (Table 3) in which strains of *Atractiella*, particularly PMI 95, often produced zones of inhibition when paired with other genera (Table 3). In most interactions explored, *Atractiella* growth, regardless of strain, was slowed in the presence of another fungal genus (Table 3).

*Growth of Atractiella with organic versus inorganic N sources using two C:N ratios*

Most N and C:N ratio treatments had a significant effect on the growth rate of *Atractiella* relative to control treatments (Table 4). Organic versus inorganic N treatments were significantly different ($F_{6,48} = 5.46$, $p = 0.0002$; Table 4), as was the treatment, C:N ratio ($F_{1,48} = 16.69$, $p = 0.0002$; Table 4). After 15

d of growth on P5 'control' (no N source additions), the *Atractiella* strain 95 had a 76.14 mm colony diameter while $NH_4Cl$ treatments at a 10:1 C:N ratio averaged 68.79 mm (Fig. 1). In contrast, treatments at C:N 100:1 $NH_4Cl$ resulted in slightly slower growth, 76.05 mm (Fig. 1). When $NaNO_3$ was provided as the N source it increased relative colony diameters at both ratios (10:1, 100:1). When substituted with an inorganic N source $NH_4NO_3$ at 10:1 and 100:1 C:N ratios, average colony diameters were lower, relative to P5 control (75.33 mm and 75.8 mm, respectively; Fig. 1).

Glycine was not correlated with increased colony diameter of *Atractiella* at either C:N ratio (Fig. 1), and instead significantly reduced colony diameter (Fig. 1). When glycine was added at ratios of 10:1 and 100:1 C:N, the average colony diameters were 75.3 mm and 74.37 mm, respectively (Fig. 1). Glutamate at a 10:1 ratio resulted in a much slower growth rate relative to the P5 control; an average colony diameter of 66.35 mm (Fig. 1), and a 78.22 mm average colony diameter at 100:1 C:N ratio (Fig. 1).

*Competition results*

The significant interaction effect between focal fungus and competitor (Table 5) suggested that regardless of competitor identity, focal fungal growth was slowed (Fig. 2), indicative of competitive interaction. Tests of individual growth responses using Tukey's Significantly Different (TSD) test showed *Atractiella* growth was significantly and negatively impacted by putative competitors (Table 5). In addition, qualitative analyses of interactions with multiple fungal genera support the conclusion that *Atractiella*, at least *in vitro*, is not a strong competitor. Both *Mortierella* and *Fusarium* grew comparatively rapidly and overgrew the *Atractiella* they were paired with (Table 2). Conversely, many pairings led to growth stalemates (Table 2). Isolates of *Atractiella* strains often produced zones of inhibition when paired with other fungal genera, including the basidiomycete *Flagelloscypha*, while melanized barrage zones were also evident in many inter-genera pairings, e.g. with *Lechythophora* and *Ilyonectria* (Table 2), suggesting *Atractiella* is capable of recognizing and responding to the presence of other fungal genera.

When grown solo, both *F. oxysporum* and *L. chartarum* reached colony diameters of 80 mm at 16 d (Fig. 3). While there were no significant differences between *Atractiella* strains in response to the

presence of a competitor, all strains showed arrested development in the presence of different fungal genera (Tables 2. 5; Figs. 2, 3, 4). In the presence of *Atractiella*, both *F. oxysporum* and *L. chartarum* decreased in growth rate initially (at 3 d) but recovering by the 5 d (Fig. 2). In addition, both competitor fungi adjusted morphologically to the presence of *Atractiella* by producing zones of inhibition (Figs. 2, 3). *Fusarium* displayed the least inhibition in response to the presence of *Atractiella* (Fig. 2, 3; Table 5). Whether competitive interactions resulted from competition for space, substrate resources, allelopathic interactions or some other mechanism (Porter and Carter 1938, Raynaud et al. 2008, Kennedy et al. 2011, Engelmoer et al. 2014) remains to be tested. Plausible causal mechanisms for changes in growth rate and morphology include airborne or media-infused inhibitory compounds (Garrett 1950, Arora and Upadhyay 1978, Tejesvi et al. 2007, Aly et al. 2010, Dwivedi 2013), competition for resources present in the media indirectly inhibiting growth of the adjacent fungus (Johnson et al. 2010, Engelmoer et al. 2014) or faster growth leading to a spatial impediment (Porter and Carter 1938, Raynaud et al. 2008, Kennedy et al. 2011, Engelmoer et al. 2014).

