Chromobacterium RiPP-like Biosynthetic Gene Clusters May Influence Inhibition of Globisporagium ultimum

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Abstract

Globisporangium ultimum is a fungal-like organism called an oomycete that attacks germinating seedlings and causes pre- and post-emergence damping-off in many hosts. Synthetic fungicides are primarily used to control this disease, but since oomycetes are not fungi, they do not share many of the same targets that would inhibit true fungi. Therefore, there are a limited number of oomycete-active chemicals used in seed treatments, posing the need for alternative options. The soil is a natural source of bacteria that produces bioactive metabolites. A survey of soybean spermosphere microbiomes at the Old Rotation revealed that historically irrigated soil has a significantly higher concentration of purple-pigmented bacterium than non-irrigated soil. Sequencing 16S rRNA from these cultures revealed them to be Janthinobacterium, a close relative of Chromobacterium, two genera of Gram-negative bacteria known for producing purple pigment violacein, a known antifungal agent. When co-cultured with C. violaceum, the aerial hyphae of G. ultimum collapsed, and violacein was observed to travel along the oomycete hyphae over time. When subcultured to fresh agar Petri dishes, G. ultimum died, leading to the hypothesis that contact-independent and dependent interactions were lethal to G. ultimum. To test the hypothesis of contact-independent antibiosis and its potential to protect soybean seeds from infection, sixteen Chromobacterium and Janthinobacterium isolates from at least five species were tested for their ability to protect soybean. Soybeans were co-cultured with and without both G. ultimum and each bacterial isolate, then disease severity was rated for each seed. Isolates that significantly reduced disease severity were identified. Violacein production did not correlate with disease inhibition, leading to the hypothesis that other secondary metabolites are responsible for G. ultimum inhibition. Five strains

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of *Chromobacterium* had their genomes sequenced to compare differences in biosynthetic gene clusters between inhibitory and non-inhibitory strains.

Future experiments will involve testing bioactive *Chro-mobacterium* and *Janthinobacterium* strains in the growth chamber to test their inhibitory abilities in soil.

Key Words: co-culture; biosynthetic gene cluster; ribosomally synthesized and post-translationally modified peptide (RiPP)

Introduction

Globisporangium Background

Globisporangium ultimum, formerly Pythium ultimum (Nguyen et al. 2022), is an oomycete and ubiquitous plant pathogen with a wide host range (Beckerman, 2011) that causes root rot as well as pre-and post-emergence damping off. It can produce oospores that can survive in the soil for many years, making them difficult to irradicate. Damping-off occurs when G. ultimum colonizes seeds during or soon after germination, causing seed disintegration and seedling collapse. In older plants, infection can lead to root rot ("Diseases Caused by Pythium"). G. ultimum can produce zoospores that move through the water toward their host. Therefore, moist environments, such as those created by heavy rainfall or overwatering, create ideal infection conditions, especially at lower temperatures, which slows seed germination and increases exposure to pathogens. Re-planting crops in a field once infected with the pathogen can result in the re-occurrence of stand failure, even if preventive fungicide treatment is used (Groves and Smith, 2013). Crop rotation has limited effectiveness due to the wide host range of G. ultimum. Current G. ultimum control measures include using fungicides, but G. ultimum has many key

differences in molecular targets commonly attacked by fungicides. For example, ergosterol biosynthesis inhibitors are a major class of agricultural fungicides, but this synthesis pathway is not observed in oomycetes, rendering these compounds ineffective against oomycetes (Alcazar-Fuoli and Mellado, 2013). Additionally, there is concern about overreliance on the few chemical fungicides used to control oomycetes since, in some cases, they may contribute to the rise of fungicide-resistant plant pathogen populations. As such, bioactive microorganisms and their metabolites are alternatives for G. ultimum control. Several Trichoderma species have been investigated as G. ultimum biocontrol agents (Sanchez-Montesinos et al. 2019). In addition, Bacillus species have been shown to inhibit plant disease and promote plant growth by secreting secondary metabolites and phytohormones (Miljaković 2020). Understudied and unexplored bacterial species may also produce novel anti-oomycete secondary metabolites.

