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Elucidating Function in Early Diverging Non-Flagellated Fungi Using Metabolomics and Proteomics

Gregory R. Boyce

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Elucidating function in early diverging non-flagellated fungi using metabolomics and proteomics

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Dissertation submitted to the Davis College of Agriculture, Natural Resources and Design at
West Virginia University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Genetics and Developmental Biology

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Abstract
Exploring Metabolic Capacity in Two Distinct Early Diverging Fungal Symbioses
Gregory Boyce

The obligate lifestyle of many of the early diverging non-flagellated fungi have long precluded them from functional studies reserved primarily for their easily cultured, more derived counterparts. Modern –omics–based tools including transcriptomics, proteomics and metabolomics have facilitated a better understanding of many fungi but have yet to be employed in the study of early diverging non-flagellated fungi with few exceptions. Using global and targeted metabolomics and proteomics across the symbiotic spectrum, a series of studies were undertaken to better understand metabolism of three symbiotic interactions: two AM fungus-plant associations including *Rhizophagus dimorphicus* – grass, *R. clarus* – legume, and one fungus-insect interaction, *Massospora* – cicada. For *Rhizophagus dimorphicus*, a previously undescribed dimorphic AM fungus, a multi –omics approach was taken to better understand the metabolic differences underlying phenotypic differences. The results of the multi –omics approach revealed a truncated proteome and metabolome being expressed in the white morphotype when compared to the dark morphotype. These differences in the proteome and the metabolome of these synanamorphs suggest a potential compartmentalization of metabolism in *R. dimorphicus*. However further investigation is required to fully understand this phenomenon. To better understand the interactions among AMF fungi, plant host, and environment, an applied metabolomics approach was used to elucidate the impact of each variable individually and combined on the primary metabolism of *Rhizophagus clarus*, another well described AMF member. Two leguminous plant species, *Robinia pseudoacacia* (black locust) and *Trifolium pretense* (red clover) were inoculated and maintained at two different pH (pH 4.5 and 7.5). Individual hosts and both soil pH yielded distinct metabolite profiles from fungal spores, with soil pH having a greater influence on overall metabolism. Pathway analysis showed significant changes in intermediates and end products of the glutamate catabolism pathway as a function of change in medium pH. The results of this study provide insight into how AMF fungi regulate the pH of their host tissues, the result of which may facilitate widespread colonization across suboptimal soil conditions. Finally, global and targeted metabolomics were used to better understand obligate entomopathogenic fungi, *Massospora* species, and compare two contemporary *Massospora* – cicada consortia, *M. cicadina*-infected periodical cicadas (*Magicicada* spp.) and *M. levispora* comb nov.-infected banger-wing cicadas (*Platypedia putnami*) to better help elucidate factors influencing host colonization and putative behavioral modification. Results of the global metabolomics uncovered a diverse assortment of secondary metabolites including psilocybin from *M. levispora* comb. nov. and cathinone from *M. cicadina*. These psychotropic compounds may enhance aggressiveness/stamina of infected cicadas to ensure continued spore dispersal despite debilitating infections that likely result in fatigue, decreased muscle strength and general malaise. Such discoveries as presented here have only begun to scratch the surface of the vast metabolic capacity of these two groups of enigmatic fungi. Using a systems biology approach has been shown to be an effective strategy to investigate the complex functionality of these two symbiotic systems.

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Chapter 1 Introduction

Metabolomics is the analysis of the organism's complete metabolite complement produced by the genome and proteome of an organism (Bhadauria et al. 2007, Zhou et al. 2012). The study of metabolomics involves the identification and quantification of small molecule metabolites. Multiple analytical tools can be implemented for the study of metabolomics, including gas chromatography mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), and liquid chromatography mass spectrometry (LC-MS) (Yoshida et al. 2009, Zhou et al. 2012, Dona et al. 2016, Markley et al. 2017). These tools allow for the qualitative or quantitative measurement of the functional properties that directly affect the metabolism of an organism. Recent increases in the availability of bioinformatics tools, metabolite databases and analytical instrumentation have made these studies possible (Shulaev 2006, Doyle 2011, Sugimoto et al. 2012).

The chemo-diversity found within Kingdom Fungi make these organisms perfectly suited to metabolic exploration. Through secondary metabolism, fungi are able to produce many different compounds that have importance in the pharmaceutical, agricultural, food, and natural products industries. The ability for fungi to produce these economically important secondary metabolites has drawn attention of biochemists and natural product scientists (Aly et al. 2010). The implementation of a robust analytical approach to understanding the metabolic processes involved in the production of these biochemicals could provide valuable insight in how to best enhance their production. LC-MS based global metabolomics analysis have been employed to

elucidate the most efficient conditions for culturing of fungi and to increase production of their secondary metabolites (Verduyn et al. 1992). Analyzing the metabolic flux within a fungal system also can provide valuable information into the response of organisms to environmental changes and to characterize physiological changes in an organism during its life cycle (Doyle 2011). The core enzymes required to complete metabolic pathways typically are highly conserved (Peregrín-Alvarez et al. 2009). However, how a specific organism utilizes and organizes these enzymes can greatly impact the end products of these pathways (Peregrín-Alvarez et al. 2009). This variability is what drives the study of metabolomics, especially in fungi, due to their vast metabolic potential (Sauer et al. 2002) and the nature of their interactions with their host(s).

The Zygomycetes are one group of fungi that could be better understood through a global metabolomics analysis are obligately biotrophic fungi found primarily within members of the Mucoromycota and Zoopagomycota. These systems are complex and are comprised of numerous unculturable fungi with restricted host ranges. The two groups from these classes of fungi that will be examined in this work will be the Entomophthorales (Zoopagomycota) and the Glomeromycotina (Mucoromycota).

The Zoopagomycota, whose members are considered the earliest diverging lineage of nonflagellated fungi, comprise 19 genera and 228 described species worldwide. Multiple systematic biological approaches have helped to elucidate some aspects of zygomycete evolution (Małagocka et al. 2015, Meier et al. 2015, Li et al. 2017). Yet many of the basic mechanisms that drive pathogenicity and behavioral variation still are not understood compared to data on their Ascomycotan counterparts (de Bekker et al. 2013). The implementation of a proven analytical

approach, such as LC-MS based metabolomics, can help elucidate these complex biological relationships.

Arbuscular mycorrhizal fungi (AMF) also are obligate fungal symbionts, but they reside in soil and form associations with over 80% of vascular plants on every continent. The first appearance of these fungi directly coincides with the appearance of land plants (Davison et al. (2015). These fungi also have been shown to form this association with plants in almost any terrestrial ecosystem (Smith and Read 2008). The association between plants and AMF improves phosphorus and water uptake, and in a few circumstances seem to provide pathogen and toxicity protection to the plant (Jeffries et al. 2003). In exchange, the fungus obtains carbon from photosynthetic output of the plant (Davison et al. 2015). The AMF association with plants has been shown to play an important role in improving species richness, biodiversity, and plant community stability (Van der Heijde et al. 2008).

Arbuscular mycorrhizal fungi encompass > 250 species described to date (<http://schuessler.userweb.mwn.de/amphylo>). AMF taxonomy originally was based on spore morphology and mode of spore formation (Gerdemann JW 1974, Morton JB 1990). More recently, molecular based approaches have been used for identification and phylogenetic classification of these fungi (Redecker et al. 2013). The study of the 18S rRNA gene (SSU) used for phylogenetic classification (SCHÜßLER et al. 2001) did not provide a complete unified result because some of the molecular data did not agree with the morphological evolution described by Morton (1990) and Schüßler (2001). The sequencing of ribosomal DNA has been the primary source for molecular based phylogeny of arbuscular mycorrhizal fungi, but other genes have been used with varying success. A study of partial sequences of the β -tubulin gene for phylogenetic analysis of 45 species of AMF showed that sequence data was not congruent with

SSU data at the order level, but provided congruence at the family and species level (Msiska and Morton 2008). The high variation of the exons found in this gene between species could account for some of the inconsistencies measured in this study. Stockinger et al (2010) reconstructed a phylogeny of AMF species using the RPB1 gene that correlated reasonably well with the phylogenies of rRNA genes. This single copy gene contains enough variable regions to provide resolution at the species level and above. Variation in intron organization between taxonomic groups has created some major challenges in finding suitable functional genes for sequencing and phylogenetic analysis. Metabolomics can provide an effective tool for understanding and identifying functional genes and their products for molecular targets.

Lisette et al. (2000) extracted protein from spores of several species and ran the extracted proteins on one dimensional polyacrylamide gels to obtain unique protein profiles for each species. These data provided the first evidence that species of AMF contain unique proteomes, but they were unable to identify these proteins and their functions from gel analysis alone. One of their most important findings was measurement of distinct variation in the concentration of total protein in spores of different AMF species. The amount of protein per spore averaged 20 ng across the *Glomus* species analyzed. Improvements in mass spectrometry and sequences of 28,232 genes in the *Rhizophagus irregularis* genome (Tisserant et al. 2013) provided more information on the identity and functional attributes of these proteins. The theoretical proteome was translated from the genome information and the resultant UniProt database allowed for searching global proteomic digest sequences to identify proteins. Still, much functional information is absent because a majority of the theoretical proteins found in this database have not yet been characterized. This issue is less apparent when studying the small molecule metabolite components of a biological system because core enzymes tend to be more conserved.

However, the manner in which a specific organism utilizes and organizes these enzymes can greatly impact the end products of these pathways (Peregrín-Alvarez et al. 2009). This variability is what drives the study of metabolomics, especially in fungi (Sauer et al. 2002).

In this study, both entomopathogenic fungi and arbuscular mycorrhizal fungi are studied at the biochemical level in their obligate interactions with their hosts (insects and plants, respectively). Each of these symbioses, defined here as close and long-term interactions between two phylogenetically distant organisms, can be parasitic or mutualistic, respectively (Douglas, 1994). Both mutualistic and parasitic symbioses have biological importance, and understanding their metabolic pathways could provide valuable insight into the functional interactions between host and fungal symbiont (Schardl et al. 2004, Smith et al. 2011). These interactions can have an impact on the metabolism of both the host and the symbiont (Russell et al. 2013). Fungal-Insect symbiotic interactions have been shown to increase the production of phenylalanine and tryptophan in both the host and the fungus (Russell et al. 2013). These two aromatic amino acids are the primary building blocks for a variety of secondary metabolites that provide defense against pathogens and predation (Siddiqui et al. 2012, Tohge et al. 2013). The plant-fungus interaction that occurs with arbuscular mycorrhizal fungi can increase the levels of the terpenoids accumulated in plant tissues (Welling et al. 2016, Sharma et al. 2017), which in turn can increase the plant's defense against herbivorous insects (Sharma et al. 2017). These mutualistic interactions can supplement and modify the host's metabolism to improve fitness.

The metabolism of the pathogenic symbiosis involves complex interactions, where the fungal pathogen often modifies its environment or the metabolism of its host in order to survive (Miyara et al. 2010). Fungal pathogens with broad host ranges have the ability to alter their own secondary metabolism in order to increase their virulence on different hosts (Ito et al. 2004).

These complex metabolic changes within both the mutualistic and pathogenic fungal symbioses can utilize metabolomic tools to elucidate mechanisms of interactions and their functional significance (Schardl et al. 2004, Smith et al. 2011, Russell et al. 2013). Moreover, analyses at the biochemical level can reveal unique as well as shared processes in contrasting mutualistic and pathogenic symbioses.

Chapter 2 *Rhizophagus dimorphicus* sp. nov., a dimorphic species in Glomeraceae (Glomeromycota), produces glomoid spores with two contrasting phenotypes and a partitioning of divergent metabolisms

Abstract

A dimorphic species of arbuscular mycorrhizal fungi, *Rhizophagus dimorphicus*, was discovered in the International Culture Collection of Arbuscular Mycorrhizal Fungi (INVAM) originating from Brazil and Florida. The two spore phenotypes were so different they were originally thought to be two distinct species, however repeated culturing of each phenotype yielded mix cultures containing both. Analysis of a 750-800 bp region near the 5' end of the 28S rRNS gene (LSU) resulted in the grouping of the two spore phenotypes into a single clade that is a sister to other *Rhizophagus* clades. These results confirmed the dimorphic nature of this single species. The two synanamorphs produced by this dimorphic species include a dark reddish-brown morph that most closely resembles *Septaglomus constrictum* and white morph that resembles *Rhizophagus clarus*. The dark morphotype forms globose to subglobose spores with three distinct layers that range in size from 60-160 µm. The subtending hyphae of the dark morph shows a constriction at the base of the subtending hyphae near the spore. The white morphotype also forms globose to subglobose spores with three layers that range in size from 60-120 µm. In addition to phylogenetic and morphological analysis a multi omics approach was taken to better understand the metabolic differences occurring within these two morphotypes. The global proteomics analysis revealed a truncated proteome being expressed in the white morphotype when compared to that of the dark morphotype. The global metabolomics showed a similar pattern, with the metabolome of the white spores lacking many of the secondary metabolites

found in the dark spores. These differences in the proteome and the metabolome of each spore morphotype suggest a potential compartmentalization of distinct metabolic processes.

Introduction

Two accessions deposited in the International Culture Collection of (VA) Arbuscular Mycorrhizal Fungi (INVAM) were deposited in 1988, cryopreserved until 2008, and then propagated in pot culture again thereafter. One culture originated from Brazil and the other from Florida. Both produced spores of identical black and white contrasting phenotypes, with the former sharing features of *Septoglomus constrictum* and the latter with some resemblance to *Rhizophagus clarus*. Both spore phenotypes were so different they were assumed to be separate species and new cultures of both strains were started from each spore phenotype. Surprisingly, both phenotypes sporulated together in each of the culture lineages and repeated attempts over the next eight years to separate the phenotypes in pure culture failed. Sporulation always was highly productive, but both morphotypes were inseparable. When cultures of each morphotype were started from single spores of the Florida strain, both phenotypes sporulated together. Genotyping using a 750-800 bp region near the 5' end of the 28S rRNA gene (LSU) grouped both morphotypes in the same clade and verified their dimorphic behavior. Two additional accessions originating in Taiwan and Colorado, USA were identified as synonymous from pot cultures started in 2005 and 2011, respectively. With four geographically distinct strains grouped by identical spore phenotypes, sporulation behavior and similarity in LSU sequences, sufficient evidence existed to group and define them as a new species, *Rhizophagus dimorphicus*.

The functional value of two spore morphotypes, especially when they are dramatically contrasting phenotypes, is worthy of investigation because fungi are modular organisms

(Dighton et al. 1992). As such, they have no defined birth-death cycles, are flexible in component parts capable of starting new individuals (e.g. spores, hyphae), and those parts often are repetitive and form asynchronously (REF). With a mobile population of nuclei that are able to migrate into different spores or hyphae at any time during the life cycle of a glomeromycotan fungus (Bever and Wang 2005), sample opportunities exist for partitioning of resources and responses to environmental perturbations. Global metabolomics and proteomics provides the tools to examine the biochemical processes occurring in the two synanamorphs of *R. dimorphicus*. In this study, the hypothesis that these processes differ between synanamorphs is tested. Also, *R. dimorphicus* is formally described.

Materials and Methods

Standardized methods perfected over 26 years of culturing more than 1000 strains of glomeromycotan species were used to grow and characterize the four strains of *R. dimorphicus* (Morton et al., 1993). Starter material of each culture consisted of chopped roots, spores and mycorrhizal roots from a pre-existing culture. This homogenized material was mixed in a growth medium consisting of coarse sand and a sandy loam soil (3:1 v/v) in a 15-cm diam pot, with pH adjusted to 6.2 and < 10 mg/kg P. The pot was seeded with *Sorghum sudanense* (L.) Pers. and grown for four months in a greenhouse with high-output fluorescent lighting (photon flux density of $345 \mu\text{mol m}^{-2} \text{s}^{-1}$, measured at the soil line) and a daylength of 14 h. Ambient temperature varied from 22 - 30° C for duration of the culture period.

For single spore cultures, the same growth medium was used as that described above, but the medium was placed in 3.8 x 21 cm (150 cm³) SC10 Supercell “cone-tainers” (Stuewe and Sons, Inc., Corvallis, OR). Spores were extracted from a mature pot culture by wet-sieving and sucrose gradient centrifugation (Gerdemann and Nicolson 1963), washed, collected under a dissecting

microscope and surfaced cleaned by sonication in distilled water. Individual spores of each spore phenotype were selected randomly from the extracted population and pipetted onto intertwined roots of three 10-12 day-old sorghum (*Sorghum sudanense* L.) seedlings. Plants were transplanted into cone-tainers and grown in a growth room with $420 \mu\text{E m}^{-2} \text{sec}^{-1}$ light intensity for a 14-hour day period and air temperature ranging from 25-28 C. Spores were sampled from these cone-tainers three months after transplanting and the populations viewed microscopically. Reculturing of these lineages was done using bulk inocula described above.

Morphological analysis

Spores collected from the field and from all pot cultures were preserved in 0.05% sodium azide and vials were deposited at the Oregon State University Herbarium and in the voucher collection of INVAM. Spores also were mounted permanently on glass slides in polyvinyl alcohol-lactic acid-glycerol (PVLG, Koske and Tessier, 1983) and in PVLG with Melzer's agent (1:1 v/v). Spores were broken to varying degrees by applying pressure to the coverslip and examined with a Nikon Optiphot compound microscope equipped with differential interference contrast (DIC) optics. Selected slides also were deposited at the Oregon State University Herbarium and in the voucher collection of INVAM. Color photographs of mycorrhizae and spores can be viewed on the World Wide Web at <http://invam.wvu.edu>.

Morphological characters of the black and white synanamorphs of *Rhizophagus dimorphicus* overlapped enough with those described for *Septoglomus constrictus* and *Rhizophagus clarus* to conduct a comparative analysis with reference strains in INVAM. All

comparisons were done using fresh spores extracted from actively growing pot cultures since some artefacts can arise in stored material.

DNA extraction, amplification and sequencing

Single sonicated spores of each synanamorph were selected for each amplification. A spore was crushed in a 0.2 mL microcentrifuge tube containing 14 μ L 10X *Taq* polymerase buffer (New England Biolabs, Ipswich, MA) with a UV sterilized micropestle (Kontes Pellet Pestle). Tube contents were transferred immediately to 94 °C water bath for 4 min, snap chilled and stored on ice.

Two rounds of 50 μ l PCR amplification were performed to generate enough product of a 700-750bp LSU fragment for cloning (vanTuinen *et al.* 1998). The first round of amplification utilized primer pair ITS1 and NDL22, followed by a nested amplification with primers LR1 and FLR2 (White *et al.* 1990; van Tuinen *et al.* 1998; Trouvelot *et al.* 1999). The first PCR amplification contained 4 μ l spore DNA, 3 μ l 10x PCR buffer (New England BioLabs, Ipswich, MA), 0.2 mM of each dNTP, 1.5 mM MgCl₂, 5 pM of each primer, and 0.1 μ l of *Taq* DNA polymerase (New England BioLabs, Ipswich, MA). The second round of PCR was similar except that only 1 μ l of first amplification product was used as template in 7 μ l 10x PCR buffer.

Cycling parameters for both PCR reactions entailed initial denaturation at 94 °C for 4 min, 30 cycles at 94 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. Amplicons (8 μ l) were stained with ethidium bromide and electrophoresed on a 1.5% agarose gel and visualized by UV illumination, purified with QIAquick PCR Purification kit (Qiagen USA, Valencia, CA), cloned with pCR®4-TOPO plasmid vector and transformed using One Shot® TOP10 chemically competent cells

(Invitrogen, Carlsbad, CA). Positive transformants were verified with colony PCR using LR1/FLR2 primers. Plasmid DNA containing the insert was purified using QIAprep Miniprep kit and sequenced at Davis Sequencing (Davis, CA, USA) using M13 primers. LSU sequences were deposited in NCBI XXXXXX. The sequence alignment is available on the INVAM website (<http://invam.wvu.edu/>).

Phylogenetic analyses

Sequence chromatographs were inspected in Biological Sequence Alignment Editor (BioEdit, www.mbio.ncsu.edu/BioEdit/bioedit.html) and subjected to a search on the NCBI nucleotide Basic Local Alignment Search Tool (nBLAST) to verify homology with other glomeromycotan fungal sequences. Nucleotide sequences were aligned using MUSCLE (Edgar 2004), inspected and edited in MEGA6 (Tamura *et al.* 2013). Phylogenetic trees were reconstructed using the maximum likelihood (ML) method with 1000 replications to assess bootstrap support and MEGA6 to determine the best evolutionary model and nucleotide substitution pattern.