While all *Atractiella* colonies remained translucent when grown on P5 media, thicker aerial growth developed in the presence of some N sources and concentrations. Previous reports indicated bioavailability and resulting fungal biomass generated *in vitro* is based on N-source present in the media (Baar et al. 1997, Digby et al. 2010). Experiments with liquid media to compare fungal biomass produced in response to N manipulations are warranted. Growth in sterile, soilless and soil mix, using ergosterol techniques to measure fungal biomass, is another means of unravelling the impacts of N source and concentration directly and indirectly on *Atractiella*'s growth (Zill et al. 1988, Ekblad et al. 1998, Mille-Lindblom et al. 2004, Kennedy et al. 2005). Additional lines of research include isotopic analysis to determine how much of the N from various N sources is directly taken up by *Atractiella*'s hyphae, and how alterations in substrate or media pH correlate with fungal biomass production with and without competition from plant roots (Six et al. 2002, Treseder et al. 2014).

*Metabolomic profiles resulting from competitive interactions between either F. oxysporum or L. chartarum in the presence of Atractiella*

To test for plausible competitive mechanisms, GC-MS was used to explore metabolite signatures. We focused the exploratory metabolite analysis on putative microbial inhibiting metabolites. Interestingly, *Atractiella* down-regulated all of the metabolites determined to be present (Table 6) when grown with *F. oxysporum*, but down-regulated metabolites by orders of magnitude in the presence of *L. chartarum*. Metabolite profiling indicated increased production of glycosides (Table 6) by *Atractiella* in response to the presence of other fungi and *F. oxysporum* and *L. chartarum* in response to the presence of *Atractiella* (Table 6). *Leptosphaerulina chartarum* and *Atractiella* both produced greatly elevated levels of 2,3-butanediols, glycosides and uric acid when interacting with another fungal genera, especially *F. oxysporum* (Table 6). Glycosides are involved in a variety of metabolic activities (KEGG Orthology; http://www.genome.jp/kegg/ko.html), and have been specifically identified in plant-pathogen interactions (PATH:ko04626) involving fungal pathogen-associated molecular pattern (PAMP), and specifically PAMP-triggered immunity (Das et al. 2013). *Atractiella* and *F. oxysporum* both increased *a-e*-diaminopimelic acid when interacting (Table 6), with the highest level closest to *F. oxysporum*, suggesting that this fungus was likely the major source of the metabolite. These results suggest that *Atractiella* strain 95 is stressed by the presence of *F. oxysporum* and *L. chartarum*, and also provide initial data with which to explore specific metabolites plausibly causal to competitive interactions through the PAMP pathway.

There were four related benzoxazin-3-one-like metabolites, including 2,4-dihydroxy-5-methoxy-2H-1,4-benzoxazin-3-one (tentative ID), and three unknowns, including those with retention time and key mass-to-charge (m/z) ratios of 12.52 234 324 194 249, 13.52 324 412 163 193 222, and 11.08 234 193 180 91 (Table 6). These were produced by all three fungi, but comparatively more so by *F. oxysporum* (Table 6). *Fusarium oxysporum* and *L. chartarum* also produced 3-deoxy-D-ribo-hexitol in general, which appeared to be upregulated when interacting with *Atractiella* (Table 6). Specific fungal metabolic activities could potentially include ustilagic acid biosynthesis (Eveleigh et al. 1964, Teichmann et al. 2010) and antibacterial effects (Tian et al. 2016), as well as cell wall remodeling and reproduction (van

Munster et al. 2012, van Munster et al. 2015), all common responses of fungi to stress (Fuchs and Mylonakis 2009).

In contrast, *Fusarium oxysporum* secreted several metabolites in response to the presence of *Atractiella* (Table 6). These metabolites, which include glycosides, butanediols and 2,4-dihydroxy-5-methyl-2H-1,4-benzoxazin-3-one, are not secreted when *F. oxysporum* is incubated alone (Table 6), indicating these were secreted in direct response to *Atractiella*. Benzoxazin compounds are involved in plant stress responses and provide plants with pathogen and pest resistance (Niemeyer 1988, Morrissey and Osbourn 1999, Niemeyer 2009). There is a possibility that these same compounds serve a similar function in competitive interactions between *F. oxysporum* and *Atractiella*. This response may be indicative of a broader defense compound arsenal, which would explain the faster growth rate of *F. oxysporum* and positive competitive performance *in vitro* (Figs. 2, 3; Table 5).

