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GENETIC AND BIOCHEMICAL CHARACTERIZATION OF ERGOT ALKALOID SYNTHESIZING FUNGI AND THEIR SYMBIONTS

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ABSTRACT

Genetic and biochemical characterization of ergot alkaloid synthesizing fungi and their symbionts

Matthew Maust

Ergot alkaloids are fungal tryptophan derived toxins which affect mammalian circulation and neurotransmission. These compounds are biosynthesized by a conserved genetic pathway, known as the ergot alkaloid synthesis (EAS) pathway by fungi belonging to the ascomycete families Trichocomaceae and Clavicipitaceae. Several *Ipomoea* species and related plants in the morning glory family harbor vertically transmitted symbiotic fungi in the genus *Periglandula,* also members of Clavicipitaceae, that produce ergot alkaloids. Metabolomic analysis of seeds identified a previously uncharacterized glycoside form of the pharmaceutically important ergot alkaloid, ergonovine. Several species belonging to the fungal genus *Metarhizium* have recently been shown to have the capacity to express lysergic acid derived compounds. *Metarhizium* species are prolific entomopathogens and have the capacity to form beneficial relationships with plants by colonizing their roots. Proteomics analysis showed that wildtype and knock out strains of *Metarhizium brunneum* infected insects had different antimicrobial peptide and protein expression profiles based on the presence of ergot alkaloids. Metabolomics analysis found that unlike with insects, *M. brunneum* does not produce ergot alkaloids when grown in conjunction with plants and factors known to promote microbial symbiosis and stress-response in plants were upregulated. Fungi from Trichocomaceae (genera include *Penicillium)* diverge from fungi in Clavicipitaceae at a middle step of the ergot alkaloid synthetic (EAS) pathway to produce fumigaclavines and related compounds. *Penicillium biforme* is a known producer of rugulovasine A/B, which has never been observed in *Penicillium camemberti*. Data presented here suggest that the ancestor of modern *P. camemberti* had the capacity to synthesize rugulovasines and other ergot alkaloid precursors but lost this capability due to a V13G mutation on the protein. Analysis of the genomes from *P. camemberti* and *P. biforme* revealed that the two species contain the same cluster of EAS genes, and both organisms express mRNA from these genes in specific culture conditions. Metabolomics analysis confirmed that the regulatory elements needed for EAS gene expression are functional in *P. camemberti.* These results show how genetic techniques and biochemical analysis can provide new insights into these organisms.

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CHAPTER 1
Introduction

Since ancient times, humans have had mostly negative interactions with the class of mycotoxins now known as ergot alkaloids. Humans have a long history of interaction with ergot alkaloids with regards to agriculture. *Claviceps purpurea*, a pathogen of rye, has been implicated in the contamination of livestock feed and food for humans. As a result of consumption, mammals typically experience conditions ranging from psychoactive effects, such as hallucination, to impacts related to the toxins' vasoconstrictive properties such as gangrene. Therefore, many countries monitor harvested grains for the presence of fungal structures associated with ergot alkaloid production (Belser-Ehrlich et al., 2013). In more recent times these compounds have been harnessed in medicine. This group of specialized metabolites have a commonality in that they are tryptophan-derived indoles, and share structural similarities with the mammalian signaling molecules serotonin, dopamine and noradrenaline (Wallwey & Li, 2011). Because of this, ergot alkaloids have found a role as pharmaceutical agents for a number of human conditions, such as migraines, Parkinson's disease and to control bleeding during childbirth (Bonuccelli et al., 2009; Howard et al., 1964; P. C. Tfelt-Hansen & Koehler, 2008).

These compounds are biosynthesized by a conserved fungal genetic pathway, known as the ergot alkaloid synthesis (EAS) pathway. Early steps in the pathway are highly conserved, with later steps employing different enzymes based on the species, leading to the diversity of structures that are known (Gerhards et al., 2014). Pathway intermediates, side-products and end-products can all be commonly observed in any particular synthesizing fungus, as the pathway is known to be relatively inefficient (Panaccione, 2011). Due to this diversity and inefficiency, a lot of work has been performed to understand what genetic and regulatory elements are involved in the production of the ergot alkaloid suite found in any fungus or fungal host.

The ergot alkaloid synthesis pathway is composed of an entire gene cluster and can be found in several Ascomycete families including Clavicipitaceae and Trichocomaceae (Robinson & Panaccione, 2015). The metabolic pathway with gene names can be seen in figure 1. Early steps of the pathway are highly conserved, with the genes *dmaW*, *easF, easC,* and *easE* found in representatives from both clades. The gene *dmaW* starts synthesis with the precursor dimethylallyltryptophan (DMAT), and the early pathway concludes with the production of chanoclavine-I by the *easC* gene.

Most species of ergot alkaloid synthesizing organisms proceed through a couple of transitional steps with enzymes encoded by *easA* and *easG*, leading to the production of festuclavine, or the related compound agroclavine. It is at this point that major differences in end-products start to occur. A large proportion of these variations are found within the Clavicipitaceae family. These compounds can be classified as clavines, lysergic acid amides and ergopeptines (Florea et al., 2017). Species representing this clade include *Periglandula ipomoeae*, *Metarhizium brunneum*, and *Epichloë inebrians.* A separate group of modifications to the ergot alkaloid synthesis pathway, the fumigaclavines, occurs in a group of fungi from the family Trichocomaceae. Representatives from this family include *Penicillium camemberti, Penicillium biforme,* and *Neosartorya fumigata.* The production and effects of the ergot alkaloids produced by *P. ipomoeae*, *M. brunneum* and *P. biforme* (and the lack thereof by *P. camemberti*) form the basis for study for the interactions investigated in this work. These compounds are utilized by these fungi to provide competitive advantages in their respective niches.

Many ergot alkaloid producing fungi occupy a role as endophytes of the plants they grow within. These fungi provide their plant hosts with a competitive advantage, and encourage them to maintain the relationship, by producing ergot alkaloids which punish or even outright prevent herbivory. Lambs grazing on ergot alkaloid containing pastures show signs of toxicosis and end up with lower body mass gains when compared to those grazing on ergot alkaloid free pastures (Parish et al., 2003a). A similar impact is observed in steers grazing on ergot alkaloid containing pastures (Parish et al., 2003b). This

study also showed a behavioral component, where ergot alkaloid impacted steers had a decrease in grazing habits as well as water consumption. This effect was also observed in rabbits, and was specific to the presence of ergot alkaloids (Panaccione et al., 2006). In this study, the rabbits dramatically preferred feeding on plants that contained endophytes but lacked ergot alkaloids versus plants that lacked endophytes. When ergot alkaloids accumulated in the grass, this effect was counteracted. Satiety was also evaluated in this same study, which showed that the ergot alkaloid ergovaline reduced rabbit appetites.

Ergot alkaloids also provide plants with a competitive advantage with regards to challenge from invertebrates, including insects and nematodes. The ergot alkaloid content of the endophyte infected Cloudcroft sleepygrass (*Achnatherum robustum*) protected these plants from aphids (Shymanovich et al., 2014). This effect was confirmed when oat leaves were supplemented with the ergot alkaloid ergonovine. Another study showed that ergot alkaloids contribute to caterpillar resistance by endophyte infected ryegrass (Potter et al., 2008). Ergot alkaloid containing morning glories also gain an advantage against the insect pest potato psyllid (*Bactericera cockerelli*). In one study it was observed that morning glory species which contained large quantities of ergot alkaloids killed the insects rapidly, whereas they survived to adulthood on the species which lacked ergot alkaloids (Kaur et al., 2018). In a recently published follow-up study, it was shown that even supplementing endophyte lacking leaves with even a small amount of ergot alkaloids was enough to cause a large and significant increase in psyllid mortality (Kaur et al., 2020). In another study involving morning glory roots, ergot alkaloids were shown to have a protective effect when the plants were grown in soil infested with Southern root-knot nematode (*Meloidogyne incognita*) (Durden et al., 2019).

Ergot alkaloids also help fungi compete directly as they cause infections or compete for nutrients. Waxworm larvae (*Galleria mellonella*) which were injected with ergot alkaloid deficient strains of *Neosartorya fumigata* had significantly lowered mortality rates. Fumigaclavine C, the pathway

endpoint, appears to be a particularly important virulence factor, as strains of the fungus accumulating earlier pathway intermediates had lower virulence (Panaccione & Arnold, 2017). Another study involving the entomopathogen *Metarhizium brunneum*, showed that this fungus produces high levels of ergot alkaloids when it infects living waxworm larvae (Leadmon et al., 2020). Perhaps ergot alkaloids are used as a virulence factor by the fungus when causing infection. Alternatively the compounds may be used to help the fungus secure nutritional resources in competition with other microorganisms, as some ergot alkaloids have been shown to have antibacterial properties (Eckart & Pertz, 1999).

As a result of the diversity of structures found in ergot alkaloid synthesis pathways, and due to the inefficiency of the pathway leading to relatively high-level accumulation of pathway intermediates, metabolome profiling is a powerful approach to studying these fungi. Apart from ergot alkaloids, many fungi carry in their genomes the capacity to synthesize a whole suite of specialized metabolites which play roles in human activities including agriculture, production and preservation of food, and medicine (Nisa et al., 2015). With the development of accurate mass, high resolution mass spectrometers with increasingly fast scanning speeds, experiments which profile the global metabolome of a given organism or symbiotic relationship have become possible (Schrimpe-Rutledge et al., 2016).

While molecular biology and proteomic approaches can be utilized in powerful ways to study fungal gene regulation and phenotypes, many fungi lack the characterized genomes which help enable these types of analysis. Small molecule metabolite profiling becomes an elegant solution for investigating these situations. Where a genome may include > 20,000 genes and the resulting proteome may result in > 100,000 potential proteins, a typical metabolome may only contain approximately 5,000 species (Schrimpe-Rutledge et al., 2016). This issue is further compounded when fungi are involved in symbiotic relationships (*e.g.* plant symbiosis, insect pathogenesis) or are studied in complex matrices (*e.g.* milk for cheese making). Fortunately, while this large variation in genome and proteome can lead to high levels of complexity, these same proteins and genes contribute to the function of a large

proportion of highly conserved pathways (Peregrín-Alvarez et al., 2009). The result is that variability in

the abundance (or presence/absence) of a relatively smaller number of metabolites can be linked back

the gene expression profiles. These metabolite profiles constitute the observations of phenotypes which

relate to the underlying genotypes by which they are produced and can ultimately shed light into the

nature of interactions between fungi and their hosts or their environment.

The production and effects ergot alkaloids form the basis for study for the interactions

investigated in this work.

Objectives for the work discussed in this document:

1. To investigate the impacts of *Periglandula* species infections on the host metabolomic pathway

    expression of plants belonging to the genus *Ipomoea*

    a. While many species of morning glories harbor fungal symbionts from the genus

        Periglandula, morning glories can be "cured" of fungus. While these "cured" plants

        seem to grow normally without their symbiont it is unknown how the metabolism of the

        plants changes in response to the fungus or ergot alkaloids.

2. To evaluate the metabolite profile of *Ipomoea* sp. for biomarkers indicating presence of non-

    ergot alkaloid producing fungi

    a. Ergot alkaloids can be detected in large number of species in the Convolvulaceae family

        and are used as a biomarker to indicate the presence of *Periglandula sp.* symbionts.

        That *Periglandula sp.* cannot be grown in pure culture makes studying these organisms

        difficult and has confounded attempts at generating a full genome sequence. It is

        unknown if there are species of *Periglandula*, which are symbionts of morning glories

        which lack that ability to produce ergot alkaloids.

3. To investigate the impact ergot alkaloid expression by *Metarhizium brunneum* has on the immune response of its insect hosts

   a. Fungi belonging to the genus *Metarhizium* are well characterized as entomopathogens. They are known to secrete a number of proteins and specialized metabolites which assist in colonizing insects. *M. brunneum* was recently shown to synthesize a suite of ergot alkaloids, it is unknown what role these play in virulence.

4. To investigate how plant metabolite expression changes as a response to *M. brunneum* symbiosis

   a. *M. brunneum* can grow as symbiont of plants, as a result it is used as an insect biocontrol agent for several crops. Since these fungi may alter expression of host metabolites, it is important to understand the expression of host metabolites in these conditions.

5. To investigate the biochemical mechanism(s) which prevent *Penicillium camemberti* from producing the ergot alkaloids predicted by the genes shared with its ergot alkaloid producing ancestor, *Penicillium biforme.*

   a. Like its close relative, *P. biforme, P. camemberti* transcribes genes from its EAS pathway under certain conditions. Despite this fact, the rugulovasines produced by *P. biforme* are not detected in cultures in *P. camemberti.*

   b. Neither *P. biforme* nor *P. camemberti* produce mRNA transcripts for EAS genes, or the ergot alkaloid profiles predicted by their genes, when grown in cheese making conditions.
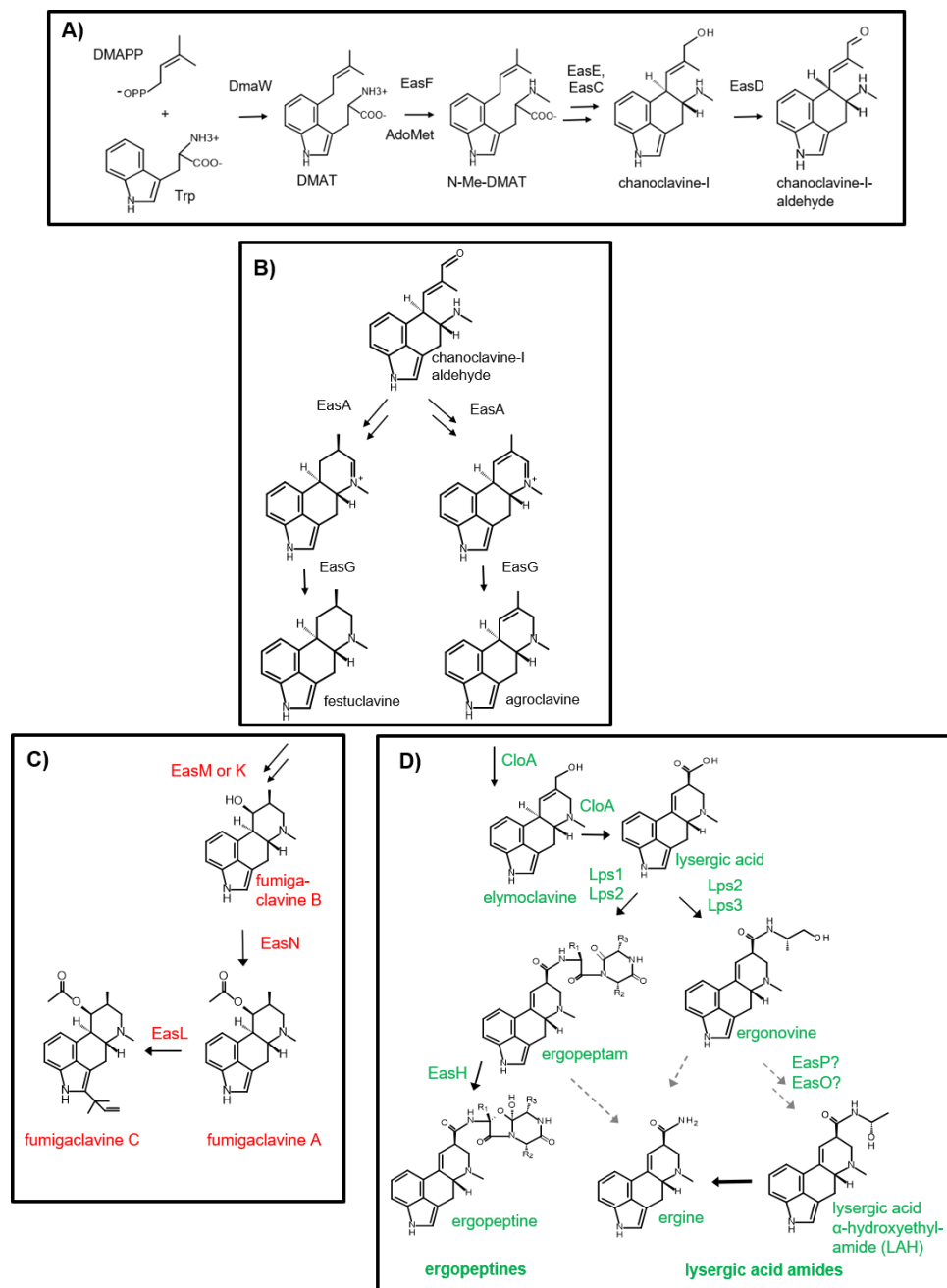
Figure 1. A map showing ergot alkaloid synthetic pathways. A) Highly conserved early precursor metabolites, B) after the middle steps, structures diverge according to lineage with those found in C) Trichocomaceae and those found in D) Clavicipitaceae. Redrawn from Robinson and Panaccione (2015).

CHAPTER 2
DISCOVERY OF A NOVEL ERGOT ALKALOID GLYCOSIDE FROM METABOLOMIC ANALYSIS OF *IPOMOEA* SPECIES

Abstract

Ergot alkaloids are fungal tryptophan derived toxins that affect human circulation and neurotransmission. Several *Ipomoea* species and related plants in the morning glory family harbor vertically-transmitted, symbiotic fungi in the genus *Periglandula* that produce ergot alkaloids. Many additional *Ipomoea* species are found to contain ergot alkaloids, indicating symbiosis with uncharacterized *Periglandula* species. A metabolomics approach was used to investigate biomarkers of fungal infection, which could indicate the presence of cryptic non-ergot producing *Periglandula* species. The metabolites of the *Ipomoea tricolor* seeds studied were collected from *Periglandula* sp.-infected plants (P+) or plants that had been cured by treatment with fungicide (P-). Seed extracts were screened for ergot alkaloids by fluorescence HPLC (LC-FLD), and total metabolites by mass spectrometry. Previously reported ergot alkaloids were present in high concentrations in P+ seeds and were not detected in P- seeds. Amino acid concentrations and detected plant stress hormones did not differ significantly between treatments. Analytes that were significantly more abundant in P+ seeds compared to P- seeds were compared to metabolomes from seed extracts of ergot alkaloid-positive and ergot alkaloid-negative seeds of *Ipomoea parasitica* and *Ipomoea pes-caprae,* as well as from nine ergot alkaloid-deficient *Ipomoea* species. Four metabolites tracked the presence of symbiont in this survey. One of these compounds identified by MS/MS analysis is a previously uncharacterized glycoside of ergonovine, a pharmaceutically important compound. The data indicate that apart from the

accumulation of ergot alkaloids, *Periglandula* species have a minimal impact on the metabolome of

seeds of their host plants. Furthermore, I found no evidence of cryptic, non-ergot alkaloid producing

Periglandula species in seeds of the nine additional *Ipomoea* species analyzed.

Introduction

Ergot alkaloids are a class of specialized metabolites biosynthesized from tryptophan by a largely conserved gene pathway expressed in fungi (Robinson & Panaccione, 2015). This class of compounds has a well-documented history of toxicity to invertebrates and mammals (Florea et al., 2017). Perhaps owing to their capacity to synthesize these compounds, Clavicipitaceous fungi occupy a variety of ecological niches. For members of the genus *Metarhizium* this takes the role of a pathogen of invertebrates (St. Leger et al., 2011). Within the genera *Epichloë* and *Claviceps* this manifests in a symbiotic relationship with monocotyledonous plants from the order Poales. One thing these genera all have in common, apart from their reliance on host organisms, is the expression of ergot alkaloids.

For years, it had been thought that the expression of ergot alkaloids was not unique to fungi, with certain plants within the family Convolvulaceae also accumulating these compounds. The potential source of these biosynthetic pathways may have been convergent evolution or acquisition through horizontal gene transfer from plant to fungi or vice versa (Steiner & Leistner, 2012). Unexpectedly, experimentation revealed these plants accumulating ergot alkaloids were infected with a Clavicipitaceous fungi of the genus *Periglandula* that were the source of the ergot alkaloids (Steiner et al., 2011). There were only two signs of the infection – the presence of ergot alkaloids and, in some species of *Ipomoea*, white fungal colonies on the surface of new unfolded leaves. Curing the plant of the fungus by fungicide treatment eliminated the ergot alkaloids along with the fungus (Kucht et al., 2004). Much of the biochemistry of the symbiosis is a mystery. It is unknown how *Periglandula* species infecting plants in the genus *Ipomoea* impact pathway expression of the host plant and whether the plant responds to the presence of the fungus as it would a pathogen. Furthermore, while it is known that ergot alkaloid expression in morning glories always accompanies infection by a *Periglandula*

species, it is unknown if there are non-ergot alkaloid producing fungi from that genus which could be present.

While some DNA sequencing has been performed on *Periglandula* species expressing ergot alkaloids, it is unknown if the symbiosis results in changes to plant or fungal gene expression. The biology of fungi in the genus *Periglandula* and their inability to be grown in pure culture has made genome sequencing challenging. A lack of a well-populated database from which to work from make RNA sequencing based approaches challenging and liquid chromatography mass spectrometry (LC-MS) proteomics based approaches impossible. Furthermore, the inability to grow *Periglandula* in pure culture makes distinction between plant and fungal genes or proteins dubious.

In recent years, developments in mass spectrometry and chromatography have enabled researchers to quantitatively profile thousands of small molecule metabolites simultaneously from a single sample. Stationary phases used in hydrophilic interaction chromatography (HILIC) allow for the reversal of mobile phases typically seen in a reversed phase chromatography gradient, allowing for improved retention of extremely polar compounds (Buszewski & Noga, 2012). While these compounds can be challenging to separate chromatographically, high resolution accurate mass spectrometry (HRAM), found in quadrupole time of flight (QToF) and quadrupole orbitrap mass spectrometers, allows for improved detection of molecules within a given range of mass channels when compared to older unit mass resolution instruments. With these techniques, individual isotopes from co-eluting compounds can be distinguished, and their chromatographic features can be resolved (Lesur & Domon, 2015). As a result of these improvements, liquid chromatography mass spectrometry metabolomic biomarker discovery based approaches have found acceptance in clinical diagnostic and drug discovery arenas (Nagy-Szakal et al., 2018; Yeung, 2018).

By leveraging the technological advances enabling HILIC-QToF mass spectrometry, the objective for this study was to investigate the impacts of *Periglandula* species infections on the host metabolomic pathway expression of plants belonging to the genus *Ipomoea*. Furthermore, since it is unknown whether non-ergot alkaloid expressing fungi are present in *Ipomoea* infections, biomarkers indicating fungal presence were also evaluated.

Methods

Biological materials and metabolite extractions

Seeds from *Periglandula* sp.-infected (P+) *Ipomoea tricolor* cv. Pearly Gates and cv. Flying Saucers as well as seeds that were cured of fungus by treatment with fungicide (P-) were acquired from the Keith Clay lab (Indiana University, Bloomington, Indiana). The P+ and P- seeds had been collected from plants demonstrated to contain or be free of *Periglandula* fungus (which has not yet been formally described; Beaulieu et al., 2013). This study made use of an extensive collection of methanolic extracts of seeds of *Ipomoea* species collected from a world-wide sampling of herbarium samples and wild plants by Keith Clay and students(Beaulieu, 2014). Previously prepared ergot alkaloid extractions from *Ipomoea* species (*I. parasitica, I. acanthi, I. barbatisepala, I. clavata, I. pes-caprae, I. pandarata, I. imperati, I. aquatica, I. tubiodes, I. carnea, I. polpha, I. leptophylla*) were weight normalized for further metabolomic analysis.

Methanol extracts of pulverized seeds from *I. leptophylla* and *I. tricolor* were screened for ergot alkaloids by high resolution accurate mass LC-MS/MS analysis. A seed was placed inside a 1-mL screw cap tube containing 10 glass beads, and tubes were shaken on a Fastprep 120 instrument (Bio101, Carlsbad, CA) at 6 m/sec for 20 seconds. Bead beating was repeated after a brief cooling period if necessary, to pulverize the seed. The seed powder was incubated at room temperature for 10 minutes

with 500 μL of HPLC grade methanol. Following incubation, solids were pelleted by centrifugation and the supernatant was removed.

Polar metabolite extraction followed a similar workflow. Metabolite extracts were weight normalized based on the weight of the intact seeds. The metabolite extraction solvent (50% methanol, 50% water) was added to yield a concentration of 10 mg of seed per 1 mL of extraction solvent. After bead beating, samples were centrifuged to pellet insoluble material, and the supernatants were removed. The supernatants were stored at -80°C until analyzed.

QToF Metabolomics

Polar metabolites were separated using HILIC chromatography, by injecting 10 μL of extracts onto a 2.1 x 100 mm, 5 μm Phenomenex Luna NH2 column (Torrance, CA). A 15 minute linear gradient, which ramped from 90% solvent B (100% acetonitrile) to 60% solvent A (10 mM ammonium acetate) at a flow rate of 300 μL/min, was used to elute the metabolites for detection by electrospray ionization mass spectrometry. Each sample was analyzed in positive and negative ionization modes. The LC-MS system used for analysis consisted of an Infinity 1290 ultra-high pressure liquid chromatography instrument (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 6530 quadrupole time of flight (QToF) mass spectrometer configured with a jet stream source for electrospray ionization. For both positive and negative ionization modes, the mass spectrometer scanned over a range from 50 to 1200 m/z with a scan speed of 2 *hz*.

Analysis of Metabolomics Data

LC-MS data were processed using Agilent Masshunter Profinder (version B.06.00) in batch mode according to polarity of acquisition. Chromatographic features were extracted which contained a minimum peak height of 600 counts and a maximum charge state of 2. The resulting chromatography was inspected for consistency between samples, with retention time and mass alignments performed on irregular features. Peak areas from these chromatographic features were exported to Mass Profiler Professional (version 2.4.3) for further filtering, quality control and statistical analysis. Differences in peak abundances of chromatographic features between ergot alkaloid containing seeds and ergot alkaloid lacking seeds (as determined by HPLC with fluorescence detection) were assessed by a Bonferonni adjusted T-test ($p < 0.05$), with a minimum fold change > 5.

Regarding Mass Spectrometry methods

A variety of instrument platforms were utilized during the course of this research. A distinction could be made between high resolution accurate mass instruments, such as the Agilent Technologies 6530 quadrupole time of flight mass spectrometer or the Thermo Scientific Q Exactive Orbitrap mass spectrometer, and unit mass resolution instruments (*i.e.* Thermo Scientific LCQDecaXP, Sciex QTrap 5500). The HRAM instruments both offer sub 10 ppm mass measurements as well as efficient fragmentation mechanisms, which are conducive for the identification of small molecules (Ichou et al., 2014; Kind et al., 2018). Under ideal circumstances, all of the mass spectrometry analysis in this study would have been carried out on such instruments. Due to uncontrollable circumstances, analytical methodologies were adapted to the instruments which were available at the time of analysis. Some common threads linking these experiments were the usage of positive ion mode analysis, reversed phase chromatography and mobile phases (following the initial discovery of ergonovine glycoside on a

HILIC chromatographic method). While precursor mass assignments from unit mass resolution instruments are not sufficient in and of themselves to confirm the identity of a peak, the selectivity afforded by retention times and tandem mass spectra were utilized to confirm the presence of the molecule(s) of interest throughout these experiments. Data in figures 2, 4, 5, and 8 were collected using a Thermo Q Exactive. Data in figure 3 were collected using an Agilent 6530 QToF. Data in 7a were collected using a Thermo LCQDecaXP, while 7b and 10 were collected using a Sciex QTrap 5500.

Q Exactive analysis of ergot alkaloid extracts

Reversed phase separations of analytes contained in the supernatant were performed by injecting 5 μL onto a 2.1 x 100 mm, 3.5 μm, Zorbax SB-C18 LC column (Agilent Technologies, Santa Clara, CA) held at 40°C. Analytes were eluted with a gradient ramping from 95% solvent A (0.1% formic acid) and 5% solvent B (0.1% formic acid, acetonitrile) to 50% solvent B over 15 minutes, which was generated using an Accela 1290 UHPLC instrument (Thermo Scientific, San Jose, CA) at a flow rate of 300 μL/min. Detection of analytes was performed by positive polarity electrospray ionization using an in-line Q Exactive hybrid quadrupole Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA), operated in a data dependent acquisition mode. A precursor scan of 200 to 600 m/z was generated using the 70,000 resolution setting. The top five most abundant ions from the precursor scan were isolated by the quadrupole (2 Da isolation window) for higher-energy collisional dissociation (HCD) set at a normalized collision energy of 30. Fragment ions were then scanned with a resolution setting of 35,000.