Global proteome analyses

A sampling of 200 spores from an extracted population of spores of each synanamorph of *R. dimorphicus* were collected for global proteomic analysis. The spores were lysed in a protein extraction buffer consisting of 10 mM ammonium bicarbonate and 0.1% Progenta non-ionic acid labile surfactant. The spores were placed into a 0.5 mL microcentrifuge tube with 50 µL of extraction buffer and crushed using a UV sterilized pestle. The spore samples then were sonicated for 20 minutes prior to a 1-hour incubation on ice. After incubation, spore samples

were centrifuged at 14,000 xG to pellet spore debris. The resulting supernatant was removed for proteolytic digestion.

Ten mM dithithreitol (DTT) was added (25 μ L) to each sample to reduce the protein. The samples were incubated for 1 hour at 37°C, after which 25 μ L of 10 mM iodoacetamide (IA) was added and the samples incubated at room temperature in the dark for one hour. Sequencing grade trypsin was prepared at a concentration of 1 μ g/ μ L and a total of 4 μ L was added to each sample for digestion. The samples were incubated for 10 hours at 37°C, after which they were acidified by adding 4 μ L of 10% formic acid and freeze dried prior to mass spectrometry analysis.

Samples were reconstituted in 50 μ L of 0.1% formic acid in H₂O for mass spectrometry analysis. For chromatography, nanoLC was performed on an Agilent 1260 Infinity Nanoflow using a ProntoSil C18, 75m x 150mm (200 Å, 3mm) (nanoLCMS) at a flow of 150nL/min. Mobile phases consisted of A) 0.1% formic acid/water and B) 0.1% formic acid /acetonitrile. Mass spectrometry was analysis was performed using a Q Exactive Orbitrap (Thermo) operating in positive ion mode with a mass range of 400 – 2000 m/z for precursor ions with Resolution set at 70,000 mass resolution in profile mode. Tandem mass spectrometry (MS/MS) spectra were collected with a mass range of 400 – 2000 m/z with resolution set at 17,500 mass resolution in profile mode. Data Dependent Acquisition mode was used for MSMS data acquisition with the top Top10 ions selected for MS/MS (Normalized Collision Energy @ NCE 27) and Dynamic Exclusion set to 20 seconds. Proteomics LC-MS/MS data was processed using Proteome Discoverer (V 2.1, Thermo). Search engines included Sequest HT and Mascot. Precursor Tolerance was set to 10 ppm and Fragment Mass Tolerance was set to 0.02 Da. Static Modifications were set to carbamindomethylation (C) and Dynamic Modifications were

designated for deamidation (N&Q) and oxidation (M). A *Rhizophagus irregularis* Fasta protein database was used for all queries. Confidence for protein identification was set to a minimum of two peptides per protein with 0.5% Decoy False Discovery Rate. Scaffold (Proteome Software, Inc.) was used for determination of fold-changes in protein expression across samples. MS/MS data was imported into Scaffold and for Label-Free Quantitation the Total Precursor Intensities were compared pairwise for each spore morphotype. All fold-changes were calculated in reference to the Total Precursor Intensities (Normalized) for the Control samples. Quantitative analysis and statistical evaluation was performed using a T-test with Benjamin-Hochberg (Multiple Test Correction). Significance was set to $p < 0.05$ for detecting fold-change differences between species.

Global metabolome analyses

A total of 500 spores from each of *R. dimorphicus* FL727N and *Rhizophagus clarus* WV235 were sonicated in methanol for 20 minutes to remove any plant or growth media related metabolites. This process was performed in triplicate. The fungal metabolome was extracted from sonicated spores of each morphotype through manual lysing in 200 μ L of extraction buffer in a 0.5 mL microcentrifuge tube with a UV sterilized micropestle. The extraction buffer consisted of 80% methanol and 20% high performance liquid chromatography (HPLC) grade H₂O. The spore lysate was then sonicated at 14,000 x g for 20 minutes at 4°C to pellet proteins and cellular debris. The resulting supernatant was then removed for global metabolome analysis.

The Agilent LC-MS system (Agilent Technologies, Santa Clara, CA, USA) included an Agilent 1290 Infinity quaternary ultra-high pressure liquid chromatography (UHPLC) pump. This LC system was coupled to an Agilent 6530 quadrupole time of flight (Q-ToF) mass

spectrometer with electrospray ionization. The chromatographic separations were performed using a Merck polymeric bead based ZIC-pHILIC column (100mmx2.1mm, 5 μ m).

The LC-MS/MS data acquired using Agilent Mass Hunter Workstation was processed in Profinder software for batch recursive analysis. The datasets were subjected to spectral peak extraction with a minimum peak height of 600 counts and the charge state for each metabolite was restricted to two. Further, retention time and mass alignment corrections were performed using Agilent Profinder software version 2.3.1 on the runs to remove non-reproducible signals. The resulting features were then exported as .cef files to Mass Profiler Professional (MPP) software version 2.4.3 (Agilent Technologies, Santa Clara, CA, USA) for statistical analysis.

Statistical Data Analysis the extracted features were imported into MPP software for statistical analysis. Principle Component Analysis (PCA) was performed to check the quality of the samples and then the data containing filtered features were processed by unpaired t-test to find the difference between the dark and white synanamorphs. Only the analytes with p values < 0.05 and fold change (FC) >5 were considered statistically significant. Additionally, multiple test corrections using Bonferroni were applied to reduce false positives and false negatives in the data. The statistically filtered data was exported to Mass Hunter for targeted MS/MS analysis.

Taxonomy

Rhizophagus dimorphicus nov. sp. Boyce, Kasson & Morton

This species produces two morphologically distinct spore phenotypes (synanamorphs), one that is dark red-brown to black and one that is white to pale amber (Fig. 1A). In all cultures propagated to date, the black synanamorph sometimes sporulated alone but usually numbered more than twice that of the white synanamorph when they sporulated together. When present, both synanamorphs sporulated together within a root segment (Figs 1B-F), although the dark

synanamorph often numbered at least twice or more that of the white synanamorph. The phenotype of each synanamorph was formed terminally from separate hyphae or branched from a shared hypha (Fig 1F-G).

Mycorrhizae of *R. dimorphicus* produce a typically dark staining reaction by 0.05% direct blue in roots of *S. sudanense*. Arbuscules and intraradical hyphae are similar in appearance to those of other species in Glomeraceae. The hyphae were mostly cylindrical and straight, ranging in width from 4-10 μm , rarely formed coils and often branched at acute angles in cortical cells. Intraradical vesicles are interspersed with thicker-walled spores of both spore phenotypes often present together (Fig1D-E). A phylogeny of the LSU gene placed *R. dimorphicus* in an equivalent sister clade of *R. clarus*, *R. intraradices*, and *R. irregularis* (Fig. X).

Dark synanamorph

Spores are smooth, globose to subglobose, 60-160 μm diam. (mean = 110 μm , n = 100; Fig. 2), and dark orange brown to black in reflected light. The median spore color is a dark reddish-black.

The spore wall consists of three layers with a composite thickness of 11.3 μm (N=50) (Fig. 3A-D). The outer layer (L1) is hyaline, 2-3 μm thick (mean = 2.2 μm , n=30), staining amber in Melzer's reagent, sometimes sloughing with age. The middle permanent layer (L2) is dark red-brown, 5-8 μm thick (mean = 7.4, n=50), and rigid. The inner permanent layer (L3) also is red-brown, 2-4 μm thick (mean = 3.2, n=50), and sometimes thin enough to produce folding in crushed spores.

The subtending hypha varies from constricted near the spore base to cylindrical, 4-5 μm wide for the former and 8-12 μm wide for the latter (Fig. 3D). The hyphal wall is concolorous with that of the outer layer of the spore wall for a short distance (10-100 μm), after which the middle (layer 2) of the spore wall becomes the wall of the subtending hypha and transitions from an amber color to hyaline. The subtending hyphal outer layer ranges from 1-3 μm thick and the inner layer 2-4 μm thick at the base of the spore. There is a thin septum that is $< 1 \mu\text{m}$ thick present separating the spore contents from the subtending hyphae.

White synanamorph

Spores of the white synanamorph are smooth, globose to subglobose, 60-120 μm diam. (mean = 107 μm , $n = 100$; Fig. 2). The spore wall consists of three layers, all of which are hyaline (Fig. 4A-D). The outer layer (L1) produces a pale to dark red dextrinoid reaction in Melzer's reagent when present, especially in immature spores (Fig. 4B). This layer generally is less than 1-2 μm thick (mean = 1.8 μm) but sloughs to create a rough surface (Fig. 4A) until it is shed completely (Fig. 4D). The middle layer (L2) is mostly rigid but has a small degree of plasticity when pressure is applied. This layer is 4-8 μm thick (mean = 6.3 μm , $n=40$) μm , permanent and continuous with the wall of the subtending hypha. It does not react in Melzer's reagent (Fig. 4C-D). The inner layer (L3) also can be flexible (with folding; Fig. 4B), is 1-3 μm thick (mean = 2.1 μm , $n=40$), and is present as part of the subtending hyphal wall near the spore base (Fig. 4D).

The subtending hypha is consistently cylindrical to slightly flared, 6-10 μm wide at the spore, consisting of two hyaline layers, with a composite thickness of 1.5-3 μm . The middle layer of the spore wall (L2) comprises the main structural wall of the subtending hyphae, with

the inner layer of the spore wall (L3) extending 1 to 5 μm from the spore base (Fig. 4D). There is a thin septum that is $<1 \mu\text{m}$ thick separating the spore contents from the subtending hyphae.

Global Proteomics Results

The global proteomics data yielded 1,506, 1,327, and 888 proteins for *R. clarus*, *R. dimorphicus* (dark synanamorph), and *R. dimorphicus* (white synanamorph) respectively. *Rhizophagus clarus* and the two synanamorphs from *R. dimorphicus* shared expression of 723 proteins. The dark morphotype of *R. dimorphicus* expressed 111 unique proteins when compared to the white morphotype and *R. clarus*, while the white morphotype expressed only 9 unique proteins. A principal component analysis revealed that the proteomes of all three spore morphotypes grouped separate of each other, while that of the two synanamorphs of *R. dimorphicus* grouped closer to each other than with that of *R. clarus* (Fig. 5). These results show that white morphotype of *R. dimorphicus* expresses a truncated proteome in comparison to its darker synanamorph and *R. clarus* (Fig. 5). The search results for the identification of the proteins detected revealed that 92% of the proteins identified were uncharacterized, so that function could not be assigned. Molecular function could be assigned to 175 proteins, with 95 proteins involved in catalytic activity, and 137 proteins involved in binding reactions. While the currently available protein database from UNIPROT is only a theoretical proteome generated from the genome sequence of *R. irregularis*, it cannot be used to effectively assign function to the detected proteins. Nevertheless, these data provide valuable information for assessing the complexity of the proteomes from these spores.

Global Metabolomics Results

The global metabolomics analysis revealed two distinct metabolomes from the two morphotypes, these differences can be best visualized in the principal component analysis in Fig. 5. There were multiple classes of metabolites detected in the global analysis including lipids, free amino acids, nucleotides, and various secondary metabolites. The total detected metabolites from both of the morphotypes was 828. Of these compounds 517 were significantly upregulated in the dark morphotype compared to the white. These significantly upregulated compounds in the dark synanamorph were primarily comprised of secondary metabolites including flavones, and alkaloids. The upregulated metabolites that were observed in the white synanamorph, were primarily comprised of phospholipids and fatty acids. A hierarchical clustering analysis was performed based on the results of the global metabolomes from the two spore types and the two morphotypes grouped into their own clades. The heat map represents the classes of metabolites and their up/down regulation between spore types. The heatmap data highlights the down regulation of the secondary metabolites that are found to be upregulated in the dark synanamorph compared to the white (Fig. 6).

Etymology: Latin *dimorphicus*, referring to the two contrasting spore phenotypes produced from a single thallus.

Distribution and Habitat: This species is defined by four strains isolated from distant geographic locations. BR947 originated from the rhizosphere of *Vitis* species near Jundiai, Sao Paulo, Brazil, contributed by Eli Lopez on 4/25/1988, cryopreserved, reconstituted in 2014. CO215 was accessed in INVAM on 3/4/2011 by Robert Bills from native grasses on the Colorado Shortgrass Prairie (SGRB site). FL727N came from Bartow, Florida and was deposited by David Sylvia in 1988 after isolating from *Styrax*. TW115 was provided by Tomoko Oba on 8/29/2005 with no accompanying information.

Mycorrhizal associations: Associated with *Vitis* species, *Styrax americanus*, *Buchloe dactyloides* and *Bouteloua gracilis* in field samples; *Paspalum notatum*, *S. sudanense*, *Trifolium pratense*, and *Robinia pseudoacacia* in greenhouse-grown pot cultures.

Collections examined: Voucher specimens and living spores from pot cultures of BR947, CO215, FL727N, TW115: 18 Nov. 2016.

Discussion

The dimorphism of *Rhizophagus dimorphicus* is unique in the genus *Rhizophagus*, but this sporulation behavior occurs with low frequency across taxa in Glomeromycota (Redecker et al. 2009). Therefore, it is a trait that has evolved multiple times and is not sufficiently unique to have evolutionary consequence. Its functional role is unknown, but metabolomic analyses currently underway with *R. dimorphicus* and *Am. leptoticha* suggests there may be some partitioning and/or compartmentalization of pathways. This partitioning is evident in the metabolomics evidence presented in the current analysis. Spores of both synanamorphs are infective and form a mycorrhizal symbiosis, as evidenced by the establishment of successful single-spore cultures from both species.

The habit of intraradical sporulation also is a convergent behavioral trait within Glomeromycota, but in *Rhizophagus* it is shared by all species in the clade. When interpreted in the context of all other morphological traits in the genus, it is a trait that further supports monophyly of the clade.

The darkly pigmented synanamorph bears closest resemblance to spores of *Septoglomus constrictum*. The similarities between these two species include the size distribution of the spores, spore color, and the presence of a constricted subtending hyphae in some spores. The dark morphotype also resembles *S. constrictum* in the L1 and L2 layers of the spore wall. The L1 layers of both species have a similar thickness and both can slough and be missing on older spores. The L2 layer of both species range from 5-9 μm in thickness, are both rigid, and are both red-brown to red-black in color (Trappe 1977).

The major difference between *S. constrictum* and the dark morphotype of *R. dimorphicus* is the presence of a third inner spore wall layer (L3) in *R. dimorphicus* that is pigmented and enhances the dark coloration of intact spores (Trappe 1977). Moreover, *R. dimorphicus* readily sporulates in roots, and this habit is rare in cultures of *S. constrictum*.

The white synanamorph mostly closely resembles the phenotype of *R. clarus* spores. The shared features between *R. clarus* and the white morphotype of *R. dimorphicus* include the three spore wall layers, the ability to sporulate prolifically within roots of their plant host, and the structure of the subtending hyphae (Nicolson and Shenck 1979). The subtending hyphal wall of *R. clarus* and the white morphotype of *R. dimorphicus* are both cylindrical with a thickness range of 7-9 μm .

Spores of *R. clarus* and the white morphotype of *R. dimorphicus* differ in their size distribution, with the latter being smaller with a narrower range of distribution. Mean spore diam. of the white morphotype of *R. dimorphicus* and *R. clarus* is widely separated at 107 μm and 182 μm , respectively. Spores of *R. clarus* also range in color from white to dark yellow because the innermost spore wall layer is yellow and coloration varies with thickness of this layer. Conversely, the innermost layer of the *R. dimorphicus* morphotype always is hyaline.

The global proteomics analysis revealed a truncated proteome in the white morphotype of *R. dimorphicus*. The unique proteins expressed by these spores were less than 25% of the unique expressed proteins from spores of the dark morphotype. While the vast majority of the proteins detected in the global proteomics analysis were shared between *R. clarus* and the two morphotypes of *R. dimorphicus*, the dark synanamorph expressed more than 100 unique proteins, which is comparable to the number of unique protein expressed in spores of *R. clarus*.

The global metabolomics data suggests that metabolism may be compartmentalized between the two morphotypes of *R. dimorphicus*. While the dark morphotype contained a more complex and diverse metabolome, with numerous secondary metabolites and intermediates from the general carbon metabolism pathway, the white morphotype harbored a greater amount of phospholipids and lipid precursors. This suggests that the functional metabolism of the two morphotypes are vastly different. Work currently is underway to analyze further both the proteome and metabolome of both synanamorphs to better characterize functional compartmentalization of metabolic processes.

Figure 1. Sporulation behavior of the two synanamorphs of *Rhizophagus dimorphicus* strain FL727. A. Synanamorphs sporulating in a sudangrass culture grown for four months. B.

Sporulation in unstained 4-mo-old sudangrass roots. C-D. Stained mycorrhizae containing both synanamorphs from a single spore culture of the dark spore phenotype. E. Stained mycorrhizae containing both synanamorphs from a single spore culture of the white spore phenotype. F. Branched hypha producing each synanamorph terminally. G. Loose cluster showing formation of both synanamorphs from a shared hypha of origin. Bar in A, C-E = 100 μm ; bar in B = 200 μm , bar in F-G = 10 μm .

Figure 2. Spore diameter size distribution of the white and black synanamorphs of *R. dimorphicus*.

Figure 3. Representative images of the dark synanamorph of *R. dimorphicus* strain FL727 showing the three-layered spore wall structure. A. Spore mounted in PVLG, with the inner layer having some flexibility (folding). B. Spore mounted in Melzer's reagent. C. Spore in Melzer's reagent showing the two-layered subtending hypha at the spore base. D. Spore in Melzer's reagent exhibiting a constricted subtending hyphae.

Figure 4. Representative images of the white synanamorph of *R. dimorphicus* strain FL727. A. Spore mounted in PVLG showing two permanent spore wall layers (L2, L3) and remnants of the

sloughing outer (L1) layer. B. Young spore mounted in Melzer's reagent showing all three spore wall layers and the dark red reaction of L1. C. Spore in Melzer's reagent showing the lack of any reaction in the inner two wall layers (L2, L3). D. Spore in Melzer's showing the continuation of both inner spore wall layers in the subtending hypha and a sloughed outer layer (L1).

Figure 5. Principal component analysis (PCA) plot showing separation between the dark and white synanamorphs of *R. dimorphicus* strain FL727 and spores of *R. clarus* WV235. The Venn diagram shows the number of shared and unique proteins expressed by all three spore types from the two species.

Figure 6. LSU maximum likelihood (ML) phylogenetic tree for *Rhizophagus dimorphicus*. Bootstrap support is indicated near each node and only values greater than 70% are shown.

Figure 7. PCA from the global metabolomics analysis of the two snynanamorphs of *R. dimorphicus* strain FL727 (top). The separation between the two morphotypes indicates a high level of variation between their metabolomes. The clustering analysis and heat map (bottom) confirm the trends in the PCA plot, showing the separation of the two morphotypes into separate

clusters. The heat map visualizes the truncated metabolome of the white morphotype, with the large area of down-regulated metabolites depicted in blue.

Chapter 3 Applied metabolomics reveals host and environmental influence on metabolism in *Rhizophagus clarus*

Abstract

Arbuscular mycorrhizal fungi (AMF) are soil-inhabiting mutualists that obligately associate with a majority of vascular plant species and show little evidence for host specificity. Their obligate lifestyle and multinucleate state have impeded traditional genetic approaches. Yet, previous studies have confirmed that the AMF spores are metabolically active, and thus can serve to explore the metabolome of these fungi independent of their plant host. An applied metabolomics approach was used to elucidate the impact of host genotype and soil pH environment individually and together on the primary metabolism of *Rhizophagus clarus*, selected because it is a fungal mutualist occurring across a diversity of habitats and locations worldwide. The specific goal was to determine the relative role of host and soil pH on global metabolite expression in new spores. Two leguminous plant species, *Robinia pseudoacacia* (black locust) and *Trifolium pratense* (red clover) were inoculated and grown at pH 4.5 and 7.5 in a growth room. Fungal spores yielded distinct metabolic profiles in each host and in soil pH environment.