Future research on *Atractiella*'s role in the plant microbiome should focus on substrate requirements by the fungus, plant phenotype responses (i.e., plant growth, biomass, and metabolic profile) to colonization both by *Atractiella* alone and in combination with pathogenic fungi, as well as potential survival responses to competition employed by *Atractiella*, which remains abundant and ubiquitous in the *Populus* microbiome despite presenting as a poor competitor. Such research will help determine the impact of *Atractiella* on host organisms, as well as move forward the understanding of how poor competitors in a diverse microbiome remain viable and abundant.

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**Fig 1** Total colony diameter of *Atractiella* 95 when plated on unmodified P5 and PDA media as well as P5 with inorganic or organic N sources. Inorganic and organic N sources are compared at the 10:1 C:N ratio (left) versus the 100:1 C:N ratio (right). Bars represent standard errors.

**Fig 2** *Atractiella*, *F. oxysporum* and *L. chartarum* growth in response to the presence of a competitor.

**Fig 3** Total colony diameter with the focal fungus indicated underneath each grouping. Diameter is shown at 8 d. The same general pattern was produced at all time points measured (data not shown). Bars represent standard errors.

**Fig 4** *Atractiella* growth inhibition was observed by 2 d measurements after the addition of the two competitor fungus. *Atractiella* is growing on the right in both images with the central 2 mm diameter plug visible. *Leptosphaerulina chartarum* is the competitor species on the left. Images are the upper (A) and lower (B) side of the plate.