LCQ Deca XP analysis of ergot alkaloid extracts

Reversed phase separations of analytes contained in the supernatant were performed by injecting 5 µL onto a 2.0 x 150 mm, 4 µm, Synergi Polar-RP LC column (Phenomenex, Torrance, CA) held at 30°C. Analytes were eluted with a gradient ramping from 86% solvent A (5% acetonitrile + 0.1% formic acid) and 14% solvent B (0.1% formic acid + 75% acetonitrile) to 100% solvent B over 20 minutes, which was generated using an Surveyor HPLC instrument (Thermo Scientific, San Jose, CA) at a flow rate of 200 µL/min. Detection of analytes was performed by positive polarity electrospray ionization using an in-line LCQ Deca XP ion trap mass spectrometer (ThermoFinnigan, San Jose, CA), operated in data dependent acquisition mode. A precursor scan of 200 to 500 m/z was generated using the 70,000-resolution setting. The top most abundant ion from the precursor scan was isolated by the quadrupole (2 Da isolation window) for collision induced dissociation (CID) set at a normalized collision energy of 35.

Sciex 5500 QTrap analysis ergot alkaloid extracts

Ergonovine and a previously unknown glycoside of ergonovine were monitored in plant extracts by positive mode electrospray ionization LC-QQQ-MS using a Sciex ExionLC AD UHPLC coupled to a Sciex QTrap 5500 linear ion trap quadrupole mass spectrometer (Sciex, Framingham, MA). Chromatographic separations were performed on 2.1 x 50 mm, 3.5 µm, Zorbax SB-C18 column (Agilent Technologies, Santa Clara, CA) using a gradient composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient ramped from 5% solvent B to 95% solvent B over 7 minutes, at a flow rate of 300 µL/min, with the column held at 40°C.

Mass spectrometry analysis was performed using multiple reaction monitoring. Ergonovine was monitored using the 326.3 → 208.0 transition. Ergonovine glycoside was monitored using the 488.3 m/z

→ 326.3 m/z transition. Analytes were fragmented using a dwell time of 50 msec and a collision energy of 35.

Extraction of ergot alkaloids from drunken horse grass (*Achnatherum inebriens*)

Three biological replicates of homogenized leaves of *Epichloë gansuense*-infected *Achnatherum inebrians* were received from the Chunjie Li lab (Lanzhou University, Lanzhou, Gansu Province, China). Homogenates (50-100 mg) were incubated in 500 µL of 50% HPLC grade methanol to extract ergot alkaloids. The supernatant was removed after centrifuging at 14,000 x g for 10 minutes to pellet solids.

Plant incubations

Leaves were removed from P+ and P- *Ipomoea tricolor* cv. Pearly Gates plants. The leaves were cut at the petiole, which was then submerged into a 2 mL microcentrifuge tube containing a 3 mg/mL solution of ergonovine (Sigma-Aldrich, St Louis, MO) in water. The tube was then covered with parafilm. The leaf was incubated in the solution for several days; ergonovine solution that was lost to transpiration by the leaf or by evaporation was replaced. Following this treatment, the leaf was then cut into 2x2 mm pieces and bead beaten in a 50% methanol extraction solution (as described above for seeds).

Leaves were removed from plants listed in Table 2. All plants were acquired from the campus of West Virginia University or the surrounding area (Morgantown, WV). The leaves were cut at the petiole or approximately three inches from the end of the leaf (for plants lacking a petiole), which was then submerged into a 15 mL centrifuge tube containing a 3 mg/mL solution of ergonovine. The tube was then capped with a covering of paraffin film. Leaves were incubated in the solution for a week, ergonovine solution that was lost to transpiration by the leaf or by evaporation was replaced. Following

this treatment, the leaf was then cut into 2x2 mm pieces and bead beaten in a 50% methanol extraction solution.

Method for PNPGal/UDP gal incubations with b-galactosidase

Ergonovine and PNPGal/Glu or UDPGal/Glu (Sigma Aldrich, St. Louis, MO) were added to a solution containing phosphate buffered saline pH 7.5 with MgCl (1 mM) and β-mercaptoethanol (5 mM). Ergonovine and activated hexoses were added in equimolar amounts for the respective incubations, for a final concentration of 10 mM. The samples were incubated for 8 hours at room tempurature. A set of PNPGal incubations were repeated with the same buffer conditions (pH 4.5 and pH 6) and with or without boiling prior to incubation. Samples were analyzed using a Sciex 5500 Qtrap, LCQ DecaXP or Q Exactive, where noted in the results.

Results

Ergot alkaloid content of *Ipomoea sp.* seeds

The infection status of *Periglandula*-infected (P+) *Ipomoea tricolor* seeds (cv. Pearly Gates) was confirmed by analyzing extracts for the same assortment of ergot alkaloids (chanoclavine-I aldehyde, chanoclavine, ergine, ergonovine, LAH) as previously reported (Beaulieu et al., 2015) (figure 1). Retention times, mass spectra and tandem mass spectra matched what is found in the related *Metarhizium brunneum* for these ergot alkaloids (Leadmon et al., 2020). Those *I. tricolor* seeds that had been cured of fungus by treatment with fungicide (P-) lacked all ergot alkaloids. Among the tested seeds from *I. acantha, I. aquatica, I. carnea, I. clavata, I. imperati, I. pandurata, I. polpha* and *I. tuboides* all individuals lacked ergot alkaloids. All seeds from *I. barbatisepala* and *I. leptophylla* contained ergot alkaloids (Beaulieu, 2014). In addition to the ergot alkaloids listed for *I. tricolor*, seeds of *I. leptophylla*

also accumulated the on-pathway intermediate chanoclavine-I aldehyde to detectable levels (figure 2). Within the collection of seeds from *I. pes-caprae,* and *I. parasitica* different individuals contained and lack ergot alkaloids (Table 1). *I. pes-caprae* also accumulated ergobalansine, which agrees with the findings of Beaulieu et al. (2015).

Metabolomic analysis of *Ipomoea sp.* seeds

Full metabolite analysis was performed on methanol seed extracts by HILIC-LC-ESI-QTOF-MS. Samples were injected twice, for analysis in positive and negative ion modes. Metabolomic analysis tracked expression of 1768 compounds in negative ion mode, and 1371 compounds in positive ion mode. Analytes that were significantly more abundant, as assessed by t-test ($p < 0.05$) in P+ seeds compared to P- seeds were compared to metabolomes from seed extracts of ergot alkaloid-positive and ergot alkaloid-negative seeds of *I. parasitica* and *I. pes-caprae*, as well as from the nine ergot alkaloid-deficient *Ipomoea* species. Among some of the compounds that were identified, no detectable changes in the expression of cinnamate, amino acids, jasmonic acid and salicylic acid (often associated with response to microbial infection) were found between P+ seeds and P- seeds. Four metabolites, with masses not associated with previously reported ergot alkaloids, tracked the presence of the *Periglandula* symbiont in this survey (figure 3). These data taken together, bolster the argument that there are no cryptic non-ergot alkaloid producing *Periglandula* symbionts of plants in the Ipomoea genus. In negative ion mode, three compounds - 551.2830 Da (putative formula: $C_{26} H_{39} N_3 O_7$), 549.2759 Da (putative formula: $C_{28} H_{33} N_5 O_4$) and 581.2974 Da (putative formula: $C_{24} H_{44} N O_9 P$) were identified as upregulated in P+ samples. In positive mode, a compound of 487.2389 Da (putative formula: $C_{25} H_{33} N_3 O_7$) was found to be upregulated in P+ samples. For all four compounds, searches of precursor masses and ms/ms spectra using databases from Biocyc and Metlin, failed to produce identifications.

Manual investigation of the ms/ms spectra for 488.2391 *m/z* (the M+H ion of 487.2389 Da) revealed

fragment ions of 326.1845 *m/z*, 283.1428 *m/z,* 223.1219 *m/z,* 208.0747 *m/z,* and 197.1064 *m/z*. When

compared to the fragmentation spectra of an authentic ergonovine analytical standard, these fragment

m/z values matched within a mass error of less than 1.0 ppm, indicating that the molecule contained

ergonovine (figure 4). Investigation of the precursor mass minus the mass of the highest abundance

fragment ion revealed a mass shift of 162.0523 Da, which is consistent with the mass of a hexose

residue (-3.09 ppm mass error).

Bioconversion capacity of *I. tricolor* tissues

To test the requirement of the *Periglandula* fungus for bioconversion of ergonovine to the

glycoside form, leaves from P+ and P- *I. tricolor* plants were incubated in an ergonovine solution and

evaluated by LC-MS. Initially, leaves from the P+ plants were evaluated, and bioconverted ergonovine to

its glycoside form (figure 5). Conversion is evidenced by the two peaks of the 488 *m/z* ion in the

chromatograph. It is unknown whether these peaks are a result of different monosaccharides

incorporated into the ergot alkaloid, or if these peaks correspond to different stereoisomers in the

ergoline scaffold. Examples of chromatographic separation of such stereoisomers can be seen in figure 2

(*e.g.* LAH, ergine, ergonovine).

When compared with a non-incubated control, ergonovine levels in the leaf tissue increased,

whereas ergonovine levels were below detectable limits in the non-incubated leaf sample. Likewise, it

was found that ergonovine glycoside accumulated to detectable levels compared to the non-incubated

control. Following this initial evaluation, a P- leaf was leaf was incubated in ergonovine, and ergonovine

glycoside was detected. Tissue was collected from the root, leaf, stem and flower of a P- *I. tricolor* plant

and homogenized in PBS, then incubated in the presence of ergonovine. While ergonovine was detected

in the homogenates, ergonovine glycoside was observed at very low levels by HPLC with fluorescence

detection (figure 1) but was below the limit of detection by mass spec (figure 6). These results indicate

that the presence of the *Periglandula* symbiont is not required for ergonovine glycoside conversion, and

that there is a structural component in the intact plant tissues which is required for efficient

bioconversion to take place.

Synthesis of ergonovine glycoside

Prior findings from *in vitro* studies showed that ergonovine-β-D-galactoside (wherein

ergometrine is a synonym for ergonovine) can be synthesized with β-galactosidase isolated from

Aspergillus oryzae and p-nitrophenyl β-D-galactoside (PNPGal) as the galactose donor (Křen et al., 1990,

1992). In my study, these conditions were repeated using UDP-glucose (UDPGlu), UDP-galactose

(UDPGal), PNPGal and p-nitrophenyl β-D-glucose (PNPGlu), to identify the individual peaks in the

doublet for 488.2391 *m/z* as seen in *I. tricolor* (figure 5)*.* LC-MS analysis of UDPGlu incubations gave two

prominent peaks of similar peak areas, eluting at 5.2 minutes and 6.9 minutes. In contrast, when the

UDPGal incubation was analyzed, the second peak (6.9 minutes) showed a large increase in peak area

relative to the peak at 5.2 minutes (figure 7). This result was confirmed with *I. tricolor* leaves, which also

showed increased synthesis of the later eluting peak, when supplemented with UDPGal. An MRM assay

revealed that the presence of UDPGal improved the glycosylation capacity of *I. tricolor* leaves (figure 8)*.*

Furthermore, the data reveal a marked increase in the abundance of the later eluting peak when

the plant leaf is incubated with UDPGal. This observation contrasts with the results of incubations with

UDPGlu which did not produce an increased abundance for either peak (figure 9a). *Epichloë inebrians*-

infected drunken horse grass samples analyzed along with the morning glory leaf UDP incubated

samples revealed two peaks, matching the abundance profile (figure 9b). PNPGal and PNPGlu were also

tested for *in vitro* enzymatic synthesis; these synthesis experiments yielded similar results to the UDPGal/Glu experiments. When β-galactosidase was deactivated with boiling prior to incubation, ergonovine glycoside was not detected (figure 9).

In both the PNP and UDP synthetic experiments, these data indicate that this enzymatic approach has a relatively low turnover in comparison to what was previously reported in *in vitro* studies both substrates were generated at a ratio of approximately 1:10 (glycoside to ergonovine) (Křen et al., 1990).

Conversion capability of a variety of plants

Ergonovine glycoside was observed in *Periglandula* containing *Ipomoea* species. Two species of plants from the Commelinidae clade (*Achnatherum inebrians* and *Achnatherum robustum*) harbor *Epichloë*. Representatives from this fungal genus are known to produce ergot alkaloids, including ergonovine. Sleepy grass (*A. robustum*) grows in the southwestern United States and is known to harbor two different types of ergot alkaloid producing fungal endophytes, an undescribed *Epichloë sp.* (ergonovine and ergine production) and *Epichloë funkii* (chanoclavine I production)(Shymanovich et al., 2014)*.* Drunken horse grass (*A. inebrians*) grows in northwestern China and has also been shown to harbor ergonovine producing *Epichloë inebrians.* Both plants species from genus *Achnatherum* which contained ergonovine also contained ergonovine glycoside (figure 10), while the data are not shown it was observed that the chanoclavine I containing plants did not produce ergot alkaloid glycosides.

Since *I. tricolor* was found to convert ergonovine to ergonovine glycoside when the fungus is not present, plants representing a variety of clades were investigated for the capacity to perform this conversion. Plants were chosen from pteridophyta and ginkgoopsida (gymnosperm) to represent non-angiosperm clades. Plants were also chosen from the angiosperm clades Asteridae (Solanales and

Asterales), Commelinidae, Dilleniidae, Hamamelididae, Liliidae, Magnoliidae and Rosidae. A full list of plants tested can be found in table 2. The MRM assay detected peaks corresponding to ergonovine glycoside from every representative tested. Plant specimens varied in the number of peaks which were detected and as well as in abundances. This may indicate variation in the types of sugar residues that were incorporated into the glycoside. Based on the results from the galactose incorporating experiments, many plants produced a peak corresponding to the retention time of ergonovine galactoside.

Discussion

The results presented indicate that apart from ergot alkaloids, *Periglandula* species have a minimal impact on the metabolome of seeds of their host plants. We also found no evidence of non-ergot alkaloid producing *Periglandula* species in seeds of the *Ipomoea* species analyzed. However, it does appear that a biochemical process within the plant is in place which modifies the ergot alkaloid ergonovine to its glycoside form. As a result of this finding, the focus of this study shifted to explore the capacity for plants to perform this modification in vivo and characterize the structure of a newly discovered form of ergonovine.

The results presented here led to the proposed structure of the most abundant compound as ergonovine with a hexose residue conjugated at the primary hydroxyl group. This structure was originally described as ergonovine-β-D-galactoside by Křen et al (1992). They produced this compound *in vitro* by incubating PNPGal along with β-galactosidase from *Aspergillus oryzae* but never observed it from a natural source. Also, in this report Křen et al. synthesized galactosides of elymoclavine, 9,10-dihydrolysergol, lysergol, and chanoclavine. An earlier report was made by Floss et al. (1976), who isolated elymoclavine-O-β-D-fructoside from a saprophytic culture of a *Claviceps* strain. In a related

study, *Claviceps purpurea* grown in saprophytic culture media supplied with O-β-D-fructofuranosyl was shown to produce elymoclavine-O-β-D-fructosides (Flieger et al., 1989).

This present report differs from previous reports, in that ergonovine glycoside accumulated *in vivo* in the *Ipomoea sp.* symbiotic with *Periglandula* sp. that were evaluated in this study. Furthermore, as shown in this report, for ergonovine to be glycosylated in plant tissues a fungal symbiont is not required. Until now this phenomenon was only observed using fungal cultures induced with high levels of sugar containing substrates or *in vitro* with enzymes derived from fungal or bacterial origin. While Křen et al. (1992) reported generating glycosides from several forms of ergot alkaloids, of the ergot alkaloids produced by *Periglandula sp.*, only ergonovine was glycosylated *in vivo*. This was the case across all the plants tested, which indicates that the enzymes required for this transformation have specificity for the chemical moieties found on ergonovine

The fragmentation pattern for the compound supports the presence of a hexose residue. Under low energy fragmentation (*i.e.* CID), it would be expected that a hexose residue connected via a glycosidic bond, would be readily dissociated from the parent ion(Yu et al., 2016). Indeed, the 326 *m/z* ion representing ergonovine is the predominant ion found in the spectra. This evidence along with the accurate mass data, agree with the structure reported here, and by Křen et al. (1992). It is worth noting that the primary hydroxyl found on chanoclavine-I, the secondary hydroxyl on LAH, the carboxylic acid on lysergic acid, and the primary amine on ergine, apparently are not suitable sites for the catalytic activity of the enzyme responsible for this conversion in *Ipomea sp.*, because glycosylated forms of these compounds were not detected. While many plant extracts contained multiple chromatographic peaks for ergonovine glycoside, the evidence suggests that one of the most abundant peaks is the galactoside form. Based on what can be found in other complex carbohydrates, it could be speculated that other hexose residues could alternately be incorporated into the structure. Perhaps other hexose residues which were not tested (*e.g.* mannose, fructose, etc.) could be responsible for other peaks present in the

chromatographs presented. The modification of ergonovine also seems to be specific to hexose incorporation, as glucuronic acid forms were not observed.

The appearance of ergonovine glycoside in plant tissues is enzyme mediated and not a spontaneous reaction. While the data are not presented here, the one animal model tested (*Galleria mellonella*) did not produce ergonovine glycoside when infected with an ergonovine producing fungus. This process is possibly not specific to plants (it was synthesized *in vitro* using a fungal enzyme and galactose; Křen et al., 1992), however glycosyltransferase function is conserved across a wide range of plant clades. While performing the plant transformation experiments, vascular tissue seemed to be important for conversion. When these structures were disrupted or not enough ergonovine solution was provided to vascular tissue, conversion was reduced or halted. One explanation for the conservation of this function could be as a mechanism for mobility within plant tissue, as part of a detoxification process or defense response against ergot alkaloids. Plants are known to use glycosyltransferases for moving specialized metabolites throughout tissues or for storage (J. Wang & Hou, 2009). This has been shown specifically with *I. tricolor*, where ergot alkaloid species are differentially allocated throughout the plant, independent of the presence of the fungal symbiont (Beaulieu et al., 2013). In *Ipomoea sp.* this may be a part of leveraging the symbiosis with its *Periglandula* symbiont. Most recently, it has been that observed the ergot alkaloids being transported throughout morning glories are a part of the defense response against nematodes (Durden et al., 2019), perhaps specialized glycosyltransferase enzymes are involved in transporting ergonovine in its glycoside form, from where it is produced by the fungus, to the infected roots.

Acknowledgments

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variety of plants used in this work.

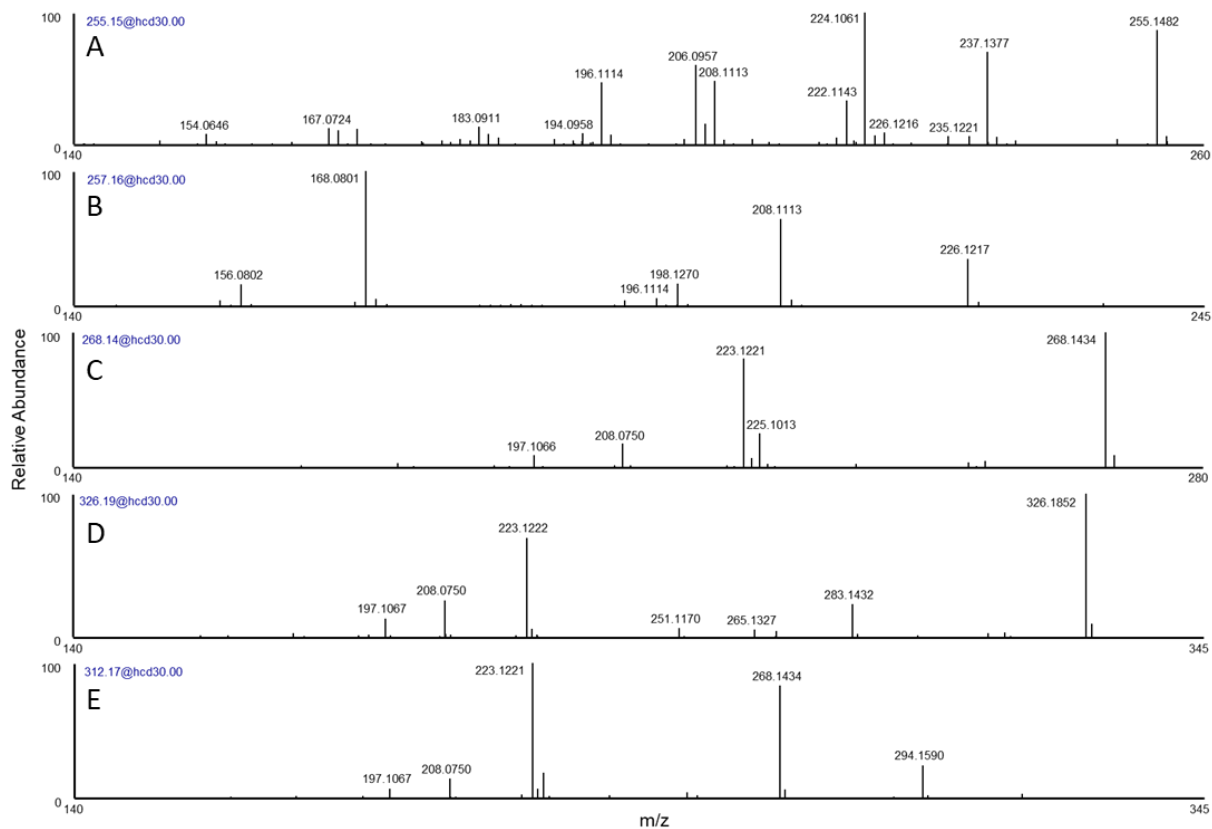# Figures
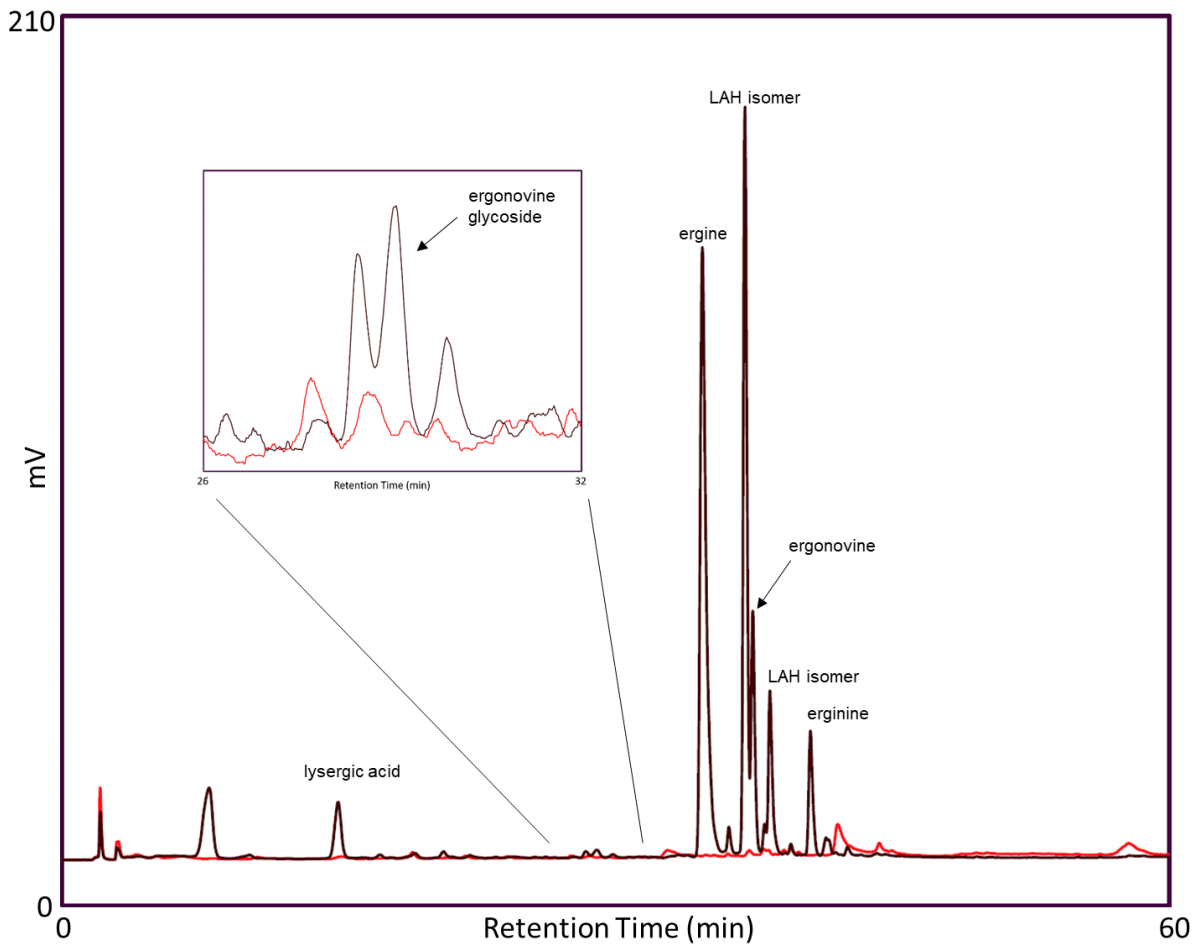
Figure 1. HPLC characterization of ergot alkaloids present in *Ipomoea tricolor* infected with *Periglandula* sp. endophyte (brown trace), or without endophyte (red). Ergot alkaloids were detected with fluorescence excitation at 310 nm and emission at 410 nm. This ergot alkaloid profile matches what was previously observed in this symbiotic relationship (Beaulieu et al., 2013).
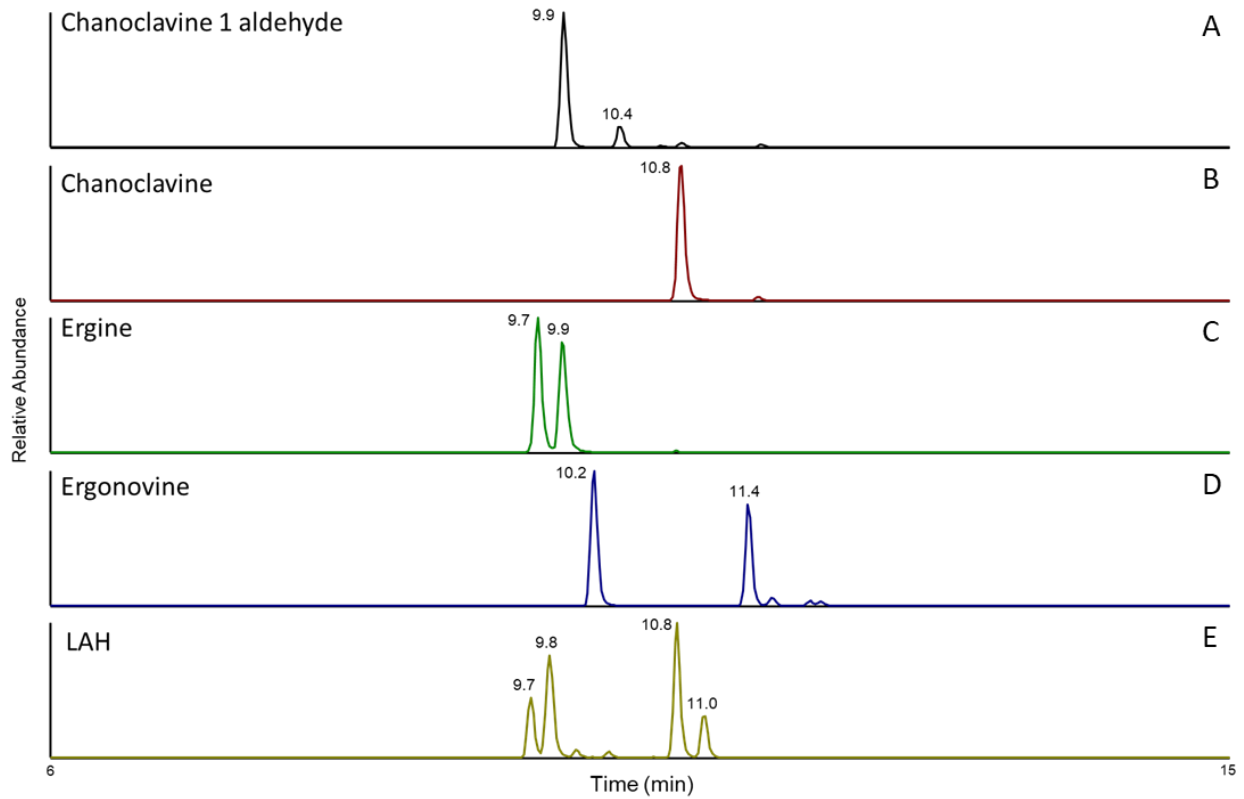
Figure 2a. Extracted ion chromatography, from a Q Exactive, of ergot alkaloids observed in *I. leptophylla*.