However, soil pH caused a greater influence on overall spore metabolism. Multiple classes of metabolites were detected, with 44 unique metabolites expressed as a result of a change in pH compared to 11 unique metabolites expressed in response to a change in plant host. Fifteen unique metabolites were attributed to a combined change of host and pH. Pathway analysis showed significant changes in intermediates and end products of the glutamate catabolism pathway as a function of change in soil pH. These results suggest that the capacity of *R. clarus* spores to catabolize glutamate into fumarate and ammonia contributes to regulation of pH in the rhizosphere. Metabolite expression in spores following mycorrhizal development provides new clues as to how an AMF fungus can regulate the pH of its host or soil environment and thus facilitate widespread colonization across a range of conditions.

Introduction

Arbuscular mycorrhizal fungi (AMF) are soil-inhabiting fungi that form mutualistic associations with over 80% of vascular plants, and are found on every continent. The first appearance of these fungi coincides with the appearance of land plants (Davison et al. 2015). These fungi also form this association with plants in almost any terrestrial ecosystem with little evidence of host plant preference (Smith and Read 2008). The association between plants and AMF improves phosphorus and water uptake as well as some level of pathogen and toxicity protection to the plant (Jeffries et al. 2003). In exchange, the fungus obtains carbon from photosynthetic output of the plant (Davison et al. 2015). The AMF symbiosis with plants has been shown to play an important role in improving species richness, biodiversity, and plant community stability (Van der Heijde et al. 2008).

This association has been shown to increase production of secondary metabolites by the plant (Chen et al. 2013) and also improve the host plant's nitrogen and phosphorus metabolism

(Mensah et al. 2015). While the metabolic changes induced by the fungal symbiosis in the host plant have been explored, there has been little investigation of the changes in fungal metabolism occurring during this symbiosis. The fungal symbiont is able to consume up to 20% of the photosynthetic product in the form of carbohydrates (Bago et al. 1999). The carbon in the form of hexoses such as glucose and fructose are delivered to the fungus at the arbuscule, where it is then transported into the mycelia and used to synthesize lipids (Bago et al. 1999). The lipids then are sent to the extraradical mycelia where they are utilized in spore production and as storage compounds in spores (Pfeffer et al. 1999).

Newly-formed spores of arbuscular mycorrhizal fungi are metabolically active. The enzymes involved in tricarboxylic acid cycle, such as malate dehydrogenase, are more active in germinating spores than in intra-radical or extra-radical hyphae of *Gigaspora margarita* (Saito 1995). Enzyme activity of glucose-6-phosphate dehydrogenase also is greater in germinated spores than in somatic hyphae, while the activity of phosphofructokinase differed little between spores and hyphae. Therefore, the physiology of AMF spores can be used experimentally to explore the metabolome of the fungus separate from that of its plant host. While approaches have been taken to explore targeted enzyme activity within the spores of AMF (Bago et al. 2000), global metabolic activity of spores has not yet been investigated.

Fungi are known to have that ability to thrive in a wide range of environmental pH (Rousk et al. 2009, Martin et al. 2012, Yee et al. 2016). Arbuscular mycorrhizal fungi also are able to tolerate a range of metal impacted and soil pH environments (Coughlan et al. 2000). While species diversity may change in fungal populations exposed to varied soil pH conditions (e.g., Del Val et al. 1999), evidence indicates little impact on functionality of the symbiosis (Coughlan et al. 2000). When examining the effects of soil chemistry changes on sporulation and spore

germination of AMF communities in saline soils, a range in soil pH environments had little impact (Aliasgharzadeh et al. 2001). The dominant AMF species in these high soil saline communities were from the genus *Rhizophagus*, which consist of species that tolerate a wide range of habitats worldwide (Savary et al. 2017). The metabolic processes occurring within the fungi in response to these environmental changes have not been explored. Therefore, *Rhizophagus clarus* was chosen as the model species for this work due to its pandemic distribution and tolerance to many environments. The goal of this work was to explore the global metabolome in spores of *Rhizophagus clarus* produced after the fungus was grown on two highly mycotrophic phylogenetically distant leguminous plant hosts in soils at pH 4.5 and 7.5. Bioinformatics tools were used to discover functional metabolic pathways impacted by this combination of host-soil pH environments.

Materials and Methods

Experimental Material

The fungal isolate used in this study, *Rhizophagus clarus* WV235, was isolated originally from an abandoned strip mine site in Monongalia county West Virginia and obtained from International Culture Collection of Arbuscular and Vesicular Mycorrhizal Fungi (INVAM, West Virginia University).

Experimental Design

Pot culture inoculum of *R. clarus* WV235, which consisted of a sand and soil mixture (3:1 v/v), mycorrhizal root fragments of *Sorghum sudanense*, asexual spores and external fungal hyphae was diluted 1:10 (v/v) with the same presterilized sand-soil potting medium with pH

equilibrated to 4.5 and 7.5 using calcium chloride. Six sterilized *Robinia pseudoacacia* (black locust) and six sterilized *Trifolium pratense* (red clover) seeds were sown into four replicate 4 x 21 cm cone-tainers™ (Stuewe and Sons Inc., Corvallis, Oregon) and grown in a growth room under sodium vapor lamps with a photon flux density of $245 \mu\text{mol PAR m}^{-2}\text{s}^{-1}$ at pot level with a 14-hour photoperiod for 10 weeks. Ambient temperature ranged from 22-30°C for the duration of the experiment.

After 10 weeks, the mycorrhizal plant in each cone-tainer was surrounded with a 40 μm nylon mesh transplanting into the center of a 15-cm diameter pot. The mesh allowed fungal hyphae to spread into the surrounding soil, but prevented spores from passing through. The soil around the periphery of the mesh-encased transplant was seeded with the same host, 4 plants per pot. Spores produced from the peripheral plants were collected at 6 and 10 weeks after emergence. Spores were extracted by wet-sieving through nested sieves with 500 μm and 38 μm openings, followed by sucrose-density gradient centrifugation. The step gradient consisted of a 20% and 60% sucrose and was centrifuged at 900 g for 2 minutes. Suspended spores were then removed and washed with tap water, placed in a petri dish, and manually collected using a Pasteur pipette and a stereomicroscope.

Spore metabolite extraction

Extracted spores were placed into 0.5 mL tubes and sonicated for 10 minutes. Following sonication, the spores were washed three times with 50:50 methanol and water to remove any residual plant metabolites from the outside surface of the spore. Washed spores then were manually lysed using a pestle in 50 μL of a chilled 100% methanol to extract polar metabolites. Spore lysates were centrifuged at 14,000x g for 15 minutes at 4°C to pellet proteins and spore debris. The supernatant was removed for mass spectrometry analysis.

Normalization of samples by spore protein content

Samples were normalized based on total protein concentration. The spore lysate was taken through a bicinchoninic acid assay (BCA assay) to determine protein concentration. The Pierce BCA Protein Assay Kit was used for total protein quantitation following the kit's recommended protocol. A volume totaling 500 µg of total protein from each treatment was used to extract metabolites.

Analytical procedure and LC-MS Conditions

The Agilent LC-MS system (Agilent Technologies, Santa Clara, CA, USA) included an Agilent 1290 Infinity quaternary ultra-high pressure liquid chromatography (UHPLC) pump. This LC system was coupled to an Agilent 6530 quadrupole time of flight (Q-ToF) mass spectrometer with electrospray ionization. The chromatographic separations were performed using a Merck polymeric bead based ZIC-pHILIC column (100mmx2.1mm, 5µm).

Data Processing and Metabolite identification

The LC-MS/MS data acquired using Agilent Mass Hunter Workstation (*.d files) was processed in Profinder software for batch recursive analysis. The datasets were subjected to spectral peak extraction with a minimum peak height of 600 counts and the charge state for each metabolite was restricted to two. Further, retention time and mass alignment corrections were performed using Agilent Profinder software version 2.3.1 on the runs to remove non-reproducible signals.

The resulting features were then exported as *.cef files to Mass Profiler Professional (MPP) software version 2.4.3 (Agilent Technologies, Santa Clara, CA, USA) for statistical analysis.

Statistical Data Analysis

The extracted features were imported into MPP software for statistical analysis. Principle Component Analysis (PCA) was performed to check the quality of the samples, after which the data containing filtered features were analyzed by unpaired t-test to determine the differences between the control and roundup groups. Only the analytes with p values < 0.05 and fold change (FC) > 2 were regarded as statistically significant. Additionally, multiple test corrections using Bonferroni were applied to reduce false positives and false negatives in the data. The statistically filtered data was exported to Mass Hunter for targeted MS/MS analysis.

Pathway Analysis

A metabolomics pathway experiment was performed on the statistically significant data for the control and treatment groups using MPP software. Curated pathways such as BioCyc, KEGG and WikiPathways were used to map expression of significant compounds.

Results

Rhizophagus clarus at two different soil pH conditions (4.5 and 7.5) and on two phylogenetically distant legume plant hosts (*Robinia pseudoacacia* and *Trifolium pratense*) yielded distinct metabolite profiles from fungal spores. While both the host and soil pH impacted the global metabolome of *R. clarus* spores, pH had the greater impact. A total of 715 distinct metabolites were detected from the global metabolomics analysis, of which 44 reached detectable levels as a result of a change in pH of the growth medium (Fig. 1). Only 11 unique metabolites were detected in response to a change in plant host, whereas 15 unique metabolites accumulated to detectable levels with a combined change of host and pH. While qualitative differences in metabolites were detected in each of these treatments, some metabolites were shared among treatments but varied quantitatively depending on the environment in which the fungus was growing. The majority of the changes in metabolome occurred as an effect of a change in soil pH. The effect of the host on changes in the spore metabolome was greater at pH 7.5.

The impact of environmental effects on the global metabolome of *R. clarus* spores were evident in a principal component analysis (PCA) plot (Fig. 2), wherein pH of the growth medium provided the greatest separation between treatments. Principal component 1 contributed 27.75% of the variation and consisted mostly of amino acids and secondary metabolites, while principal component 2 contributed 14.13% of the variation and consisted primarily of lipids and lipid precursors. A hierarchical clustering analysis performed on the normalized intensity values from the complete data set (Fig. 2) using a Euclidean similarity measurement showed the metabolomes of *R. clarus* spore samples clustered together based on pH of the growth media rather than the host on which they were grown.

Figure 1. Venn diagram showing the number of significantly different unique metabolites detected in the metabolome of *Rhizophagus clarus* spores produced in response to three

treatment variables: host species (*Trifolium pratense*, *Robinia pseudoacacia*), pH (4.5, 7.5) of a sand-soil (3:1 v/v) growth medium, and host-pH interaction.

Figure 2. Variation in the global metabolome in *R. clarus* spores formed in three-month-old pot cultures of *Robinia pseudoacacia* and *Trifolium pratense* at two divergent pH environments (4.5 and 7.5) in a sand-soil (3:1 v/v) growth medium. Left: Principal component analysis of 715 detected metabolites. Right: A global hierarchical clustering analysis of these metabolites.

Multiple classes of metabolites were detected, including amino acids, nucleotides, nucleotide sugars, TCA cycle intermediates, secondary metabolites, lipids, and lipid precursors. Selected metabolites from each of these classes were targeted for further analysis based on a preliminary pathway analysis. The pathway analysis showed significant changes in intermediates and end products of the glutamate catabolism pathway as a function of change in medium pH (Fig. 3). Alpha-ketoglutarate and fumarate were upregulated in spores produced in both host species at pH 4.5 compared to levels of expression measured at pH 7.5. Conversely, significantly lower concentrations of glutamate, glutamine, and aspartate were measured in the pH 4.5 samples compared to those at pH 7.5. The absolute quantification data (Fig. 3) matched the trends observed from the full scan fold change data (Table 1) and show this pathway was up-regulated in the lower soil pH environment.

Figure 3. Illustration of the glutamic acid catabolism pathway together with absolute concentrations (ng/mL) of metabolites detected in this pathway from extracts of *R. clarus* spores sampled from three-month-old pot cultures of *Robinia pseudoacacia* and *Trifolium pratense* growing in a growth medium of sand-soil (3:1 v/v) equilibrated at pH 4.5 and 7.5. Error bars represent standard deviation of samples from three replicate pot cultures of each treatment.

Table 1. Absolute concentrations (ng/mL) and standard deviation of targeted metabolites expressed in spores of *R. clarus* extracted from three-month old *Trifolium* and *Robinia* species growing in a sand-soil growth medium (3:1 v/v) at two pH 4.5 and 7.5.

Metabolite	Fold change pH 4.5 vs. 7.5	<i>T. pretense</i> pH 4.5	<i>T. pretense</i> pH 7.5	<i>R. pseudoacacia</i> pH 4.5	<i>R. pseudoacacia</i> pH 7.5
Ethanolamine	2.4	14.4 ± 0.7	BLQ	9.3 ± 0.7	0.9 ± 0.3
myo-Inositol	-3.5	4.3 ± 0.8	8.7 ± 0.9	6.6 ± 0.9	9.3 ± 0.8
Choline	1.2	8.9 ± 0.5	9.3 ± 0.6	9.7 ± 0.7	8.9 ± 0.6
Lactate	1.8	1.5 ± 0.2	1.2 ± 0.1	1.6 ± 0.2	1.1 ± 0.2
Citrate	-1.4	4.3 ± 0.3	5.5 ± 0.3	4.5 ± 0.3	5.2 ± 0.4
Fumarate	3.1	8.2 ± 0.2	2.7 ± 0.1	7.2 ± 0.3	1.9 ± 0.3
α-ketoglutarate	2.3	14.6 ± 0.6	9.8 ± 0.9	12.6 ± 0.6	8.6 ± 0.4
Alanine	-4.3	23.1 ± 1.2	47.8 ± 0.3	32.6 ± 0.9	38.6 ± 0.7
Arginine	-1.8	621.6 ± 16.4	755.3 ± 23.7	585.6 ± 14.8	685.6 ± 15.2
Aspartate	-1.2	80.8 ± 1.9	111.3 ± 2.8	90.6 ± 1.3	92.4 ± 1.5
Glutamate	-2.1	150.6 ± 5.6	188.3 ± 2.4	165.2 ± 4.5	195.6 ± 3.2
Glutamine	-4.6	57.8 ± 5.7	81.6 ± 0.5	65.3 ± 3.3	76.5 ± 1.3
Lysine	-2.3	86.8 ± 8.1	151 ± 8.5	110 ± 27.2	121 ± 6.4
Phenylalanine	3.1	1.3 ± 0.03	0.2 ± 0.1	1.6 ± 0.1	0.6 ± 0.1
Proline	1.6	7.9 ± 0.2	5.2 ± 0.2	8.7 ± 0.6	6.9 ± 0.4
Tyrosine	1.6	10.2 ± 0.2	9.8 ± 0.6	9.7 ± 0.7	8.7 ± 0.9
Tryptophan	-1.2	3.1 ± 0.6	4.2 ± 0.2	6.2 ± 0.6	5.2 ± 0.4

BLQ, below limit of quantification

Discussion

The observed upregulation of the glutamate catabolism pathway as a response to the lower pH of the growth medium shows that *Rhizophagus clarus* spores have the ability to catabolize glutamate into fumarate and ammonia, which may contribute to regulation of pH in the rhizosphere. Pathogenic fungi have been shown to regulate the pH of their host tissues through the secretion of ammonium to elevate the pH to modulate the host's immune response and breakdown tissue (Alkan et al. 2009). *Colletotrichum coccodes* virulence of tomato fruit has been directly tied to the amount of ammonium secreted by the pathogen (Alkan et al. 2009). The secreted ammonium has been shown to increase the levels of tissue-induced H₂O₂, which increased tissue necrosis in *Solanum lycopersicum*. Ammonium is a key biological molecule used by fungi for the alkalization of their environment. Ammonia can be secreted by the fungi and then subsequently converted by ureases to NH₄⁺ in the environment (Vylkova 2017). The significant fold changes in the intermediates of this pathway were confirmed in the targeted quantification experiments, providing evidence that the glutamate catabolism was highly upregulated in the pH 4.5 samples compared to expression in the pH 7.5 samples.

Rhizophagus clarus may release secondary metabolites that alter plant rhizosphere pH. (Orozco-Patiño and Medina-Sierra 2013) found that *R. clarus* grown in association with *Pueraria phaseoloides* (kudzu) at pH 8.5 and pH 6.0 altered the pH of the growth medium, lowering and raising it, respectively, to a pH of 7.0. *Rhizophagus clarus* is able to successfully colonize plant hosts at pH levels as low as 4.0 (Clark et al. 1999). The ability for the *R. clarus* to change its metabolic processes to manipulate the rhizosphere could help explain the ability of this fungi to persist in such a wide range of soil pH environments. The upregulation of the

glutamate catabolism pathway in spores suggests a possible metabolic mechanism that contributes to the manipulation of rhizosphere pH.

The four-fold decrease in number of significantly changing metabolites observed between the two host species compared to the soil pH change may be due in part to the absence of host specificity by arbuscular mycorrhizal fungi (Albrecht et al. 1999, Davison et al. 2015). This lack of any major host impact on the metabolism of the spores of *R. clarus* suggests that the level of metabolic pressure on the fungus by the host plant is less than that of the soil environment. This result further strengthens existing evidence of wide host range and little host specificity (Bolan 1991).

The results of this experiment could help guide future experiments exploring the metabolism of the arbuscular mycorrhizal fungi. The lack of host influence on the metabolome of the fungi allows metabolomics experiments to be performed on any host that will accept the fungal association. The significant impact of the environmental changes on the fungal metabolome provides a new technique for investigating the function of these obligate symbionts. The ability to manipulate the environmental conditions in which these fungi are cultured to elicit different metabolic responses can be used to test new hypotheses about their functional capacity. These metabolic function experiments could be used to assign function to uncharacterized portions of the genome.

Chapter 4 Metabolomics of the cicada pathogen *Massospora* confirms psilocybin and cathinone production in early diverging non-flagellated fungi

Abstract

The obligate lifestyle and large genome size of many of the entomopathogenic members of the Zoopagomycota have long precluded their study. Modern “omics”-based tools, including metagenomics, transcriptomics and metabolomics, have facilitated a better understanding of these fungi despite barriers that still prevent traditional lab-based studies. *Massospora* is one of several members of the Entomophthorales for which a systems biology approach has yet to be employed to elucidate factors influencing host colonization and putative behavioral modification. A comparison of global and targeted metabolomes of two contemporary *Massospora* – cicada consortia, *M. cicadina*-infected periodical cicadas (*Magicicada* spp.) and *M. platypediae*-infected banger-wing cicadas (*Platypedia putnami*) uncovered a diverse assortment of secondary

metabolites. These included psilocybin from *M. platypediae* and *M. levispora* and cathinone from *M. cicadina*. Fragmentation of pooled samples further validated their identity. Targeted quantification confirmed absolute concentration of psilocybin and psilocin, which are biologically significant as psychotropic compounds that could enhance aggressiveness/stamina of infected cicadas. Such a benefit would ensure continued spore dispersal despite debilitating infections that likely result in fatigue, decreased muscle strength and general malaise. Phylogenetic studies showed *M. platypediae* and *M. levispora*, both of which harbored psilocybin, grouped as a single clonal lineage and therefore are synonymized as *M. levispora* comb. nov. This study provides an initial view of the metabolic capacity of these enigmatic and cryptic fungi and offer clues of phylogenetic relationships heretofore unknown.

Introduction

The Entomophthorales (Zoopagomycota) comprise some of the most important insect- and arthropod-destroying “zygomycetes,” including well-known examples such as *Entomophthora muscae*, causal agent of “summit disease” of flies, *Entomophaga maimaiga*, a virulent pathogen and biological control of gypsy moth, and *Massospora cicadina*, a behavior-modifying pathogen of periodical (13- and 17-year) cicadas (Cooley et al. 2017; Gryganskyi et al. 2017; Hughes et al. 2016; Spatafora et al. 2017). Many of these entomopathogens were formally described first by Thaxter (1888) and other early mycologists more than a century ago. Only recently have these species been phylogenetically resolved, including *Arthropaga myriapodina*, a recently described lethal summit pathogen of polydesmid millipedes (Gryganskyi et al. 2012, 2013; Hodge et al. 2017).