## Figures

**Fig 1**

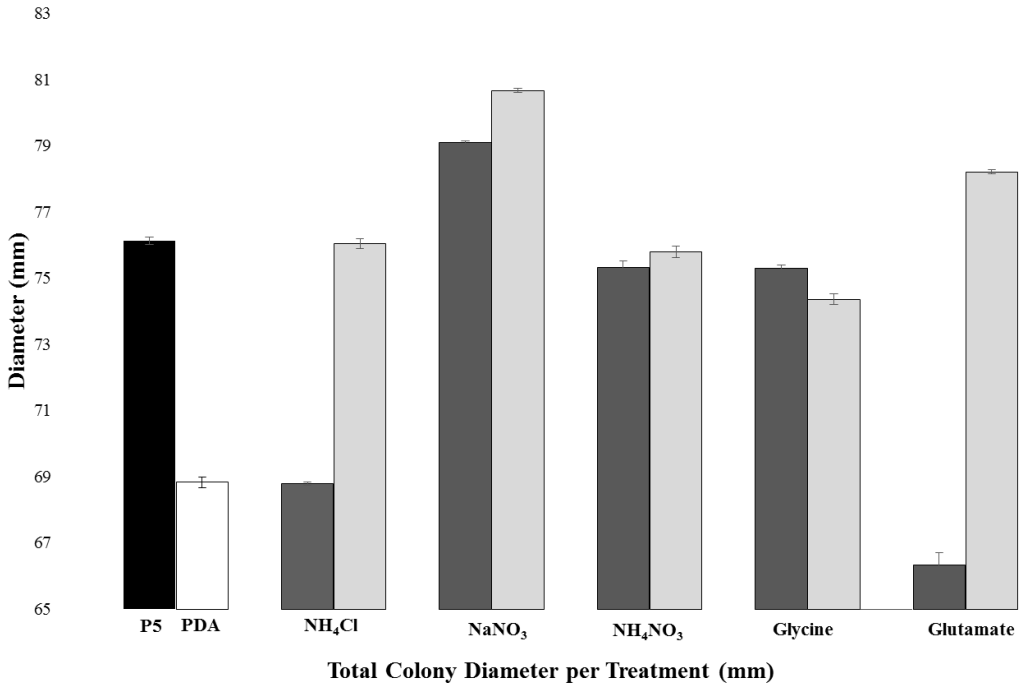


**Fig 2**

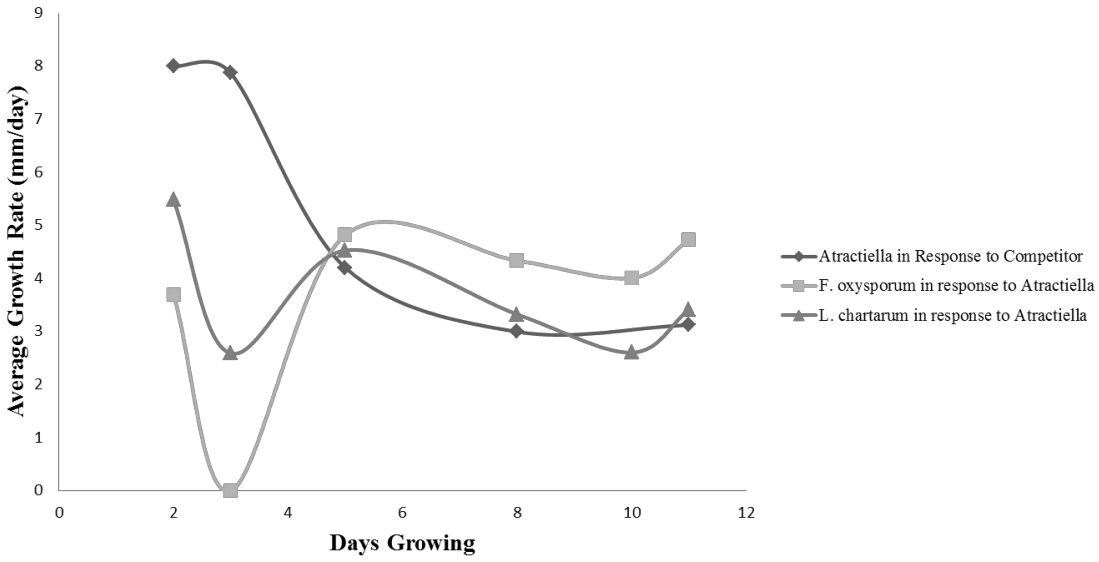
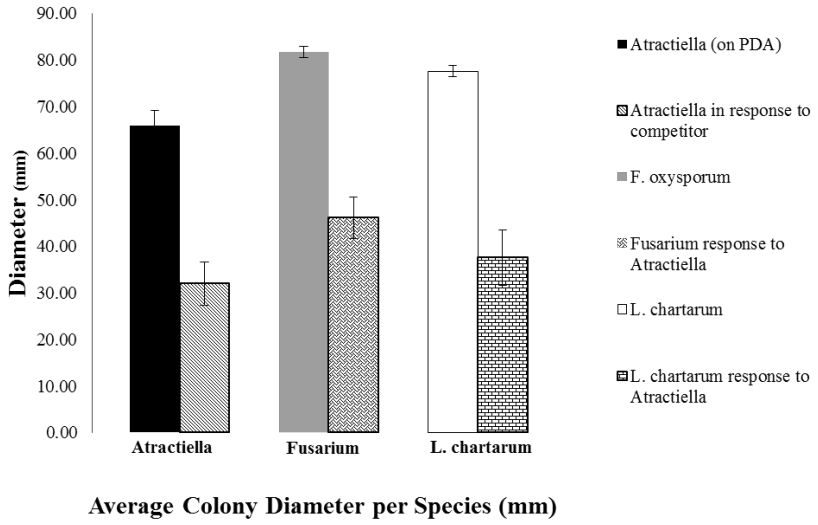
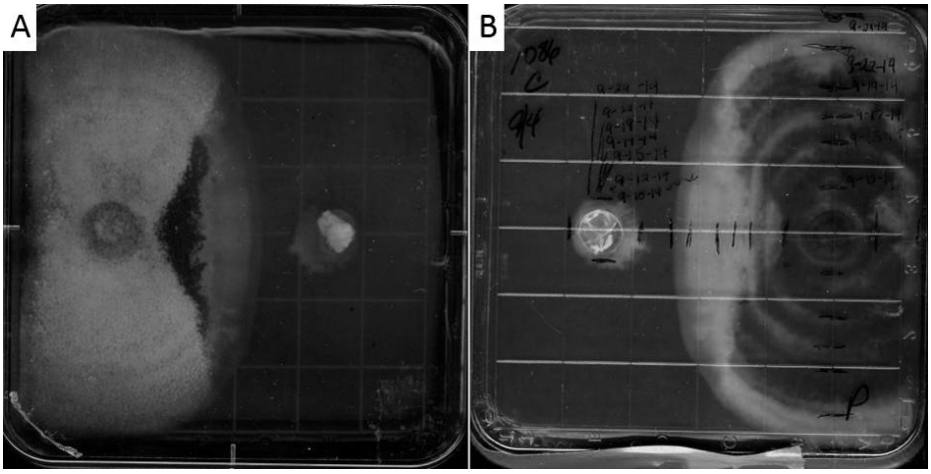