(A) chanoclavine 1 aldehyde, 255.1492 *m/z*, (B) chanoclavine, 257.1649 *m/z*, (C) ergine, 268.1445, *m/z*,

(D) ergonovine, 326.1863 *m/z*, (E) D-lysergic acid α-hydroxyethylamide (LAH), 312.1707 *m/z*. All ions

were extracted with a 10 ppm mass window centered on the theoretical monoisotopic M+H. The

multiple peaks observed in ergine, ergonovine and LAH are chromatographically resolved stereoisomers.

Figure 2b. High resolution, accurate mass HCD fragmentation spectra, from a Q Exactive, for ergot alkaloids detected in *I. leptophylla*. (A) chanoclavine 1 aldehyde, (B) chanoclavine, (C) ergine, (D) ergonovine, (E) LAH. Early pathway intermediates (A, B) show common fragment ions – 196.111 *m/z*, 208.111 *m/z* and 226.122 *m/z*, while later pathway intermediates (C-E) show common fragment ions – 197.107 *m/z*, 208.075 *m/z,* and 222.122 *m/z*.
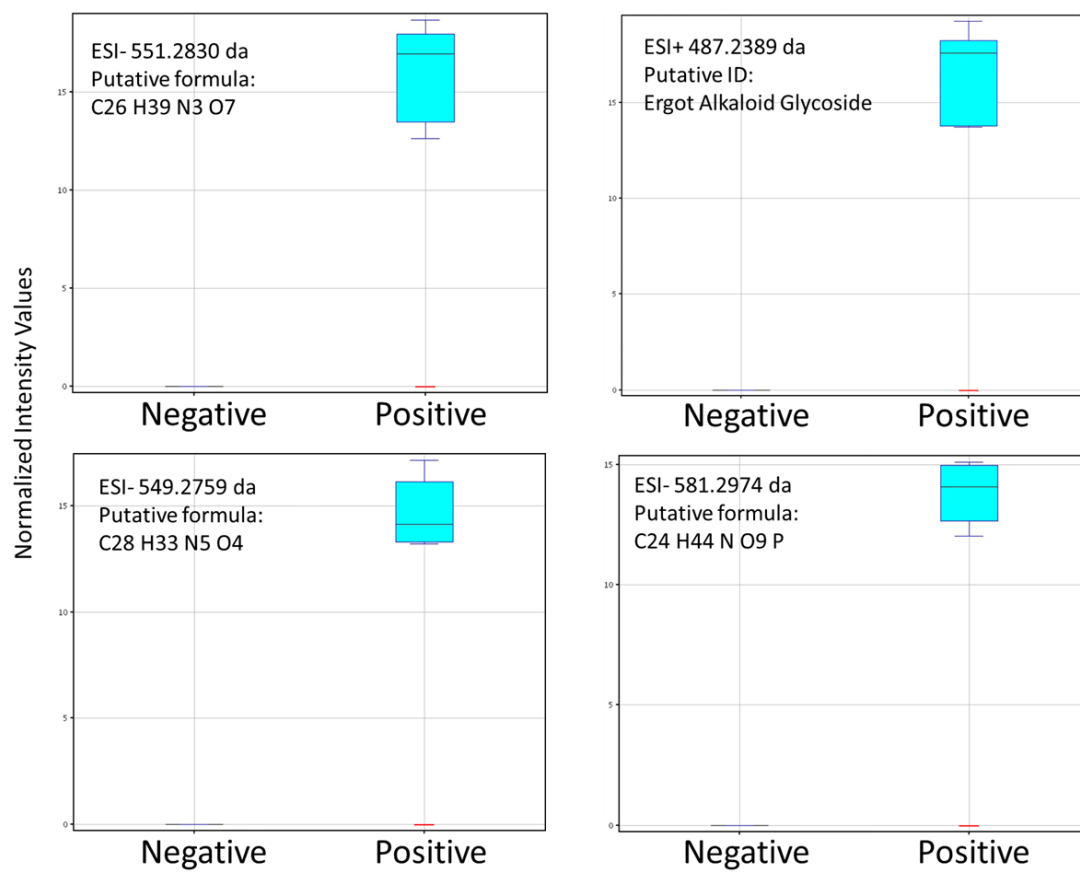
Figure 3 – Abundance profiles of four different metabolites from *Ipomoea sp.* grouped in ergot positive and ergot negative cohorts.
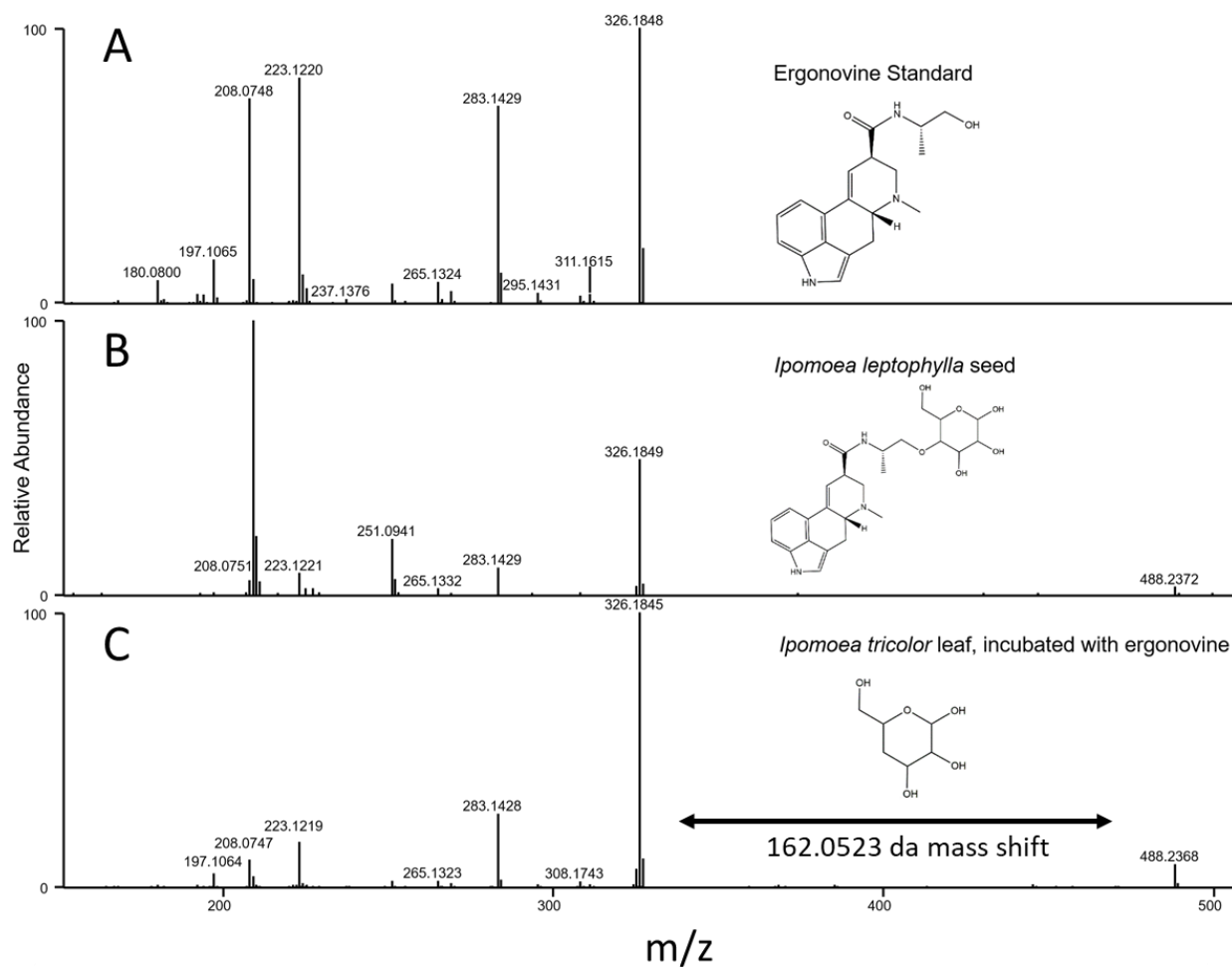
Figure 4. Q Exactive MS/MS spectra (RT 9.6 min) from ergonovine (A), an ergonovine-like precursor from an *I. leptophylla* seed (B) and *I. tricolor* leaf incubated in an ergonovine solution (C). The mass shift seen in B and C, from 488.237 m/z to 326.185 m/z, is consistent with the mass of a hexose residue (-3.1 ppm).
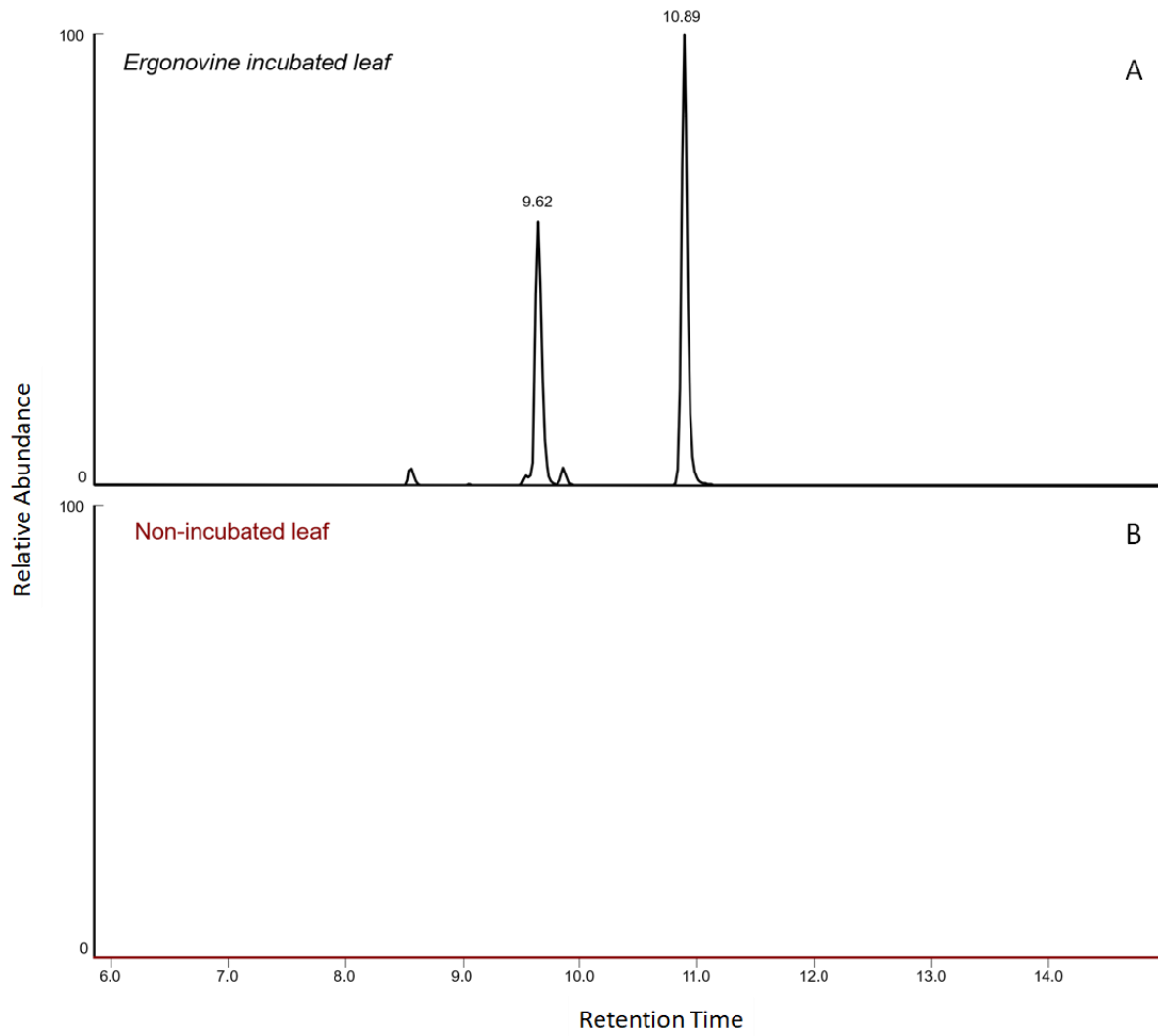
Figure 5. Extracted ion chromatogram (488.239 m/z) generated on a Q Exactive, from an ergonovine incubated *I. tricolor* leaf (A), and a non-incubated leaf (B).
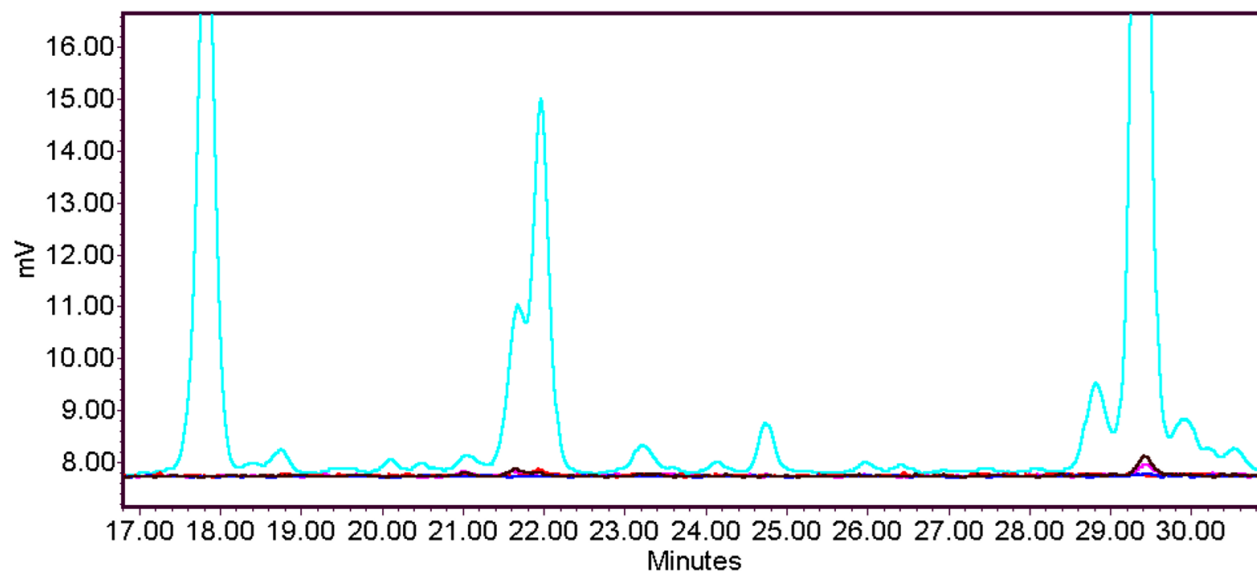
Figure 6. Fluorescence chromatography of ergot alkaloids extracted from morning glory leaves. The ergonovine glycoside retains at 29.5 minutes with this method. The teal trace shows an intact leaf which was incubated in an ergonovine solution. The black trace shows a P+ leaf which was homogenized in a phosphate buffered saline solution containing ergonovine. The magenta trace shows a P- leaf which was homogenized in phosphate buffered saline containing ergonovine. The cyan and red traces show P+/P- leaves homogenized in PBS.
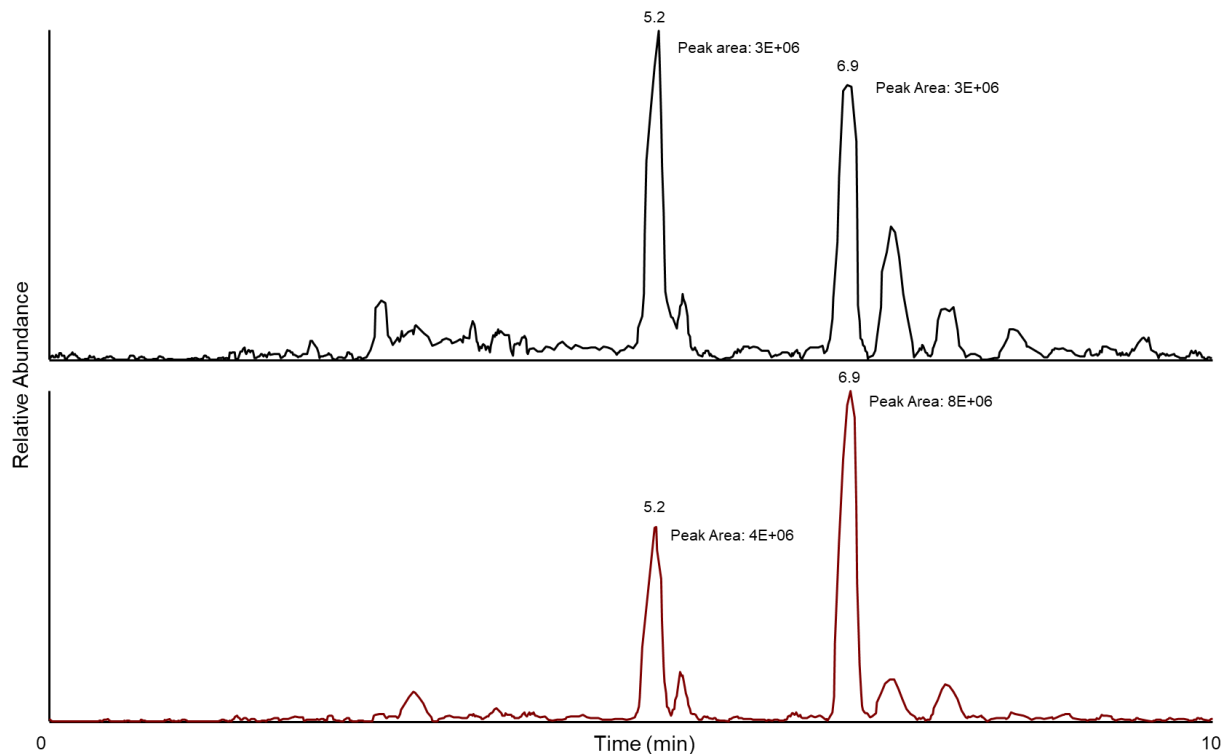
Figure 7a. Extracted ion chromatograms of 488.5 *m/z,* generated on a LCQDecaXP, from enzymatic synthesis of ergonovine glycoside. (A) UDP-glucose incubated with β-galactosidase, (B) UDP-galactose incubated with β-galactosidase. The peak area at 6.9 minutes grew a half order of magnitude in the sample incubated with UDP-galactose.
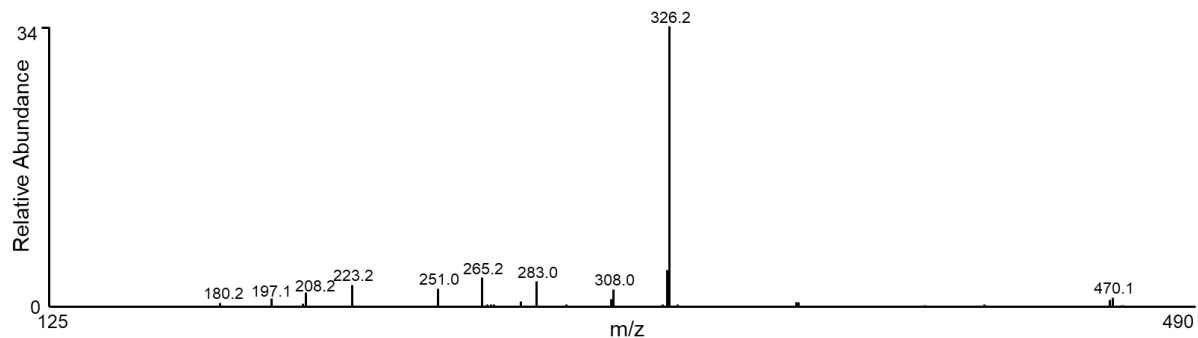
Figure 7b. Representative CID fragmentation spectrum, from a LCQDecaXP, of ergonovine glycoside. Due to the lower energy of the fragmentation technique, most of the abundance of the spectrum is represented by the 326.2 *m/z* ergonovine fragment. The spectra is zoomed to show the lower mass peaks, matching those ions found in the HCD fragmentation spectrum.
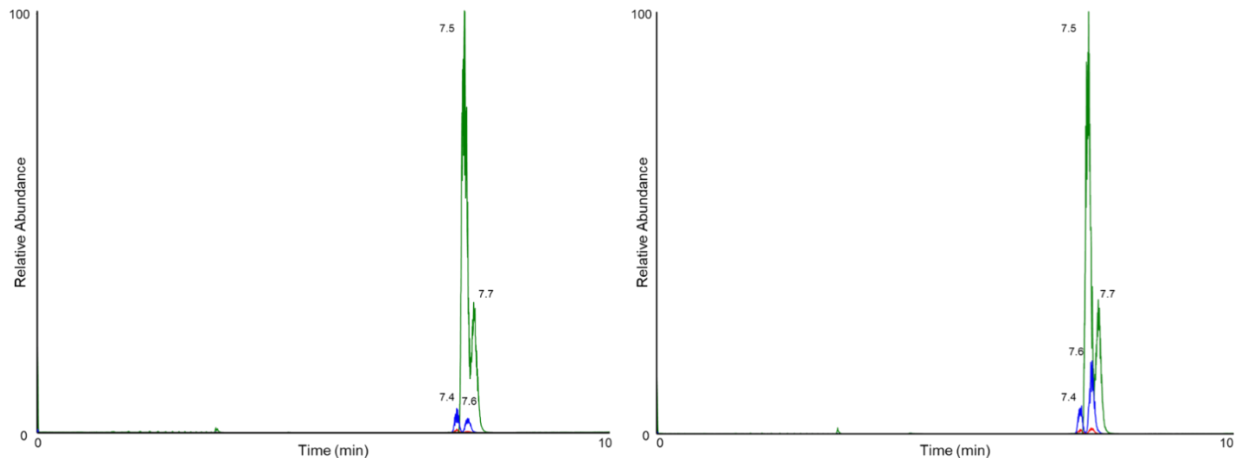
Figure 8. Rresults from a targeted LC-MS assay, generated on a 5500 QTrap, which monitors ergonovine (green trace) and the glycoside of ergonovine (blue). The trace on the left shows the result of incubating *I. tricolor* leaves with UDP-glucose and ergonovine, while the trace on the right shows *I. tricolor* leaves incubated with UDP-galactose and ergonovine. While the peak at 7.4 minutes remains at the same level between the two experiments, the peak at 7.6 minutes grows when UDP-galactose is added.
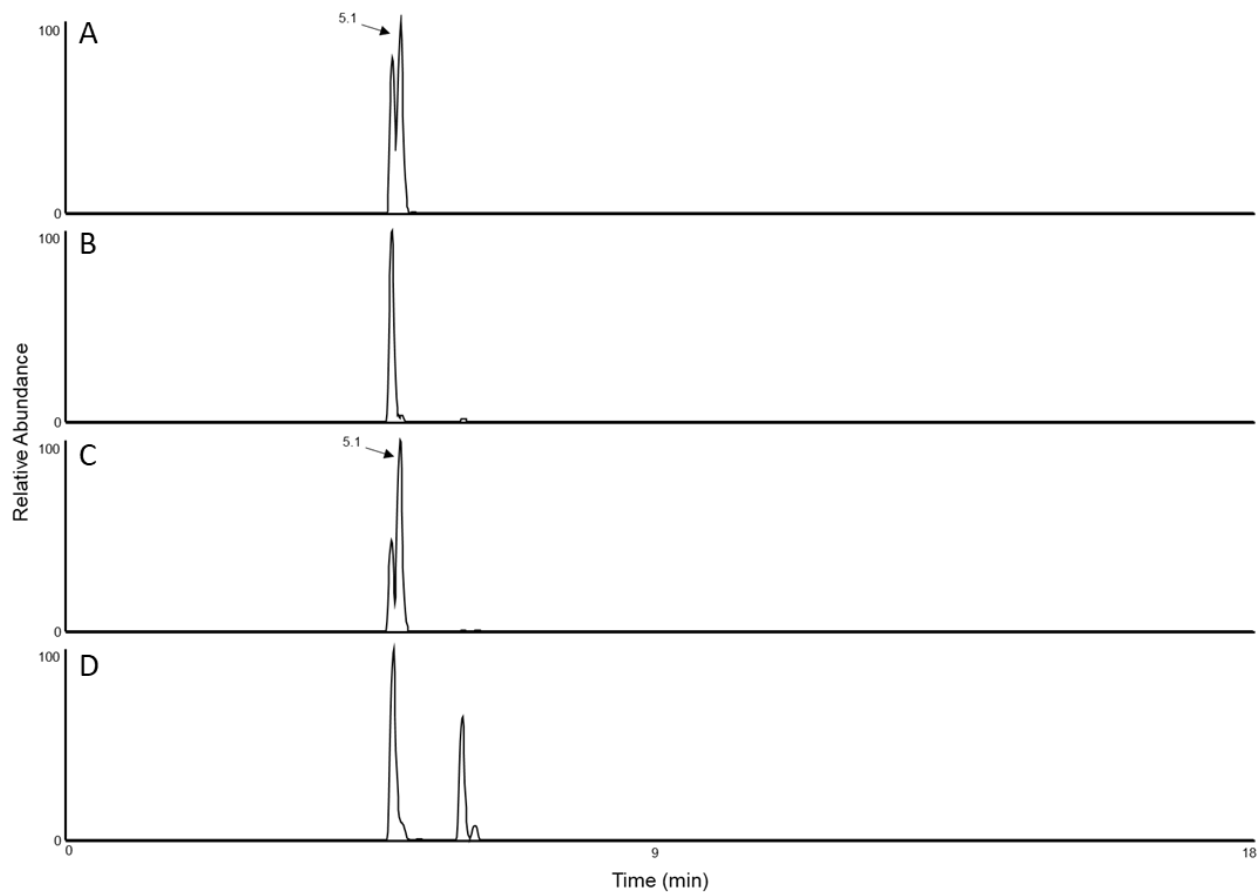
Figure 9. Extracted ion chromatograms showing 488.2931 *m/z*, from LC-MS analysis, on a Q Exactive, of four different β-galactosidase incubations. A peak matching the previously observed fragmentation spectrum for ergonovine glycoside was observed at 5.1 minutes. (A) β-galactosidase was incubated along with PNPGal and ergonovine at pH 4.5 (B) β-galactosidase was incubated along with PNPGal and ergonovine at pH 4.5, the solution was heated at 90°C for five minutes prior to incubation. (C) β-galactosidase was incubated along with PNPGal and ergonovine at pH 6. (D) β-galactosidase was incubated along with PNPGal and ergonovine at pH 6, the solution was heated at 90°C for five minutes prior to incubation.
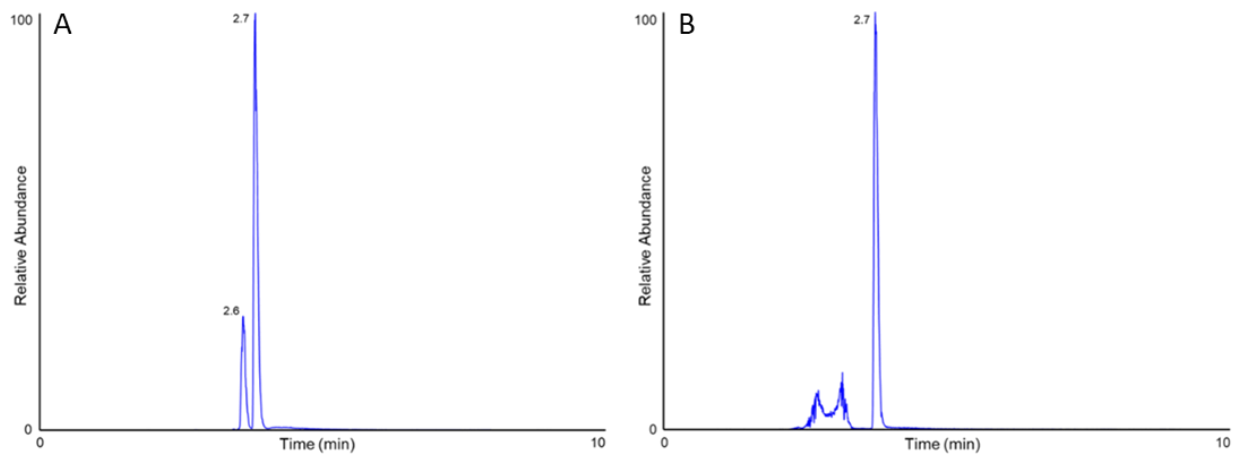
Figure 10. (A) *Achnatherum inebrians*, (B) *Achnatherum robustum*; Targeted LC-MS analysis (488 *m/z* → 326 *m/z*) using a 5500 QTrap, of ergot alkaloid extracts from two plants harboring ergonovine producing *Epichlöe sp.* A peak corresponding to ergonovine galactoside (2.7 min) can be seen in both extracts, *A. inebriens* may contain an earlier eluting alternate glycoform (2.6 min).