The Zoopagomycota are considered the earliest diverging lineage of nonflagellated fungi, comprising 19 genera and 228 described species worldwide. Because of their large genome size and obligate lifestyle, they are underrepresented in GenBank with fewer than three publically available genomes and 125 DNA sequences representing only about 1–10% of described species (Gryganskyi et al. 2017; Spatafora et al. 2017). *Massospora cicadina*, for example, is represented by only four DNA sequences (three 18S and one 28S) from a single isolate (ARSEF 374). The overreliance on sequences derived from a single specimen poses risks to resolving species phylogenetically, and, at the same time, emphasizes the need for extensive taxa sampling to accurately characterize relationships among other members of the Entomophthorales (Gryganskyi et al. 2012, 2013). Relationships between *M. cicadina* and the other twelve described *Massospora* species (Soper 1963, 1974, 1981) are unexplored because the group is so underrepresented. Powerful genomic, transcriptomic, and metabolomic approaches have helped elucidate some aspects of zygomycete evolution (Li et al. 2017; Małagocka et al. 2015; Meier et al. 2015; Sun et al. 2012; Zhou et al. 2014), yet many of the basic mechanisms that drive pathogenicity and behavioral manipulation, particularly among members of the Zoopagomycota (Cooley et al. 2017), are poorly understood compared to their Ascomycotan counterparts (de Bekker et al. 2013).

Despite gaps in Zoopagomycota systematics, much has been learned about their epizootiology. *Massospora* infections are conspicuous: the destruction of the intersegmental membranes of the cicada's abdomen incites a progressive sloughing off of the abdominal sclerites, revealing a clay-like expanding mass of infective conidia emerging from the posterior end of the insect. Over time, just the head and thorax of the living adult cicada remain and mass of conidia are passively disseminated during flight, feeding, and mating attempts. The latter

renders the fungus sexually transmissible (Riley 1885, Butler 1886, Hopkins 1898, Goldstein 1929, Cooley et al. 2017). The lifecycle of the fungus is associated mostly with the later developmental stages of the cicada. Mature nymphs become infected by resting spores encountered underground during construction of their vertical emergence burrows, emergence, and eclosure into adults. Lastly, over a period of days, the fungus produces an exposed conidial mass that serves as inoculum for secondary infections of healthy cicadas. These infections give rise to azygospores that are reincorporated into the soil to start the cycle again.

Within the Entomophthorales, complex infection and transmission strategies manipulate host behavior, such as the summit disease caused by ?????, where infected individuals move towards an elevated position to optimize spore dispersal (Gryganskyi et al. 2017, Hodges et al. 2017; Hughes et al. 2017, Roy et al. 2006). Species in *Massospora* and *Strongwellsea*, *Entomophthora*, *Erynia*, and *Entomophaga* are unique in that these fungi keep their hosts alive and interact in ways to control flight and/or dispersal behavior, thus ensuring that infected insects become a moving vector for spore dispersal (Cooley et al. 2017).

Several studies have examined behavior of *Massospora*-infected cicadas (Table S1). Most notably, Cooley and colleagues (2017) recently reported male *M. cicadina*-infected periodical cicadas with conidial infections exhibit precisely timed wing-flick signaling that attracts copulation attempts from conspecific males. This behavior normally is observed only in sexually receptive female cicadas. Likewise, Cooley (1999) reported *Massospora levispora*-infected male *Okanagana rimosa* exhibited similar sexual behavioral changes, resulting in bisexual behavior. Similar modifications in sexual behaviors have been observed in *Diceroprocta* spp. infected by *M. diceroprocta* (Jon Hastings, personal communication). *Massospora platypediae*-infected wing-banger cicadas (*Platypedia putnami*) also exhibited

sexually ambiguous courtship behavior, moving towards other signaling cicadas regardless of sex and making frequent abdomen to abdomen contact (Murphy and Redden 2003). Together, these observations indicate that *Massospora* infections can give rise to an extended phenotype that hijacks cicadas, turning them into vectors for fungus transmission at the expense of the cicadas' own interests (Cooley et al. 2017).

The inability to culture a majority of the Zoopagomycota means that characterizing their activity, especially pathogenicity (e.g. secretion of secondary metabolites), requires approaches involving naturally infected individuals. Complications can arise because these individuals can be subject to secondary invasion by opportunistic fungi, bacteria, nematodes, and other microorganisms. Nonetheless, we used global and targeted metabolomics and metagenomics to compare two contemporary *Massospora* – cicada consortia, *M. cicadina*-infected periodical cicadas (*Magicicada* spp.) and *M. platypediae*-infected banger-wing cicadas (*Platypedia putnami*) to better understand factors influencing host colonization and putative behavioral modification.

This study was conducted to accomplish four objectives. The first was to reconstruct a phylogeny of three species of *Massospora* from annual and periodical cicadas and to explore their haplotypic diversity. Second, identification of species was validated by comparing spore morphology of specimens for which DNA sequences exist to that in published descriptions. Third, the total metabolic profiles of two contemporary *Massospora* – cicada consortia were compared to identify candidate metabolites important to entompathogenicity and behavioral modification. Last, targeted metabolomics were used to validate important up-regulated candidate metabolites from *Massospora*-infected cicadas.

Methods and Materials

Sampling fungus-infected cicadas

From May 28 to June 6, 2016, newly emerged Brood V 17-year periodical cicadas were visually inspected for *Massospora* fungal infections on the campus of West Virginia University in Morgantown, West Virginia, USA (39°38' 01"N, 79° 57' 02"W). Individuals with visible fungal plugs were transferred individually to sterile 20 mL vials, transported to the laboratory, and stored in a -20 C freezer until processing. Samples were obtained from 95 infected cicadas, of which 47 were from *M. cassini*, 41 from *Magicicada septendecim*, and seven from *M. septendecula*. A similar number of males and females were sampled across the three Brood V species, with 53 from females and 50 from males. Sampling was carried out on WVU property and did not require permits.

On June 1, 2016, additional geographically separated Brood V cicada populations were visually inspected for fungal infections. Seven infected specimens of *M. septendecim* and one of *M. cassini* were recovered from Hocking Co., OH. Concurrently, a collaborator provided an additional 34 infected Brood V specimens of *M. septendecim* collected from across five OH counties including Ashland, Cuyahoga, Hocking, Licking, and Lorain counties. Eight female, *Massospora*-infected, Brood VI specimens of *Magicicada septendecim* were collected in June, 2017 from North Carolina. All samples were collected and stored as previously described.

Twenty-three *M. cicadina*-infected specimens of *Magicicada* collected from 1978-2002 were provided from Dr. Chris Simon at the University of Connecticut, where they had been stored at room temperature, dried and pinned, since their collection. Dr. Gene Kritsky from Mount St. Joseph University provided fungal plugs from seven *M. cicadina* infected specimens

of *Magicicada* collected from 1989-2008, three of which were thirteen year cicadas and four were 17-year cicadas.

In addition to periodic cicadas, two species of annual cicadas also were sampled and included in this study. On June 4, 2017, newly emerged banger-wing cicadas (*Platypedia putnami*) were inspected for *Massospora* infections 12 km northeast of Gallina, NM along the Continental Divide Trail. Sixteen infected individuals and one outwardly asymptomatic individual (to serve as a negative control) were identified and sampled as described above.

Nine *Massospora levispora*-infected specimens of *Okanagana rimosa* were collected by John Cooley from the University of Michigan Biological Station, Cheboygan and Emmet Counties, MI on June 20, 1998 (Cooley 1999). Infected individuals as well as outwardly asymptomatic individuals were placed immediately in individual 20-ml screw-cap vials containing 95% ethanol. Specimens were completely submerged and stored at 4 C until October 2017, when specimens were sent to WVU for processing.

All fungus-infected and negative control cicadas were assigned a two letter code corresponding to the latin binomial (e.g. *Massospora cicadina*, MC) of the infecting strain followed by a unique number. Negative controls were designated NC1 for each species. Cicadas were processed aseptically, with half of the visible fungal plug removed with a sterile scalpel and transferred to a 1.5 ml microcentrifuge tube for DNA extraction. Controls were processed similarly, where half of the most posterior segments as well as underlying host tissues were sampled. A majority of the remaining plug from infected individuals were used for global metabolomics analyses. Dry plugs were transferred aseptically to sterile 1.5 mL microcentrifuge tubes and stored at 20 C. A small sampling of spores was retained for morphological studies, although not all specimens were examined.

Morphological studies

Given the scarcity of *Massospora* sequences from NCBI GenBank, morphological comparisons were necessary to determine if data on putatively assigned *Massospora* species agreed with that of previously reported measurements for conidial and azygospore stages (Soper 1963, 1974). A portion of select fungal spore masses was harvested with a sterile scalpel and mounted in lactophenol for examination with light field microscopy. Slip covers were fastened with nail polish to allow slides to be archived and reexamined when necessary. Mean conidial/azygospore length x width and cicada host provided the main criteria to differentiate *Massospora* species. Slides were examined and photographed using a Nikon Eclipse E600 compound microscope (Nikon Instruments, Melville, New York) equipped with a Nikon Digital Sight DS-Ri1 high-resolution microscope camera. A sampling of 25 spores from each slide mount were measured using Nikon NIS-Elements BR3.2 imaging software. A total of 51 *Massospora*-infected cicadas were examined including 26 *M. cicadina* (15 conidial and 11 azygospore isolates), nine *M. levispora* (eight conidial and one azygospore isolates), and 15 *M. platypediae* (14 conidial and one azygospore isolates).

DNA extraction, amplification, sequencing, and phylogenetic analyses

Fungal genomic DNA was extracted from harvested fungal plugs using a modified Wizard kit (Promega, Madison, WI, USA) as previously described (Short et al. 2015). DNA was suspended in 75 µl Tris-EDTA (TE) buffer and stored at -20 C. Portions of the nuclear 18S (SSU) and 28S (LSU) ribosomal DNA regions were amplified with the primer combinations

NS24/NSSU1088R (Hodge et al. 2017) and LR0R/LR5 (Vilgalys and Hester 1990) using BioLine PCR kits (Bioline USA Inc., Taunton, MA, USA). PCR conditions were as follows. LSU: initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 51.1°C for 45 seconds, and elongation at 72°C for 90 seconds, and final elongation at 72°C for 5 minutes; SSU: initial denaturation at 94°C for 5 minutes, 36 cycles of denaturation at 94°C for 30 seconds, annealing at 49°C for 30 seconds, and elongation at 72°C for 60 seconds, and final elongation at 72°C for 7 minutes. Amplified products were visualized with SYBR gold (Invitrogen, Grand Island, NY, USA), which then were loaded onto a 1-1.5%, wt/vol agarose gel made with 0.5-1% Tris-borate-EDTA buffer (Amresco, Solon, OH, USA). All products were purified with ExoSAP-IT (Affymetrix, Inc., Santa Clara, CA, USA) and submitted to Eurofins MWG Operon (Huntsville, AL, USA) for Sanger sequencing. Chromatograms were assembled and inspected in MEGA 7 (Kumar et al. 2016).

Phylogenetic trees were reconstructed using LSU and SSU sequences generated from 18 and 21 *Massospora* sequences, respectively, representing three *Massospora* species, *M. cicadina*, *M. levispora* and *M. platypediae* (Table X). The concatenated set consisted of four sequences, two from *M. cicadina* and one each from *M. levispora* and *M. platypediae*. Thirteen additional published sequences spanning the known diversity of the Entomophthorales were included to help resolve relationships among *Massospora* and its close allies (Hodge et al. 2017). One isolate of *Conidiobolus pumilus* (strain ARSEF 453) served as an outgroup. Sequences were aligned using CLUSTAL-W (Larkin et al. 2007). Alignments were visually inspected, after which optimized nucleotide substitution model (Tamura 3-parameter + G) was selected using ModelTest X.X (Posada and Crandall, 1998). Partial deletion with site coverage cutoff of 95%

was used. Maximum likelihood (ML) trees were estimated using Mega 7. Phylogenetic support was assessed by bootstrap analysis with 1000 replicates using MEGA 7.

Fungal metabolite extraction

Fungal plugs were weighed and transferred into sterile 1.5 mL microcentrifuge tubes. Metabolite extracts from each fungal sample were normalized to a final concentration of 10 mg of fungal tissue to 1 mL and polar metabolites were extracted in a buffer consisting of 80% HPLC grade methanol and 20% water. Samples then were sonicated for 10 minutes. The sonicated samples were centrifuged at 14,000 x g for 15 minutes at 4°C to pellet proteins and cellular debris. The supernatant was removed and dried down using lyophilization prior to analysis. Samples were reconstituted in 100 µL of 50% methanol and 50% water for mass spectrometry analysis.

Analytical procedure and LC-MS Conditions

The Agilent LC-MS system (Agilent Technologies, Santa Clara, CA, USA) included an Agilent 1290 Infinity quaternary ultra-high pressure liquid chromatography (UHPLC) pump. This LC system was coupled to an Agilent 6530 quadrupole time of flight (Q-ToF) mass spectrometer with electrospray ionization. The chromatographic separations were performed using a Merck polymeric bead based ZIC-pHILIC column (100mmx2.1mm, 5µm).

Mobile phase A consisted of 10 mM ammonium acetate and mobile phase B consisted of 100% acetonitrile. Metabolites were separated on a gradient that went from 95% mobile phase A to 5% mobile phase B over 20 minutes with 7-minute re-equilibration at the end of the chromatographic run.

The mass spectrometer was operated in both positive and negative ion mode for the analysis. Technical replicates were acquired for each sample. Data dependent MS/MS was performed on triplicate pooled samples for fragmentation and library searching.

Data Processing and Metabolite identification

The LC-MS/MS data acquired using Agilent Mass Hunter Workstation (*.d files) were processed in Agilent Profinder software version 2.3.1 for batch recursive analysis. The datasets were subjected to spectral peak extraction with a minimum peak height of 600 counts and the charge state for each metabolite was restricted to two. Further, retention time and mass alignment corrections were performed using Agilent Profinder software version 2.3.1 on the runs to remove non-reproducible signals. The resulting features then were exported as *.cef files to Mass Profiler Professional (MPP) software version 2.4.3 (Agilent Technologies, Santa Clara, CA, USA) for statistical analysis.

Statistical Data Analysis

The extracted features were imported into MPP software for statistical analysis. Principle Component Analysis (PCA) was performed to check the quality of the samples after which the data containing filtered features were processed by one way ANOVA to ascertain differences between control and fungal groups. Only the analytes with p values < 0.05 and fold change (FC) > 20 were treated as statistically significant. Additionally, multiple test corrections using Bonferroni were applied to reduce false positives and false negatives in the data.

Targeted Metabolites

The absolute quantification of psilocin and psilocybin from the *M. cicadina* and *M. platypediae* fungal plug metabolite extracts described above, *M. levispora* extracts that were stored long term were not able to be analyzed for global metabolite changes were able to be analyzed in this targeted experiment. This analysis was performed using a 5500 QTRAP mass spectrometer (SCIEX Inc., Concord, Ontario, Canada). Chromatographic separations were performed using an HPLC system consisting of two Shimadzu LC 20 AD pumps that included a degasser and a Shimadzu SIL 20 AC auto sampler (Shimadzu Corp. Kyoto, Kyoto Prefecture, Japan). Analytes were separated on a 3.0x150 mm Imtakt Scherzo SW-C18 column (Imtakt USA, Portland, Oregon). The HPLC column oven was set to 40°C for the analysis, with an 8 µL injection volume for each sample. Mobile phase A consisted of HPLC grade H₂O with 0.1% formic acid, while mobile B consisted of acetonitrile with 0.1% formic acid. The LC gradient started 5% for 0.5 minutes and then increased to 95% B over 5 minutes followed by a re-equilibration to starting conditions for a period of 2.1 minutes. The flow rate was set to 0.3 mL/minute.

Targeted metabolites were quantified using multiple reaction monitoring. The transitions that were monitored for psilocybin were m/z 284.5→205.2 and 240.1. Psilocin was monitored at m/z 205.2→116.3. Mass spectrometer source conditions were set to the following: curtain gas = 15, ion spray voltage= 4800 V, temperature= 600 °C, gas 1= 45, gas 2= 50. Calibration samples were prepared in 50 % methanol/H₂O. Calibration concentrations ranged from 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, to 10 ng/mL of both psilocin and psilocybin. In order to assess variation in the assay duplicate standard curves were analyzed at the beginning and at the end of the analytical

run. Absolute quantification data was processed using Analyst software ver. 1.6.3 (SCIEX Inc., Concord, Ontario, Canada).

Results

A total of 176 specimens of *Magicicada* species infected with *M. cicadina* were sampled in this study, of which 31 were from historic collections and 144 were collected from 2016 - 2017. A total of six of seven known *Magicicada* species were sampled, including three 17-year cicadas (*M. cassini*, *M. septendecim*, *M. septendecula*) and three 13-year cicadas (*M. tredecassini*, *M. tredecim*, *M. tredecula*). Conidial infections were present in 164 of the *Magicicada* specimens, with resting spore infections in the remaining twelve individuals (Fig. 1). The disparity likely is a consequence of highly varied sampling times.

Of the 16 *M. platypediae*-infected specimens of *Platypedia putnami* examined, 15 had conidial infections. The other infected *P. putnami* specimen had an azygospore infection, which is a first observation for this species (Fig. 1). Of the nine *M. levispora*-infected specimens of *Okanagana rimosa*, only one infection producing azygospores was sampled (Fig. 1). Representatives for each *Massospora* species and mode of sporulation across numerous broods and geographic locations are listed in Tables S2-S3.

Morphological studies

Morphology of azygospores was examined in 12, 1 and 1 specimens of *M. cicadina*, *M. platypediae*, and *M. levispora*, respectively. Morphology of conidia were studied in 15, 14, and 8 respective species (Table S2). The conidial specimens of *M. cicadina*-infected cicadas spanned ten Broods and six of the seven *Magicicada* species (Table S3).

Mean conidia ($14.76\ \mu\text{m} \times 16.65\ \mu\text{m}$) and azygospore ($42.42\ \mu\text{m}$) dimensions for *M. cicadina* overlapped with previously reported measurements with one exception (Table S2). Mean width for conidia from ARSEF specimen 374 ($17.4\ \mu\text{m}$) barely exceeded the reported range of $10\text{--}17\ \mu\text{m}$ for this species. Despite the mean width and length values falling within the known reported range for this species, the range of conidial measurements for individual isolates, ($9.7\text{--}20.5\ \mu\text{m} \times 11.3\text{--}25.5\ \mu\text{m}$), far exceeded dimensions ($10\text{--}17\ \mu\text{m} \times 14\text{--}20\ \mu\text{m}$) reported by Soper (Fig. 1, Table S2). All azygospore measurements fell within the previously reported range for this species: $33.5\ \mu\text{m} \times 47.5\ \mu\text{m}$ ($40.5\ \mu\text{m}$).

Mean conidial ($8.78\ \mu\text{m} \times 14.26\ \mu\text{m}$) and azygospore ($40.7\ \mu\text{m}$) measurements for *M. levispora* overlapped with previously reported conidial ($6.0\text{--}11.0\ \mu\text{m} \times 9.5\text{--}23.0\ \mu\text{m}$ ($8.0 \times 15.0\ \mu\text{m}$) and azygospore ($34.0\ \mu\text{m}$) measurements (Fig. 1, Table S2). In contrast, spore dimensions in the majority of *M. platypediae* specimens fell completely outside the reported range for the species (Fig. 1; Table S2). Two additional specimens produced spores with at least one dimension outside the known range. Spore dimensions of four isolates overlapped with those previously reported (Table S2). Interestingly, all the *M. platypediae* measurements fell within the reported range for *M. levispora*, including the single resting spore specimen that was recovered (Fig. 1).

Figure 1. Mean A) conidia and B) azygospore dimensions for three *Massospora* species infecting . White bars indicate overall mean values while black bars indicate the range of variation in spore dimensions. Depending on the spore stage, 25 conidia or azygospores were measured for each specimen except *M. platypediae* azygospores, in which 50 were measured.

Molecular identification and phylogenetic diversity in *Massospora*

Massospora previously was represented in NCBI Genbank by a single isolate (ARSEF 374) of *M. cicadina*. GenBank blastn results of LSU sequences from *M. cicadina*, *M. levispora*, and *M. platypediae* DNA templates were 86-88% identical to *Eryniopsis caroliniana* strain ARSEF 640 (EF392387), but only 82-84% identical to *Massospora cicadina* strain ARSEF 374. Independent DNA extraction and sequencing of ARSEF 374 fungal plug tissue failed to generate a high quality LSU sequence and therefore it was not included in the LSU phylogeny. GenBank blastn results of SSU sequences from *M. cicadina*, *M. levispora*, and *M. platypediae* DNA templates were 96-97% identical to *Entomophthora schizophora* (AF052402) and *Entomophthora thripidum* (AF296755). Despite a deposited SSU reference sequence for *M. cicadina*, none of the *Massospora* species sequenced in this study had a reportable BLAST hit to *M. cicadina* ARSEF 374. In contrast, successful amplification of SSU from the resampled ARSEF 374 specimen did yield a usable sequence 100% identical to other *M. cicadina* strains from this study using pairwise BLAST searches and 97% identical to strains of *E. schizophora* and *E. thripidum*.