Chromobacterium

Chromobacterium is an eleven-species genus of motile Gram-negative proteobacteria known for producing violacein, a purple-pigmented secondary metabolite with exhibited in vitro inhibition of bacteria, viruses, protozoa, and even tumors (De Souza 1999; Andrighetti-Fröhner, 2003; Leon, 2001; Melo, 2016). Violacein helps protect the bacterium from oxidative stress (Konzen et. al. 2006). The outer membrane of Chromobacterium exhibits greater endotoxicity and enhanced resistance to phagocytosis than other Gram-negative species (Bennett, 2020). Janthinobacterium, like Chromobacterium, is Gram-negative, motile, and produces violacein. One species, J. lividum, suppresses fungal growth on amphibian skin (Becker et al. 2009). The author also describes significant in vitro inhibition of Fusarium graminearum when co-cultured with violacein-producing Janthinobacterium.

Existing Bacterial Genomes

Both *Chromobacterium* and *Janthinobacterium* lack comprehensive genomic analysis in the literature as neither genus has complete genomes, and available gene cluster analyses widely focus on the violacein operon. Complete bacterial genomes are realistic with long-read third-generation sequencing like Oxford Nanopore combined with short-read and accurate Illumina sequencing. When biosynthetic gene clusters can be hundreds of thousands of base pairs long, adding long-read sequencing helps discover new and complete biosynthetic gene clusters that may encode novel antifungals. Therefore, complete genomes for other species that may contain novel biosynthetic gene clusters have yet to be explored.

Ribosomally Synthesized and Post-Translationally Modified Peptide (RiPP) Biosynthetic Gene Clusters

RiPPs have been recently discovered via genome sequencing to be a fifth class of natural biosynthesized products, in addition to the previously known classes of terpenoids, alkaloids, polyketides, and non-ribosomal peptides (Arnison, et. al. 2013). RiPPs have been identified among Bacteria, Archaea, and Eukarya. Since the discovery of RiPPs and the characterization of their nomenclature and properties, further research into the gene clusters that synthesize these peptides has been ongoing.

Preliminary Experiments

Preliminary experiments characterizing the soybean spermosphere microbiome from the Auburn University Old Rotation (Auburn, AL, USA) in irrigated versus non-irrigated soil were done (Harrison et. al., 2022). Twelve isolates of Chromobacterium were transferred from cryotubes stored at -80°C with a sterile loop to 10mL of tryptic soy broth and grown in the shaker at 28 degrees Celsius at 250 rpm for 72 hours. After three days of growth, 10 microliters of isolate broth were pipetted at the points of a concentric pentagon on quarter-strength potato dextrose agar plates (Figure 1). Soybeans were sterilized in a 10% bleach solution, rinsed with sterile water, and allowed to air dry in a biosafety cabinet. Sterilized soybeans were then placed on top of each of the isolates, and equally sized plugs of G. ultimum were placed at the center of each plate. Each treatment had three replicates. Positive controls contained no bacteria, only G. ultimum. Negative controls contained neither bacteria nor G. ultimum. Co-cultures were incubated at room temperature 72 hours, and the disease ratings of each seed per treatment and replicate were taken according to Noel et al. 2019 (Table 1).



Fig. 1. Rendering of co-culture setup with *G. ultimum*, bacterial sample, and sterilized soybean. Created with BioRender.

Table 1: Disease severity chart for seed ratings adaptedfrom Noel et al. 2019.

Disease Severity Rating	Description	
	0 germinated healthy seedling	
	1 germinated, limited to no discoloration	
-	2 germinated, non-coalesced lesions present	
S2.C	3 germinated, coalesced lesions present	
Care -	4 not germinated and/or seed fully colonized	

Preliminary findings indicated *C. sphagnii* and *C. vacinni* had a statistically significant amount of disease inhibition against *G. ultimum*, and *C. subtsugae* showed significant disease inhibition to a lesser degree.

In this experiment, we observed an increased concentration of purple-pigmented bacteria in irrigated soil. Sequencing the 16S rRNA gene revealed these bacteria to be *Janthinobacterium*. To test potential disease inhibition, *Janthinobacterium* isolated from irrigated soil was co-cultured with *G. ultimum* and monitored. At four days post-inoculation, *G. ultimum* aerial hyphae collapse was observed. At seven days post-inoculation, further hyphae collapse was noted, and violacein was observed traveling across the hyphae of *G. ultimum*.



Fig. 2. Left- *Janthinobacterium* and *Globisporangium ultimum* at four days post-inoculation. Right- *Janthinobacterium* and *G. ultimum* at seven days post-inoculation.