Table 1. Profiling of ergot alkaloids in Morning glory seeds by reversed phase fluorescence HPLC

Ipomoea species	Ergot alkaloids
I. acantha	-
I. aquatica	-
I. barbatisepala	+
I. carnea	-
I. clavate	-
I. imperati	-
I. leptophylla	+
I. pandurata	-
I. parasitica [a]	-/+
I. pes-caprae [a]	-/+
I. polpha	-
I. tricolor (P+) [b]	+
I. tricolor (P-) [c]	-
I. tuboides	-

[a] of the seeds profiled, certain individuals contained or lacked ergot alkaloids
[b] wild type seeds
[c] seeds from a lineage of plants that were cured of *Periglandula*

Table 1. Seeds of various species of genus *Ipomoea* were tested by fluorescence HPLC for retention times matching known ergot alkaloids. Three species had individuals that contained ergot alkaloids and others that lacked ergot alkaloids. Those from *I. tricolor* that lacked ergot alkaloids were from a line of plants that had been cured of *Periglandula ipomoeae* infection.

Table 2. Species tested for ergonovine conversion to glycoside

Species	Common name	Clade
Achillea milleform	Yarrow	Asteridae, Asterales
Solanum lycopersicum	Tomato	Asteridae, Solanales
Ipomoea tricolor	Morning glory	Asteridae, Solanales
Tradescantia virginiana	Spiderwort	Commelinidae
Secale cerleale	Rye	Commelinidae
Zea mays	Corn	Commelinidae
Tilia cordata	European linden	Dilleniidae
Ginko biloba	Ginko	Ginkoopsida
Castanea mollissima	Chinese chestnut	Hamameliddae
Hemerocallis sp.	Day lily	Liliidae
Iris croatica	Bearded iris	Liliidae
Magnolia virginiana	Sweetbay magnolia	Magnoliidae
Equisetum arvense	Horsetail	Pteridophyta
Cannabis sativa	Industrial hemp	Rosidae, Rosales

CHAPTER 3
THE IMPACT OF ERGOT ALKALOIDS ON *METARHIZIUM BRUNNEUM* SYMBIOSIS WITH INSECTS AND PLANTS

Abstract

Ergot alkaloids are a group of specialized metabolites expressed by several fungi including species from

the Clavicipitaceae. As members of the family Clavicipitaceae, several species belonging to the fungal

genus *Metarhizium* have recently been shown to express lysergic acid derived compounds. *Metarhizium*

species are prolific entomopathogens and have the capacity to form beneficial relationships with plants

by colonizing their roots. A major goal of this study was to investigate the impact ergot alkaloid

expression by *Metarhizium brunneum* had on the immune response of an insect host. A second goal was

to determine how plant metabolite expression changes as a response to fungal symbiosis. *Galleria*

mellonella larvae were injected with conidia selected from *M. brunneum* ARSEF 9354 and a strain of *M.*

brunneum which had an ergot alkaloid synthesis gene knocked out. Insects infected with the knockout

strain showed an altered melanization response compared to the wild type and control. LC-MS

proteomic analysis showed that wild type and knock out strain infected insects had different protein

expression profiles compared to each other as well as the control. Of particular interest, several

antimicrobial peptides and proteins were downregulated in the ergot alkaloid expressing wildtype

fungus. Metabolomics analysis demonstrated that stachydrine and ϒ-aminobutyric acid (GABA), among

other compounds, were upregulated in the roots. These metabolites are known to promote microbial

symbiosis and to promote stress-responses in plants. The results presented here, help to support the

concept of *M. brunneum* as a biocontrol agent. This is the first report which suggests a potential

mechanism for ergot alkaloids as a virulence factor for insect infection.

Some of the results presented in this chapter have been published as part of Leadmon et al (2020). In this chapter, I have excerpted and expanded upon my original contributions to that published study.

Introduction

Ergot alkaloids are a group of specialized metabolites expressed by several fungi from the Clavicipitaceae family. Representative genera from this family include *Claviceps spp., Epichloë spp., Balansia spp.,* and *Periglandula spp.* (Florea et al., 2017; Robinson & Panaccione, 2015). These fungi produce ergot alkaloids derived from lysergic acid, which have played a role in the development of medicine. As pharmacological understanding of these compounds has improved over time, synthetic derivatives of lysergic acid have been used to mitigate symptoms related to dementia, hyperprolactinemia, Parkinson's disease and migraines (Baskys & Hou, 2007; Donnet et al., 2016; Perez-Lloret & Rascol, 2010; Peer C. Tfelt-Hansen, 2013). Furthermore, these fungi have been shown to grow in close association with plants, either as mutualistic symbionts or as parasites. This plays a significant role in agriculture since lysergic acid derived compounds have been shown to cause toxic pathologies in humans and livestock resulting from the consumption of contaminated grains and forage crops (Craig et al., 2015; Florea et al., 2017; Haarmann et al., 2009; Klotz, 2015). While several studies have shown that ergot alkaloids can deter herbivory from mammals and insects, more research is needed to fully understand the role of ergot alkaloids in these fungal-plant relationships (Kaur et al., 2018; Panaccione et al., 2006; Parish et al., 2003a; Parish et al., 2003b; Potter et al., 2008).

As members of the family Clavicipitaceae, several species belonging to the fungal genus *Metarhizium* have recently been shown to have the capacity to express lysergic acid derived compounds (Leadmon et al., 2020). The ergot alkaloid gene cluster found in *M. brunneum* is composed of the same genes required in the pathway for the synthesis of LAH in *Periglandula ipomoea*e (Beaulieu et al., 2015; Hu et al., 2014; Schardl et al., 2013). Like other members in this family, *Metarhizium* species have the capacity to form beneficial relationships with plants by colonizing their roots. Furthermore, these fungi are also prolific entomopathogens, owing this lifestyle to their suite of virulence factors including

hydrolytic enzymes and destruxins (Boldo et al., 2009; Duan et al., 2009; St. Leger et al., 1996, 2011; B. Wang et al., 2012). Because of the interaction between these two lifestyles, humans have been able to harness these fungi as biocontrol agents for agricultural applications (Faria & Wraight, 2007).

Several studies have been published which investigated the gene expression response of insects infected by a variety of fungi, including *Metarhizium sp.* In the case of *Metarhizium sp.* It is well established that a variety of excreted proteins and metabolites from this genus, especially destruxins, modulate the insect immune response during infection (Mc Namara et al., 2017). Panaccione and Arnold (2017) found that ergot alkaloids contributed to the ability of *N. fumigata* to infect waxworms (*Galleria mellonella*). Since ergot alkaloid expression was only recently shown, it is unknown what role ergot alkaloids play as a virulence factor for *Metarhizium sp.* during insect infection. The goal of the study presented here was to investigate the impact ergot alkaloid expression by *Metarhizium brunneum* had on the immune response of its insect hosts, whether ergot alkaloids were produced in plant associations and how plant metabolite expression changes as a response to fungal symbiosis.

Methods

Culture conditions and ergot alkaloid extraction

Petri dish cultures of *M. brunneum* (ARSEF 9354) were grown on sucrose yeast extract agar composed of 20 g of sucrose, 10 g yeast exact, 1 g magnesium sulfate heptahydrate, 2 mL of trace elemental solution (Hutner et al., 1950), and 15 g agar (totaling to 1 L with water) for 7 days at room temperature.

LC-MS analysis of ergot alkaloid extracts

Ergot alkaloids were extracted from 50-mm^2 agar-cutouts placed in methanol. These extracts were agitated and then centrifuged to pellet debris; supernatants were concentrated using a vacuum concentrator. A 2.1 x 100 mm, 3.5 µm particle size Zorbax SB-C18 LC column (Agilent Technologies, Santa Clara, CA) held at 40°C was used for reversed phase separations of 5 µL injections of ergot alkaloid extracts. The analytical gradient ramped from 95% solvent A (0.1% formic acid) and 5% solvent B (0.1% formic acid, acetonitrile) to 50% solvent B over 15 minutes, which was generated using an Accela 1290 UHPLC instrument (Thermo Scientific, San Jose, CA) at a flow rate of 300 µL/min. A top five data dependent positive polarity electrospray ionization experiment using an in-line Q Exactive hybrid quadrupole Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) was used to detect and identify ergot alkaloids. Precursor scans were performed at 70,000 resolution over a detection window of 200 to 600 m/z. The top five most abundant ions from the precursor scan were isolated by the quadrupole (2 Da isolation window) for higher-energy collisional dissociation (HCD) set at a normalized collision energy (NCE) of 30. Fragment ions were then scanned with a resolution setting of 35,000.

Waxworm hemolymph protein extraction and digestion

Waxworm (*Galleria mellonella*) larvae were inoculated with 20 µL PBS modified to contain 0.01% tween 20 and 10 ug/mL rifampin, or 20 µL of modified PBS containing 800,000 conidia of *M. brunneum easO* knockout or wild type and then incubated at room temperature for 24 hours in quintuplicate. The extraction was performed according to the procedure that was previously described in the melanization assay, and then diluted to 100 µl with PBS. Proteins were precipitated by adding 20°C acetonitrile at a 6:1 ratio (vol acetonitrile: vol hemolymph+PBS). Samples were then centrifuged at

15,000 for 15 minutes at 4°C. The protein pellet was resuspended in 50 mM Tris, 1 mM CaCl$_2$ buffered at

pH 8.0 (digestion buffer). Protein concentrations were determined using A280 measurement on a

NanoDrop One UV-Vis spectrophotometer (ThermoFisher Scientific, Waltham, MA). Protein

concentrations were then normalized using digestion buffer. Cysteine bonds were disrupted by

incubating in 1 M dithiothreitol at 37°C for 60 minutes and were subsequently alkylated with 2 M

iodoacetamide in the dark at room temperature for 30 minutes. Trypsin was added at a 1:50 ratio

(enzyme to substrate) and incubated at 37°C for 8 hours, and then acidified with 5 µl of 1 N HCl.

LC-MS sequencing and quantification of *Galleria mellonella* hemolymph proteins following

***Metarhizium* infection**

Peptides were introduced to the Q Exactive hybrid quadrupole Orbitrap mass spectrometer

using a reversed phase gradient separation from an Acquity BEH C18, 130Å, 1.7 µm, 1 mm X 150 mm

UPLC column (Waters Corporation, Milford, MA) on an Accela 1290 UHPLC instrument. The column was

equilibrated with 98% mobile phase A (0.1% formic acid, water) and then a gradient ramped from 2%

mobile phase B (0.1% formic acid, acetonitrile) to 38% B over 54 minutes at a flow rate of 150 µL/min

held at 45°C. The mass spectrometer was operated in positive ionization mode with a data dependent

acquisition scheme. Precursor ions were monitored between 300-1750 m/z at 70,000 resolution. The

top 10 most abundant multiply charged ions (charge state 2-6) were selected for sequencing by MS/MS

with a 20 second dynamic exclusion list. Precursor ions were selected using a 2.0 m/z isolation width and

were fragmented by and HCD setting of 28 NCE. Fragment ions were scanned at 17,500 resolution.

Peptide searches and relative quantification were performed using Proteome Discoverer 2.4

(Thermo Scientific, Waltham, MA) and the Sequest HT search engine against the Uniprot *Galleria*

mellonella TrEMBL database. The search was configured in the following manner: required fully tryptic

sequence; up to two missed cleavages; fixed modification of carbamidomethylation (C) and dynamic modifications of oxidation (M) and N-terminal acetylation; precursor mass tolerance set to 10 ppm and a fragment mass tolerance of 0.02 Da. Protein and peptide results were filtered based on a minimum of two unique peptides and a 1% FDR calculated from the number of hits in the search against a concatenated database of reverse protein sequences. Minora Feature Detector was used to align precursor ion peaks in a 10 ppm window, linked to peptide spectral matches with a high confidence level (q < 0.01). The peak areas of the two most abundant peptides for each protein were averaged to generate an abundance value normalized against the total abundance of all peptides from each analytical run. Sample group protein abundance ratios were generated pairwise verses each treatment type and filtered based on a minimum Log2 fold change of one. Quantitative significance was assessed on the individual protein level by ANOVA (p < 0.05) with a Benjamini-Hochberg adjustment to compensate for multiple comparisons.

Hemolymph melanin measurement

Hemolymph was extracted from waxworm larvae inoculated with PBS, *M. brunneum* easO knockout or wildtype and then incubated at room temperature for 6 hours in triplicate. The extraction was performed according to the procedure that was previously described the proteomic study. Hemolymph was centrifuged at 10,000 rpm for 10 minutes at 4°C to remove cellular debris and then transferred to 96 well flat bottom plate for analysis. Melanization was measured by absorbance at 470 nm in a Synergy HTX plate reader (BioTek, Winooski, VT). Differences in absorbance were assessed using JMP (SAS, Cary, NC). A Brown-Forsythe test was used to check for unequal variances, while means were assessed with ANOVA and a post-hoc Tukey's Test.

LC-MS metabolomics of *Zea mays* inoculated with *Metarhizium sp.*

Plants used in the following studies were prepared according to the procedures described in Leadmon et. al (2020). Polar metabolites were separated using HILIC chromatography, by injecting 10 µL of extracts onto a 2.1 x 100 mm, 5 µm Phenomenex Luna NH2 column (Torrance, CA). A 15 minute linear gradient, which ramped from 90% solvent B (100% acetonitrile) to 60% solvent A (10 mM ammonium acetate) at a flow rate of 300 µL/min, was used to elute the metabolites for detection by electrospray ionization mass spectrometry. Each sample was analyzed in positive and negative ionization modes. The LC-MS system used for analysis consisted of an Infinity 1290 ultra-high pressure liquid chromatography instrument (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 6530 quadrupole time of flight (QToF) mass spectrometer configured with a jet stream source for electrospray ionization. For both positive and negative ionization modes, the mass spectrometer scanned over a range from 50 to 1200 m/z with a scan speed of 2 hz.

Analysis of Metabolomics Data

LC-MS data were processed using Agilent Masshunter Profinder (version B.06.00) in batch mode according to polarity of acquisition. Chromatographic features were extracted which contained a minimum peak height of 600 counts and a maximum charge state of 2. The resulting chromatography was inspected for consistency between samples, with retention time and mass alignments performed on irregular features. Peak areas from these chromatographic features were exported to Mass Profiler Professional (version 2.4.3) for further filtering, quality control and statistical analysis. Differences in peak abundances of chromatographic features between treatment types were assigned significance by a Bonferonni adjusted T-test ($p < 0.05$), with a minimum fold change > 5.

Compound Identity confirmation by tandem MS analysis

Those compounds identified as significantly changing in the previous step were re-analyzed using MS/MS fragmentation. Metabolite extracts were pooled according to sample type, and 20 µL of metabolite extract were injected onto an Accela 1290 UHPLC instrument configured for HILIC chromatography (according to the previously described separation), coupled to a Q Exactive hybrid quadrupole Orbitrap mass spectrometer. Injections were repeated for analysis in positive and negative ionization modes. The instrument was operated in a data dependent acquisition mode with an inclusion list generated from the significant compounds in the QToF analysis, a match tolerance of 10 ppm was used. A precursor scan of 80 to 1200 m/z was generated using the 70,000-resolution setting. The top ten most abundant singly charged ions from the precursor scan were isolated by the quadrupole (2 Da isolation window) for HCD fragmentation at an NCE of 30. Fragment ions were then scanned with a resolution setting of 35,000. The resulting data were searched using Compound Discoverer 2.1 (Thermo Scientific, Waltham, MA) against the KEGGS, BioCyc and Metlin metabolite libraries using a mass match tolerance of 10 ppm.

Results

Confirmation and identification of ergot alkaloids expressed by *Metarhizium brunneum*

High-resolution mass spectrometry data confirm the accumulation of chanoclavine, lysergic acid α-hydroxyethylamide (LAH), ergonovine, and ergine (spontaneously formed from LAH) in *M. brunneum* cultures (figure 1a), consistent with HPLC and unit mass resolution LC-MS data (Leadmon et al., 2020). Also observed for the first time was ergotryptamine, an early pathway spur product synthesized through the activities of DmaW, EasF, and EasC (Ryan et al., 2013, 2015). All compounds were observed within a narrow 10 ppm mass window, and fragmentation patterns match the expected patterns for all compounds (figure 1b).

LAH synthesizing *M. brunneum* modulates the melanization response in waxworms

To test the effect of LAH as a virulence factor for *M. brunneum*, waxworms were injected with conidia selected from *M. brunneum* ARSEF 9354 and a hypervirulent strain of *M. brunneum* which had *easO* knocked out, the gene for the committed biosynthetic step for LAH production. In addition to its lack of ergot alkaloids, the *easO* mutant kills insects more rapidly than the wild type but does not emerge from the corpse of the insect (Tyo and Panaccione, 2018). In these ways the *easO* mutant provides a stark contrast to the wild type strain. Hemolymph was extracted six hours after injection and tested for the melanization response by measuring absorbance at 470 nm. The insects that were injected with the *easO* knockout strain of *M. brunneum* demonstrated a significantly lower level of melanization as compared to the PBS control and the wild type fungus (P < 0.001, ANOVA) (figure 2). Insects were screened by HPLC analysis to confirm that absence of LAH in *easO* knockout infected insects. These data suggest that ergot alkaloids play a role in the regulation of insect melanization as a response to fungal infection.

Waxworm antimicrobial proteome expression is suppressed by LAH synthesizing *M. brunneum*

Hemolymph was recovered from *G. mellonella* larvae for assessment of differences in protein expression between the PBS control and the two types of *M. brunneum* infections (*easO* knockout and wild type). Analysis of these proteomes yielded unique protein expression profiles based on treatment type. PCA analysis generated from protein abundance profiles (P <0.05) showed that samples from each treatment type group together within their respective cohorts but separately from the other treatment types (figure 3). Furthermore, when comparing normalized abundance profiles of significantly changing proteins (P < 0.05) using Euclidean farthest neighbor hierarchical clustering, the wildtype infections, and the PBS control treatments cluster together and apart from *easO* knockout strain infections (figure 4).

Volcano plots, contrasting log2 fold change with significance were generated to identify individual proteins which had their expression significantly changed by the treatment types (figure 5). A total of 148 proteins were quantified among the three groups. Of the proteins monitored, this present study provides evidence at the protein level for the expression of 44 genes, which were previously only observed by mRNA sequencing. A total of 21 proteins were found to be differentially expressed in at least one of the three treatment groups (table 1), with 6 of these proteins being differentially expressed between the *easO* knockout infected larvae and the wild type-infected larvae. These expression profiles of the wild type-infected larvae showed a decrease in abundance for *G. mellonella* orthologs of four (or is it three?) antimicrobial peptides (figure 6) - gloverin (-3.1 $\log_2$ fold change), moricin (-1.2 $\log_2$ fold change), proline rich antimicrobial peptide (-2.7 $\log_2$ fold change) and peptidoglycan recognition protein (-0.8 $\log_2$ fold change).

M. brunneum colonization alters the metabolome of *Zea mays*

Since plant roots are also an ecological niche occupied by Metarhizium species, I analyzed how colonization by *Metarhizium* species affected the metabolome of corn (*Zea mays*). When the roots of filter-paper sprouted corn seedlings were exposed to inocula from *M. brunneum* and *M. flavoviride,* fungal mycelia were observed growing on the surface of the plants. Since homogenates of these plants were negative for ergot alkaloids when tested using an FLD-HPLC assay (Leadmon et al., 2020), metabolomics analyses also provided a means for demonstrating colonization of the plants by the fungi. Global metabolite profiling was performed on homogenized plant tissues using HILIC-LC-MS analysis. Of the >1000 chromatographic features which were monitored (ergot alkaloids also were not detected in these independent analyses), 66 had altered expression profiles between plants inoculated with *M. brunneum*, *M. flavoviride*, or non-inoculated control plants (table 2). The identities of these compounds

and their relative abundance profiles can be found in table 3. Of particular note, ϒ-aminobutyric acid

was observed with a 20-$\log_{10}$ fold higher abundance in roots infected with *M. brunneum* and a 18-$\log_{10}$

fold higher abundance in roots infected with *M. flavoviride* ($P < 0.05$).

Discussion

The accurate mass results presented here confirm the ergot alkaloid profile produced by *M.*

brunneum as previously reported (Leadmon et al., 2020). These ergot alkaloids were shown to

accumulate in *M. brunneum* infected *G. mellonella*, with lysergic acid α-hydroxyethylamide being the

most abundant. A role for ergot alkaloids in modulating an insect's immune response to *Metarhizium*

sp. has not been investigated, until now. The genetically modified ergot alkaloid deficient strain of *M.*

brunneum proved to be a useful tool for probing these interactions. An important point to note

regarding the *easO* mutant of *M. brunneum* used in the present study is that its chemotype is a lack of

all ergot alkaloids. Based on genotype, an *easO* deficient strain of *M. brunneum* would be expected to

accumulate ergot alkaloids up through and including ergonovine. The lack of all ergot alkaloids in the

easO mutant used here indicates further mutations or a change in gene regulation. Nonetheless, the

strains lack of ergot alkaloids and hypervirulent phenotype provide an altered background against which

the wild type of *M. brunneum* can be compared.

Melanization is a well characterized physiological pathway in insects, whereby they respond to a

variety of stimuli, including infection (Nakhleh et al., 2017). The melanization assay performed here was

used as a measure of the insect immune response. These data suggest that the regulation of insect

immunity as a response to infection by a fungal pathogen is modified by ergot alkaloids and point to a

role for these compounds as virulence factors.

Global hemolymph proteome analysis was used to further investigate the role of ergot alkaloids in *Metarhizium* virulence. Principal component analysis (figure 3) of the observed changing proteins confirms what was seen in the melanization assay. The protein expression profiles cluster together according to infection type, with the wild-type infections separating away from the *easO* knockout infections, with the PBS control separating into its own distinct group. When this is taken into consideration with the hierarchical clustering analysis (figure 4), the proteomic differences indicate that while the *M. brunneum* infected waxworms do mount some response to the presence of the fungus. Indeed, several antimicrobial peptides and defense related proteins were measured and were expressed at similar levels between the two types of infections (table 1). However, the overall insect proteome in wild-type *M. brunneum* infections is more like what is found in a healthy individual than an individual that has been infected by the hypervirulent strain of *M. brunneum* lacking ergot alkaloids, with several immune related peptides and proteins being downregulated. This difference in immune response is not surprising, as different microorganisms have been shown to elicit differential immune peptide responses in *G. mellonella* from different pathogens (Mak et al., 2010).

The interesting part, that may point to a role for ergot alkaloids, is that six proteins were differentially regulated between *Metarhizium brunneum* infections with and without ergot alkaloids (figure 6). Among these proteins gloverin, moricin and the proline-rich peptide are known as antimicrobial peptides and have been shown to be involved in insect immune response (Mc Namara et al., 2017; Vogel et al., 2011). The *Manduca sexta* (tobacco hornworm) homolog of the gloverin-like protein, has been shown in to bind cellular components of a variety of microbes and present antimicrobial activity against bacteria and fungi alike (X. X. Xu et al., 2012). The proline-rich peptide identified here may be related to proline-rich peptides from other lepidopteran species, which are known to have antimicrobial activity against gram-negative and gram-positive bacteria, as well as fungi (Yi et al., 2014). A recent study where *G. mellonella* was challenged by *Candida albicans* infection

showed elevated peptidoglycan recognition-like protein as a response (Sheehan & Kavanagh, 2018).

Moricins are a group of antimicrobial peptides originally identified in the lepidopteran insect *Bombyx mori* (Hara & Yamakawa, 1995), more recently *G. mellonella* was shown to have its own unique repertoire of moricin-like peptides (Brown et al., 2008). In that study moricin A (also identified in this present study) was shown to be especially active against the filamentous fungi *Fusarium graminearum, Fusarium oxysporum, Ascochyta rabiei,* and *Leptosphaeria maculans*. All of this together suggests that the waxworm immune response is limited in scope when challenged by a fungus using ergot alkaloids as a virulence factor. Indeed, a similar conclusion was reached by Panaccione and Arnold (2017) for the distantly related fungus Neosartorya fumigata and its ergot alkaloids. This study suggests that the actual protein targets of ergot alkaloids are regulatory elements upstream of both the melanization response and the antimicrobial peptides identified as changing in this study. Further research could be performed to associate genes in the waxworm with this response.

While *Metarhizium brunneum* is effective as an entomopathogen, it also readily grows in association with plants. However, one major distinction can be made with regards to that association – unlike with insects, *M. brunneum* does not produce ergot alkaloids when grown in conjunction with plants. Despite this, the metabolomic data presented here show that *M. brunneum* does not grow as a passive participant with its plant host, several metabolites were identified as changing when the plant was grown with the fungus present. Two of these metabolites, stachydrine and Y-aminobutyric acid (GABA) are particularly interesting. Stachydrine has been reported as being involved in promoting plant nodulation and microbial symbiosis, as it is released from the seeds of *Medicago* sp. and in turn activates *nod* genes in the symbiotic bacteria *Sinorhizobium meliloti* (Cooper, 2007). As for GABA, plants use this compound as a signaling molecule to regulate growth and development (Ramesh et al., 2017). Several reports have been released in recent years which indicate that supplementing a plant with GABA

helps to promote responses to stress, including low-light conditions and high-salinity (Li et al., 2017;

Vijayakumari & Puthur, 2016; J. Xu et al., 2019).

The results presented here, help to support the concept of *M. brunneum* as a biocontrol agent.

The ability of *M. brunneum* to grow in an association with a plant host, and promote certain key growth

factors, while not producing ergot alkaloids make it suitable to be used with food crops. Furthermore,

while it has already been shown that *M. brunneum* uses ergot alkaloids as a virulence factor in its role as

an entomopathogen, this is the first report which suggests a potential mechanism.