Phylogenetic analysis of the combined dataset using maximum likelihood (ML) resolved all *Massospora* in a strongly supported monophyletic group sister to a clade containing *Entomophthora* and *Arthropaga* (Fig. 2). *Massospora levispora* and *M. platypediae* were not resolved as distinct monophyletic clades. Instead, they grouped together in a single sister clade to

M. cicadina (Fig. 2). Therefore, these data support resolution of a single species, *M. levispora* comb. nov. that encompasses a broader host range.

ML bootstrap analysis of the individual LSU (Fig. S1) and SSU (Fig. S2) partitions revealed strong support for two *Massospora* species although a polytomy in the SSU tree could not fully resolve the relationships among *M. cicadina*, *M. levispora* comb. nov., a clade containing *Entomophthora* and a fourth clade containing *Arthrophaga myriapodina* and *Eryniopsis ptycopterea*.

Figure 2. Concatenated LSU+SSU maximum likelihood (ML) tree consisting of *Massospora* species and related species in the *Entomophthorales*. *M. levispora* comb. nov. accommodates both *M. levispora* sensu strictu and *M. platypediae*. Bootstrap support is indicated near each node and only values greater than 70% are shown.

Global Metabolomics

The global metabolomes of fungal plugs of *M. cicadina* and *M. platypediae* from infections of *P. putnami* yielded over 2000 unique chromatographic and mass to charge peaks in the combined positive and negative polarities. Univariate statistics were performed on all the detected ions to separate unique fungal plug metabolites from the insect host metabolome. Each *Massospora* species and healthy cicada controls yielded unique metabolomes, with the two fungal species displaying more similar metabolite signatures (Fig. 3). The results of these statistical analyses revealed 678 and 489 fungal plug-specific metabolites in positive and negative ion modes, respectively (Tables S4-S5). These metabolites measured a >20 log fold change and all differed significantly in expression levels ($p < 0.01$) compared to the healthy cicada control. These highly varying compounds included free amino acids, phospholipids, ceramides, alkaloids, and a diverse assortment of secondary metabolites.

Figure 3. Representative base peak chromatogram (BPC) from the global metabolomics analysis of samples from *M. cicadina*, *M. levispora* (as specimens of *M. platypediae*), and a healthy

control of (cicada species?), comparing the metabolome profiles among fungal plugs and with the non-infected cicada control.

Among the secondary metabolites recovered from the fungal plugs, monoamine alkaloids were abundant and diverse across both *Massaspora* species and they included substituted tryptamines and substituted amphetamines (Table 1). Psilocybin (a substituted tryptamine) was among the top upregulated metabolites detected in *M. levispora*, with > 16-fold higher level of expression than both the non-infected control and *M. cicadina* in fungal plugs (Table 1). However, no intermediates in the psilocybin biosynthesis pathway were detected in the global metabolomics dataset. Fragmentation of psilocybin was performed on pooled samples and expected fragmentation patterns were observed (m/z 245.0 and 205.1), thereby verifying the identity of psilocybin (Figure S3). Other tryptamine-containing natural products were recovered from both *M. cicadina* and *M. levispora* at significantly higher levels than the negative controls. In *M. levispora*, two lysergic acid pathway product intermediates, 4-Dimethylallyl-L-tryptophan (4-DMAT) and agroclavine (ergot alkaloid), were expressed at 17.6x and 16.8x log fold higher change than in *M. cicadina* and in the non-infected control, respectively. Aspidospermine, another tryptamine-containing alkaloid, was expressed at a 16.8x and 14.5x log fold change higher in *M. cicadina* and *M. levispora* compared to the uninfected control. Nb-lignoceroyltryptamine also was measured in *M. cicadina* at a 16.8x log fold change higher than *M. levispora* and the negative control. None of these alkaloids were confirmed via fragmentation. Furthermore, LC/UV analysis failed to confirm the expression of agroclavine.

A second class of monoamine alkaloids, substituted amphetamines, also were detected, but only from *M. cicadina* plugs (Table 1). Cathinone and two intermediates from its biosynthesis pathway also were found at significantly higher levels than in *M. levispora* and the uninfected

control (Table 1, Fig. 4). Cathinone, trans-cinnimac acid, and benzoic acid were measured at a 17.1x, 17.9 x, and 18.1 x fold change higher, respectively. Benzaldehyde, a non-pathway specific intermediate, was upregulated in both *M. levispora* (16.1x) and *M. cicadina* (15.0x) compared the negative control. Fragmentation of cathinone was performed on the pooled samples for both conidial and azygospore isolates and expected fragmentation patterns (m/z 260.09, 179.07, and 132.08) were ... (Fig. S4) (The fragmentation of Cathinone is currently underway and will be completed by next week) Slaframmine, a piperidine alkaloid, also showed a 13.6x log fold change increase in *M. cicadina* compared to expression in *M. levispora* and the control.

Table 1. Fold change and up/down regulation of naturally occurring alkaloids from *Massospora* fungal plugs in infected cicadas using global metabolomics.

Compound	<i>M. cicadina</i> (Mc)		<i>M. platypediae</i> (Mp)		<i>Mc</i> vs. <i>Mp</i>	
	Fold-change ^a	Directionality	Fold-change	Directionality	Fold-change	Directionality
Monoamine alkaloids						
<i>Substituted amphetamines</i>						
Trans-cinnimac acid	17.9 ± 1.0	Up	NSD	–	17.9 ± 1.0	Up
Benzaldehyde	15.0 ± 0.6	Up	16.1 ± 1.1	Up	NSD	–
Benzoic acid	18.1 ± 2.1	Up	NSD	–	18.1 ± 2.1	Up
Cathinone	17.1 ± 0.4	Up	NSD	–	17.1 ± 0.4	Up
<i>Substituted tryptamines</i>						
Nb-Lignoceroyltryptamine	17.9 ± 1.4	Up	NSD	–	17.9 ± 1.4	Up
Aspidospermine	16.8 ± 0.9	Up	14.5 ± 1.2	Up	NSD	–
4-Dimethylallyl-L-tryptophan (4-DMAT)	NSD	–	17.6 ± 0.3	Up	17.6 ± 0.3	Down

Agroclavine	NSD	-	16.8 ± 2.4	Up	16.8 ± 2.4	Down
Psilocybin	NSD	-	16.9 ± 0.6	Up	16.9 ± 0.6	Down
Piperidine alkaloids						
Slaframine	13.6 ± 0.6	Up	NSD	-	13.6 ± 0.6	Up

Fold change ± standard error based on comparisons with control. Value is average of five biological replicates.

Figure 4. Non-Beta Oxidation pathway for the biosynthesis of S-Cathinone with fold-change results from global metabolomics of *M. cicadina* and *M. levispora* plugs in infected cicadas.

In addition to alkaloids, other noteworthy compounds were differentially upregulated in *M. levispora* compared to *M. cicadina* (Fig. S4). These included 2-Hydroxyestradiol, an endogenous

steroid and metabolite of estradiol and 3-O-acetylecaldysone 2-phosphate, a metabolite of ecdysone, a steroidal prohormone of the major insect molting hormone 20-hydroxyecdysone .

Lastly, six other fungus-derived compounds were recovered from *M. cicadina* at significantly higher levels than those in the *M. platypediae* samples of *M. levispora* comb. nov. (Table S6). These including two mycotoxins (4-Propanoyl-HT2 toxin and Aflatoxin b1 triolcohol) previously described from Ascomycotan fungi (REF?) and one Basidiomycotan toxin, Ustiloxin C (REF?). Two other fungus-derived compounds (*Sarcodon scabrosus* Depsipeptide and Azoxystrobin) were differentially upregulated in *M. platypediae* (Table S6). While these compounds were found to be upregulated, a high quality MS/MS fragmentation spectrum was not able to be generated for either.

Targeted Metabolomics

Based on the results of the global metabolomics, targeted LC-MS/MS absolute quantification was performed on extracts of plugs from fungal infections of all three cicada species. Due to submersion in ethanol prior to analysis and inability to properly normalize the samples for global analysis, only targeted quantification of specific metabolites was performed.

Both psilocybin and its biologically relevant metabolite psilocin were monitored in the targeted quantification analysis. The dynamic range of the LC-MS quantification for psilocin and psilocybin encompassed 2.5 orders of magnitude. Signal for these analytes that fell below the lower limit of quantitation, but a peak still was visible, was used to determine presence or absence. Psilocybin was present in 10 of 11 *M. platypediae* plugs and seven of eight *M. levispora* plugs (Table 2, Fig. 4). Of these, psilocybin was only quantifiable in 6 of 7 *M. levispora* samples, with concentrations ranging from 1.2 to 3.2 ng/mL of ethanol extract (Table 2). Psilocin was

detected only in 9 of 11 extracts from *M. platypediae* at concentrations ranging from 0.02 to 4.65 µg/mL (Table 2, Fig. 4).

Note: This section on targeted metabolomics is logically incomprehensible. The reason(s) for targeting only psilocybin and psilocin and the disparity between qualitative versus quantitative data are poorly presented. Clarify!!

Table 2. Quantification of psilocybin and psilocin from *Massospora* fungal plugs using targeted LC-MS.

Specimen ID	Spore type	Psilocybin [m/z 285]		Psilocin [m/z 205]	
		Present	Conc. [ng/mL]	Present	Conc. [µg/g]
MC139	Azygospore	ND	–	ND	–
MC140	Azygospore	ND	–	ND	–
MC141	Azygospore	ND	–	ND	–
MC142	Azygospore	ND	–	ND	–
MC23	Conidia	ND	–	ND	–
MC33	Conidia	ND	–	ND	–
ML03	Azygospore	ND	–	ND	–
ML01	Conidia	Yes	1.2	ND	–
ML02	Conidia	Yes	2.3	ND	–
ML04	Conidia	Yes	1.8	ND	–
ML05	Conidia	Yes	2.2	ND	–
ML06	Conidia	Yes	BLQ	ND	–
ML07	Conidia	Yes	3.2	ND	–
ML08	Conidia	Yes	2.8	ND	–
MP01	Conidia	Yes	–	Yes	2.59
MP02	Conidia	Yes	–	Yes	1.16
MP03	Conidia	Yes	–	Yes	0.18
MP04	Conidia	Yes	–	No	BLQ
MP05	Conidia	Yes	–	No	BLQ
MP06	Conidia	Yes	–	Yes	0.02
MP07	Conidia	Yes	–	Yes	0.28
MP08	Conidia	Yes	–	Yes	4.65
MP09	Conidia	Yes	–	Yes	1.38
MP10	Conidia	Yes	–	Yes	0.68
MP14	Conidia	Yes	–	Yes	0.48
MCNC1	noninfected	ND	–	ND	BLQ
MLNC1	noninfected	ND	–	ND	BLQ
MPNC01	noninfected	ND	–	ND	BLQ

*ND, not detected; ‡ BLQ, below limit of quantification. Need to define your acronyms pertaining to specimen ID.

Figure 4. Ion chromatograms of monoamine alkaloids extracted from conidial plugs produced by *M. cicadina*, *M. levispora* and *M. platypediae* infections in adult cicada specimens of *M. septendecim*, *O. rimosa* and *P. putnami* respectively. ND = none detected.

Discussion

Phylogenetic analyses reveal that *M. levispora* and *M. platypediae* are not distinct species and therefore are combined. Morphology and common metabolite pathways further support their shared lineage. Such combinations of data will be necessary to clarify phylogeny and functional fungus-host interactions in Zoopagomycota because of their obligate nutritional relationships (Hodge et al 2017, Cooley et al. 2017).

The endogenous production of monoamine alkaloids such as psilocybin by both lineages of *M. levispora* (which includes *M. platypediae*) and cathinone by *M. cicadina* reported here for the first time have opened the door for broader investigations of secondary metabolite production across the diversity of this biologically unique fungal phylum and provides some preliminary insight into the mechanisms of the interplay between microbe and host. Another member of the Entomophthorales, *Strongwellsea*, causes a similar disease in flies (REF?) resulting in abdominal

openings that aid in spore dispersal and this species is a potential candidate to assess the extent to which these pathways are conserved phylogenetically.

Even though global metabolomics are comprehensive and provide a robust qualitative snapshot of all the metabolites within an organism or biological system at one point in time, comprehensive databases recording compound identity are largely limited to metabolites of *Saccharomyces cerevisiae* because of technological and/or financial restraints (Xu et al. 2014). Numerous fungal secondary metabolites have been recovered based on accurate mass, but the lack of MS/MS spectral libraries has limited fragmentation identification, a necessary intermediate validation between global metabolomics and targeted quantification using standards.

The recent observations on *Massospora*-infected cicadas provide data for generating hypotheses on how these fungi manipulate its cicada host. *Massospora*, like *Hypocrealean* entomopathogens, hijacks the infected individual to optimize spore dispersal at the expense of the cicada's own interests (Cooley et al 2017). *Massospora* renders the cicada more promiscuous as a means of spreading infective spores to both conspecific females and males. The monoamine alkaloids identified in this study may contribute to such hijacking behavior. Several studies have investigated the actions of hallucinogens on arthropods including ants and spiders. Frischknecht and Waser (1978) administered amphetamine, LSD, and THC to the black-backed meadow ant (*Formica pratensis*). Low doses (1 or 10~ug/ant) of amphetamine caused an increase in intraspecific aggression compared to LSD where doses of 20 ng or more typically impaired locomotion. Although amphetamines didn't seem to influence aggressiveness in spiders, a dose of 1 mg or about 6.7 g/kg resulted in irregular web building with frequently interrupted radii and some erratic strands by adult female *Araneus diadematus* 12-hours after receiving the dose (Witt

1971). Cathinone is chemically similar to ephedrine, cathine, methcathinone and other amphetamines. The higher levels of cathinone in *M. cicadina* may confer enhanced aggressiveness that benefit continued spore dispersal despite debilitating infections that likely result in fatigue, decreased muscle strength and general malaise. Similar responses by cathinone have. Similarly, psilocybin and its dephosphorylated metabolite psilocin, a serotonin receptor agonist, may be acting similarly in *M. levispora*-infected cicadas by increasing euphoria and dispersal despite debilitating infections. Alternatively, psilocybin/psilocin may act as a repellent against predation. Reynolds et al (2017) postulate a similar role by these metabolites in hallucinogenic mushrooms. Masiulionis et al. (2013) report that basidiocarps of *Psilocybe coprophila* growing on cow dung were actively collected by workers of leaf-cutter ants, *Acromyrmex lobicornis*, in Santa Fé province, Argentina. While not measured, psychotropic compounds often found in these mushrooms may serve to protect the colony.

Psilocybin causes perceptual distortions in infected animals and may cause similar effects in insects. Reported behavioral observations on *Massospora*-infected cicadas indicate increased sexual receptivity with greater tolerance to contact by an approaching male (Table S1). These data support the hypothesis that expression of psilocin causes delayed flight initiation and flight orientation in cicadas, which can result in immobile individuals becoming copulatory targets (White et al. 1983). The measurable absence of intermediates in the biosynthetic pathway of psilocybin in this study suggest rapid degradation/conversion within the cicada host.

Another plausible explanation for behavioral bisexuality in infected males is the sharp increase in steroids observed in *M. platypediae* infected cicadas. 2-Hydroxyestradiol, a catechol estrogen, was significantly elevated in *M. platypediae*-infected male cicadas (only 1 female was recovered from the collection). Such sharp increases in estrogen in males may be due, in part, to

castration by *Massospora*. However, similar outcomes were not obtained in global metabolomics runs of both females and males of *M. cicadina*.

Together, the identified targeted secondary metabolites identified in this study represent a small fraction of the total metabolites detected. Given the strong body of literature surrounding many of these natural products and their pathways, significant headway is likely to be made in understanding their role in pathogen-cicada interactions have been measured in ants.

Supplementary Materials

Figure S1. LSU maximum likelihood (ML) phylogenetic tree for *Massospora* spp. and close allies in the Entomophthorales. *M. levispora* comb. nov. accommodates both *M. levispora* sensu

strictu and *M. platypediae*. Bootstrap support is indicated near each node and only values greater than 50% are shown.

Figure S2. SSU maximum likelihood (ML) phylogenetic tree for *Massospora* spp. and close allies in the Entomophthorales. *M. levispora* comb. nov. accommodates both *M. levispora* sensu strictu and *M. platypediae*. Bootstrap support is indicated near each node and only values greater than 50% are shown.

Figure S3. MS/MS fragmentation spectrum of psilocybin (m/z 285.0098) from pooled *M. platypediae* fungal plug extracts. An asterisk above a peak indicates predicted fragments from psilocybin fragmentation.

Figure S4. Differential fold change and regulation of 2-Hydroxyestradiol and 3-O-acetylcyclopentanone 2-phosphate expression in fungal plugs of *M. platypediae* and *M. cicadina* using global metabolomics. The former metabolite is an endogenous steroid and the latter is a product of ecdysone, a steroidal prohormone of the major insect molting hormone 20-hydroxyecdysone.

Table S1. Changes in cicada behavior as a result of *Massospora* infection compared to outwardly healthy counterparts.

<i>Massospora</i> – Cicada consortia	Flight						Singing/crepitations	Wing-flick signals by males in response to male courting calls	Sexual receptivity by tolerance of contact by an approaching male
	Distance	Endurance	Initiation	Orientation	Speed				
<i>M. cicadina</i> – <i>Magicalada</i> spp.*	<	None	Delayed	Deviated	None		None	+	[x] males, [x] females
<i>M. sp.</i> – <i>Meimuna boninensis</i> †	–	–	–	–	–		Weaker	–	–
<i>M. diceroprocta</i> – <i>Diceroprocta</i> spp.‡	–	–	–	–	–		>	–	+
<i>M. levispora</i> – <i>Okanagana rimosa</i> *	–	–	None	–	–		None	–	+
<i>M. platypediae</i> – <i>Platypedia putnami</i> §	–	–	None	–	–		Intermediate	+	+

Based on the results of four studies: * Cooley et al. 2017; ‡ Hastings, personal communication; § Murphy and Redden 2003; † Ohbayashi et al. 1999; * White et al. 1983).

Table S2.