Average Growth Rate (mm/day)
Days Growing
0
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10
12
Atractiella in Response to Competitor
F. oxysporum in response to Atractiella
L. chartarum in response to Atractiella

**Fig 3**




**Average Colony Diameter per Species (mm)**

**Fig 4**



**Tables**

Table 1: Geographic origin and *Populus* trap plant hosts from which the Atractiella strains were collected from root tissues. Strains used in this study are bolded.

| Strain | Origin of Soil | Trap Plant Host |
|---|---|---|
| **95** | North Carolina (with *P. deltoides*) | *P. deltoides* |
| 152 | North Carolina (with *P. deltoides*) | *P. deltoides* |
| **1054** | California (with *P. trichocarpa*) | *P. trichocarpa* |
| **1086** | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1114 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1119 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| **1138** | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1140 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1142 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1145 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1148 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1152 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1165 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1165.2 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1176 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1180 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| 1199 | California (with *P. trichocarpa*) | *P. trichocarpa* |
| **1210** | California (with *P. trichocarpa*) | *P. trichocarpa* |

| | | |
|---|---|---|
| **1580** | North Carolina (with *P*. *deltoides*) | *P*. *ponderosa* |
| **1587** | North Carolina (with *P*. *deltoides*) | *P*. *trichocarpa* |
| **1645** | North Carolina (with *P*. *deltoides*) | *P*. *trichocarpa* |
| **1649** | North Carolina (with *P*. *deltoides*) | *P*. *trichocarpa* |
| 1656 | North Carolina (with *P*. *deltoides*) | *P*. *trichocarpa* |
| 1678 | North Carolina (with *P*. *deltoides*) | *P*. *trichocarpa* |

Table 2: All strains of *Atractiella* produced significantly different growth rates in response to media type.

| Effect | Num df | den df | F | P |
|---|---|---|---|---|
| Time | 5 | 50 | 12.88 | <0.0001 |
| Strain | 6 | 10 | 4.63 | 0.0166 |
| Time X Strain | 30 | 50 | 1.35 | 0.1733 |
| Media | 1 | 10 | 228.08 | <0.0001 |
| Strain X Media | 6 | 10 | 4.2 | 0.0226 |

Table 3: A summary of the single pair interactions performed between *Atractiella* and co-isolated fungal taxon from *Populus*. A + designation indicates *Atractiella* out-competed the co-isolate, a – indicates *Atractiella* was out-competed by the co-isolate, and 0 indicates no observed effect on growth of either *Atractiella* or the co-isolate.

| Single Pair Interactions Table of *Atractiella* versus a Co-Isolate | | | |
|---|---|---|---|
| Paired Species | Atractiella - PMI 152 | Atractiella - PMI 95 | Atractiella - PMI252 |
| *Pleosporales* - PMI 150 | - | - | - |
| *Ilyonectria* - PMI 151 | 0 | 0 | 0 |
| *Ilyonectria* - PMI 153 | + | + | 0 |
| *Ilyonectria* - PMI 154 | + | + | + |
| *Pleosporales* PMI 155 | - | 0 | - |
| *Phialocephala* - PMI 193 | + | + | + |
| *Mortierella* PMI 93 | - | - | - |
| *Thelephoraceae* PMI 130 | + | + | + |
| *Sordariomycete* PMI 145 | + | 0 | 0 |
| *Pleosporales* - PMI 146 | + | + | + |
| *Fusarium* - PMI 11 | - | - | - |
| *Umbellopsis* - PMI 12 | 0 | 0 | 0 |
| *Corticiales* - PMI 14 | 0 | 0 | 0 |
| *Ilyonectri*a - PMI 122 | - | 0 / - | - |
| *Mariannaea* - PMI 123 | 0 | 0 | 0 |
| *Sordariomycete* - PMI 1 | 0 | 0 | 0 |
| *Sordariomycete* - PMI 3 | 0 | 0 | 0 |
| *Sordariales* - PMI 4 | 0 | 0 | 0 |
| *Ilyonectria* - PMI 6 | - | - | - |