Figures
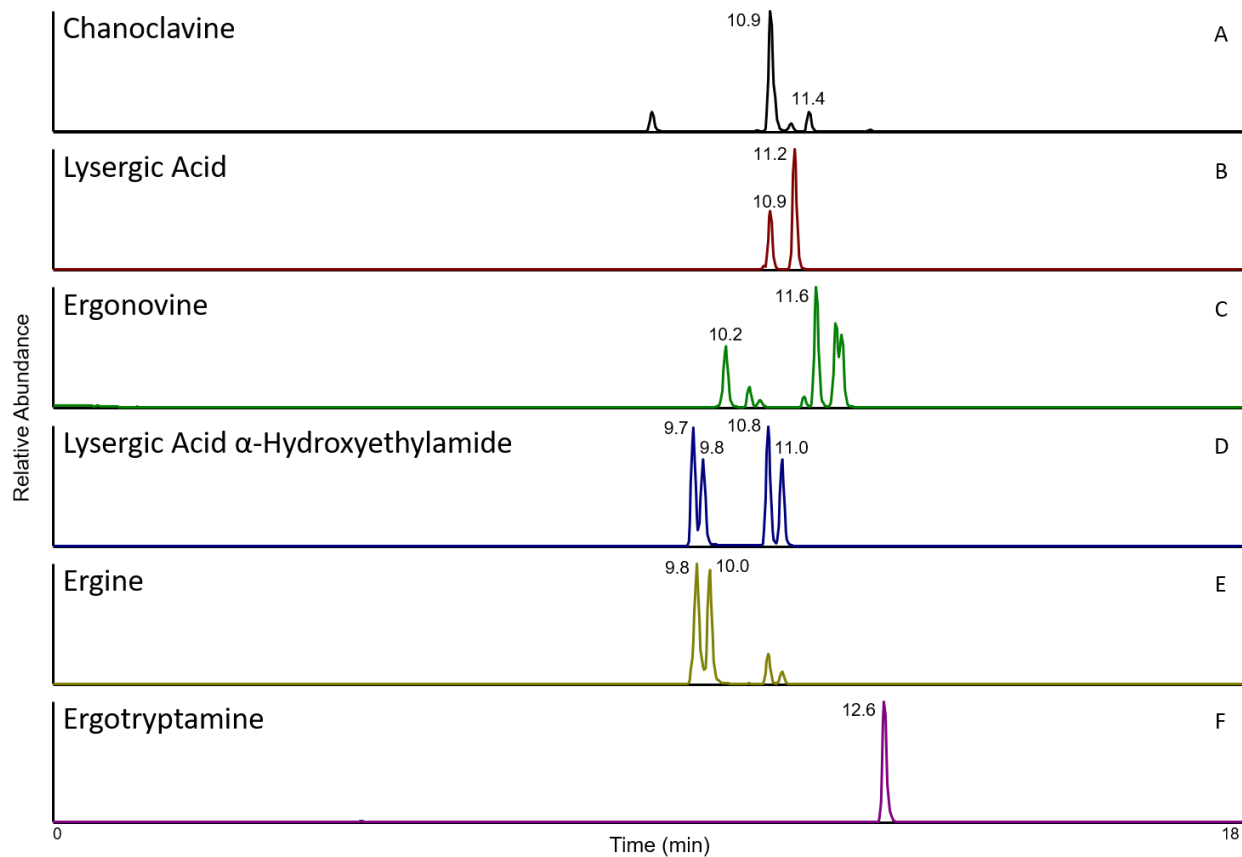
Figure 1a. Extracted ion chromatography, from a Q Exactive, of ergot alkaloids observed in M. *brunneum*. (A) chanoclavine, 257.1649 *m/z*, (B) lysergic acid, 269.1285 *m/z*, (C) ergonovine, 326.1863 *m/z*, (D) D-lysergic acid α-hydroxyethylamide (LAH), 312.1707 *m/z*, (E) ergine, 268.1445 *m/z*, (F) ergotryptamine, 259.1805 *m/z*. All ions were extracted with a 10 ppm mass window centered on the theoretical monoisotopic M+H. The multiple peaks observed in most compounds are chromatographically resolved stereoisomers. Tandem MS spectra were used to assign identities for the labeled retention times.
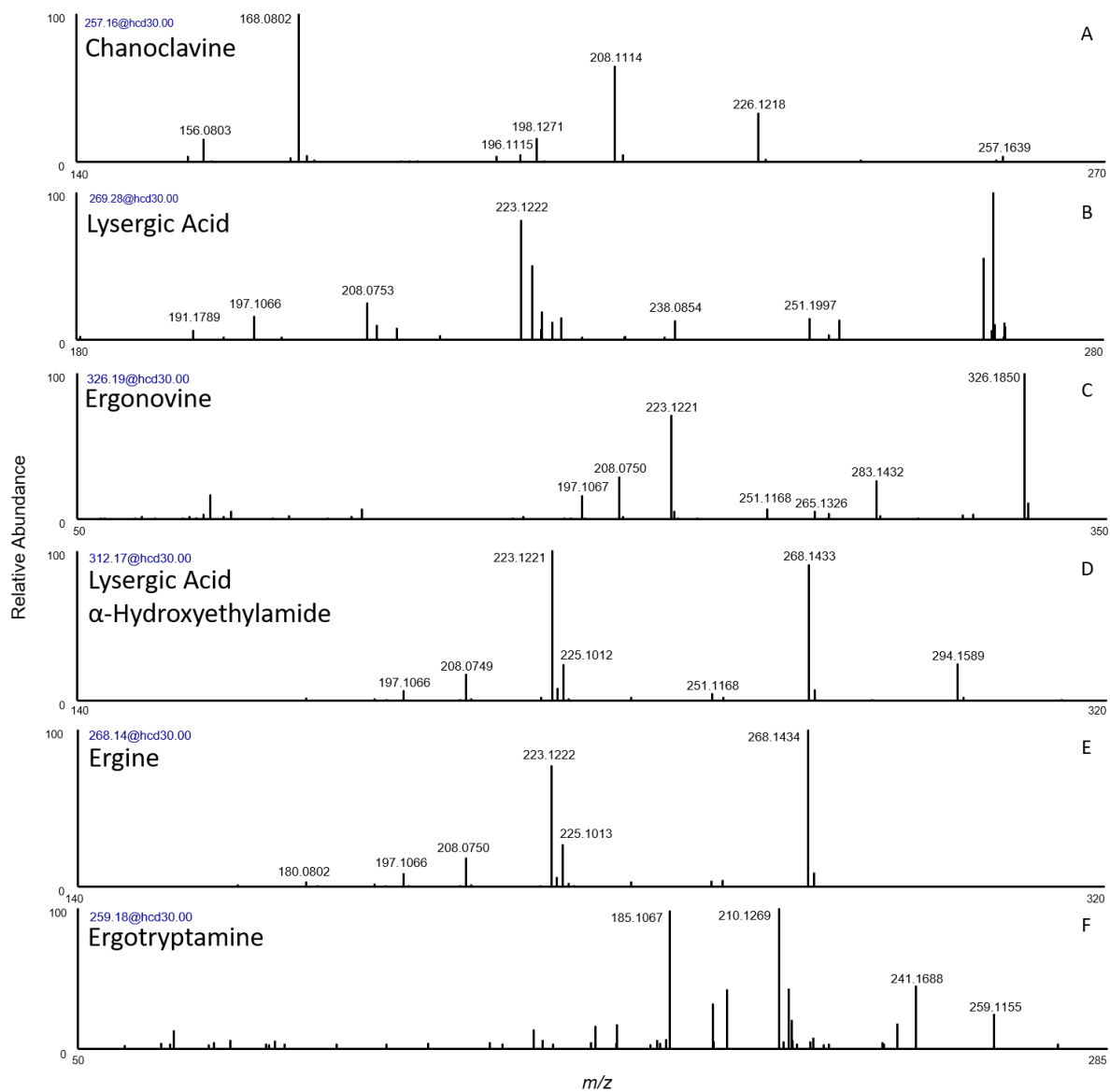
Figure 1b. High resolution, accurate mass HCD fragmentation spectra, for ergot alkaloids detected in *M. brunneum*. (A) chanoclavine, (B) lysergic acid, (C) ergonovine, (D) D-lysergic acid α-hydroxyethylamide (LAH), (E) ergine, (F) ergotryptamine. Later pathway intermediates (B-E) show common fragment ions – 197.107 *m/z*, 208.075 *m/z,* and 223.122 *m/z*.
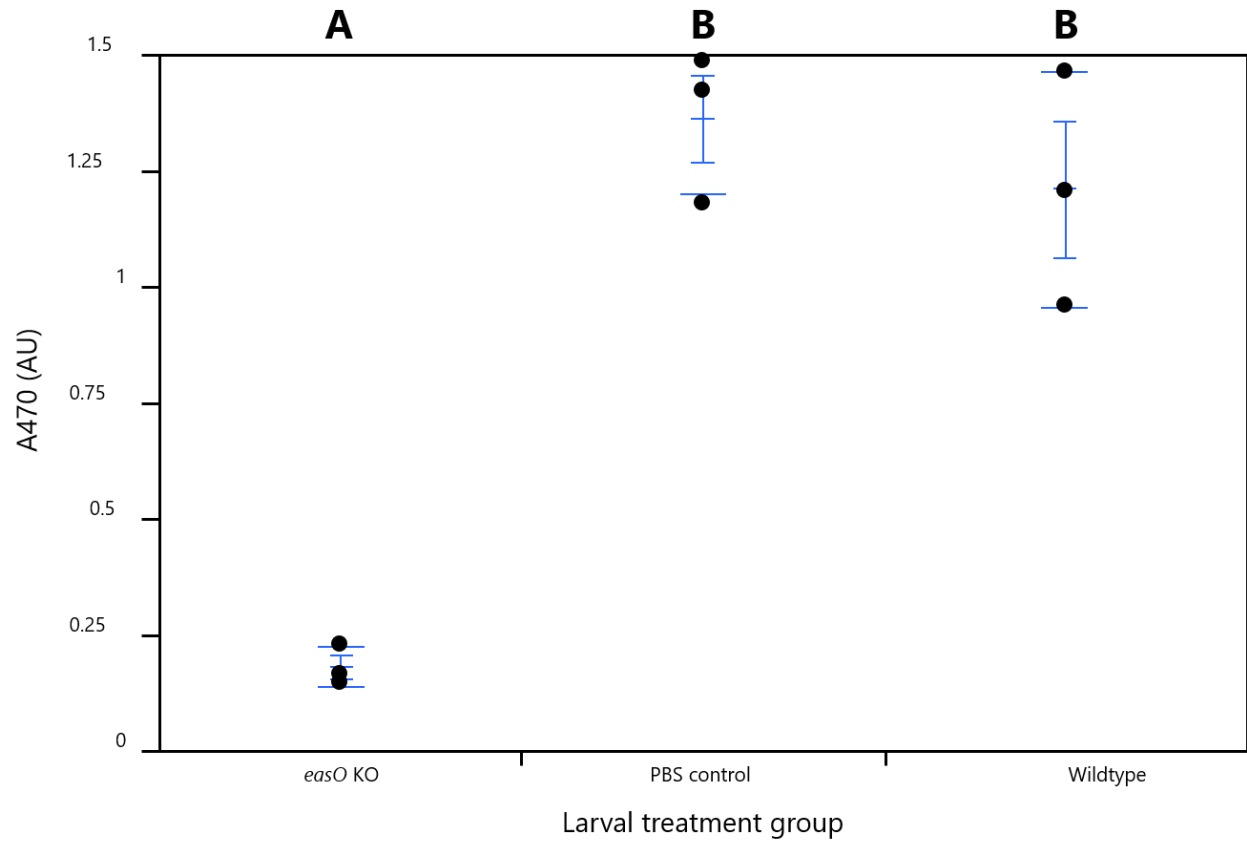
Figure 2. Abundance DOPA (measured by absorbance at 470 nm) as a proxy for melanization responses for waxworm larvae injected with *easO* knockout *M. brunneum,* wild-type *M. brunneum*, and sterile phosphate buffered saline control. A comparison of all pairs using Tukey-Kramer HSD, showed that mean abundance response for the PBS control and wild-type fungus were similar (B), and both differed from the response for the *easO* knockout (A).
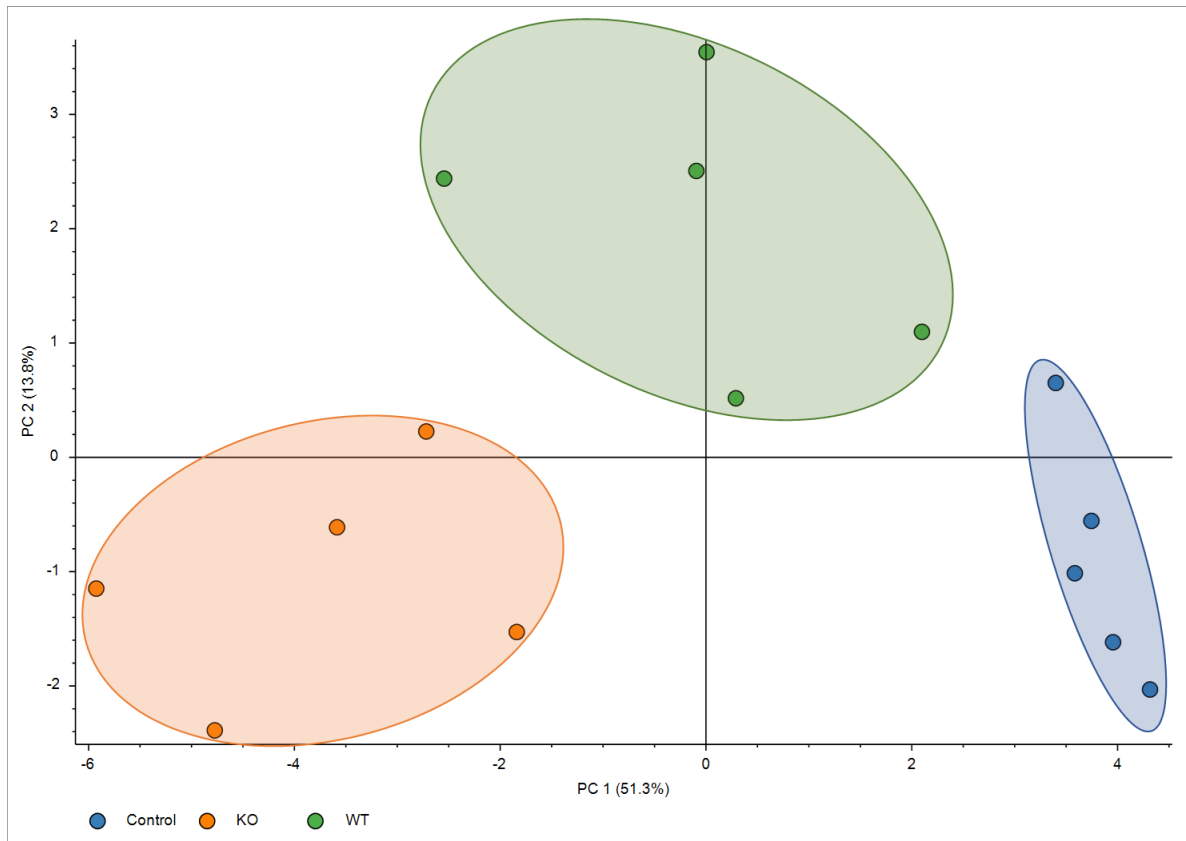
Figure 3. Principal component analysis plot showing separation between the three conditions used to challenge *G. mellonella* larvae. The control, *easO* knockout *M. brunneum* and wildtype *M. brunneum* separate into three different groups, suggesting different protein expression profiles.
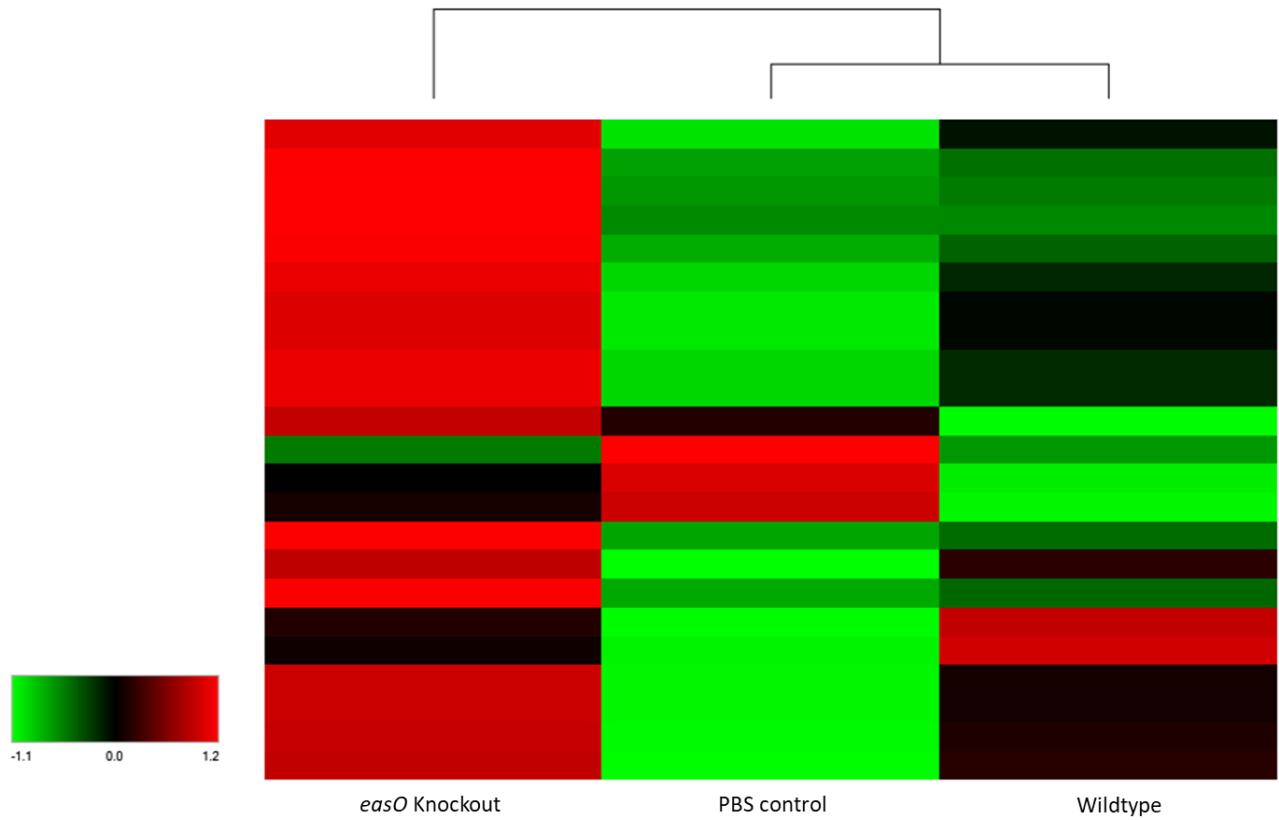
Figure 4. A Euclidean farthest neighbor hierarchical cluster generated from grouped protein abundances, scaled after clustering. These protein expression patterns indicate that general hemolymph protein content is more similar between the wild type infection and the PBS control than either is to the *easO* knockout infection.
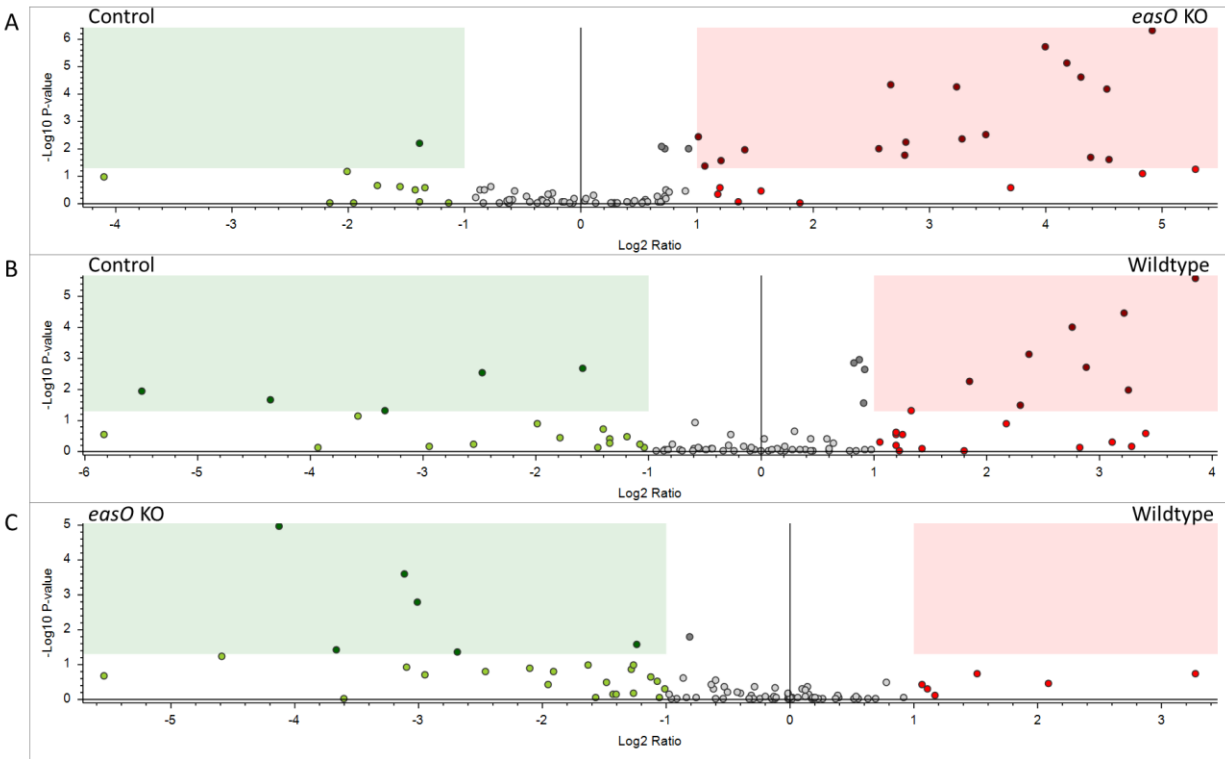
Figure 5. Volcano plots showing protein abundance differences in all identified proteins based on relative abundance changes in the three treatment types. Proteins are arranged based on their p value (log10 p-value, y-axis) and fold change (log2 average label free quantitation difference in abundance, x-axis). Proteins found in the red and green boxes are considered to be statistically significant and having a log2 fold change greater than 1.
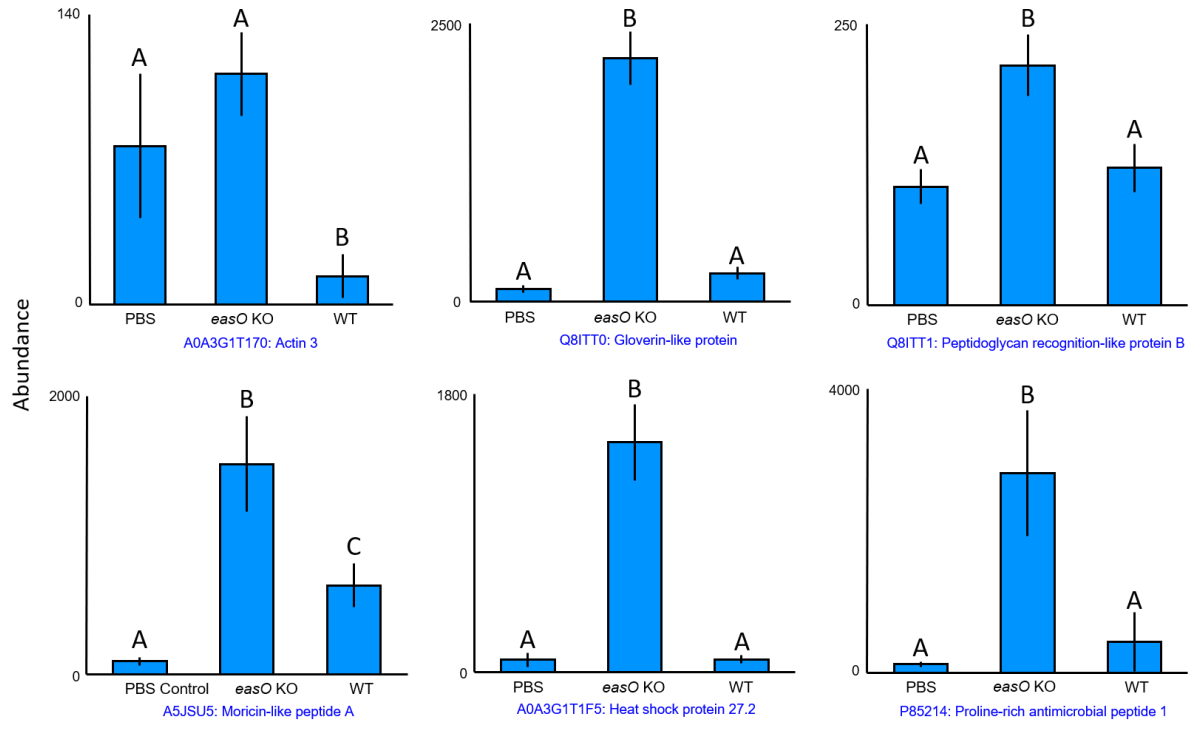
Figure 6. Bar graphs showing proteins that were differentially expressed between the *easA* knockout and wild-type strain infected *G. mellonella*. Moricin-like peptide A was the only protein which was differentially expressed between all three sample groups.

Table 1. Proteins with altered expression profiles (minimum two log2-fold change; $P<0.05$) in waxworms infected with *Metarhizium brunneum* wildtype and *easO* knockout compared to PBS inoculated controls (n=5 biological replicates)

Uniprot Accession	Protein Description	Number of unique peptides	easA KO vs Control	Wildtype vs Control	Wildtype vs easA KO
A0A3G1T170‡	Actin 3	23	—	-2.5	-3.0
A0A5C0E4H9‡	Chemosensory protein 15	6	2.6	3.3	—
P85215	Defensin-like peptide	4	3.5	2.9	—
A0A3G1T1H0‡	Fatty acid binding protein 1	14	—	-5.5	—
O62605	Fibrohexamerin	10	4.4	—	—
Q7M468	Fibroin heavy chain PG-2' (Fragments)	2	4.6	—	—
Q8ITT0	Gloverin-like protein (Fragment)	3	4.3	—	-3.1
A0A3G1T1F5‡	Heat shock protein 27.2	2	4.2	—	-4.1
A0A3G1T1I1‡	Heat shock protein 70	15	-1.4	-1.6	—
C7ASJ3	Hemolin	35	1.4	—	—
P82176	Inducible metalloproteinase inhibitor protein	6	1.2	0.9	—
P85211	Lebocin-like anionic peptide 1	5	3.2	2.4	—
P82174	Lysozyme OS=Galleria mellonella	15	2.8	2.3	—
A5JSU5	Moricin-like peptide A	2	4.0	2.8	-1.2
A5JSU6	Moricin-like peptide B	1*	4.7	—	-2.7
A5JSU8	Moricin-like peptide C2	2	4.9	3.9	—
A5JSV2	Moricin-like peptide D	4	2.7	3.2	—
Q8ITT1	Peptidoglycan recognition-like protein B (Fragment)	7	1.0	—	-0.8
M1FW43	Peptidoglycan-recognition protein	8	2.8	—	—
P85214	Proline-rich antimicrobial peptide 1	5	4.5	1.9	-2.7
P85212	Proline-rich antimicrobial peptide 2	5	3.3	—	—

Table 2. Numbers of compounds that varied between *Metarhizium* species-infected corn roots compared to uninoculated corn roots by a minimum two log-fold difference (*P* < 0.05; n = 3 biological replicates).