<i>Massospora</i> species	Fungal ID	Spore type	Spore measurements ^a [width range x length range (mean width x mean length)]
<i>M. platypediae</i>	MP11	Azygospore	40.5 µm x 41.4 µm (40.9 µm)
<i>M. platypediae</i>	Holotype	Conidia	5.2 – 7.7 µm x 6.5 – 12.9 µm (6.0 x 9.9 µm)
<i>M. platypediae</i>	MP01	Conidia	6.4 – 9 µm x 10.49 – 19.7 µm (8.0 x 15.0 µm)
<i>M. platypediae</i>	MP02	Conidia	7.7 – 10.5 µm x 10.3 – 17.1 µm (9.1 x 13.1 µm)
<i>M. platypediae</i>	MP03	Conidia	5.6 – 9.2 µm x 8.3 – 17.7 µm (<u>7.2 x 12.0 µm</u>)
<i>M. platypediae</i>	MP04	Conidia	6.8 – 9.4 µm x 9.2 – 14.7 µm (<u>7.7 x 11.7 µm</u>)
<i>M. platypediae</i>	MP05	Conidia	7.0 – 9.1 µm x 11.7 – 17.1 µm (7.9 x 13.8 µm)
<i>M. platypediae</i>	MP06	Conidia	6.8 – 9.3 µm x 9.2 – 16.2 µm (8.2 x <u>12.4 µm</u>)
<i>M. platypediae</i>	MP07	Conidia	5.5 – 8.8 µm x 12 – 19.3 µm (<u>7.1 x 15.2 µm</u>)
<i>M. platypediae</i>	MP08	Conidia	5.2 – 7.5 µm x 8.9 – 15.1 µm (<u>6.5 x 11.6 µm</u>)
<i>M. platypediae</i>	MP09	Conidia	7.2 – 10.1 µm x 10.3 – 16.9 µm (8.1 x 13.3 µm)
<i>M. platypediae</i>	MP10	Conidia	6.8 – 10.8 µm x 9.6 – 19.4 µm (9.0 x 14.6 µm)
<i>M. platypediae</i>	MP16	Conidia	6.4 – 9.1 µm x 9.0 – 13.6 µm (<u>7.7 x 10.9 µm</u>)
<i>M. platypediae</i>	MP13	Conidia	5.4 – 10.6 µm x 10.3 – 18.7 µm (8.0 x 14.2 µm)
<i>M. platypediae</i>	MP14	Conidia	7.4 – 10.2 µm x 9.9 – 18.9 µm (8.6 x 13.9 µm)
<i>M. platypediae</i>	MP15	Conidia	7.1 – 10.9 µm x 9.6 – 20 µm (8.8 x 13.7 µm)
<i>M. levispora</i>	Holotype	Azygospore	27.5 µm x 40.5 µm (34.0 µm)
<i>M. levispora</i>	ML03	Azygospore	38.8 µm x 40.7 µm (<u>39.8 µm</u>)
<i>M. levispora</i>	Holotype	Conidia	6.0 – 11.0 µm x 9.5 – 23.0 µm (8.0 x 15.0 µm)

<i>M. levispora</i>	ML01	Conidia	7.5 – 10.5 μm x 10.5 – 17.4 μm (<u>8.8 x 12.9 μm</u>)
<i>M. levispora</i>	ML02	Conidia	7.4 – 10.8 μm x 12.4 – 20.9 μm (<u>9.2 x 16.6 μm</u>)
<i>M. levispora</i>	ML04	Conidia	7.5 – 9.9 μm x 10.4 – 16.3 μm (<u>8.8 x 13.4 μm</u>)
<i>M. levispora</i>	ML06	Conidia	7.6 – 10.8 μm x 11.7 – 19.5 μm (<u>8.7 x 14.1 μm</u>)
<i>M. levispora</i>	ML07	Conidia	7.1 – 9.5 μm x 11.3 – 17.9 μm (<u>8.6 x 14.2 μm</u>)
<i>M. levispora</i>	ML08	Conidia	7.0 – 10.2 μm x 11.8 – 19.6 μm (<u>8.6 x 14.4 μm</u>)
<i>M. levispora</i>	ML10	Conidia	7.2 – 9.6 μm x 10.8 – 16.5 μm (<u>8.2 x 13.2 μm</u>)
<i>M. levispora</i>	ML05	Conidia	8.5 – 10.6 μm x 13.2 – 18.6 μm (<u>9.4 x 15.3 μm</u>)
<i>M. cicadina</i>	Holotype	Azygospore	33.5 μm x 47.5 μm (40.5 μm)
<i>M. cicadina</i>	MC150	Azygospore	
<i>M. cicadina</i>	MC138	Azygospore	39.2 μm x 41.3 μm (<u>40.2 μm</u>)
<i>M. cicadina</i>	MC142	Azygospore	42.1 μm x 43.5 μm (<u>42.8 μm</u>)
<i>M. cicadina</i>	MC141	Azygospore	39.8 μm x 41.0 μm (<u>40.4 μm</u>)
<i>M. cicadina</i>	MC139	Azygospore	41.8 μm x 42.8 μm (<u>42.3 μm</u>)
<i>M. cicadina</i>	MC140	Azygospore	39.7 μm x 41.0 μm (<u>40.4 μm</u>)
<i>M. cicadina</i>	MC145	Azygospore	42.1 μm x 43.3 μm (<u>42.8 μm</u>)
<i>M. cicadina</i>	MC143	Azygospore	43.0 μm x 45.1 μm (<u>44.1 μm</u>)
<i>M. cicadina</i>	MC144	Azygospore	43.7 μm x 45.5 μm (<u>44.6 μm</u>)
<i>M. cicadina</i>	MCH17	Azygospore	41.0 μm x 42.6 μm (<u>41.8 μm</u>)
<i>M. cicadina</i>	MCH05	Azygospore	42.8 μm x 43.7 μm (<u>43.2 μm</u>)
<i>M. cicadina</i>	MCH06	Azygospore	43.1 μm x 45.0 μm (<u>44.1 μm</u>)
<i>M. cicadina</i>	Holotype	Conidia	10 – 17 μm x 14 – 20 μm (15.5 x 18.5 μm)
<i>M. cicadina</i>	MCH21	Conidia	9.7 – 14.4 μm x 11.3 – 18.1 μm (<u>12.1 x 14.4 μm</u>)
<i>M. cicadina</i>	ARSEF 374	Conidia	14.1 – 20.5 μm x 16.2 – 24.5 μm (<u>17.4 x 19.9 μm</u>)
<i>M. cicadina</i>	MCH14	Conidia	12.4 – 17.3 μm x 13.4 – 19.1 μm (<u>14.7 x 16 μm</u>)
<i>M. cicadina</i>	MCH12	Conidia	13.8 – 18.2 μm x 15.3 – 22.7 μm (<u>15.7 x 18.4 μm</u>)
<i>M. cicadina</i>	MCH16	Conidia	12.1 – 17.3 μm x 12.2 – 20.8 μm (<u>14.6 x 16.6 μm</u>)
<i>M. cicadina</i>	MC46	Conidia	11.5 – 15.1 μm x 11.8 – 18.5 μm (<u>13.7 x 15.4 μm</u>)
<i>M. cicadina</i>	MC95	Conidia	12.9 – 15.6 μm x 13.3 – 17.3 μm (<u>14.3 x 15.4 μm</u>)
<i>M. cicadina</i>	MC94	Conidia	13.3 – 16.9 μm x 13.6 – 18.5 μm (<u>14.6 x 16.2 μm</u>)
<i>M. cicadina</i>	MC33	Conidia	11.8 – 15.9 μm x 12.8 – 17.3 μm (<u>13.7 x 14.9 μm</u>)
<i>M. cicadina</i>	MC136	Conidia	13.7 – 18.9 μm x 14.4 – 25.5 μm (<u>16.5 x 19.4 μm</u>)
<i>M. cicadina</i>	MCH15	Conidia	11.1 – 15 μm x 13.6 – 19.7 μm (<u>13.4 x 16.2 μm</u>)
<i>M. cicadina</i>	GK05	Conidia	12.5 – 16.3 μm x 13.4 – 18.5 μm (<u>14.6 x 16.2 μm</u>)
<i>M. cicadina</i>	GK04	Conidia	12.3 – 17 μm x 12.6 – 19.3 μm (<u>14.3 x 16.1 μm</u>)
<i>M. cicadina</i>	GK01	Conidia	14 – 19.4 μm x 14.3 – 20.1 μm (<u>15.9 x 17.3 μm</u>)
<i>M. cicadina</i>	GK02	Conidia	13.6 – 17.7 μm x 14.8 – 19.7 μm (<u>16 x 17.4 μm</u>)

^a Conidial and azygospore measurements based on the average of 25 measured spores per fungal isolate. Underlined mean values in parentheses denote that mean length and width fell within the previously reported ranges for that given species

Table S3. *Massospora* isolate histories and the type of analyses performed.

Fungus (<i>Massospora</i> sp.)	Fungal ID	Cicada host	Gender	Brood	Location	Year collected	Analyses performed ^a
<i>M. platypediae</i>	MP01	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, TM, MS, PS
<i>M. platypediae</i>	MP02	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, TM, MS, PS
<i>M. platypediae</i>	MP03	<i>Platypedia putnami</i>	Female	n/a	NM, USA	2017	GM, TM, MS
<i>M. platypediae</i>	MP04	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, TM, MS
<i>M. platypediae</i>	MP05	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, TM, MS
<i>M. platypediae</i>	MP06	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, TM, MS
<i>M. platypediae</i>	MP07	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, TM, MS
<i>M. platypediae</i>	MP08	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, TM, MS, PS
<i>M. platypediae</i>	MP09	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, TM, MS
<i>M. platypediae</i>	MP10	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, TM, MS
<i>M. platypediae</i>	MP16	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, MS
<i>M. platypediae</i>	MP13	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, MS, PS
<i>M. platypediae</i>	MP14	<i>Platypedia putnami</i>	Female	n/a	NM, USA	2017	GM, TM, MS
<i>M. platypediae</i>	MP15	<i>Platypedia putnami</i>	Female	n/a	NM, USA	2017	GM, MS
<i>M. platypediae</i>	MP11	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, MS, PS
<i>M. platypediae</i>	MP12	<i>Platypedia putnami</i>	Male	n/a	NM, USA	2017	GM, TM
<i>M. levispora</i>	ML01	<i>Okanagana rimosa</i>	Female	n/a	MI, USA	1998	TM, MS, PS
<i>M. levispora</i>	ML02	<i>Okanagana rimosa</i>	Female	n/a	MI, USA	1998	TM, MS, PS
<i>M. levispora</i>	ML04	<i>Okanagana rimosa</i>	Female	n/a	MI, USA	1998	TM, MS, PS
<i>M. levispora</i>	ML06	<i>Okanagana rimosa</i>	Female	n/a	MI, USA	1998	TM, MS
<i>M. levispora</i>	ML07	<i>Okanagana rimosa</i>	Female	n/a	MI, USA	1998	TM, MS
<i>M. levispora</i>	ML08	<i>Okanagana rimosa</i>	Female	n/a	MI, USA	1998	TM, MS
<i>M. levispora</i>	ML10	<i>Okanagana rimosa</i>	Female	n/a	MI, USA	1998	MS
<i>M. levispora</i>	ML03	<i>Okanagana rimosa</i>	Female	n/a	MI, USA	1998	TM, MS
<i>M. levispora</i>	ML05	<i>Okanagana rimosa</i>	Female	n/a	MI, USA	1998	TM, MS
<i>M. cicadina</i>	MC138 (BVI-01)	<i>Magisicada septendecim</i>	Female	VI	NC, USA	2017	GM, TM, MS, PS
<i>M. cicadina</i>	MC142 (BVI-05)	<i>Magisicada septendecim</i>	Female	VI	NC, USA	2017	GM, TM, MS
<i>M. cicadina</i>	MC141 (BVI-06)	<i>Magisicada septendecim</i>	Female	VI	NC, USA	2017	GM, TM, MS
<i>M. cicadina</i>	MC139 (BVI-07)	<i>Magisicada septendecim</i>	Female	VI	NC, USA	2017	GM, TM, MS
<i>M. cicadina</i>	MC140 (BVI-08)	<i>Magisicada septendecim</i>	Female	VI	NC, USA	2017	GM, TM, MS
<i>M. cicadina</i>	MC138 (BVI-03)	<i>Magisicada septendecim</i>	Female	VI	NC, USA	2017	MS
<i>M. cicadina</i>	MC143 (BVI-02)	<i>Magisicada septendecim</i>	Female	VI	NC, USA	2017	MS
<i>M. cicadina</i>	MC144 (BVI-04)	<i>Magisicada septendecim</i>	Male	VI	NC, USA	2017	MS
<i>M. cicadina</i>	MCH17	<i>Magisicada tredecim-like</i>	Female	XIX	IL, USA	1998	MS
<i>M. cicadina</i>	MC150	<i>Magisicada tredecim</i>	?	XIX	IL, USA	1998	MS, PS
<i>M. cicadina</i>	MCH05	<i>Magisicada tredecim</i>	Male	XIX	IL, USA	1998	MS
<i>M. cicadina</i>	MCH06	<i>Magisicada tredecim</i>	Female	XIX	IL, USA	1998	MS
<i>M. cicadina</i>	MCH21	<i>Magisicada septendecula</i>	Female	I	WV, USA	1978	MS
<i>M. cicadina</i>	ARSEF 374	<i>Magisicada septendecim</i>	?	II	NY, USA	1979	MS, PS
<i>M. cicadina</i>	MCH14	<i>Magisicada cassini</i>	Female	II	NY, USA	1979	MS
<i>M. cicadina</i>	MCH12	<i>Magisicada septendecim</i>	Female	II	CT, USA	1979	MS

<i>M. cicadina</i>	MCH16	<i>Magიცicada septendecula</i>	Male	IV/XIX	MO, USA	1998	MS
<i>M. cicadina</i>	MC46	<i>Magიცicada septendecula</i>	Male	V	WV, USA	2016	MS, PS
<i>M. cicadina</i>	MC95	<i>Magიცicada cassini</i>	Male	V	WV, USA	2016	GM, MS
<i>M. cicadina</i>	MC94	<i>Magიცicada cassini</i>	Female	V	WV, USA	2016	GM, MS
<i>M. cicadina</i>	MC33	<i>Magიცicada septendecula</i>	Male	V	WV, USA	2016	GM, TM, MS, PS
<i>M. cicadina</i>	MC136	<i>Magიცicada septendecim</i>	Female	V	OH USA	2016	MS
<i>M. cicadina</i>	MCH15	<i>Magიცicada septendecim</i>	Male	VIII	PA, USA	2002	MS
<i>M. cicadina</i>	GK05	<i>Magიცicada cassini</i>	Male	X	OH, USA	2004	MS, PS
<i>M. cicadina</i>	GK04	<i>Magიცicada septendecula</i>	Female	XIV	OH, USA	2008	MS, PS
<i>M. cicadina</i>	GK01	<i>Magიცicada tredecula</i>	Female	XXIII	IL, USA	1989	MS
<i>M. cicadina</i>	GK02	<i>Magიცicada tredecassini</i>	Female	XXIII	IN, USA	2015	MS, PS
<i>M. cicadina</i>	MCH19	<i>Magიცicada septendecula</i>	Female	I	WV, USA	1978	MS, PS
<i>M. cicadina</i>	MCH22	<i>Magიცicada septendecula</i>	Female	XIX	IL, USA	1998	MS
<i>M. cicadina</i>	MC19	<i>Magიცicada cassini</i>	Female	V	WV, USA	2016	GM, MS
<i>M. cicadina</i>	MC23	<i>Magიცicada septendecula</i>	Male	V	WV, USA	2016	GM, TM, MS, PS
<i>M. cicadina</i>	MC104	<i>Magიცicada septendecim</i>	Male	V	OH, USA	2016	PS
<i>M. cicadina</i>	MC130	<i>Magიცicada septendecim</i>	Female	V	OH, USA	2016	PS
No fungus control	MCNC1	<i>Magიცicada septendecim</i>	Female	VI	NC, USA	2017	GM, TM
No fungus control	MPNC01	<i>Platypedia putnami</i>	?	n/a	NM, USA	2017	GM, TM
No fungus control	MLNC01	<i>Okanagana rimosa</i>	?	n/a	MI, USA	1998	GM, TM

^a GM, global metabolomics; TS, targeted metabolomics for psilocybin, psilocin, agronovine, and cathinone; MS, morphological studies; PS, Phylogenetic studies.

Table S4. Positive ion mode metabolites from where?.

Compound Name	Compound Class	Log FC ([Control] vs [MC]) : Normalized	Regulation ([Control] vs [MC])	Log FC ([MC] vs [MP]) : Normalized	Regulation ([MC] vs [MP])
(+)-Syringaresinol O-beta-D-glucoside	n/a	0	down	- 16.535217	down
20-HDoHE	very long-chain fatty acids	- 15.599645	down	- 0.3088932	down
(1R)-N-Acetyl-L-cysteinyl-(2R)-hydroxy-1,2-dihydronaphthalene	n/a	0	down	- 14.853127	down
(24R)-24,25-dihydroxyvitamin D2 / (24R)-24,25-dihydroxyergocalciferol	n/a	0	down	- 19.344164	down
(2S)-2-([1-(R)-Carboxyethyl]amino)pentanoate	n/a	- 17.463285	down	17.463285	up
(5-D-ribonyl)hopane	Hopanoids	- 16.858719	down	16.858719	up
(Z)-22-Hentriacontene-2,4-dione		0	down	- 16.545946	down
[6]-Gingerdiol 4'-O-beta-D-glucopyranoside	beta-diketones phenolic glycosides	0	down	- 15.264545	down
1-(10-methyl-hexadecanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine	1-acyl,2-alkylglycerophosphoethanolamines	0	down	- 15.86329	down
1-(2-Thienyl)-1-heptanone	aryl alkyl ketones.	- 14.632663	down	14.632663	up
1,2-Dihydroxy-3-keto-5-methylthiopentene	alpha-branched alpha,beta-unsaturated ketones	- 14.0850525	down	-1.43892	down
1,4-Benzenediol, 2,6-bis(1-methylethyl)-, 4-(hydrogen sulfate)	phenylpropanes	0	down	- 15.932431	down
10,11-Epoxy-3,7,11-trimethyl-2E,6E-tridecadienoic acid	Branched fatty acids	- 16.30103	down	16.30103	up
10E,12Z-Tetradecadienyl acetate	Wax monoesters	- 15.077946	down	15.077946	up
11,11-Difluoro-9Z-dodecenyl acetate	Wax monoesters	- 16.299614	down	16.299614	up
11?-(4-dimethylaminophenyl)-1?,25-dihydroxyvitamin D3 / 11?-(4-dimethylaminophenyl)-1?,25-dihydroxycholecalciferol	n/a	19.723108	up	0	down
14-Hydroxypergolide glucuronide	n/a	0	down	- 15.432378	down

15-Lipoxygenase Inhibitor 1	n/a	- 17.0148 16	down	17.01481 6	up
18-acetoxy-1?,25-dihydroxyvitamin D3 / 18-acetoxy-1?,25- dihydroxycholecalciferol	n/a	0	down	- 14.71124 9	down
1-Piperidinecarboxaldehyde	piperidines	- 17.0109 63	down	17.01096 3	up
2,2,11,13,15-pentachloro-docosane- 1,14-disulfate	Other Fatty Acyls	14.6707 17	up	0	down
26-Glucosyl-1,3,11,22- tetrahydroxyergosta-5,24-dien-26- oate	tetrahydroxy bile acids, alcohols and derivatives.	0	down	- 16.23875	down
2alpha-Hydroxypyracrenic acid	triterpenoids	- 17.6331 96	down	17.63319 6	up
2alpha-Hydroxypyracrenic acid nullnullnull 1.0360001		- 15.7736 635	down	15.77366 35	up
3'-(2''-Galloylglucosyl)- phloracetophenone	phenolic glycosides	16.1955 97	up	0	down
3',4'-Dihydroxy-5,6,7- trimethoxyflavone	Flavones and Flavonols	0	down	- 15.39277 65	down
3,7-Dihydroxy-2-[4-hydroxy-3-(4- hydroxy-3-methylbutyl)phenyl]-5,6- dimethoxy-4H-1-benzopyran-4-one	Flavones and Flavonols	- 18.8368 78	down	18.83687 8	up
3?-Hydroxy-4,4-Bisnor-8,11,13- Podocarpatriene	n/a	0	down	- 14.90055 6	down
32:4(17Z,20Z,23Z,26Z)	Unsaturated fatty acids	0	down	- 15.45768 1	down
3-Methylthiopropylamine	n/a	- 20.2151 32	down	20.21513 2	up
4- (Hydroxymethyl)benzenediazonium(1+)	benzyl alcohols	- 19.4882 49	down	19.48824 9	up
4a-Hydroxytetrahydrobiopterin	biopterins and derivatives	- 21.2010 44	down	21.20104 4	up
4'-Hydroxy-7-methoxy-8- methylflavan	Flavans, Flavanols and Leucoanthocyanidins	- 15.3423 78	down	15.34237 8	up
4-Propanoyl-HT2 toxin	trichothecenes	- 16.8662 32	down	16.86623 2	up
5-(1-Hydroxyethyl)-4-methylthiazole	n/a	- 19.6665 88	down	19.66658 8	up
5,7,4'-Trihydroxy-6,8,3'- trimethoxyflavone 7-glucoside	flavonoid o-glycosides	0	down	- 16.01759 5	down
5-Acetoxypalisadin B	sesquiterpenes	0	down	- 14.95966 6	down
5-Aminoimidazole-4-carboxamide-1- ?-D-ribofuranosyl 5'- monophosphate		- 15.9506 83	down	15.95068 3	up