| | | | |
|---|---|---|---|
| *Ilyonectria* - PMI 7 | - | - | - |
| *Ilyonectria* - PMI 83 | - | 0 | 0 / - |
| *Mortierella* PMI 85 | - | 0 / - | - |
| *Mortierella* PMI 86 | - | - | - |
| *Ilyonectria* - PMI 91 | 0 | 0 | 0 |
| *Heliotiales* - PMI 350 | + | + | + |
| *Clavulina* - PMI 390 | 0 / - | 0 | 0 / - |
| *Leptodontidium* - PMI 454 | 0 | 0 / + | 0 |
| *Sordariomycete* - PMI 493 | 0 / - | 0 | 0 / - |
| *Flagelloscypha* - PMI526 | 0 / - | 0 / - | 0 / - |
| *Sordariomycete* - PMI 527 | 0 | 0 | 0 |

Table 4: All effects were significant separately and in combination, with the C:N ratio having the largest impact on *Atractiella* growth rate.

| Effect | DF | F Value | p Value |
|---|---|---|---|
| Treatment | 6 | 5.46 | 0.0002 |
| C:N Ratio | 1 | 16.69 | 0.0002 |
| Treatment x C:N Ratio | 4 | 3.51 | 0.0137 |

Table 5: Comparison of growth rate in response to identity of focal fungus (*Atractiella*, *F. oxysporum* or *L. chartarum*) and competitor presence or absence.

| Effect | df | T | Tukey's adjusted p |
|---|---|---|---|
| *Atractiella*/*F. oxysporum* versus *Atractiella* alone | 10 | -2.76 | 0.0201 |
| *F. oxysporum*/*Atractiella* versus *F. oxysporum* alone | 10 | -2.49 | 0.0322 |
| *Atractiella*/*L. chartarum* versus *Atractiella* alone | 10 | -3.3 | 0.008 |
| *L. chartarum*/*Atractiella* versus *L. chartarum* alone | 10 | -2.52 | 0.0303 |
| *Atractiella* alone | 10 | -2.4 | 0.037 |
| *F. oxysporum* alone | 10 | -3.71 | 0.004 |
| *L. chartarum* alone | 10 | -4.29 | 0.0016 |

Table 6: Metabolite prevalence in mg $g^{-1}$ as determined by GC-MS analysis. Determination of significant differences was not tested on account of a small total sample size.

| | Identified Metabolites | | | | | | Unidentified Metabolites | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample ID | 3-deoxy-D-ribo-hexitol | 2,4-dihydroxy-5-methyl-2H-1,4-benzoxazin-3-one | uric acid | a-e-diaminopimelic acid | butanediols | 523 538 glycosides | 1[a] | 2[b] | 3[c] |
| *F. oxysporum/Atractiella* | **50.4** | **17.3** | 1.7 | 19 | 25.9 | 143 | **33.9** | **27.1** | 3.9 |
| *Atractiella/F. oxysporum* | 21 | 8.4 | 1 | **37.1** | **56.4** | 76.9 | 14.2 | 12.4 | 1.6 |
| *F. Oxysporum* | 15.1 | **62.5** | 0 | 0 | 0 | 0 | **113.9** | **120.8** | **31.3** |
| *L. chartarum/Atractiella* | 13.3 | 0 | 0.3 | 0 | 3 | 424 | 0 | 0.2 | 0 |
| *Atractiella/L. chartarum* | 3.7 | 0 | 0.3 | 0 | 2.3 | 426.7 | 0 | 0.2 | 0 |
| *L. chartarum* | 6 | 1.1 | 0.3 | 0 | 15.4 | 109.8 | 1.1 | 1.2 | 0.3 |
| *Atractiella* | 0 | 0.9 | 0.1 | 0 | 0.4 | 0.5 | 1.3 | 0.8 | 0.2 |

[a]Unidentified metabolite 1 ID number: 12.52 234 324 194 249. [b]Unidentified metabolite 2 ID number: 13.52 324 412 163 193 222. [c]Unidentified metabolite 3 ID number: 11.08 234 193 180 9.