Response	Positive ionization mode	Negative ionization mode	Total
Up in *M.b.*[a]; up in *M.f.*[b]	11	28	39
Up in *M.b.*; unchanged in *M.f.*	2	12	14
Up in *M.b.*; down in *M.f.*	0	2	2
Down in *M.b.*; up in *M.f.*	0	0	0
Down in *M.b.*; unchanged in *M.f.*	1	1	2
Down in *M.b.*; down in *M.f.*	1	2	3
Unchanged in *M.b.*; up in *M.f.*;	2	0	2
Unchanged in *M.b.*; down in *M.f.*;	4	0	4

[a] *M.b.*, corn roots infected with *M. brunneum* ARSEF 9354

[b] *M.f.*, corn roots infected with *M. flavoviride* BC 1163

Table 3. Compounds altered in concentration (minimum two log-fold change; *P*<0.05) in corn roots infected with *Metarhizium* species compared to uninoculated corn roots (n=3 biological replicates)

Ionization mode	Mass	Log fold change *M.b.*[a] vs Control	Regulation *M.b.* vs Control	Log fold change *M.f.*[b] vs Control	Regulation *M.f.* vs Control	Identification
negative	60.0232	15.9	up	14.9	up	unknown
negative	103.0661	19.8	up	17.7	up	Y-aminobutyric acid (GABA)
negative	114.0345	18.9	up	18.4	up	4-hydroxy-5-methyl-3(2H)-furanone
negative	138.0404	-18.6	down	-1.2	unchanged	salicylic acid or 4-nitroaniline or 2-aminonicotinic acid
negative	150.0578	16.7	up	17.0	up	unknown
negative	155.0729	20.7	up	20.3	up	histidine
negative	166.0514	-2.3	down	-0.1	unchanged	unknown
negative	166.0514	17.2	up	0.0	unchanged	unknown
negative	177.0559	17.8	up	17.7	up	unknown
negative	180.0287	17.6	up	18.6	up	unknown
negative	181.0724	20.6	up	0.0	unchanged	tyrosine
negative	188.0852	13.4	up	16.4	up	unknown
negative	194.0447	16.1	up	0.0	unchanged	7-carboxy-7-deazaguanine
negative	199.1034	16.4	up	15.8	up	unknown
negative	201.0675	14.9	up	15.3	up	unknown
negative	205.0578	4.0	up	3.0	up	unknown
negative	208.0941	14.3	up	15.8	up	dambonitol
negative	215.0779	17.2	up	18.1	up	succinyl proline
negative	239.0793	15.4	up	0.0	unchanged	unknown
negative	240.0874	19.4	up	19.4	up	unknown
negative	244.0654	3.4	up	3.1	up	unknown
negative	250.0988	5.3	up	4.6	up	(3S,6S)-3-(4-Hydroxybenzyl)-6-(hydroxymethyl)-2,5-piperazinedione
negative	254.0653	15.0	up	0.0	unchanged	unknown
negative	266.1074	17.0	up	15.7	up	unknown
negative	272.0684	18.6	up	0.0	unchanged	unknown
negative	272.1166	17.4	up	15.8	up	unknown
negative	279.1368	21.6	up	19.8	up	N-(1-deoxy-1-fructosyl)leucine
negative	306.0063	16.6	up	15.8	up	unknown
negative	310.1848	19.3	up	0.0	unchanged	potential botrydial
negative	317.1282	15.2	up	0.0	unchanged	unknown

negative	326.1942	5.6	up	4.1	up	heptaethylene glycol or 2-dodecylbenzenesulfonic acid
negative	354.1038	-20.5	down	-20.5	down	asarinin
negative	358.0975	16.3	up	16.3	up	unknown
negative	368.1164	15.0	up	14.2	up	3-O-feruloyl-D-quinic acid (O-feruloylquinic)
negative	388.1620	4.8	up	-18.2	down	unknown
negative	411.0667	16.3	up	0.0	unchanged	unknown
negative	482.1381	18.2	up	0.0	unchanged	unknown
negative	490.2749	2.4	up	0.0	down	unknown
negative	502.3154	-14.2	down	-14.2	down	unknown
negative	596.2847	23.7	up	23.9	up	unknown
negative	688.4750	21.7	up	18.3	up	unknown
negative	734.4921	3.3	up	-1.6	unchanged	unknown
negative	746.5172	2.9	up	2.2	up	unknown
negative	810.6056	14.3	up	0.0	unchanged	unknown
negative	812.6355	18.8	up	16.9	up	unknown
negative	856.5146	4.9	up	4.0	up	unknown
positive	124.0640	13.9	up	15.1	up	2-methoxy-3-methylpyrazine
positive	131.0945	20.6	up	19.7	up	leucine/isoleucine
positive	131.1307	15.9	up	14.8	up	unknown
positive	143.0945	20.2	up	17.6	up	stachydrine
positive	173.1049	19.1	up	18.2	up	N-acetyl-L-leucine
positive	216.1108	17.5	up	17.7	up	tert-butyl 3-amino-1-methyl-2,3-dioxopropylcarbamate
positive	255.0745	-17.8	down	-17.8	down	D-ribosylnicotinate
positive	276.0989	0.0	unchanged	15.2	up	Glu-Glu
positive	283.1210	-1.4	unchanged	-16.6	down	ambiguous: ceramide or n-trans-p-coumaroyl tyramine
positive	308.2238	-1.5	unchanged	-17.8	down	7-(1-ethoxyethoxy)-4,10-dimethyl-3,5,9,11-tetraoxatridecane
positive	311.1219	14.8	up	15.2	up	N,N-dimethylguanosine
positive	345.1224	0.0	unchanged	15.5	up	unknown
positive	349.3177	14.7	up	0.0	unchanged	unknown
positive	352.1310	-13.7	down	0.3	unchanged	potential 1-(alpha-D-glucopyranosyluronosyl)-3-[(2S)-1-methyl-5-oxo-2-pyrrolidinyl]pyridinium
positive	401.3491	1.8	unchanged	-14.0	down	unknown
positive	422.1051	15.9	up	17.2	up	potential tetracenomycin A2
positive	511.0720	25102.9	up	16.7	up	unknown
positive	533.4801	36944.1	up	2.6	up	unknown
positive	577.3516	262910.0	up	19.5	up	unknown

| positive | 603.2449 | 201676.2 | up | 0.0 | unchanged | unknown |
| positive | 911.6445 | 1.6 | unchanged | -14.5 | down | unknown |

CHAPTER 4
INVESTIGATION OF *EASC* AND EXPRESSION OF THE ERGOT ALKALOID SYNTHESIS PATHWAY IN *PENICILLIUM CAMEMBERTI*

Abstract

The Trichocomaceous fungus *Penicillium camemberti* is used in the production of camembert and brie cheeses. Ergot alkaloids are specialized metabolites synthesized by fungi belonging to the ascomycete families Trichocomaceae and Clavicipitaceae. The ergot alkaloid synthetic (EAS) pathway of fungi from Trichocomaceae (genera include *Penicillium*) diverges from fungi in Clavicipitaceae at a middle step to produce fumigaclavines and related compounds. *Penicillium biforme* is a known producer of rugulovasine A/B, which have never been observed in *Penicillium camemberti*. Analysis of the genomes from *P. camemberti* and *P. biforme* revealed that the two species contain the same cluster of EAS genes, and both organisms express mRNA from these genes in specific culture conditions. Heterologous gene expression of *P. camemberti easC* in a *Neosartorya fumigata easC* knockout strain, along with LC-MS analysis of *P. biforme* and *P. camemberti* cultures were used to test the regulation and the gene function of *P. camemberti easC*. When grown on succinate and glucose containing media *P. biforme* produced rugulovasines, while the *P. camemberti* EAS pathway stopped at the step prior to EasC and accumulated N-Me-dimethylallyltryptophan. Along with mRNA expression of *easC*, this confirms that the regulatory elements needed for *eas* gene expression are functional in *P. camemberti.* When compared to *P. biforme*, *P. camemberti easC* contains a single nucleotide mutation. As a result, *P. camemberti easC* was unable to complement *Neosartorya fumigata easC* knockout in a transformed strain expressing the

mRNA for the introduced gene. The data presented here suggest that the ancestor of modern *P. camemberti* had the capacity to synthesize rugulovasines and other ergot alkaloid precursors but lost this capability due to a Gly to Val mutation at position 13 on the protein.

Some of the results presented in this chapter have been published as part of Fabian et al (2018). In this chapter, I have excerpted and expanded upon my original contributions to that published study.

Introduction

Ergot alkaloids are specialized metabolites synthesized by fungi belonging to the ascomycete families Trichocomaceae and Clavicipitaceae. The psychoactive, convulsive, and circulatory system effects of these compounds have been observed since the middle ages. In modern times, these compounds have been harnessed as precursors for medicines used to treat a variety of disorders including migraines, Parkinson's disease, and to prevent blood loss during childbirth (Haarmann et al., 2009). These effects are mostly attributed to lysergic acid related ergot alkaloids derived from Clavicipitaceae species, with clavine related compounds arising from Trichocomaceae being less understood (Florea et al., 2017).

Five enzymes involved in the early steps of the ergot alkaloid synthetic biochemical pathway are highly conserved across fungi and are contained in the ergot alkaloid synthesis (*eas*) gene cluster (Robinson & Panaccione, 2015). Figure 1 shows a summary of these reactions. The gene *dmaW* encodes dimethylallyltryptophan (DMAT) synthase and begins the pathway with prenylation of tryptophan. Following this, the enzyme encoded by *easF* methylates the amino group of tryptophan, and a couple of reactions, involving enzymes coded by *easC* and *easE,* decarboxylate and oxidize the methylated DMAT and yield chanoclavine-I (Goetz et al., 2011; Lorenz et al., 2010). The primary alcohol on this closed-ring ergot alkaloid is then oxidized to an aldehyde by the product of the *easD* gene, yielding chanoclavine-I aldehyde (Wallwey et al., 2010). With this step, the conserved portion of the pathway is completed, with later products being modified forms of the chanoclavine-I aldehyde scaffold. Fungi from the Clavicipitaceae (genera include *Metarhizium*, *Claviceps, Periglandula, Epichloë)* perform modifications to form lysergic acid and related compounds, while fungi from Trichocomaceae diverge to produce fumigaclavines and related compounds (genera include *Aspergillus* and *Penicillium*) (Young et al., 2015).

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The genome sequence of the Trichocomaceous fungus *Penicillium camemberti*, used in the production of camembert and brie cheeses, was recently made publicly available (Cheeseman et al., 2014; Ropars et al., 2015). Analysis of its genome revealed that *P. camemberti* contains a cluster of ergot alkaloid synthesis (*eas*) genes. Interestingly, the closely related, ancestral species *P. biforme* contains a similar cluster organized in the same manner. Furthermore, the *eas* cluster from both species contain several genes with homology to the *Neosartorya fumigata eas* gene cluster (*dmaW, easF, easC, easE, and easD*), along with two additional genes, *easH* and *easQ* encoding activities associated with specialized metabolites (Fabian et al., 2018). A comparison of the *N. fumigata* and *P. camemberti* gene clusters can be seen in figure 2.

Attempts by Fabian et al. (2018) to screen *P. biforme* and *P. camemberti* cultures using fluorescence (FLD) HPLC for the presence of ergot alkaloids were unsuccessful. Following successful transformation of chanoclavine-I aldehyde accumulating *N. fumigata* knockout strains with *P. camemberti easH/easQ*, cultures were screened by LC-MS and by FLD-HPLC for the presence of ergot alkaloids. LC-MS analysis revealed the presence of a 269.1 *m/z,* with high abundance counts in the mass spectrometer, and no signal in the FLD-HPLC analysis (excitation/emission wavelengths of 272 nm/372 nm and 310 nm/410 nm). While 269.1 *m/z* is consistent with lysergic acid, the lack of FLD-HPLC signal hinted at a different structure. Indeed, LC-MS analysis of *P. biforme* malt extract cultures yielded a similar precursor mass and fragmentation spectra as that found in the *P. camemberti easH/easQ* transformed *N. fumigata* knockout strain (figure 3). *P. biforme* is a known producer of rugulovasines A/B, which have an accurate mass of 268.1206 Da and is consistent with the observed 269.1 M+H ion observed.

Fabian et al. (2018) investigated and found by LC-MS analysis that ergot alkaloids were produced by *P. biforme* and not *P. camemberti,* when grown on malt extract agar. Furthermore, both *Penicillium* species produced mRNA transcripts for all the *eas* genes in the cluster. A heterologous

complementation strategy along with *N. fumigata eas* gene knockout strains was used to investigate the functionality of several genes from the *P. camemberti eas* gene cluster. This work revealed that *easH, easQ, dmaW* and *easE* complemented *N. fumigata* knockout strains*.* By deduction and by chemical analyses described herein, I hypothesized that *P. camemberti easC* contains a defect, as a result this fungus has lost the ability to produce ergot alkaloids downstream of the *easC* gene product. Heterologous gene expression of *P. camemberti* in a *N. fumigata easC* knockout strain, along with LC-MS analysis of *P. biforme* and *P. camemberti* cultures were used to test this hypothesis.

Methods

Culture conditions and ergot alkaloid extraction

Petri dish cultures of *P. biforme* (NRRL 885), *P. camemberti* (NRRL 874 and 875), and *N. fumigata easC* knockout (Goetz et al., 2011) were grown on malt extract agar composed of 6 g of malt extract, 6 g dextrose, 1.8 g maltose, 1.2 g yeast extract, 15 g agar, totaling to 1 L with water), for 7 days at 22°C. Cultures were also grown in a modified Czapek's medium (2 g sodium nitrate, 1 g dipotassium phosphate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, 0.01 ferrous sulfate, totaling to 1 L with water), supplemented with succinate, glucose, glycine or glycerol as sole carbon sources at 10 mM. Ergot alkaloids were extracted from agar-based petri dishes, by repeatedly washing the culture with 4 mL of methanol. A portion of these methanol washes was concentrated using a vacuum concentrator.

Transformation gene construct formation

Restriction digested fusion PCR products were inserted in plasmids, to be used for heterologous gene expression. Phusion 5x high-fidelity PCR buffer (100 mM KCl, 20 mM Tris-HCl, 1.5 mM MgCl$_2$,

buffered at pH 7.4; Thermo Scientific, Waltham, MA) was combined with distilled/deionized water (11

μL), Phusion HF polymerase (0.5 μL, Thermo Scientific), 1.25 mM deoxynucleoside triphosphates (4 μL),

20 μM each of forward and reverse primer (1.25 μL of both), template DNA (2 μL). The sequences used

for the forward and reverse primers can be found in table 1. The *P. camemberti easC* coding sequence

was amplified with primer set 1, and the *N. fumigata easA-easG* promoter was amplified with primer set

2. These products were then combined by a fusion PCR reaction using primer set 3. The PCR reactions

followed a temperature-cycling program which consisted of an initial denaturing step at 98°C for 30 s,

then 35 cycles of denaturing at 98°C for 15 s, annealing at the prescribed temperature, and extension at

72°C for the prescribed length of time, with a final extension of 72°C for 60 s. The annealing

temperatures and extension times can be found in table 1. The resulting PCR products were purified

using a Zymogen DNA Clean & Concentrator kit (Zymo Research Corp., Irvine, CA).

The fusion PCR product was ligated into the pBCphleo plasmid obtained from the Fungal

Genetics Stock Center (Kansas State University, Manhattan, KS). Ligation was performed using T4 DNA

ligase (New England BioLabs, Ipswich, MA), and the product was transformed into *Escherichia coli*. The

resulting plasmids were recovered using a Zippy plasmid miniprep kit (Zymo Research Corp., Irvine, CA).

A portion was analyzed by agarose gel electrophoresis to confirm successful construct formation.

N. fumigata recombinant DNA transformation

The *N. fumigata easA/easG* promoter – *P. camemberti easC* fusion pBCphleo plasmid was

transformed into the *N. fumigata easC* knockout strain according to established methods (Fabian et al.,

2018, Bilovol and Panaccione, 2016). The recipient *N. fumigata* culture was incubated overnight in malt

extract broth. The collected mycelia were incubated at 22°C for 2 hours in 15 mL of 0.7 M NaCl along

with 40 mg of lysing enzyme (Sigma-Aldrich, St. Louis, MO) and 1 g of VinoTastePro (Gusmer Enterprises

Inc., Mountainside, NJ). The resulting protoplasts were filtered, washed by repeated centrifugation and

resuspension, and then incubated with the *N. fumigata* promoter-*P. camemberti easC* pBCphleo plasmid

construct as described previously (Bilovol and Panaccione, 2016). Phleomycin-resistant transformed

protoplasts were transferred to malt extract agar containing phleomycin. Single spore cultures were

then selected, to ensure nuclear purity, and cultured for genotype confirmation by DNA extraction with

PCR (using primer set 4 from table 1) and phenotype confirmation by LC-MS.

mRNA extraction and cDNA generation

Transformants containing-*P. camemberti easC* and that had been verified for their ergot alkaloid

phenotype were re-cultured as mycelial mats in malt extract broth for 3 days at room temperature.

Mats were extracted and flash frozen in liquid nitrogen and pulverized via mortar and pestle. A Qiagen

RNeasy plant kit (Qiagen, Germantown, MD) was used to extract RNA from the cultures; DNA was

removed using on-column Dnase I digestion. The resulting RNA sample was reverse transcribed using

SuperScript IV reverse transcriptase (Thermo Scientific, Waltham, MA) and an oligo(dT) primer. A PCR

reaction using primer set 4 from table 1 was used to amplify *easC* cDNA, which was then analyzed by

agarose gel electrophoresis for length. Sanger sequencing (Eurofins Genomics, Louisville, KY) was used

to confirm the sequence of the PCR product.

LC-MS analysis of ergot alkaloid extracts

High-resolution mass spectra were collected on a Thermo Scientific Q Exactive mass

spectrometer coupled to a Thermo Accela 1250 UHPLC system. The mass spectrometer was operated in

positive ion mode using data dependent acquisition settings. Precursor scans were acquired at 70,000

resolution (at *m/z* = 200) over an 80 to 1200 *m/z* mass range. The 10 most abundant ions from each

precursor scan were selected for HCD fragmentation (NCE = 30) and were analyzed at 35,000 resolution.

Separations were performed on a 2.1 mm by 100 mm Zorbax Eclipse XDB-C18 column (Agilent, Santa

Clara, CA) using a gradient composed of mobile phase A (water, 0.1% formic acid) and mobile B

(acetonitrile, 0.1% formic acid). The sample was loaded at 95% A + 5% B and held for one minute before

ramping linearly to 40% A + 60% B at 20 minutes using a flow rate of 300 μL/min.

Unit mass resolution spectra (figure 5) were collected on a Thermo LCQ Deca XP Plus mass

spectrometer coupled to a Thermo Surveyor HPLC system (Thermo Scientific, Waltham, MA). The mass

spectrometer was configured and operated in the manner according to what is described in Ryan et al.

(2013, 2015). Separations were performed on a 2 mm by 150 mm Phenomenex 4-μm polar RP column

using a gradient composed of mobile phase A (5% acetonitrile, 0.1% formic acid) and mobile phase B

(75% acetonitrile, 0.1% formic acid) at 200 μl/min. Samples were loaded at 14% B, and eluted with a

linear gradient which ramped to 100% B over 20 minutes.

Results

P. camemberti eas pathway is expressed under certain culture conditions

Investigation into culture conditions of *P. camemberti* and *P. biforme* revealed differences in

ergot alkaloid production. Fabian et al. (2018) demonstrated that *P. camemberti eas* genes were

transcribed when the organism was grown on malt extract agar, but not when grown in conditions used

for cheese making. To test for differences between *P. biforme* and *P. camemberti* nutritional regulation

of the *eas* pathway, both species were grown on a modified Czapek's media, and supplied alternately

with glucose, succinate, glycerol and glycine as sole carbon sources. Those *P. biforme* cultures grown on

glucose and succinate containing media (but not the other listed carbon sources) produced

rugulovasines, whereas *P. camemberti* cultures were not able to produce rugulovasines under any

culture conditions. However, under conditions which induced rugulovasines in *P. biforme*, *P. camemberti*

produced N-methyl DMAT (figure 4, figure 5). Cheese samples (commercial and home-made) were

analyzed for N-Me-DMAT, but none was detected.

Rationale for investigating *easC*

Fabian et al. (2018) showed that three *eas* genes of *P. camemberti* (*dmaW, easC, easE*) had

genetic polymorphisms when compared to functional homologs from *P. biforme*. Moreover, they

showed, by heterologous expression of *P. camemberti eas* genes in the corresponding *N. fumigata*

knockout strains, that *P. camemberti easH, easQ, easE* and *dmaW* complemented or augmented (in the

case of *easH* and *easQ*) the corresponding *N. fumigata* mutant and thus encode functional enzymes.

Furthermore, it has been demonstrated that *eas* pathway regulation is intact for *P. camemberti* under

the correct culture conditions (figure 4, figure 5), as evidenced by the presence of N-Me-DMAT and gene

transcripts for *eas* genes. It can be reasoned that there must exist a defect in a gene downstream of N-

Me-DMAT in *P. camemberti*. Since the product of *easC* uses N-Me-DMAT as a substrate, I hypothesized

that *easC* of *P. camemberti* (which had not previously been functionally analyzed but did contain a SNP

relative to the functional allele in *P. biforme*) was dysfunctional (figure 6).

***P. camemberti easC* does not restore *N. fumigata easC* knockout strain's mutant phenotype**

A construct for expressing *easC* in *N. fumigata* was prepared by joining the promoter from the

N. fumigata easA gene to the coding sequences and 3' untranslated sequences of *P. camemberti easC*

(figure 7). When a previously constructed *N. fumigata easC* knockout strain (Goetz et al., 2011) was

transformed with the *N. fumigata easA* promoter + *P. camemberti easC* fusion construct, fumigaclavine

C was not produced. Figure 8 shows the results of a PCR experiment using promoter combination 4 in

table 1*,* where a fragment of genomic DNA was amplified, matching the size of the *P. camemberti easC*

construct (1391 bp). Sanger sequencing of this product confirmed the identity of the amplified gene,

verifying that the gene had been inserted into the *N. fumigata easC* knockout strain (figure 8).

N. fumigata **transcribes and correctly splices the** **P. camemberti easC** **gene**

Figure 6 shows the predicted location of introns and exons for *P. camemberti easC*. The *P.*

camemberti easC genomic sequence was aligned with *easC* from *N. fumigata* and the allele from

another well-characterized ergot alkaloid producer, *Claviceps purpurea* (figure 9). The overall layout of

the *easC* gene is similar among the three species. To confirm that *P. camemberti easC* was transcribed

and properly processed by *N. fumigata*, a reverse transcription-PCR experiment was performed to verify

the presence of correctly spliced mRNA corresponding to *P. camemberti easC*. Figure 8 shows RT-PCR

analysis using primers for *P. camemberti easC.* This analysis revealed a cDNA amplification product with

a reduction in length corresponding to the predicted size of the intron (68 bp). Correct splicing of the

intron was confirmed by Sanger sequencing of the cDNA. Shown in figure 8 is the absence of the intron

with the two exons adjacent to one another in the cDNA. These results indicate that the *P. camemberti*

gene was transcribed and spliced in a manner that was predicted and is consistent with how *N. fumigata*

and *C. purpurea* splice their *easC* genes.

A point mutation in *P. camemberti easC* causes it to lose function

Also confirmed by these Sanger sequencing results (figure 10), is a point mutation of G → T at position 199 of the gene. This results in a GTC codon instead of a GGC codon and causes *P. camemberti* to express valine at position 13 instead of the glycine residue at the same position in the homologous protein from *P. biforme*. Amino acid alignments of *easC* homologs from *P. biforme, N. fumigata, C. purpurea, Periglandula ipomoeae, Metarhizium anisopliae* and *Epichloe inebriens* reveal a conserved polar amino acid residue (Gln, Lys, or Asp) in that position for the other species surveyed (figure 11). *P. biforme easC* codes for a Gly residue at that position, with *P. camemberti* coding for a Val residue. Since this mutation was the only difference between the alleles from *P. camemberti* and *P. biforme*, and because *P. camemberti* accumulated the substrate for EasC (N-Me-DMAT), these results indicate that the mutation of a Gly to a Val residue causes the *P. camemberti* gene to lose the ability to catalyze decarboxylation of methylated DMAT to yield the precursor to chanoclavine-I. While, N-Me-DMAT is not the direct substrate for EasC, the substrate is likely the oxidized diene product of EasE (284 Da). This compound was not directly observed, it was proposed that EasE and EasC form a complex, so that the oxidized diene of N-Me-DMAT is a transient form (Goetz et al., 2011).

Discussion

The inability of *P. camemberti easC* to restore the *N. fumigata easC* knockout strain's production of ergot alkaloids correlates well with the LC-MS and transcriptome results previously reported (Fabian et al., 2018; Lessard et al., 2014). Fabian et al. detected no ergot alkaloids of any kind in cheese cultures of *P.camemberti* and *P. biforme* of varying ages and sources. Also detected were *P. camemberti* gene transcripts for *eas* genes in certain culture conditions but not in cheese cultures. While *P. camemberti*

cannot synthesize rugulovasines, the presence of N-Me-DMAT suggests that regulatory elements needed for *eas* gene expression are still functional. Furthermore, there is a nutritional regulatory element regarding the expression of *eas* genes in both *Penicillium* sp. studied. The fact that succinate and glucose were the only carbon sources that allowed the fungi to produce ergot alkaloids suggests regulatory elements related to the citric acid cycle (succinate enters metabolism as a citric acid cycle intermediate). Most importantly, the data suggest that *easC* is defective in *P. camemberti*.

Residue 13 of EasC from both *Penicillium* spp. differs from the conserved polar amino acid residues seen in other ergot alkaloid synthesizing fungi. Considering this, it is noteworthy that only *P. biforme* (as opposed to *P. camemberti*) retained a functional *easC* gene. Betts and Russell (2007) discuss the consequences of substituting amino acid residues - while substituting polar residues carries little to no penalty in most cases, substitution of a hydrophobic valine residue generally carries a relatively harsh penalty (as in *P. camemberti*). Due to the bulky side chain on valine, this residue can have a restrictive impact on protein backbone folding conformations. In the case of *P. biforme*, the presence of a substituted glycine may impact activity if it occurs at the catalytic site, but otherwise with regards to structure it contributes little more than flexibility due to its lack of side chain. This mutation likely modifies the higher-order structure of the protein in such a way as to impact the catalytic site or to modify the required interaction of EasC with some other protein (*e.g.* EasE).