5-Hydroxy-7-methoxy-2-tritriacontyl-4H-1-benzopyran-4-one		0	down	-	down
	chromones			16.160921	
5-Methyl-2-phenyl-2-hexenal		0	down	-	down
	phenylacetaldehydes			17.042318	
6,8-Di-C-methyl-luteolin 7-methyl ether	n/a	0	down	-	down
				18.718008	
6?-Naltrexol-3-glucuronide	n/a	0	down	-	down
				15.416902	
7-Aminocephalosporanic acid	n/a	0	down	-	down
				16.204536	
7-Methoxyflavanone	Flavones and Flavonols	-	down	-	down
		19.392294		1.034029	
9,12,15-Hexadecatrienoic acid	Unsaturated fatty acids	-	down	15.016484	up
		15.016484			
9-Thiastearic Acid	n/a	0	down	-	down
				18.152506	
Acutilol A	C20 isoprenoids (diterpenes)	-	down	1.3257074	up
		14.823766			
Agavasaponin C	steroidal saponins	-	down	16.457964	up
		16.457964			
Akeboside Stf	n/a	-	down	18.423658	up
		18.423658			
Alkaloid A6		0	down	-	down
	acridones			17.398186	
Allocain-S		-	down	15.886935	up
		15.886935			
Amastatin	n/a	-	down	15.267987	up
		15.267987			
Arginyl-Hydroxyproline	dipeptides	0	down	-	down
				15.646347	
AS-605240	n/a	0	down	-	down
				16.08645	
Asp Glu Trp	n/a	0	down	-	down
				17.373049	
Asp Tyr Asn	n/a	-	down	-	down
		16.571829		0.9135399	
Asp Val Asp	n/a	-	down	16.514227	up
		16.514227			
Benzo[a]fluoranthene	n/a	-	down	18.111872	up
		18.111872			
beta-Obscurine	n/a	-	down	15.421987	up
		15.421987			

C-6 Ceramide		- 15.1379 42	down	15.13794 2	up
C9:2n-5,7	Unsaturated fatty acids	- 16.3549 98	down	16.35499 8	up
Calyculin A	n/a	- 16.6401 44	down	16.64014 4	up
Capsaicin	methoxyphenols.	- 15.1512 83	down	15.15128 3	up
CAY10499		0	down	- 15.50116 6	down
Cer(d14:1(4E)/20:0(2OH))	n/a	0	down	- 17.34730 1	down
Cer(d18:2/16:0) null 1.028	n/a	0	down	- 17.08793 8	down
Chalcomoracin	2-arylbenzofuran flavonoids	- 18.2254 5	down	18.22545	up
Chlorophyll d	metallotetrapyrroles.	- 15.6948 9	down	15.69489	up
Cinnamyl phenylacetate	styrenes	- 15.3839 6	down	15.38396	up
Conchosin B	n/a	- 16.2712 02	down	16.27120 2	up
CPA(16:0/0:0)	-acyl-sn-glycerol-2,3-cyclic-phosphates	0	down	- 15.52623 5	down
Cyclohexane	cycloalkanes	- 18.2230 3	down	- 1.045982 4	down
Dehydrotremetone		- 15.5912 32	down	- 1.835561 8	down
Descinolone	n/a	- 16.8006 27	down	16.80062 7	up
DG(12:0/17:2(9Z,12Z)/0:0)[iso2]	Diacylglycerols	- 16.8496 91	down	16.84969 1	up
DG(16:1(9Z)/20:3(5Z,8Z,11Z)/0:0)	1,2-diacylglycerols	- 15.5278 46	down	- 1.174689 3	down
DG(18:1(11Z)/22:5(7Z,10Z,13Z,16Z,19Z)/0:0)	1,2-diacylglycerols	0	down	- 15.23056 7	down
DG(18:4(6Z,9Z,12Z,15Z)/15:0/0:0)	linoleic acids and derivatives	- 15.0531 27	down	15.05312 7	up
DG(21:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)[iso2]	Diacylglycerols	- 16.3507 79	down	1.550512 3	up

Dicloriparaside C	Flavones and Flavonols	- 15.2438 76	down	15.24387 6	up
Dihomomethionine	n/a	- 17.8169 38	down	17.81693 8	up
Dihydrocorynantheine	oxindole alkaloids	- 15.9955 24	down	0.135066 03	up
Dihydromikanolide		- 15.1403 84	down	15.14038 4	up
Dimethylarsinous acid		0	down	- 16.48190 9	down
Dioxybenzone	organoarsinous acids. n/a	- 16.3440 48	down	0.045965 195	up
Dolastatin 12	cytotoxic analogue of dolastatins	- 15.7254 61	down	15.72546 1	up
Dulcoside A	steviol glycosides	- 16.8576 93	down	16.85769 3	up
Ecalcidene		0	down	- 15.82866 4	down
Enterocin 900	2-arylbenzofuran flavonoids	0	down	- 15.43837 3	down
Epiarzelechin 3-O-gallate	Flavans, Flavanols and Leucoanthocyanidins	- 16.8139 29	down	16.81392 9	up
Epimodoside D	n/a	0	down	- 16.03729 2	down
Erythromycin C	aminoglycosides	- 15.9164 86	down	15.91648 6	up
Ether	n/a	- 17.8074 04	down	- 0.056489 944	down
Fasciculol E	triterpenoids	- 17.8298 91	down	17.82989 1	up
FKGK 11	n/a	- 18.1131 94	down	18.11319 4	up
Fluorene	n/a	- 18.8844 91	down	0.501478 2	up
Gaillardin	Sesquiterpene Lactone	0	down	- 14.81690 5	down
Gallamine	n/a	0	down	- 16.16862 7	down
GalNAc?1-4[Fuc?1-3]GlcNAc?-Sp	n/a	0	down	- 14.83810 9	down

gamma-L-Glutamyl-butyrosin B	n/a	0	down	-	down
				18.284414	
GlcN?1-6Ins-1-P-Cer(t18:0/26:0)	n/a	-	down	17.011175	up
		17.011175			
Gln Met Met	n/a	-	down	16.705587	up
		16.705587			
Gln Pro Glu		0	down	-	down
				15.573043	
Glu Asn Trp	n/a	-	down	14.990135	up
		14.990135			
Gly Gly Gln		-	down	13.359711	up
		13.359711			
Glyceollidin II	furanoisoflavonoids	-	down	16.22299	up
		16.22299			
Glycochenodeoxycholic acid 3-glucuronide	steroid glucuronide conjugates	0	down	-	down
				16.339136	
Gravacridonetriol glucoside	glycosylglycerols	0	down	-	down
				16.60066	
GS 1500	n/a	0	down	-	down
				16.654623	
Hebevinoside XIV	cucurbitacin glycosides	-	down	18.102667	up
		18.102667			
His Trp	n/a	-	down	15.37111	up
		15.37111			
His Trp null 7.188		0	down	-	down
				15.512599	
HQL-79	n/a	15.733234	up	0	down
Idebenone Metabolite	n/a	-	down	19.119688	up
(Benzenebutanoic acid, 2,5-dihydroxy-3,4-dimethoxy-6-methyl-)		19.119688			
Insmine	n/a	0	down	-	down
				14.893486	
Istamycin C1	n/a	-	down	16.409029	up
		16.409029			
JZL 195		0	down	-	down
				15.526435	
KDNalpha2-3Galbeta1-4Glcbeta-Cer(d18:1/16:0)	n/a	-	down	16.41869	up
		16.41869			
L-Menthone 1,2-glycerol ketal		15.533434	up	-	down
				18.385904	
Lycoperoside D	phenyl-1,3,4-triazoles steroidal saponins.	-	down	20.311625	up
		20.311625			

Lys Arg Ser		- 18.0401 52	down	4.537039 8	up
Lys Trp	n/a	0	down	- 19.64739 4	down
m-(beta-Acetyl-alpha-ethyl-p-hydroxyphenethyl)benzoic acid	fatty alcohols.	-16.3235	down	16.3235	up
Madlongiside C	triterpenoids	- 15.3356 4	down	15.33564	up
Majoroside F4		0	down	- 17.03136 6	down
Methional	triterpenoids n/a	- 21.8872 43	down	21.88724 3	up
Methionyl-Glycine		0	down	- 16.90062 1	down
Methyl 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-p-toluate	dipeptides	- 1.09191 9	down	15.78313 1	up
Methyl 4,6-di-O-galloyl-beta-D-glucopyranoside	tannin	16.4605 96	up	0	down
Met-Nap-OH	n/a	- 16.2445 83	down	16.24458 3	up
Met-Nap-OH nullnullnull 0.762	n/a	- 16.7163 33	down	16.71633 3	up
MIPC(t18:0/18:0(2OH))	Ceramide phosphoinositols	- 16.5925 56	down	16.59255 6	up
MIPC(t18:0/20:0)	Ceramide phosphoinositols	- 15.7124 8	down	15.71248	up
N-(2-hydroxyethyl)henicosanamide	n/a	0	down	- 14.55800 2	down
N-(naphthalen-1-yl)benzamide	n/a	- 17.3373 87	down	17.33738 7	up
N2-Succinylglutamic acid	n/a	- 19.2768 78	down	19.27687 8	up
N-AcetylCilastatin	n/a	- 21.4773 7	down	21.47737	up
N-AcetylCilastatin nullnullnull 0.97800004	n/a	- 19.5298 96	down	19.52989 6	up
N-arachidonoyl glutamic acid	N-acyl amines	- 18.3753 22	down	0.516498 57	up
Natsudaiddain	7-o-methylated flavonoids	0	down	- 15.07212 5	down
Nb-Lignoceroyltryptamine	3-alkylindoles	- 16.1395 85	down	16.13958 5	up

NNAL-N-glucuronide	n-glucuronides	15.2966 46	up	- 15.50921 6	down
NNAL-N-glucuronide nullnullnull 3.236	n/a	- 16.9751 07	down	- 0.839931 5	down
N-stearoyl glutamine	N-acyl amines	- 14.4030 77	down	- 2.618130 7	down
OH-Spheroidenone	C40 isoprenoids (tetraterpenes)	0	down	- 15.33619 6	down
Oleoside dimethyl ester	terpene glycosides	- 17.3813 6	down	17.38136	up
PA(18:0/13:0)	Diacylglycerophosphates	- 15.5367 05	down	0.329556 47	up
PA(18:3(6Z,9Z,12Z)/18:2(9Z,12Z))	1,2-diacylglycerol-3- phosphates	- 15.4614 37	down	0.231830 6	up
PA(20:1(11Z)/0:0)	Monoacylglycerophosphate s	- 16.6810 82	down	2.415017 1	up
PA(O- 16:0/20:5(5Z,8Z,11Z,14Z,17Z))	1,2-diacylglycerol-3- phosphates	- 15.7705 19	down	- 1.293642	down
Palustrine	alkaloid	0	down	- 15.92930 3	down
Palustrinoside	Flavones and Flavonols	- 16.5030 23	down	- 1.389999 4	down
PC(2:0/O-16:0)[U]	n/a	20.9827 38	up	- 18.96531 1	down
PC(2:0/O-16:0)[U] null 1.164	n/a	20.1629 6	up	- 17.59570 3	down
p-Cresol, a-[2- (dimethylamino)ethoxy]-a-o-tolyl-	n/a	- 16.1764 1	down	16.17641	up
PE(18:1(9Z)/0:0)[U]	n/a	19.6810 84	up	0	down
PE(19:1(9Z)/20:3(8Z,11Z,14Z))		0	down	- 17.92779 5	down
PE(20:4(5Z,8Z,11Z,14Z)/21:0)	phosphatidylethanolamines n/a	0	down	- 19.90756 2	down
PE(22:2(13Z,16Z)/19:0)	phosphatidylethanolamines	13.4101 19	up	0	down
PE(P-20:0/0:0)	1Z- alkenylglycerophosphoetha nolamines	19.2309 07	up	0	down
PE-Cer(d14:2(4E,6E)/23:0)	n/a	15.2398 49	up	- 16.02854 3	down
Pentaglutamyl folate	oligopeptides	17.2797 58	up	0	down

PG(20:4(5Z,8Z,11Z,14Z)/22:0)	Diacylglycerophosphoglycerols	- 16.550886	down	2.0446234	up
PG(22:1(11Z)/20:5(5Z,8Z,11Z,14Z,17Z))	phosphatidylglycerol	- 16.57528	down	16.57528	up
PG(O-16:0/15:1(9Z))	1-alkyl,2-acylglycerophosphoglycerols	- 15.591332	down	2.1477852	up
Phe Pro Ser	n/a	0	down	- 14.83011	down
Phe Trp Arg	n/a	- 16.39403	down	16.39403	up
Phenylalanyl-Methionine	dipeptides	- 16.552847	down	16.552847	up
Phenylboronic acid	n/a	- 19.632034	down	19.632034	up
PI(20:3(8Z,11Z,14Z)/18:3(6Z,9Z,12Z))	phosphatidylinositols	- 16.374325	down	16.374325	up
PI(20:3(8Z,11Z,14Z)/18:4(6Z,9Z,12Z,15Z))	Diacylglycerophosphoinositols	- 18.55739	down	18.55739	up
PI(21:0/0:0)	Monoacylglycerophosphoinositols	- 17.557116	down	17.557116	up
PI(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	Monoacylglycerophosphoinositols	- 17.040262	down	17.040262	up
PI(O-18:0/18:4(6Z,9Z,12Z,15Z))	1-alkyl,2-acylglycerophosphoinositols	- 19.230669	down	19.230669	up
PPM-18	naphthoquinone derivative	0	down	- 14.727916	down
Primaquine	aminoquinolines and derivatives.	- 16.091436	down	16.091436	up
Primaquine null 4.851	n/a	0	down	- 14.58673	down
Pro Met His		- 15.126072	down	15.126072	up
PS(19:0/20:2(11Z,14Z))	phosphatidylserines.	0	down	- 19.07366	down
PS(19:0/20:3(8Z,11Z,14Z))	phosphatidylserines	14.114278	up	- 17.609865	down
PS(P-18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	glycerophosphoethanolamines	0	down	- 16.020344	down
Psilocybin		0	down	- 16.992338	down
PU-H71	tryptamines and derivatives	0	down	- 15.311356	down

Pyropheophytin b	chlorins	- 16.15795	down	16.15795	up
Ribavirin 5'-diphosphate	n/a	0	down	- 16.917831	down
Ribavirin 5'-diphosphate null 6.763		0	down	- 15.448505	down
Rosmic acid	terpene lactone	0	down	-15.97	down
S-Adenosylhomocysteine-d4	5'-deoxy-5'-thionucleosides	0	down	- 15.725578	down
Sarcodon scabrosus Depsipeptide		0	down	- 16.350218	down
Scutellarein 4'-methyl ether 7-glucuronide	cyclic depsipeptides Flavones and Flavonols	0	down	- 15.166385	down
Scutellarein 6,7,4'-trimethyl ether 5-(6'''-acetylglucosyl)(1-3)-galactoside	n/a	- 17.156542	down	17.156542	up
Sintaxanthin	triterpenoids	- 13.298961	down	- 4.256095	down
Styrene		0	down	- 16.652166	down
Sudan I	styrenes	0	down	- 15.360576	down
Sulfacytine	aminobenzenesulfonamides	- 15.367604	down	15.367604	up
Tetracenomycin D3	n/a	- 17.418566	down	- 0.77059174	down
Theaspirone A	cyclohexenones	- 14.974149	down	14.974149	up
Thr Glu	n/a	- 17.402258	down	17.402258	up
Tingenone	Quinone-methide triterpenes	- 18.540436	down	18.540436	up
Trp Trp Ser	n/a	0	down	- 17.816412	down
Trp Tyr Asp	n/a	- 15.798955	down	- 0.4765749	down
Vasicinone	quinazoline alkaloid.	- 17.962984	down	17.962984	up
Viscidulin II	n/a	0	down	- 18.26062	down
Viscidulin II null 5.105	n/a	0	down	- 16.216606	down

Xanthosine	purine nucleosides	- 15.7810 51	down	15.78105 1	up
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Table S5. Negative ion mode metabolites from where?.

Compound Name	Compound Class	Log FC ([Control] vs [MC]) : Normalized	Regulation ([Control] vs [MC])	Log FC ([MC] vs [MP]) : Normalized	Regulation ([MC] vs [MP])
Pyruvophenone	acetophenones	- 17.85754 6			
(+/-)-trans- and cis-4,8-Dimethyl-3,7-nonadien-2-ol	acyclic monoterpenoids	- 15.71630 2	down	0	up
Chlorzoxazone	benzoxazolones	- 17.42078 4	down	0	up
Rodiasine	bisbenzylisoquinoline alkaloid	- 19.09365		- 0.698144 9	
Propyl 1-(propylthio)propyl disulfide			up	- 22.28711 7	down
epi-Derriflavanone	dialkyldisulfides	0	down	- 18.09542 7	down
Spinosin B	diprenylated flavanones	0	down	- 16.74723	down
Trilobatin	flavonoid c-glycosides flavonoid o-glycosides	16.74723	up	- 13.35097 5	down
Biocytin		19.35042	down	- 18.94593	up
Kuwanon V	l-alpha-amino acids linear diarylheptanoids.	0 - 17.26686 9	down	0	down
4-(2-Nitroethyl)phenyl primeveroside	phenolic glycosides	18.13805 8	down up	18.13805 8	up down
Tetrahydro-2-methylthiophen-3-ol	secondary alcohols	- 15.90013 1			
DL-Tyrosine	tyrosine and derivatives	- 19.69170 4	down	0	up
PG(P-16:0/20:5(5Z,8Z,11Z,14Z,17Z))	1-(1z-alkenyl),2-acyl- glycerophosphocholine s	- 17.18546 - 18.60773 7	down up	- 2.198892 6	up down
DG(20:1(11Z)/22:5(4Z,7Z,10Z,13Z,16Z)/0:0)	1,2-diacylglycerols	- 18.60773 7		0	up
LPA(18:2(9Z,12Z)/0:0)		15.90013 1	down	- 7.801830 3	up down
Sampangine	1-acylglycerol-3- phosphates 1-azaoxaporphines	14.05884 7 - 16.46670 3	up down	0	up
LPA(0:0/18:0)		- 13.66439 4		- 19.41344 5	
Neomycin B	2-acylglycerol-3- phosphates 4,5-disubstituted 2- deoxystreptamines.	- 13.66439 4 0	down down	- 20.11220 6	down down

Garcinone C				-	
	8-prenylated xanthenes	0	down	17.283474	down
Neoacrimarine G				-	
				16.539125	
	acridones.	0	down	5	down
Phloracetophenone	acylphloroglucinols and derivatives	18.36511	up	18.36511	down
Juliflorine	alkaloid	-		-	
		15.259037	down	17.241135	down
Tecostanine	alkaloid			-	
				16.001368	
		0	down	8	down
Desmethyldescarbamoylnovobiocin				-	
	aminocoumarin antibiotics	0	down	17.807512	down
Ranitidine N-oxide				-	
				18.107185	
	aralkylamines	0	down	5	down
Disulfoton				-	
				19.115257	
	aralkylamines	0	down	7	down
Aspidospermine	aspidospermatan-type alkaloids derived from the fusion of tryptamine and a terpene unit	-			
		18.85817	down	0	up
MIPC(t18:0/16:0(2OH))	Ceramide	-			
	phosphoinositols	17.187817	down	0	up
6-[2,3-Dihydroxy-1-(hydroxymethyl)propyl]-1,2-dihydro-7-hydroxy-9-methoxy-cyclopenta[c][1]benzopyran-3,4-dione	coumarins and derivatives	-			
		18.046638	down	0	up
Ustiloxin C	cyclic peptides	-			
		19.157667	down	0	up
Vignatic acid B		18.642282		18.642282	
	cyclic peptides	2	up	2	down
(+)-Quercitol				-	
				16.335718	
	cyclohexanols.	0	down	8	down
Dipyridamole				-	
				16.820997	
Dinoseb	dialkylarylamines	0	down	7	down
				-	
				20.705442	
	dinitrophenols	0	down	2	down
gamma-L-Glutamyl-butirosin B				-	
				18.145292	
	dipeptides	0	down	2	down
Poloxalene				-	
		15.229734		7.2477417	
	epoxides	4	up	7	down
2-Hydroxyestradiol				-	
	estrogens and derivatives.	0	down	18.531717	down
(R)-1-O-b-D-glucopyranosyl-1,3-octanediol	fatty acyl glycosides of mono- and disaccharides	18.146881	up	18.146881	down