At seven days post-inoculation, *G. ultimum* was subcultured from close, mid, and far distances from the *Janthinobacterium*. In each subculture, *G. ultimum* did not grow, indicating the oomycete had died in the presence of *Janthinobacterium*.

Hypothesis

We hypothesized that other violacein-producing species, like *Chromobacterium*, produce metabolites that may be inhibitory toward *G. ultimum* and that there are differences in biosynthetic gene clusters encoding these metabolites between inhibitory and non-inhibitory strains. Therefore, the objectives of this paper were to identify inhibitory and non-inhibitory strains of *Chromobacterium*, sequence their genomes, and analyze the differences in biosynthetic gene clusters across strains. We analyzed in vitro relationships between *Chromobacterium* species and *G. ultimum* and describe the genomic relationships of the observed inhibitory effects described.

Methods

Differential Time to Production of Violacein

Over incubation, *Chromobacterium* colonies start clear or opaque yellow, then will develop violacein as

the colonies grow in broth. Complete violacein development was experimentally defined as the entire broth tube having deep purple pigment. We incubated strains of *Chromobacterium* in TSB for five weeks and identified when violacein production was generated.

Genome Sequencing of Chromobacterium

Complete genomes of five *Chromobacterium* strains with differential inhibition toward *G. ultimum* were sequenced with a combination of short-read Illumina and long-read Oxford Nanopore sequencing. Tryptic soy agar plates of *Chromobacterium* were sent to SeqCenter (Pittsburgh, PA), the genomic DNA was extracted, and the DNA sequenced. Genome sequence assembly was conducted by SeqCenter staff. Prediction of biosynthetic gene clusters was performed with AntiSmash v.6.1.1 (Blin et. al. 2021). Taxonomic identity based on genomic data was confirmed using the MiGa pipeline (Rodriguez-R et. al. 2018, 2020).

Results

Differential Inhibition Based on *Chromobacterium* Species and Violacein Production

Each species represented had varying degrees of inhibition within and between themselves. Figure 5 shows two inhibitory strains, CHV and CHB-4, and one non-inhibitory strain, CHA-B. CHV shows clear inhibition zones surrounding the seeds, while CHB-4 did not have a clear zone of inhibition (ZOI) but had healthy seeds. CHA-B is a non-inhibitory strain that did not show clear ZOIs or healthy seed germination.



Fig. 3. DSI averages across isolates for co-culture experiments.



Fig. 4. Co-culturing plates of CHV and CHB (inhibitory strains) and CHA-B (non-inhibitory strain) alongside positive (right) and negative (left) controls

Means were separated based on a one-way ANOVA with Tukey HSD.

Analysis of the disease severity indexes in preliminary experiments (Figure 3) shows that *Chromobacterium amazonense* and one *Chromobacterium sphagnii* isolate had no significant difference in DSI against the positive control. On the other hand, two other *Chromobacterium sphagnii* isolates, alongside Chromobacterium vacinni and one *Chromobacterium substugae* isolate, did not have any significant difference in DSI against the negative control. One *Chromobacterium substugae* had no statistically significant difference in DSI between the positive and negative control.

It was thought at the beginning of the experiment that violacein would be responsible for disease inhibition against *G. ultimum*, but some of the most inhibitory strains, including CHB-4, did not produce violacein under laboratory conditions. Some of the least inhibitory strains also vigorously produced violacein. This led to the hypothesis that other secondary metabolites were responsible for *G. ultimum* inhibition. Significant

variation in the time from incubation to complete violacein development across isolates was also observed.

Genome Sequencing of Chromobacterium

CHV, CHA-1, CHS-2, CHB-4, and CHA-B were sent for genome sequencing. To examine biosynthetic gene clusters encoding for secondary metabolites, each genome was analyzed in antiSMASH. Of the 52 gene clusters identified across the five strains, only six were returned with 75% or greater similarity to a known gene cluster. 18 returned with no identifiable similar biosynthetic gene cluster, indicating they are novel (Table 2).

 Table 2: Overview of biosynthetic gene clusters by isolate.