The data presented here suggest that the ancestor of modern *P. camemberti* had the capacity to synthesize rugulovasines and other ergot alkaloid precursors. While there is a lack of information regarding the toxicity of rugulovasines, they have been shown to be deadly to day-old poultry and to decrease blood pressure of cats (Meurant, 1981). Clearly the presence of these compounds in cheese would be a public health concern. Fortunately, we have shown that two factors prevent the accumulation of these compounds from *P. camemberti*, the fungus used to ripen camembert cheese.,

Due to its use in controlled domesticated processes, and that the end cheese product was destined for

human consumption, there likely was a lack of selective pressure for *P. camemberti* to maintain *easC.*

Figures
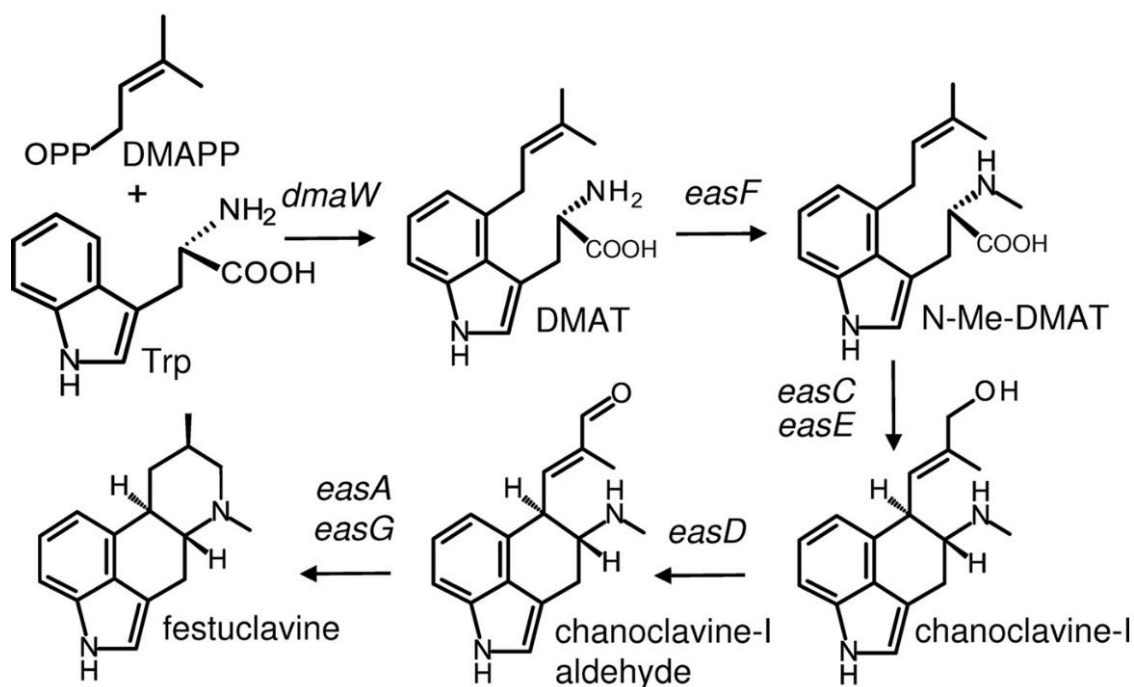
Figure 1. The ergot alkaloid synthesis pathway found in *Neosartorya fumigata*. The steps catalyzed by

dmaW, *easF*, *easC*, *easE*, and *easD* are conserved across many fungi. These genes are found in

Penicillium camemberti and *Penicillium biforme*, which diverge after the easD step. This figure was

originally presented by Fabian et al. (2018).
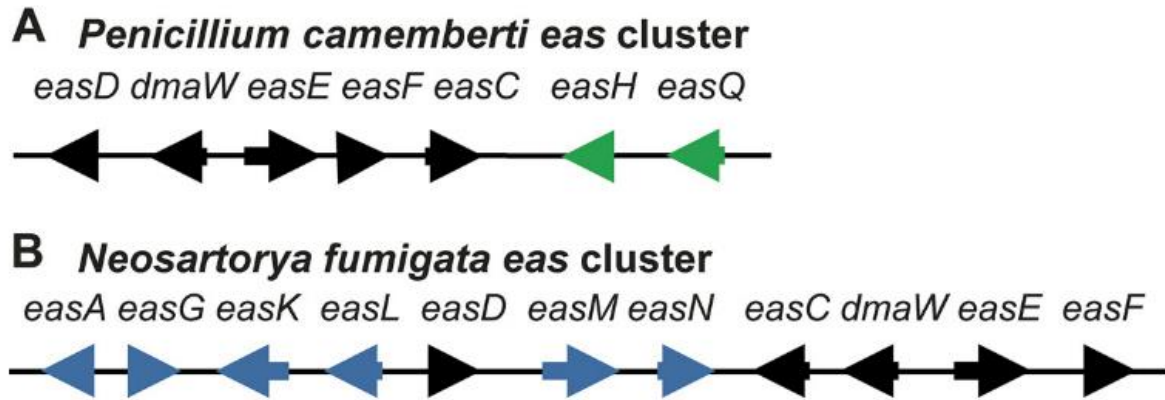
Figure 2. The alignment of ergot alkaloid synthesis genes between *P. camemberti* and *N. fumigata*. The first five genes are conserved between *N. fumigata*, *P. biforme*, and *P. camemberti* (shown in black), while two genes are unique (shown in green) to *P. camemberti* and six genes (shown in blue) are unique to *N. fumigata*. This figure was originally presented by Fabian et al. (2018).
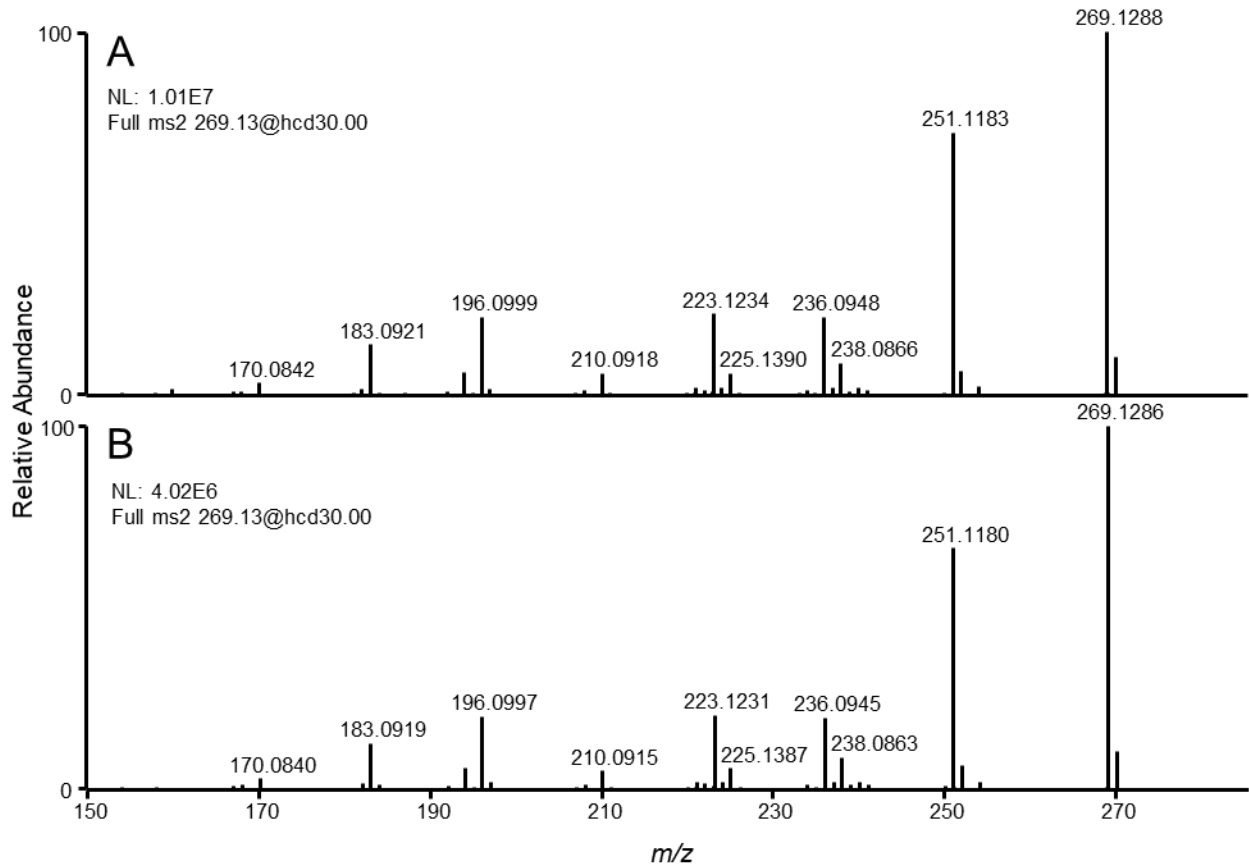
Figure 3. High-resolution mass spectra of ions resulting from fragmentation of the parent ion with a mass consistent with rugulovasine (269.13 *m/z*) in (**A**) *easH/easQ* transformed *N. fumigata easA* knockout and (**B**) *P. biforme*. Data were collected on a Thermo Q Exactive mass spectrometer operated in positive mode.
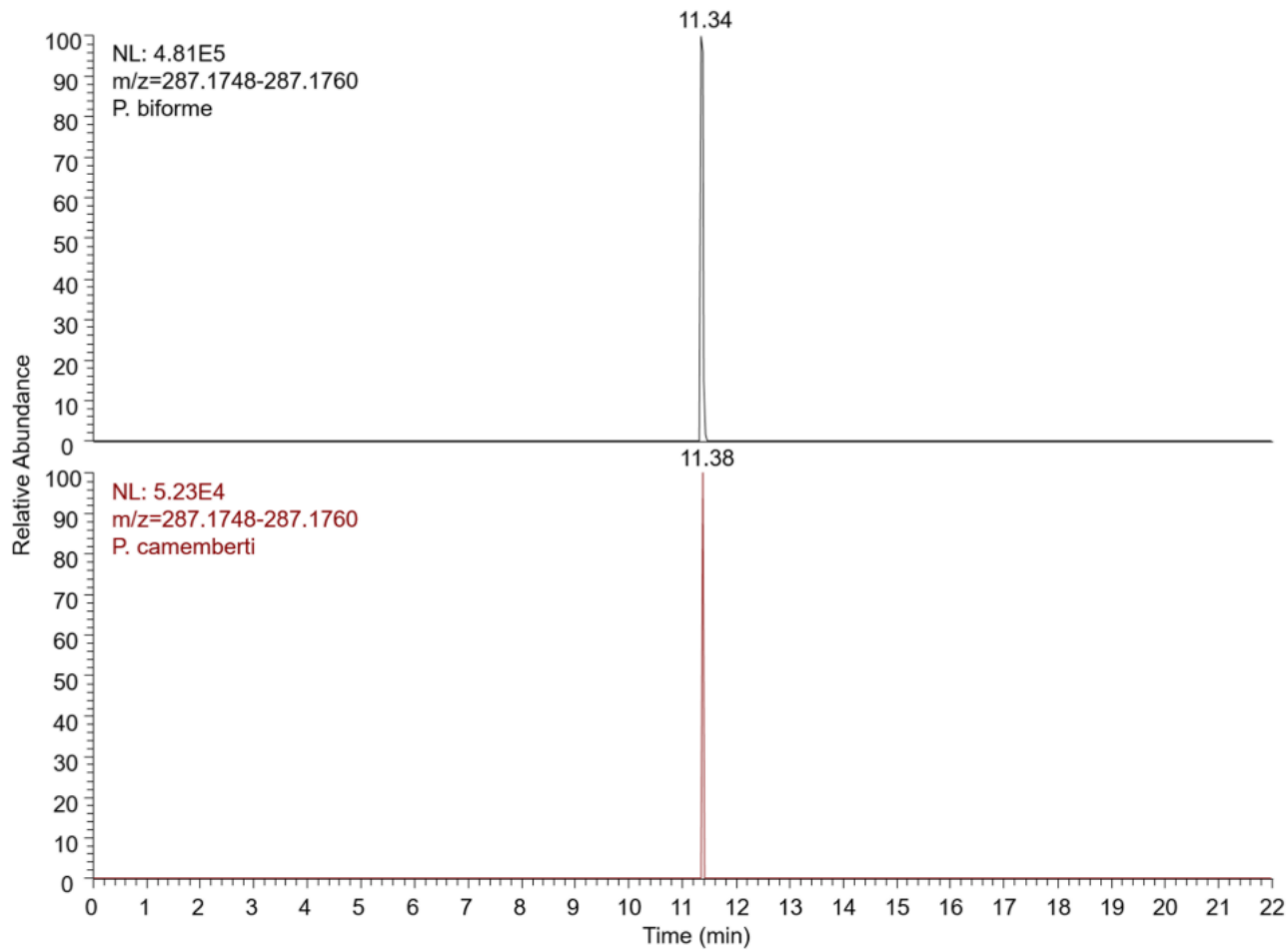
Figure 4. The presence of N-Me-DMAT *in vitro* from *P. biforme* and *P. camemberti* cultures. The chromatogram for 287.1754 *m/z* was extracted using a 2 ppm mass error extraction window. These data were generated using a Thermo Scientific Orbitrap high resolution accurate mass spectrometer.
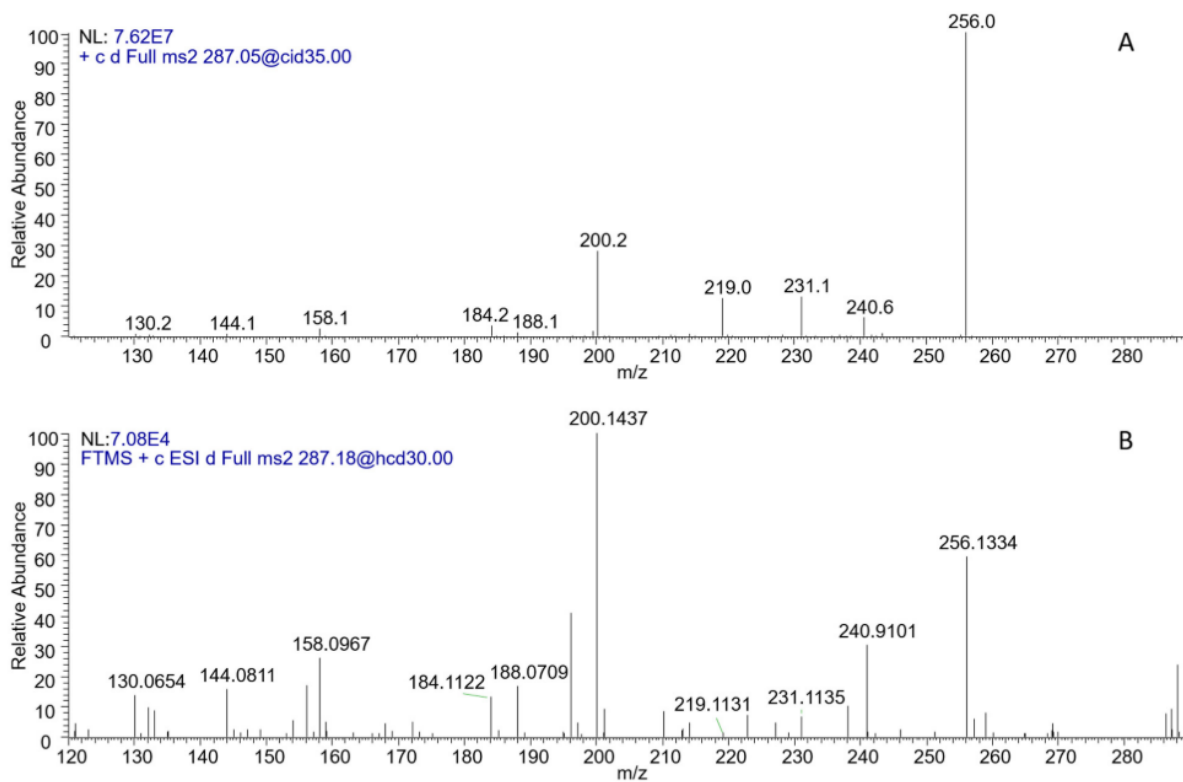
Figure 5. A CID fragmentation spectrum, of parent ion 287.1 *m/z*, was previously acquired from a N-Me-

DMAT standard, using a Thermo Scientific ion trap mass spectrometer (A). The resulting HCD MS/MS

spectrum of the extracted ion (287.1754 *m/z*) at 11.3 minutes from figure 4, generated on a Thermo

Scientific Orbitrap mass spectrometer (B).
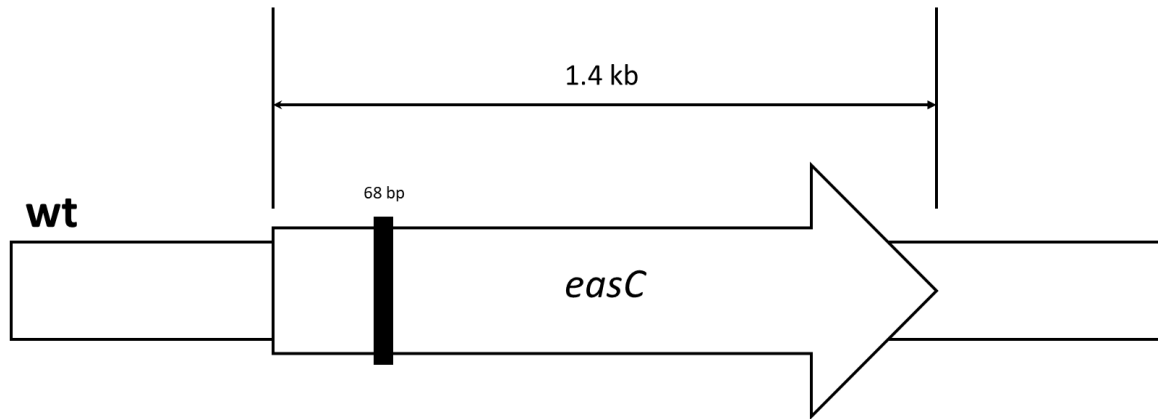
Figure 6. The ergot alkaloid synthesis gene cluster in *P. camemberti* and *P. biforme* contain the *easC* gene the product of which, along with the product of *easE*, decarboxylates methylated DMAT to yield chanoclavine-I. The gene is approximately 1.4 kb long, and contains a putative intron (marked with black) of 68 bp.
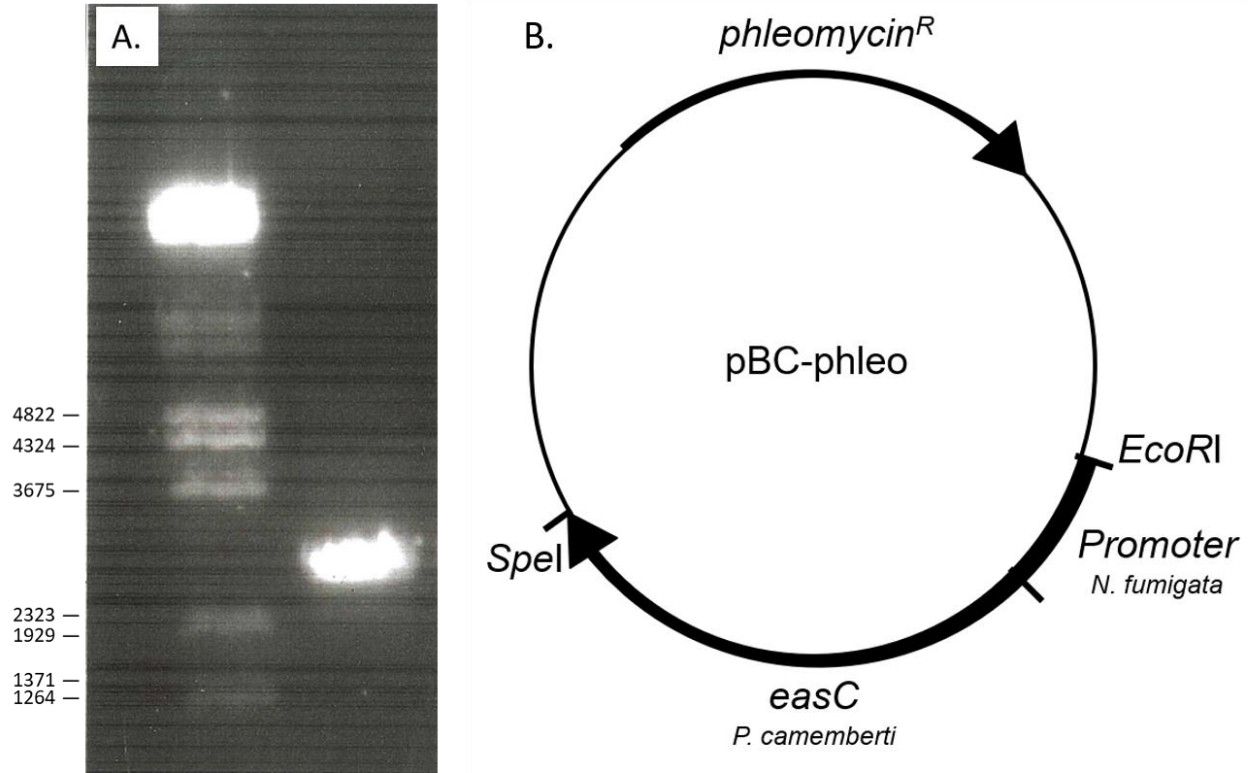
Figure 7. The *easA-easG* promoter from *N. fumigata* is joined to the *P. camemberti easC* gene by fusion PCR (A). The resulting product was inserted in the pBCphleo plasmid as a vector to complement the *N. fumigata easC* KO strain (B).
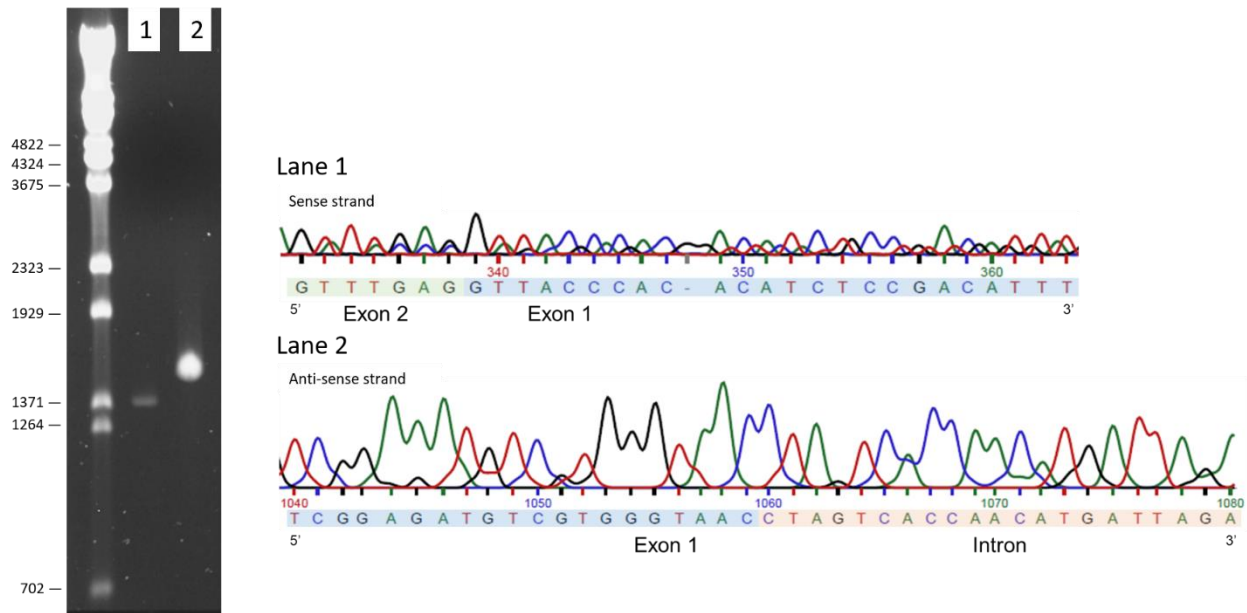
Figure 8. The same primers for *P. camemberti easC* were used to amplify cDNA (lane 1) and genomic

DNA (lane 2) from *N. fumigata easC* knockout transformed with the *P. camemberti easC* gene. Agarose

electrophoresis of the two PCR products shows that the cDNA product is shorter than the genomic DNA

product. Sanger sequencing of the product in lane 1 shows exon 1 (blue) directly adjacent to exon 2

(green), while sequencing of the product in lane 2 shows exon 1 (blue) directly adjacent to the intron.
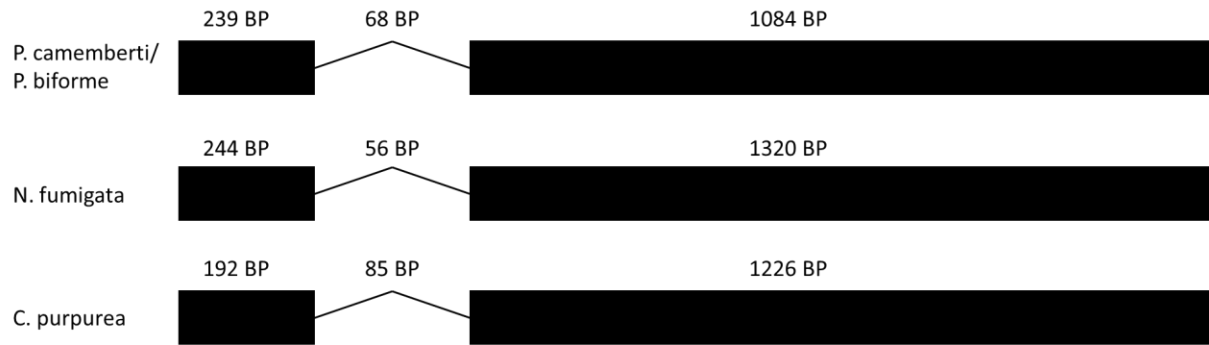
Figure 9. The splicing scheme for *easC* from *P. camemberti* is consistent with the splicing scheme from the homologous gene found in *P. biforme*, *N. fumigata* and *C. purpurea*.
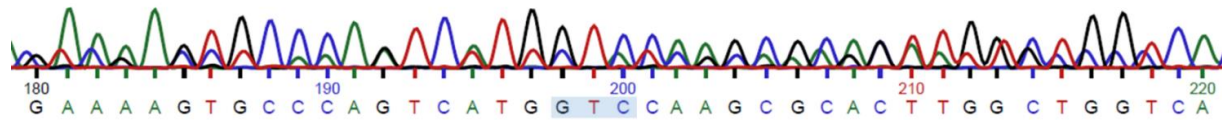
Figure 10. Sanger sequencing results of *P. camemberti easC* cDNA as expressed in *N. fumigata easC*

knockout demonstrate a point mutation (highlighted in blue). This results in a GTC codon (valine) instead

of a GGC codon (glycine) at that position.
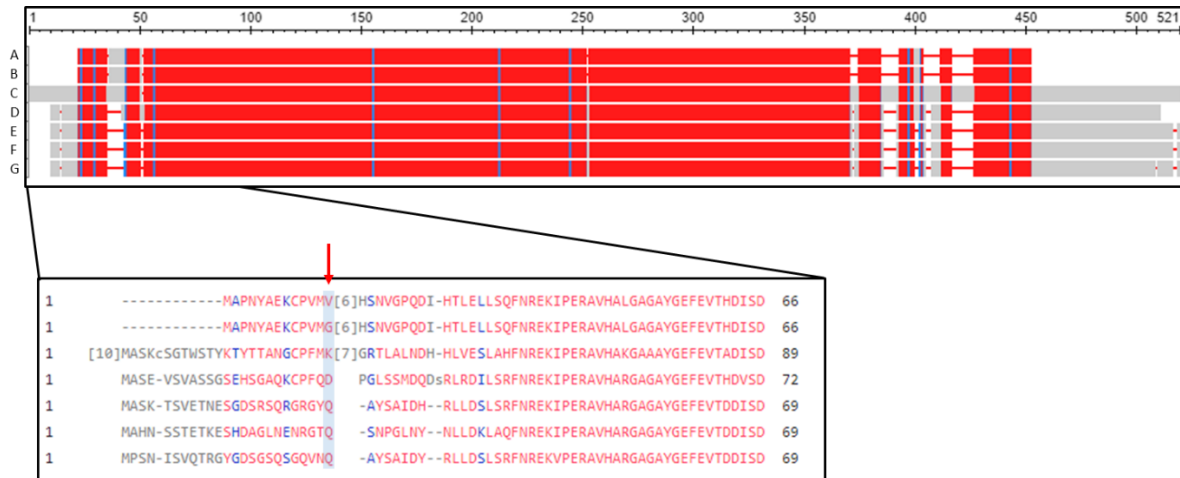
Figure 11. COBALT multiple protein sequence alignment of the *easC* gene from seven fungi – (A)

Penicillium camemberti, (B) *Penicillium biforme*, (C) *Neosartorya fumigata*, (D) *Claviceps purpurea*, (E)

Periglandula ipomoeae, (F) *Metarhizium anisopliae*, (G) *Epichloë inebrians*. Amino acid residues in red

are conserved, while those in blue are not. The valine at position 13 in the *P. camemberti* sequence has

been highlighted. This position is occupied by a large polar residue (> 115 Da) in five of the seven

species, while *P. biforme* has a small glycine (57 Da) and *P. camemberti* has a valine which is larger (99

Da) and non-polar.

Table 1. PCR primers and method

Primer Set	Primer sequence (5' to 3')	Product and length (bp)	Annealing temperature (°C), Extension time (s)
1	GAGTAGGCACTCCGCACCATGATCTCAATAGATCATATTCC + GTGGCCTTGAGTTAATCTGAAGC	*easC* with *N. fumigata* promoter extension (2,098)	59, 40
2	GGAATATGATCTATTGAGATCATGGTGCGGAGTGCCTACTC + GCTTCTAATCCACCAAGTACTTGG	*N. fumigata* promoter with *easC* extension (809)	59, 40
3	CTCGGAATTCGCTTCTAATCCACCAAGTACTTGG + GAGTACTAGTGTGGCCTTGAGTTAATCTGAAGC	N. fumigata promoter – *easC* fusion (2,886)	59, 90
4	GCCTGGAGCAAGTCAAGTTCTCA + TGATTCGACGATTCTCTCCACC	*easC* cDNA (1,323), *easC* genomic DNA (1,391)	62, 45

CHAPTER 5
SUMMARY

Literature until now focused primarily on *Periglandula* sp. as ergot alkaloid producers and as endophytes of *Ipomoea* sp. Since *Periglandula* sp. cannot be grown in pure culture, DNA sequencing has been limited and genetic manipulation could not be conducted. This has so far prevented tracking of these fungal species through molecular means and has prevented the advancement of ergot alkaloid biosynthesis studies like those that have been conducted with other ergot alkaloid producing species. The first objective of this study was to investigate the impacts of *Periglandula* species infections on seed metabolite profiles of plants belonging to the genus *Ipomoea.* The second goal of this work was to investigate whether there are non-ergot alkaloid biomarkers of fungal presence in seed extracts. The results of the metabolomics data presented indicate that apart from ergot alkaloids, *Periglandula* species have a minimal impact on the metabolome of seeds of their host plants. We also found no evidence of non-ergot alkaloid producing *Periglandula* species in seeds of the *Ipomoea* species analyzed. This may suggest that, along with their plant embryo hosts, the *Periglandula* sp. endophytes are metabolically dormant at this stage.

One unexpected and novel observation from this study was the presence of the glycoside form of ergonovine. A major form of this compound, synthesized through plant vascular processes, is likely a galactoside but chromatography data suggest that there may be other hexose residue forms. That plants from a variety of clades produce this ergonovine glycoside suggests that plants such as *Ipomoea* sp. and *Achnatherum* sp. (which harbor ergonovine producing endophytes) have harnessed this process to gain a competitive advantage in their ecological niche. Of the ergot alkaloids described in *Ipomoea* species, only ergonovine was found to be present in a glycoside form. Given ergonovine's activity against

invertebrates, more work could be done to evaluate the toxicity of this compound (Durden et al., 2019,

Kaur et al., 2020). Perhaps plants harboring ergonovine-producing endophytes are using this form of the

molecule to mobilize it throughout their tissues or a particularly potent toxin in their fight against

invertebrates who would consume them.