Illicifolinoside A	fatty acyl glycosides of mono- and disaccharides	18.14392	up	2.830016	
(-)-epicatechin-3'-O-glucuronide	flavonoid o-glycosides	-		1	down
		15.99141			
		4	down	0	up
1,1,1,3,3,3-Hexafluoro-2-hydroxypropane				-	
	fluorohydrins.	0	down	18.78052	
Araliacerebroside	glycosphingolipids	16.75924		7	down
		3	up	16.75924	
Psychosine		16.56660		3	down
	glycosphingolipids	8	up	16.56660	
GlcCer(d18:1/26:0)	glycosyl-n-acylsphingosines			8	down
		0		-	
			down	17.02177	
Perlolyrine	harmala alkaloids.	-		4	down
		16.61299			
		7	down	0	up
Leukotriene D4-d5	hydroxyeicosapentaenoic acids	-			
		17.52999	down	0	up
4,8-Dihydroxyquinoline	Hydroxyquinolines	-			
		24.12721			
		8	down	0	up
Methyl nigakinone	indolonaphthyridine alkaloids	-			
		17.05901			
		3	down	0	up
O-Phospho-4-hydroxy-L-threonine	l-alpha-amino acids	-			
		19.82952	down	0	up
11?-Acetoxylkivorin		-			
	limonoid	17.69851			
		1	down	0	up
trans-trans-Muconic acid		-		-	
	medium-chain fatty acids	19.29056		19.13881	
		4	down	3	up
7-hydroxy-octanoic acid	medium-chain hydroxy acids and derivatives	-		-	
		15.71697		0.484127	
		4	up	04	down
PA(P-16:0/0:0)	monoalkylglycerophosphates	15.52372		15.52372	
		3	up	3	down
Enicoflavine	monoterpene alkaloid	16.58462		16.58462	
		3	up	3	down
DL-Tyrosine nullnullnull 2.075	n/a	-			
		21.29210			
		7	down	0	up
Gly Gln Ala	n/a	-			
		20.59899			
		7	down	0	up
C-6 NBD-dihydro-Ceramide	n/a	-			
		20.46825			
		2	down	0	up
Caohuoside D	n/a	-			
		18.50788			
		7	down	0	up
Ceranib-2	n/a	-			
		18.46154	down	0	up
Phe Ile Tyr	n/a	-			
		18.45981			
		6	down	0	up
Phe-Tyr-OH	n/a	-			
		18.31223			
		1	down	0	up

MIPC(t20:0/18:0(2OH))	n/a	-			
		18.16525			
		8	down	0	up
N-Acetyl-O-demethylpuromycin	n/a	-			
		18.00672	down	0	up
Kenusanone E	n/a	-17.7087	down	0	up
PI(22:4(7Z,10Z,13Z,16Z)/0:0)	n/a	-			
		17.68889			
		8	down	0	up
(22E)-(25R)-26,26,26-trifluoro-1?,25-dihydroxy-22,23-didehydrovitamin D3 / (22E)-(25R)-26,26,26-trifluoro-1?,25-dihydroxy-22,23-didehydrocholecalciferol	n/a	-			
AG-494	n/a	3.367296		14.29869	
		2	down	5	up
		-			
		17.65835			
		6	down	0	up
PI(18:4(6Z,9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z))	n/a	-			
		17.62696			
		8	down	0	up
Methyl 2-benzamidoacetate	n/a	-			
		17.61426			
		7	down	0	up
Methylthiobenzoylglycine	n/a	-			
		17.28078			
		7	down	0	up
N-docosahexaenoyl glutamic acid	n/a	-			
		3.133718		14.10179	
		5	down	3	up
5-O-Methylvisamminol	n/a	-			
		16.67175			
		5	down	0	up
Arachidonoyl Glycine-d8	n/a	-			
		15.58379			
		5	down	0	up
2-Butanone, 4-[6-(sulfooxy)-2-naphthalenyl]-	n/a	-			
		22.33132		19.81743	
		4	down	8	up
Bromotheophylline	n/a	-			
		17.69727		17.57853	
		9	down	5	up
Dihydromethanophenazine	n/a	21.26796		21.26796	
		3	up	3	down
Dihydromethanophenazine null 1.495	n/a	19.69452		19.69452	
		7	up	7	down
Methyl 18-bromo-9E,17E-octadecadien-5,7,15-triynoate	n/a	16.72260		16.72260	
		9	up	9	down
PE(17:0/20:2(11Z,14Z))	n/a	17.77072		17.77072	
		5	up	5	down
PG(16:1(9Z)/0:0)	n/a	16.39646		16.39646	
		5	up	5	down
PS(19:0/18:3(9Z,12Z,15Z))	n/a	17.35170		17.35170	
		2	up	2	down
SB 431542	n/a	17.38518		17.38518	
		1	up	1	down
Desmethylnaproxen-6-O-sulfate	n/a	-			
		18.02491		18.17587	
		2	down	7	down
Carbamazepine N-Glucuronide	n/a	-			
		16.87698		17.54890	
		2	down	8	down

Baicalein 6-methyl ether 7-glucuronide	n/a	-	-	-	-
		18.42616		19.35340	
		8	down	5	down
SB 216763	n/a	-		-	
		17.33455		-	
		5	down	18.47603	down
GlcCer(d15:2(4E,6E)/20:0)	n/a	-		-	
		18.23884		19.91450	
		6	down	9	down
6a,12b-Dihydro-3,10,11,12-tetrahydroxy-6-(3,4,5-trihydroxyphenyl)-[2]benzopyrano[3,4-c]benzopyran-8(6H)-one	n/a	-		-	
		15.30608		17.09463	
		8	down	3	down
24,24-difluoro-1?,25-dihydroxy-26,27-dimethylvitamin D3 / 24,24-difluoro-1?,25-dihydroxy-26,27-dimethylcholecalciferol	n/a	-		-	
		17.38068		-	
		2	down	19.27862	down
PG(O-16:0/14:0)	n/a	-		-	
		17.05489		19.55880	
		3	down	7	down
Cer(d14:1(4E)/20:0(2OH))	n/a	-		-	
		14.92497		18.55976	
		7	down	5	down
13-Dihydrocarminomycin	n/a	-		-	
		14.39703		18.55342	
		8	down	9	down
Acutilol A	n/a	-		-	
		20.08313		24.65843	
		8	down	8	down
PS-NAc(18:1(9Z)/18:1(9Z)/19:0)	n/a	16.54720		1.458258	
		7	up	6	down
PI(18:4(6Z,9Z,12Z,15Z)/19:1(9Z))	n/a	-		-	
		-		16.01741	
		0	down	8	down
2,8-Dihydroxy-3,4,7-trimethoxydibenzofuran	n/a	-		-	
		15.24904		1.426755	
		1	up	9	down
Tyr Asn Cys	n/a	-		-	
		-		16.77047	
		0	down	5	down
3?,12?,16?-Trihydroxy-5?-cholan-24-oic Acid	n/a	-		-	
		16.03835		0.910104	
		3	up	75	down
4-Hydroxy-6-methylpretetramide	n/a	-		-	
		-		17.12250	
		0	down	7	down
Azoxystrobin	n/a	-		-	
		-		17.13818	
		0	down	2	down
2-(9R-(15Z-docosenoyloxy)-3-methyl-2Z-decenoyloxy)-ethanesulfonic acid	n/a	-		-	
		-		17.15935	
		0	down	5	down
N-palmitoyl alanine	n/a	-		-	
		-		17.17243	
		0	down	4	down
Desmethylsufentanil	n/a	0	down	-17.1738	down
Dide-O-methyl-4-O-alpha-D-glucopyranosylsimmondsin	n/a	-		-	
		-		17.18972	
		0	down	6	down
3beta-Hydroxy-16-phosphonopregn-5-en-20-one monoethyl ester	n/a	-		-	
		-		17.35540	
		0	down	6	down

Lappaol B	n/a			-	
		0	down	17.39488	
Glu Phe His	n/a			6	down
				-	
		0	down	17.53063	
4-(3-Methylbut-2-enyl)-L-tryptophan	n/a			4	down
				-	
		0	down	17.60410	
3-[(2,6-Dichlorobenzylidene)amino]-6H-dibenzo[b,d]pyran-6-one	n/a			9	down
		15.62045		-	
Zolazepam	n/a	7	up	1.987859	down
				-	
		0	down	17.65824	
Dihydro Isorescinnamine	n/a			3	down
				-	
		0	down	17.71333	
GalCer(d18:0/26:0)	n/a			9	down
				-	
		0	down	17.78198	
AG-82	n/a			4	down
				-	
		0	down	17.79048	
?-Glutamyl-?-aminopropiononitrile	n/a			7	down
				-	
		0	down	17.82238	
DG(20:4(5Z,8Z,11Z,14Z)/0:0/20:4(5Z,8Z,11Z,14Z)) (d5)	n/a			2	down
				-	
		0	down	17.87259	
Glibornuride M5	n/a			1	down
				-	
		0	down	17.89309	
?-Galactosyl Ceramide	n/a			7	down
				-	
		0	down	17.89432	
Pyrromycin	n/a			5	down
				-	
		0	down	17.89547	
Phenytoin-N-glucuronide	n/a			2	down
				-	
		0	down	17.90303	
6,7-Dimethyl-8-(1-D-ribityl)lumazine null 7.931	n/a			2	down
				-	
		0	down	17.91775	
4-Chlorophenylacetic acid	n/a			9	down
				-	
		0	down	18.00651	
Glu Lys Pro	n/a			-	
		0	down	18.10652	
5,6,7,4'-Tetrahydroxyflavanone 6,7-diglucoside	n/a			-	
				18.11968	
		0	down	8	down
PI(21:0/19:0)	n/a			-	
				18.29637	
		0	down	7	down
2H-1-Benzopyran-2-one, 6-(1,2-dihydroxyethyl)-7-hydroxy-8-methoxy-glucuronide	n/a			-	
		0	down	18.31748	
1,7-Dideacetoxy-1,7-Dioxo-3-Deacetylkhivorin	n/a			4	down
				-	
		0	down	18.33088	
				1	down

Gly Asn Asn null 11.792	n/a			-	
				18.34774	
		0	down	2	down
Ethofumesate	n/a			-	
				18.51498	
		0	down	6	down
N-Acetyldemethylphosphinothricin null 3.208	n/a			-	
				18.54405	
		0	down	2	down
Glu Ala Tyr	n/a			-	
				18.55849	
		0	down	8	down
His Pro Arg	n/a			-	
				18.61038	
		0	down	4	down
o-Monocarboxylfinasteride null 1.851	n/a			-	
				18.64088	
		0	down	4	down
PI(19:1(9Z)/19:0)	n/a			-	
				18.65833	
		0	down	9	down
Pseudolycorine	n/a			-	
				18.68149	
		0	down	2	down
PS(P-18:0/14:1(9Z))	n/a	14.63165		-	
		7	up	4.105221	down
Gly Thr Ser	n/a			-	
		0	down	18.81537	down
5-Acetoxypalisadin B	n/a			-	
				19.10119	
		0	down	6	down
N-Acetyldemethylphosphinothricin	n/a			-	
		0	down	19.11608	down
PE(P-18:0/18:2(9Z,12Z))	n/a			-	
				19.14023	
		0	down	8	down
Tridesacetoxylkivorin null 1.8360002	n/a			-	
				19.29956	
		0	down	2	down
Taccalonolide A	n/a			-	
				19.33803	
		0	down	7	down
35-aminobacteriohopane-32,33,34-triol	n/a			-	
				19.72942	
		0	down	4	down
Leucomethylene blue (Reduced Methylene Blue)	n/a			-	
				19.87034	
		0	down	8	down
Met Ala Cys	n/a			-	
				20.28167	
		0	down	5	down
Tridesacetoxylkivorin	n/a			-	
				20.29859	
		0	down	4	down
Arg Lys Asn	n/a			-	
				20.48858	
		0	down	5	down
p-Nitroglutethimide	n/a			-	
				20.86267	
		0	down	7	down

Val His Arg	n/a	-			
		20.92421			
		0	down	2	down
2-Deoxystreptidine null 8.170999	n/a	-			
		20.93384			
		0	down	7	down
3-O-acetylcaldysone 2-phosphate	n/a	-			
		0	down	21.10803	down
Sunitinib	n/a	-			
		21.55778			
		0	down	7	down
19'-Butanoyloxyfucoxanthin	n/a	-			
		21.64800			
		0	down	8	down
Glu Lys Ile	n/a	-			
		6.900758			
		14.76116	up	7	down
Val Trp Phe	n/a	-			
		21.76959			
		0	down	8	down
PA(19:1(9Z)/0:0)	n/a	-			
		22.04645			
		0	down	7	down
Sunitinib null 1.1020001	n/a	-			
		0	down	22.33835	down
1,24-Dihydroxy-25-fluorovitamin D3 null 1.847	n/a	-			
		22.43374			
		0	down	6	down
Etioporphyrin III	n/a	-			
		23.20528			
		0	down	4	down
Trp-HoPhe-OH	n/a	-			
		17.23235			
		3	down	0	up
Gln Leu Ser	n/a	-			
		17.20439			
		3	down	0	up
N-palmitoyl threonine	n/a	-			
		16.02768			
		5	down	0	up
Phloracetophenone null 1.147	n/a	15.14120		15.14120	
		8	up	8	down
1-Guanidino-1-deoxy-scylo-inositol 4-phosphate	n/a	-			
		1.238485			
		17.5149	up	3	down
3,5-Pyridinedicarboxylic acid, 4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethyl-, mono(1-methylethyl) ester	n/a	-			
		0.247882			
N-Carbamoyl-2-amino-2-(4-hydroxyphenyl)acetic acid	n/a	19.24247	up	84	down
	n-carbamoyl-alpha amino acids.	-			
		20.02152			
Isopropyl apiosylglucoside	o-glycosyl compounds	0	down	4	down
		-			
		19.12184			
		7	down	0	up
Osmundalin	o-glycosyl compounds	-			
		17.66294			
		1	down	0	up
Validamycin A	o-glycosyl compounds	-			
		16.70888			
		5	down	0	up

Ethyl beta-D-glucopyranoside				-	
				19.81764	
	o-glycosyl compounds	0	down	4	down
Leonoside A	oligosaccharides	17.73085		17.73085	
		4	up	4	down
Succinic anhydride	oxolanes.	-			
		17.69932			
		7	down	-17.6205	up
13,14-DiHDPE	Oxylipin (?)	17.97746		0.642614	
		3	up	36	down
N-Acetyl-leucyl-leucine				-	
				20.01952	
	peptides.	0	down	2	down
(S)-2,3-Dihydro-3,5-dihydroxy-2-oxo-3-indoleacetic acid 5-[glucosyl-(1-4)-b-D-glucoside]	phenolic glycosides			-	
		0	down	16.76307	down
Tirofiban	phenylalanine and derivatives	0	down	-	
		0	down	17.75036	down
Desmethyl rosuvastatin	phenylpyrimidines.			-	
				17.66713	
		0	down	7	down
Vanilpyruvic acid	phenylpyruvic acid derivatives	18.23601		18.23601	
		5	up	5	down
PE(20:5(5Z,8Z,11Z,14Z,17Z)/22:4(7Z,10Z,13Z,16Z))	phosphatidylethanolamines	-			
		0.857939		15.15122	
		7	down	7	up
PIP2(18:0/16:0)	phosphatidylinositol-4,5-bisphosphates				
		17.97717	up	17.97717	down
PS(18:3(6Z,9Z,12Z)/18:4(6Z,9Z,12Z,15Z))	phosphatidylserine	-			
		20.09565			
		7	down	0	up
PS(18:4(6Z,9Z,12Z,15Z)/0:0)	phosphatidylserines			-	
		0	down	17.59632	
				9	down
4,7-Didehydroneophysalin B	physalins and derivatives.			-	
		0	down	16.91787	
		-		7	down
Kolanone	polyisoprenylated benzophenone	-		-	
		13.52813		16.94585	
		8	down	6	down
all-trans-pentaprenyl diphosphate				-	
	polyprenyl diphosphates			19.07077	
		0	down	8	down
11-O-Demethylpradinone II	Pradimicins	-			
		17.58487			
		1	down	0	up
PGH2-EA	prostaglandins and related compounds	-		-	
		16.88987		18.96077	
		5	down	2	down
1-Methylinosine	purine nucleosides	-			
		20.17225			
		8	down	0	up
Blasticidin S				-	
	pyranoid amino acids and derivatives			17.02934	
		0	down	3	down
Risperidone				-	
				18.81150	
	pyridopyrimidines.	0	down	2	down
Vitamin B6	pyridoxamine 5'-phosphates	-			
		17.17117			
		1	down	0	up

dCMP	pyrimidine 2'- deoxyribonucleoside monophosphates	- 17.67785	down	- 17.82958	down
1-Pyrroline-2-carboxylic acid	pyrrolines	- 19.41933	down	4	up
Licochalcone C	retrochalcones	4 -	down	0 -	up
3-Hydroxy-4-methylanthranilate	S-adenosyl-L- methionine:3- hydroxyanthranilate 4- C-methyltransferase	19.03966 9	down	18.09954 3	up
p-Hydroxybenzylsulphoglucosinolate	S-glycosyl compound	- 18.01253	down	0	up
Progesterone 3-biotin	steroid hormone	5 -	down	0	up
gamma2-Solamarine	steroidal saponins	13.93598 5	down	0	up
Stemmadenine		- 17.96057	down	0	up
D-Glycerate 3-phosphate	strychnos alkaloids sugar acids and derivatives	17.02527 6	up	17.02527 6	down
Lactobionic acid	sugar acids and derivatives	0	down	16.68015 9	down
(R)-2-Amino-N-(2,2,4,4-tetramethyl-3- thietanyl)propanamide		0	down	- 18.06686	down
Pitheduloside D	thietanes triterpene saponins	0 -	down	6	down
Momordin le	triterpene saponins	19.16912 -	down	0	up
Licoricesaponin D3	triterpene saponins	18.82578 8	down	0	up
Convallasaponin A	triterpenoids	17.76941 9	up	17.76941 9	down
Ganoderic acid H		- 17.71367	down	0 -	up
Protoveratrine B	triterpenoids Veratrum Alkaloids	0 17.50435	down	19.02263 5	down
trans-Brassicic acid		3	up	17.50435 3	down
Fucoxanthin	very long-chain fatty acids	- 0	down	- 18.51780	down
4'-Hydroxy-5,7-dimethoxyflavanone 4'- [2-(5-Cinnamoyl)-beta-D- apiofuranosyl]glucoside	xanthophylls	0 -	down	17.94895 7	down
		18.50231 6	down	0	up

Table S67. Dual polarity measurements for fungus-specific metabolites recovered from *M. cicadina* and the *M. platypediae* samples of *M. levispora* comb. nov.

Compound	Ion mode	Compound Class	Previously confirmed fungal host	Fungal phylum	Log FC ([Contr ol] vs [MC]) : Normalized	Regulation ([Contr ol] vs [MC]) : Normalized	Log FC ([MC] vs [MP]) : Normalized	Regulation ([MC] vs [MP]) : Normalized
Sarcodon scabrosus Depsipeptide	Positive	cyclic depsipeptides	<i>Sarcodon scabrosus</i>	Basidiomycota	0	down	- 16.350 218	down
4-Propanoyl-HT2 toxin	Positive	trichothecenes	<i>Fusarium sporotrichioides</i>	Ascomycota	- 16.866 232	down	16.866 232	up
Fasciculol E	Positive	triterpenoids	<i>Naematoloma fascicular e</i>	Basidiomycota	- 17.829 891	down	17.829 891	up
Hebevinoside XIV	Positive	cucurbitacin glycosides	<i>Hebeloma vinosophyllum</i>	Basidiomycota	- 18.102 667	down	18.102 667	up
4-(Hydroxymethyl)benzene diazonium(1+)	Positive	benzyl alcohols	<i>Agaricus bisporus</i>	Basidiomycota	- 19.488 249	down	19.488 249	up
6-[2,3-Dihydroxy-1-(hydroxymethyl)propyl]-1,2-dihydro-7-hydroxy-9-methoxy-cyclopenta[c][1]benzopyran-3,4-dione	Negative	coumarins and derivatives	<i>Aspergillus flavus</i>	Ascomycota	- 18.046 638	down	0	down
Ustiloxin C	Negative	cyclic peptides	<i>Ustilaginoidea virens</i>	Basidiomycota	- 19.157 667	down	0	down
Azoxystrobin	Negative	n/a		Basidiomycota	0	down	- 17.138 182	down

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