Isolate	MiGA Species ID	Co- culture DSI Average	Number of BGCs	Unique BGC Types
CHV	Chromobacterium amazonense	25.83	8	6
CHA-1	Chromobacterium vaccinni*	65.83	9	8
CHS-2	Chromobacterium. vaccinni	27.5	11	9
CHA-B	Chromobacterium subtsugae	65.83	12	9
CHB-4	Chromobacterium substugae	37.5	12	9

The co-culture DSI average was calculated across several rounds of co-culture experiments beyond the preliminary rounds.

The co-culture DSI average was calculated across several rounds of co-culture experiments beyond the preliminary rounds.

There was a total of 18 different BGCs between the five strains. Comparison of BGC types between strains indicated that neither the number of unique BGC types nor overall BGCs correlated to disease inhibition.

RiPP-like BGCs are Twice as abundant in Inhibitory Strains

The three most inhibitory by DSI average had 31 BGCs, while the two least inhibitory had 19 BGCs. In particular, the inhibitory strains had double the number of ribosomally synthesized and post-translationally modified peptides (RiPP)-like BGCs. Each of these clusters returned with 0% similarity to clusters described in antiSMASH, indicating they were novel.

Discussion

In this study, we investigated the inhibitory effect of violacein-producing bacteria in the genus Chromobacterium against the plant pathogen *G. ultimum*. The most important results indicated that violacein production did not necessarily correlate with the inhibition of *G. ultimum* as initially hypothesized, meaning that other metabolites may be responsible for the inhibitory responses observed. The genomes of the *Chromobacterium* strains indicated a diverse set of novel BGCs that may be responsible or partially responsible for the inhibition observed. Inhibitory strains had twice the number of RiPP-like BGCs than non-inhibitory strains. The metabolites that these BGCs produce may be exploited for further characterization and use as alternative compounds against *G. ultimum*.

There are a few options for delivering these metabolites into the soil for seed protection, one being alginate beads. When bacteria or metabolites are mixed with alginate and pipetted dropwise into sterile 2.0% calcium chloride on a stir plate, uniform spherical beads are created, in which the bacteria and metabolites can survive for months, improving their shelf life. Future testing of different formulations for inclusion in the soil or as a seed dressing is needed.

The phylogenies of the RiPP-like BGCs were studied using NCBI BLAST. They were largely similar between isolates with similar DSIs, but the RiPP-like BGCs from non-inhibitory isolates with larger DSIs did not appear to be as closely related. Given that most of the BGCs found in the isolates studied were novel, further examination into characterizing the chemical structure and effect on G. ultimum growth is needed. Ideally, the metabolites encoded by these BGCs will be isolated, extracted, and tested for disease inhibition individually. Isolation of these metabolites and mass production into a seed treatment is ideal, but methods for the storage and delivery of *Chromobacterium* into the soil or as seed dressings are needed. Further research into the environmental determinants of metabolite production is needed to understand differences in disease inhibition within Chromobacterium species. The long-term goals of this research are to produce a biological control method that is commercially available for farmers to use.

Acknowledgements

Thank you to Dr. Martin Chilvers at Michigan State University for providing the *Globisporangium ultimum*, Dr. Mary Mendonça for providing several of the *Chromobacterium* and *Janthinobacterium* isolates, and the Auburn University Undergraduate Research Fellowship and Auburn University Honors College for providing funding for this project.

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Authors Biography



Charis Harrison is a Fall 2022 graduate with a B.S. in microbiology and a minor in public health. She was an undergraduate researcher in the Noel Lab in the Department of Entomology and Plant Pathology starting in January 2021. In July 2023, she will begin a joint MD/MSPH program at the University of Alabama at Birmingham with a concentration in outcomes research.



Laura Rodriguez obtained a bachelor's degree in Environmental Microbiology from the University of Puerto Rico Arecibo. Interested in learning more about plant-fungal interaction decided to pursue a Master's in the Plant Pathology Department at Auburn University. Here, she mainly works with the detection of fungi that can alleviate water stress in peanut plants and the identification of changes in peanut soil microbial communities under different water gradients.



Oluwakemisola Olofintila is a scientist focused on plant microbiology and plant biotechnology. As a research assistant in plant pathology, she conducted research on the assembly of the crop microbiome and biocontrol of fungal/ oomycete pathogens using molecular biology techniques and bioinformatic tools. She currently works on the development of transgenic crops as a research associate at the Donald Danforth Plant Science Center.



Zachary Noel is an assistant professor of plant health and microbiomes in the Entomology and Plant Pathology department at Auburn University.