 Metarhizium spp. are known to harbor a wide range of specialized metabolites as virulence

factors in their role as entomopathogens (Freimoser et al., 2003). With *M. brunneum* shown to produce

ergot alkaloids (Leadmon et al., 2020), this was the first study to investigate the activity of these

compounds as a virulence factor for this fungus. Additionally, the development of a hypervirulent ergot

alkaloid deficient *M. brunneum* mutant (Tyo et al., 2018) provided a useful resource to test the effect of

ergot alkaloids as virulence factors. One of the stark differences between the two strains of *M.*

brunneum was that the ergot alkaloid deficient strain would not produce conidia after killing its insect

host (Tyo et al., 2018). The results of protein expression analysis and of the melanization analysis point

to ergot alkaloids as immune system suppressants in these infections. As an entomopathogen, *M.*

brunneum is walking a narrow path of consuming its host, but not killing it too fast as to prevent

creating an environment non-conducive to its own reproduction. As the hypervirulent strain of *M.*

brunneum kills its host so fast that it cannot conidiate, it prevents its own spread which makes it a worse

pathogen from an ecological perspective. An ergot alkaloid competent strain of *M. brunneum* is able to

kill its host slowly enough that it does not prevent the conidiation process, which allows the next

generation of *M. brunneum* spread to other insects, making it ultimately a more efficient pathogen.

 The observation of ergot alkaloids in fungi infecting insects and significant roles for those ergot

alkaloids is in stark contrast with the observations of *M. brunneum* growing in conjunction with a plant

host, where it produced no ergot alkaloids (Leadmon et al., 2020). This contrast perhaps points to *M.*

brunneum regularly growing alongside plants but changing its metabolism to infect insects when the

opportunity presents itself. Indeed, the phylogeny of Clavicipitaceous fungi shows that the ancestral

lifestyle for many of these fungi was as entomopathogens, and that some interactions with plants led to species adopting endophyte lifestyles (Spatafora et al., 2007). Given this perspective, it is possible that an ancestor of fungi like *Epichloë* spp. and *Periglandula* spp. accumulated enough mutations that they became poor entomopathogens and adapted to a strictly endophyte lifestyle. These interactions provided a benefit to their plant hosts whom they helped to protect from mammalian and insect herbivores. Furthermore, if *Metarhizium brunneum* symbiosis is an example, perhaps fungi promote metabolic shifts in plants which help the plant to tolerate other stressful conditions.

Ergot alkaloid synthesis likely does carry a cost for the fungus, especially considering that the synthetic pathways are inefficient and accumulate many intermediates. Apparently in the case of the domesticated fungus *Penicillium camemberti* this cost was prohibitive for the long-term maintenance of a pathway that was not needed. In the absence of competitors in the cheese making process, a mutation which inactivated the early step of the ergot alkaloid synthesis pathway could persist. This shows that there is some evolutionary benefit to species such as *P. ipomoea* and *M. brunneum* to maintain these inefficient pathways. As discussed in the introduction, an ergot different ergot alkaloid compounds have different activities against different organisms (*e.g.* mammals, insects, bacteria). Perhaps the advantage for these fungi to maintain such an inefficient pathway allows them to produce compounds with a central scaffold. This central scaffold can be modified in a way that gives the compounds activity in the wide range of circumstances the fungi find themselves in and help them or their symbionts to compete in their ecological niches. While this is not a new idea, it was proposed by Panaccione (2005), the new information provided in this dissertation help to provide evidence for that central concept regarding the ecological function of ergot alkaloids and their synthesis.

REFERENCES

Baskys, A., & Hou, A. C. (2007). Vascular dementia: pharmacological treatment approaches and perspectives. In *Clinical interventions in aging*.

Beaulieu, W. T. (2014). *Chemical, ecological and co-evolutionary interactions between Periglandula and Convolvulaceae*. Indiana University.

Beaulieu, W. T., Panaccione, D. G., Hazekamp, C. S., Mckee, M. C., Ryan, K. L., & Clay, K. (2013). Differential Allocation of Seed-Borne Ergot Alkaloids During Early Ontogeny of Morning Glories (Convolvulaceae). *Journal of Chemical Ecology*, *39*(7), 919–930. https://doi.org/10.1007/s10886-013-0314-z

Beaulieu, W. T., Panaccione, D. G., Ryan, K. L., Kaonongbua, W., & Clay, K. (2015). Phylogenetic and chemotypic diversity of Periglandula species in eight new morning glory hosts (Convolvulaceae). *Mycologia*, *107*(4), 667–678. https://doi.org/10.3852/14-239

Belser-Ehrlich, S., Harper, A., Hussey, J., & Hallock, R. (2013). Human and cattle ergotism since 1900: Symptoms, outbreaks, and regulations. In *Toxicology and Industrial Health*. https://doi.org/10.1177/0748233711432570

Betts, M. J., & Russell, R. B. (2007). Amino-Acid Properties and Consequences of Substitutions. In *Bioinformatics for Geneticists: A Bioinformatics Primer for the Analysis of Genetic Data: Second Edition*. https://doi.org/10.1002/9780470059180.ch13

Boldo, J. T., Junges, A., do Amaral, K. B., Staats, C. C., Vainstein, M. H., & Schrank, A. (2009). Endochitinase CHI2 of the biocontrol fungus Metarhizium anisopliae affects its virulence toward the cotton stainer bug Dysdercus peruvianus. In *Current Genetics*. https://doi.org/10.1007/s00294-009-0267-5

Bonuccelli, U., Del Dotto, P., & Rascol, O. (2009). Role of dopamine receptor agonists in the treatment of early Parkinson's disease. *Parkinsonism and Related Disorders*. https://doi.org/10.1016/S1353-8020(09)70835-1

Brown, S. E., Howard, A., Kasprzak, A. B., Gordon, K. H., & East, P. D. (2008). The discovery and analysis of a diverged family of novel antifungal moricin-like peptides in the wax moth Galleria mellonella. *Insect Biochemistry and Molecular Biology*, *38*(2), 201–212. https://doi.org/10.1016/j.ibmb.2007.10.009

Buszewski, B., & Noga, S. (2012). Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique. *Analytical and Bioanalytical Chemistry*, *402*(1), 231–247. https://doi.org/10.1007/s00216-011-5308-5

Cheeseman, K., Ropars, J., Renault, P., Dupont, J., Gouzy, J., Branca, A., Abraham, A. L., Ceppi, M., Conseiller, E., Debuchy, R., Malagnac, F., Goarin, A., Silar, P., Lacoste, S., Sallet, E., Bensimon, A., Giraud, T., & Brygoo, Y. (2014). Multiple recent horizontal transfers of a large genomic region in cheese making fungi. *Nature Communications*. https://doi.org/10.1038/ncomms3876

Cooper, J. E. (2007). Early interactions between legumes and rhizobia: Disclosing complexity in a molecular dialogue. In *Journal of Applied Microbiology*. https://doi.org/10.1111/j.1365-

2672.2007.03366.x

Craig, A. M., Klotz, J. L., & Duringer, J. M. (2015). Cases of ergotism in livestock and associated ergot alkaloid concentrations in feed. *Frontiers in Chemistry*. https://doi.org/10.3389/fchem.2015.00008

Donnet, A., Braunstein, D., Pradel, V., Sciortino, V., Allaria-Lapierre, V., Micallef, J., & Lanteri-Minet, M. (2016). Ergot Use and Overuse: A Pharmacoepidemiology Retrospective Cohort Study. *Headache*. https://doi.org/10.1111/head.12776

Duan, Z., Shang, Y., Gao, Q., Zheng, P., & Wang, C. (2009). A phosphoketolase Mpk1 of bacterial origin is adaptively required for full virulence in the insect-pathogenic fungus Metarhizium anisopliae. *Environmental Microbiology*. https://doi.org/10.1111/j.1462-2920.2009.01961.x

Durden, L., Wang, D., Panaccione, D., & Clay, K. (2019). Decreased Root-Knot Nematode Gall Formation in Roots of the Morning Glory Ipomoea tricolor Symbiotic with Ergot Alkaloid-Producing Fungal Periglandula Sp. *Journal of Chemical Ecology*, *November*. https://doi.org/10.1007/s10886-019-01109-w

Eckart, E., & Pertz, H. (1999). ANTIMICROBIAL AND ANTITUMOR EFFECTS OF ERGOT ALKALOIDS AND THEIR DERIVATIVES. In *Ergot: the genus Claviceps* (pp. 441–449).

Fabian, S. J., Maust, M. D., & Panaccione, D. G. (2018). Ergot alkaloid synthesis capacity of Penicillium camemberti. *Applied and Environmental Microbiology*, *84*(19), 1–14. https://doi.org/10.1128/AEM.01583-18

Faria, M. R. d., & Wraight, S. P. (2007). Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control*. https://doi.org/10.1016/j.biocontrol.2007.08.001

Flieger, M., Zelenkova, N. F., Sedmera, P., Kren, V., Novàk, J., Rylko, V., Sajdl, P., & Rehacek, Z. (1989). Ergot alkaloid glycosides from saprophytic cultures of claviceps, I. Elymoclavine fructosides. *Journal of Natural Products*, *52*(3), 506–510. https://doi.org/10.1021/np50063a007

Florea, S., Panaccione, D. G., & Schardl, C. L. (2017). Ergot alkaloids of the family clavicipitaceae. *Phytopathology*, *107*(5), 504–518. https://doi.org/10.1094/PHYTO-12-16-0435-RVW

Floss, H. G. (1976). Biosynthesis of ergot alkaloids and related compounds. *Tetrahedron*, *32*(8), 873–912. https://doi.org/10.1016/0040-4020(76)85047-8

Gerhards, N., Neubauer, L., Tudzynski, P., & Li, S. (2014). Biosynthetic Pathways of Ergot Alkaloids. *Toxins*, *6*, 3281–3295. https://doi.org/10.3390/toxins6123281

Goetz, K. E., Coyle, C. M., Cheng, J. Z., Connor, S. E., & Panaccione, D. G. (2011). Ergot cluster-encoded catalase is required for synthesis of chanoclavine-I in Aspergillus fumigatus. *Current Genetics*, *57*(3), 201–211. https://doi.org/10.1007/s00294-011-0336-4

Haarmann, T., Rolke, Y., Giesbert, S., & Tudzynski, P. (2009). Plant diseases that changed the world: Ergot: from witchcraft to biotechnology. *Molecular Plant Pathology*, *10*(4), 563–577. https://doi.org/10.1111/j.1364-3703.2009.00548.x

Hara, S., & Yamakawa, M. (1995). Moricin, a novel type of antibacterial peptide isolated from the silkworm, Bombyx mori. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.270.50.29923

Howard, W. F., Mcfadden, P. R., & Keettel, W. C. (1964). Oxytocic Drugs in Fourth Stage of Labor. *JAMA:*

The Journal of the American Medical Association.
https://doi.org/10.1001/jama.1964.03070060021004

Hu, X., Xiao, G., Zheng, P., Shang, Y., Su, Y., Zhang, X., Liu, X., Zhan, S., St. Leger, R. J., & Wang, C. (2014). Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1412662111

Hutner, S. H., Provasoli, L., Schatz, A., & Haskins, C. P. (1950). Some Approaches to the Study of the Role of Metals in the Metabolism of Microorganisms. *Source: Proceedings of the American Philosophical Society*.

Ichou, F., Schwarzenberg, A., Lesage, D., Alves, S., Junot, C., MacHuron-Mandard, X., & Tabet, J. C. (2014). Comparison of the activation time effects and the internal energy distributions for the CID, PQD and HCD excitation modes. *Journal of Mass Spectrometry*, *49*(6), 498–508. https://doi.org/10.1002/jms.3365

Kaur, N., Cooper, W. R., Duringer, J. M., Badillo-Vargas, I. E., Esparza-Díaz, G., Rashed, A., & Horton, D. R. (2018). Survival and development of potato psyllid (Hemiptera: Triozidae) on Convolvulaceae: Effects of a plant-fungus symbiosis (Periglandula). *PLoS ONE*. https://doi.org/10.1371/journal.pone.0201506

Kaur, N., Cooper, W. R., Duringer, J. M., Rashed, A., Badillo-Vargas, I. E., Esparza-Díaz, G., & Horton, D. R. (2020). Mortality of Potato Psyllid (Hemiptera: Triozidae) on Host Clippings Inoculated With Ergot Alkaloids. *Journal of Economic Entomology*, *113*(5), 2079–2085. https://doi.org/10.1093/jee/toaa144

Kind, T., Tsugawa, H., Cajka, T., Ma, Y., Lai, Z., Mehta, S. S., Wohlgemuth, G., Barupal, D. K., Showalter, M. R., Arita, M., & Fiehn, O. (2018). Identification of small molecules using accurate mass MS/MS search. *Mass Spectrometry Reviews*, *37*(4), 513–532. https://doi.org/10.1002/mas.21535

Klotz, J. L. (2015). Activities and effects of ergot alkaloids on livestock physiology and production. In *Toxins*. https://doi.org/10.3390/toxins7082801

Křen, V., Flieger, M., & Sajdl, P. (1990). Glycosylation of ergot alkaloids by free and immobilized cells of Claviceps purpurea. *Applied Microbiology and Biotechnology*, *32*(6), 645–650. https://doi.org/10.1007/BF00164733

Křen, V., Sedmera, P., Havlíček, V., & Fišerová, A. (1992). Enzymatic galactosylation of ergot alkaloids. *Tetrahedron Letters*, *33*(47), 7233–7236. https://doi.org/10.1016/S0040-4039(00)60881-X

Kucht, S., Groß, J., Hussein, Y., Grothe, T., Keller, U., Basar, S., König, W. A., Steiner, U., & Leistner, E. (2004). Elimination of ergoline alkaloids following treatment of Ipomoea asarifolia (Convolvulaceae) with fungicides. *Planta*, *219*(4), 619–625. https://doi.org/10.1007/s00425-004-1261-2

Leadmon, C. E., Sampson, J. K., Maust, M. D., Macias, A. M., Rehner, S. A., Kasson, M. T., & Panaccione, D. G. (2020). Several Metarhizium Species Produce Ergot Alkaloids in a Condition-Specific Manner. *Applied and Environmental Microbiology*, *86*(14), 1–14. https://doi.org/10.1128/AEM.00373-20

Lessard, M. H., Viel, C., Boyle, B., St-Gelais, D., & Labrie, S. (2014). Metatranscriptome analysis of fungal strains Penicillium camemberti and Geotrichum candidum reveal cheese matrix breakdown and potential development of sensory properties of ripened Camembert-type cheese. *BMC Genomics*.

https://doi.org/10.1186/1471-2164-15-235

Lesur, A., & Domon, B. (2015). Advances in high-resolution accurate mass spectrometry application to targeted proteomics. *Proteomics*, *15*(5–6), 880–890. https://doi.org/10.1002/pmic.201400450

Li, Y., Fan, Y., Ma, Y., Zhang, Z., Yue, H., Wang, L., Li, J., & Jiao, Y. (2017). Effects of Exogenous γ-Aminobutyric Acid (GABA) on Photosynthesis and Antioxidant System in Pepper (Capsicum annuum L.) Seedlings Under Low Light Stress. *Journal of Plant Growth Regulation*. https://doi.org/10.1007/s00344-016-9652-8

Lorenz, N., Olšovská, J., Šulc, M., & Tudzynski, P. (2010). Alkaloid cluster gene ccsA of the ergot fungus Claviceps purpurea encodes chanoclavine I synthase, a flavin adenine dinucleotide-containing oxidoreductase mediating the transformation of N-methyl-dimethylallyltryptophan to chanoclavine I. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.00737-09

Mak, P., Zdybicka-Barabas, A., & Cytryńska, M. (2010). A different repertoire of Galleria mellonella antimicrobial peptides in larvae challenged with bacteria and fungi. *Developmental and Comparative Immunology*, *34*(10), 1129–1136. https://doi.org/10.1016/j.dci.2010.06.005

Mc Namara, L., Carolan, J. C., Griffin, C. T., Fitzpatrick, D., & Kavanagh, K. (2017). The effect of entomopathogenic fungal culture filtrate on the immune response of the greater wax moth, Galleria mellonella. *Journal of Insect Physiology*, *100*(February), 82–92. https://doi.org/10.1016/j.jinsphys.2017.05.009

Meurant, G. (1981). Roquefortines. In *Handbook of Toxic Fungal Metabolites* (pp. 528–544). https://doi.org/10.1016/c2009-0-03073-6

Nagy-Szakal, D., Barupal, D. K., Lee, B., Che, X., Williams, B. L., Kahn, E. J. R., Ukaigwe, J. E., Bateman, L., Klimas, N. G., Komaroff, A. L., Levine, S., Montoya, J. G., Peterson, D. L., Levin, B., Hornig, M., Fiehn, O., & Lipkin, W. I. (2018). Insights into myalgic encephalomyelitis/chronic fatigue syndrome phenotypes through comprehensive metabolomics. *Scientific Reports*, *8*(1), 1–11. https://doi.org/10.1038/s41598-018-28477-9

Nakhleh, J., El Moussawi, L., & Osta, M. A. (2017). The Melanization Response in Insect Immunity. In *Advances in Insect Physiology*. https://doi.org/10.1016/bs.aiip.2016.11.002

Nisa, H., Kamili, A. N., Nawchoo, I. A., Shafi, S., Shameem, N., & Bandh, S. A. (2015). Fungal endophytes as prolific source of phytochemicals and other bioactive natural products: A review. In *Microbial Pathogenesis*. https://doi.org/10.1016/j.micpath.2015.04.001

Panaccione, D. G. (2005). Origins and significance of ergot alkaloid diversity in fungi. *FEMS Microbiology Letters*, *251*(1), 9–17. https://doi.org/10.1016/j.femsle.2005.07.039

Panaccione, D. G. (2011). Ergot Alkaloids. In *Industrial Applications*. https://doi.org/10.1007/978-3-642-11458-8_9

Panaccione, D. G., & Arnold, S. L. (2017). Ergot alkaloids contribute to virulence in an insect model of invasive aspergillosis. *Scientific Reports*. https://doi.org/10.1038/s41598-017-09107-2

Panaccione, D. G., Cipoletti, J. R., Sedlock, A. B., Blemings, K. P., Schardl, C. L., Machado, C., & Seidel, G. E. (2006). Effects of ergot alkaloids on food preference and satiety in rabbits, as assessed with gene-knockout endophytes in perennial ryegrass (Lolium perenne). *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf060626u

Parish, J. A., McCann, M. A., Watson, R. H., Hoveland, C. S., Hawkins, L. L., Hill, N. S., & Bouton, J. H. (2003). Use of nonergot alkaloid-producing endophytes for alleviating tall fescue toxicosis in sheep. *Journal of Animal Science*. https://doi.org/10.2527/2003.8151316x

Parish, J. A., McCann, M. A., Watson, R. H., Paiva, N. N., Hoveland, C. S., Parks, A. H., Upchurch, B. L., Hill, N. S., & Bouton, J. H. (2003). Use of nonergot alkaloid-producing endophytes for alleviating tall fescue toxicosis in stocker cattle. *Journal of Animal Science*. https://doi.org/10.2527/2003.81112856x

Peregrín-Alvarez, J. M., Sanford, C., & Parkinson, J. (2009). The conservation and evolutionary modularity of metabolism. *Genome Biology*. https://doi.org/10.1186/gb-2009-10-6-r63

Perez-Lloret, S., & Rascol, O. (2010). Dopamine receptor agonists for the treatment of early or advanced Parkinsons disease. In *CNS Drugs*. https://doi.org/10.2165/11537810-000000000-00000

Potter, D. A., Tyler Stokes, J., Redmond, C. T., Schardl, C. L., & Panaccione, D. G. (2008). Contribution of ergot alkaloids to suppression of a grass-feeding caterpillar assessed with gene knockout endophytes in perennial ryegrass. *Entomologia Experimentalis et Applicata*. https://doi.org/10.1111/j.1570-7458.2007.00650.x

Ramesh, S. A., Tyerman, S. D., Gilliham, M., & Xu, B. (2017). γ-Aminobutyric acid (GABA) signalling in plants. In *Cellular and Molecular Life Sciences* (Vol. 74, Issue 9, pp. 1577–1603). https://doi.org/10.1007/s00018-016-2415-7

Robinson, S. L., & Panaccione, D. G. (2015). Diversification of ergot alkaloids in natural and modified fungi. *Toxins*, *7*(1), 201–218. https://doi.org/10.3390/toxins7010201

Ropars, J., Rodríguez De La Vega, R. C., López-Villavicencio, M., Gouzy, J., Sallet, E., Dumas, É., Lacoste, S., Debuchy, R., Dupont, J., Branca, A., & Giraud, T. (2015). Adaptive horizontal gene transfers between multiple cheese-associated fungi. *Current Biology*. https://doi.org/10.1016/j.cub.2015.08.025

Ryan, K. L., Akhmedov, N. G., & Panaccione, D. G. (2015). Identification and structural elucidation of ergotryptamine, a new ergot alkaloid produced by genetically modified aspergillus nidulans and natural isolates of epichlo?? species. *Journal of Agricultural and Food Chemistry*, *63*(1), 61–67. https://doi.org/10.1021/jf505718x

Ryan, K. L., Moore, C. T., & Panaccione, D. G. (2013). Partial reconstruction of the ergot alkaloid pathway by heterologous gene expression in Aspergillus nidulans. *Toxins*. https://doi.org/10.3390/toxins5020445

Schardl, C. L., Young, C. A., Hesse, U., Amyotte, S. G., Andreeva, K., Calie, P. J., Fleetwood, D. J., Haws, D. C., Moore, N., Oeser, B., Panaccione, D. G., Schweri, K. K., Voisey, C. R., Farman, M. L., Jaromczyk, J. W., Roe, B. A., O'Sullivan, D. M., Scott, B., Tudzynski, P., … Zeng, Z. (2013). Plant-Symbiotic Fungi as Chemical Engineers: Multi-Genome Analysis of the Clavicipitaceae Reveals Dynamics of Alkaloid Loci. *PLoS Genetics*, *9*(2). https://doi.org/10.1371/journal.pgen.1003323

Schrimpe-Rutledge, A. C., Codreanu, S. G., Sherrod, S. D., & McLean, J. A. (2016). Untargeted Metabolomics Strategies—Challenges and Emerging Directions. *Journal of the American Society for Mass Spectrometry*. https://doi.org/10.1007/s13361-016-1469-y

Sheehan, G., & Kavanagh, K. (2018). Analysis of the early cellular and humoral responses of galleria mellonella larvae to infection by Candida albicans. *Virulence*, *9*(1), 163–172.

https://doi.org/10.1080/21505594.2017.1370174

Shymanovich, T., Saari, S., Lovin, M. E., Jarmusch, A. K., Jarmusch, S. A., Musso, A. M., Charlton, N. D., Young, C. A., Cech, N. B., & Faeth, S. H. (2014). Alkaloid Variation Among Epichloid Endophytes of Sleepygrass (Achnatherum robustum) and Consequences for Resistance to Insect Herbivores. *Journal of Chemical Ecology*, *41*(1), 93–104. https://doi.org/10.1007/s10886-014-0534-x

Spatafora, J. W., Sung, G. H., Sung, J. M., Hywel-Jones, N. L., & White, J. F. (2007). Phylogenetic evidence for an animal pathogen origin of ergot and the grass endophytes. *Molecular Ecology*, *16*(8), 1701–1711. https://doi.org/10.1111/j.1365-294X.2007.03225.x

St. Leger, R. J., Joshi, L., Bidochka, M. J., & Roberts, D. W. (1996). Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.93.13.6349

St. Leger, R. J., Wang, C., & Fang, W. (2011). New perspectives on insect pathogens. *Fungal Biology Reviews*, *25*(2), 84–88. https://doi.org/10.1016/j.fbr.2011.04.005

Steiner, U., Leibner, S., Schardl, C. L., Leuchtmann, A., & Leistner, E. (2011). *Periglandula* , a new fungal genus within the Clavicipitaceae and its association with Convolvulaceae. *Mycologia*, *103*(5), 1133–1145. https://doi.org/10.3852/11-031

Steiner, U., & Leistner, E. (2012). Ergoline alkaloids in convolvulaceous host plants originate from epibiotic clavicipitaceous fungi of the genus Periglandula. *Fungal Ecology*, *5*(3), 316–321. https://doi.org/10.1016/j.funeco.2011.04.004

Tfelt-Hansen, P. C., & Koehler, P. J. (2008). History of the use of ergotamine and dihydroergotamine in migraine from 1906 and onward. *Cephalalgia*. https://doi.org/10.1111/j.1468-2982.2008.01578.x

Tfelt-Hansen, Peer C. (2013). Triptans and ergot alkaloids in the acute treatment of migraine: similarities and differences. In *Expert review of neurotherapeutics*. https://doi.org/10.1586/14737175.2013.832851

Vijayakumari, K., & Puthur, J. T. (2016). γ-Aminobutyric acid (GABA) priming enhances the osmotic stress tolerance in Piper nigrum Linn. plants subjected to PEG-induced stress. *Plant Growth Regulation*. https://doi.org/10.1007/s10725-015-0074-6

Vogel, H., Altincicek, B., Glöckner, G., & Vilcinskas, A. (2011). A comprehensive transcriptome and immune-gene repertoire of the lepidopteran model host Galleria mellonella. *BMC Genomics*, *12*. https://doi.org/10.1186/1471-2164-12-308

Wallwey, C., & Li, S.-M. (2011). Ergot alkaloids: structure diversity, biosynthetic gene clusters and functional proof of biosynthetic genes. *Nat. Prod. Rep.*, *28*(3), 496–510. https://doi.org/10.1039/C0NP00060D

Wallwey, C., Matuschek, M., & Li, S. M. (2010). Ergot alkaloid biosynthesis in Aspergillus fumigatus: Conversion of chanoclavine-I to chanoclavine-I aldehyde catalyzed by a short-chain alcohol dehydrogenase FgaDH. *Archives of Microbiology*, *192*(2), 127–134. https://doi.org/10.1007/s00203-009-0536-1

Wang, B., Kang, Q., Lu, Y., Bai, L., & Wang, C. (2012). Unveiling the biosynthetic puzzle of destruxins in Metarhizium species. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1115983109

Wang, J., & Hou, B. (2009). Glycosyltransferases: Key players involved in the modification of plant secondary metabolites. *Frontiers of Biology in China*, *4*(1), 39–46. https://doi.org/10.1007/s11515-008-0111-1

Xu, J., Liu, T., Yang, S., Jin, X., Qu, F., Huang, N., & Hu, X. (2019). Polyamines are involved in GABA-regulated salinity-alkalinity stress tolerance in muskmelon. *Environmental and Experimental Botany*. https://doi.org/10.1016/j.envexpbot.2019.05.011

Xu, X. X., Zhong, X., Yi, H. Y., & Yu, X. Q. (2012). Manduca sexta gloverin binds microbial components and is active against bacteria and fungi. *Developmental and Comparative Immunology*, *38*(2), 275–284. https://doi.org/10.1016/j.dci.2012.06.012

Yeung, P. K. (2018). Metabolomics and biomarkers for drug discovery. *Metabolites*, *8*(1), 10–12. https://doi.org/10.3390/metabo8010011

Yi, H. Y., Chowdhury, M., Huang, Y. D., & Yu, X. Q. (2014). Insect antimicrobial peptides and their applications. In *Applied Microbiology and Biotechnology*. https://doi.org/10.1007/s00253-014-5792-6

Young, C. A., Schardl, C. L., Panaccione, D. G., Florea, S., Takach, J. E., Charlton, N. D., Moore, N., Webb, J. S., & Jaromczyk, J. (2015). Genetics, genomics and evolution of ergot alkaloid diversity. In *Toxins* (Vol. 7, Issue 4, pp. 1273–1302). https://doi.org/10.3390/toxins7041273

Yu, J., Schorlemer, M., Gomez Toledo, A., Pett, C., Sihlbom, C., Larson, G., Westerlind, U., & Nilsson, J. (2016). Distinctive MS/MS Fragmentation Pathways of Glycopeptide-Generated Oxonium Ions Provide Evidence of the Glycan Structure. *Chemistry - A European Journal*, *22*(3), 1114–1124. https://doi.org/10.1002/chem.201503659