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Sympatric Soil Microbe Interactions between Streptomyces and Fusarium Isolates

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SYMPATRIC SOIL MICROBE INTERACTIONS BETWEEN *STREPTOMYCES* AND
FUSARIUM ISOLATES

By

Lehren Ander Olk-Szost

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SYMPATRIC SOIL MICROBE INTERACTIONS BETWEEN *STREPTOMYCES* AND
FUSARIUM ISOLATES

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ABSTRACT

SYMPATRIC SOIL MICROBE INTERACTIONS BETWEEN *STREPTOMYCES* AND
FUSARIUM ISOLATES

By
Lehren Ander Olk-Szost

Interkingdom interactions between soil bacteria and fungi may play a critical role in occurrence of disease suppressive soils, yet our understanding of these interactions remains limited. *Streptomyces* are well-known producers of antimicrobial compounds important to medicine and agriculture. Production of these secondary metabolites is often mediated by quorum sensing. Most *Streptomyces* research occurs in single species experiments, yet new metabolites have been discovered in interspecies co-culture experiments. Interspecies, intergenic, and interkingdom co-culture research will likely reveal many valuable compounds, and strengthen our understanding of complex ecological interactions in soil microbiomes. Interactions between sympatric *Streptomyces* and *Fusarium* isolates from disease suppressive soils were investigated in this study. Dual layer agar inhibition assay revealed inhibition of *Streptomyces* by *Fusarium* in all pairwise combinations, while only 46% of pairwise combinations showed *Fusarium* inhibition by *Streptomyces*. *Streptomyces* isolate S2-2 was shown to produce antifungal compounds in a population density dependent manner, likely governed by quorum sensing. Exposure of S2-2 broth culture to conditioned media which likely contained autoinducers from mature S2-2 culture was shown to cause a significant increase in antifungal production earlier than control groups. Simultaneous inoculation of S2-2 and *Fusarium* isolate F10-8 was shown to cause a significant decrease in antifungal production. Exploring these interactions is of great importance for antimicrobial drug discovery, identifying useful microbial biological control agents, and improving our ability to promote disease suppression in soils.

Keywords: *Streptomyces, Fusarium,* Antifungal, Sympatric, Microbial Biological Control

Agent, Co-culture, Disease Suppressive Soils

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Deoxynivalenol (DON)

Induced systemic response (ISR)

Copper (Cu)

Plant growth promoting microbes (PGPM)

Microbial biological control agents (MBCA)

Disease suppressive soils (DSS)

Homoserine-lactone (HSL)

γ-butyrolactone (GBL)

Oatmeal Agar (OA)

Starch casein agar (SCA)

Potato Dextrose Agar (PDA)

Potato Dextrose Broth (PDB)

Two-thirds strength tryptic soy broth (TSB)

Two-thirds strength tryptic soy agar (TSA)

Deionized sterile water (dsH$_2$O)

Polyether sulfone (PES)

Antifungal assay plates (AAP)

Percent inhibition of radial growth (PIRG)

filtrate percent inhibition (FPI)

Conditioned media (CM)

Ethyl acetate (EtOAc)

INTRODUCTION

Fusarium

     Fusarium is a genus of fungi known for their mycotoxin production and contamination of food crops. Classification of *Fusarium* relies on a polyphasic approach, with genomic, chemotaxonomic, and phenotypic information taken into account. There are over an estimated 70 species in this genus, and with a few hundred more putative species (Munkvold 2017). First described by Heinrich Friedrich Link in 1809, the criteria for categorizing species into the genus *Fusarium* has gone through many revisions over the years, with the number of species classified as *Fusarium* ranging from over 1000 described at the turn of the 20th century, down to around 30 by the 1970s, with debate over subspecies and criteria causing this number to continue to vary for decades (Zemankova & Lebeda 2001).

     Much of the original criteria were based on physiological characteristics, with the matter further confused by classification divisions surrounding preference for naming based on anamorph or teleomorph, causing *Fusarium* to often be counted in other genera such as *Haematonectria, Neocosmospora,* and *Gibberella*. The most current understanding of what makes a fungal species a *Fusarium* fungus is based on the phylogenetic species concept, along with criteria laid out in a land-mark paper in 2013 by Geiser et al., titled *One Fungus, One Name: Defining the Genus Fusarium in a Scientifically Robust Way That Preserves Longstanding Use.* The paper, with contributions from 66 authors and over a dozen universities, national lab institutes, and research centers from around the world, proposed criteria for defining *Fusarium* by anamorph classification, along with keeping widely studied *Fusarium* species in the

genus in order to prevent detrimental and confusing revisions, and lastly to make any future revisions and additions based on strong molecular phylogenetic evidence (Gieser et al., 2013). The confusion surrounding naming of *Fusarium* species is understandable when considering the wide range of habitats and morphology for this genus.

Isolates of *Fusarium* have been recovered from soil and from plants roots, shoots, stalks, and flowers. Many of these fungi lack a teleomorph, although many economically and medically important species have teleomorphs (Nesic et al., 2014). Outside of molecular genetic testing, practical methods for determining if a fungal isolate is a *Fusarium* rely on morphology and ability to hybridize with other strains. Species identification is compared by shape and size of microconidia, macroconidia, sporodochia, chlamydospores, as well as ability to cross with other strains or species, and more general characteristics such as pigment production, odor and growth rate, as well as the symptoms of disease if isolated from the site of infected plant material (Summerell 2003).

Many *Fusarium* are capable of producing mycotoxins, and are responsible for significant crop loss annually. The degree and prevalence of toxin production in crops is dependent on physical factors, chemical factors, and biological factors. Moisture and temperature are two of the most influential factors determining mold growth and toxin production (Bryden 2012). There are many types of mycotoxins produced by various *Fusarium* species, of which the negative effects on humans and animals can be both acute and chronic. The estrogenic compound zearalenone has been reported to cause negative effects in humans and animals at levels as low as 1-5 ppm in food material (Nelson et al., 1994). *Fusarium* can also produce a group of cancer-promoting metabolites called fumonisins, as well as moniliformin, which is highly toxic to chicks and rats (Nesic et al., 2014). The trichothecene mycotoxin deoxynivalenol (DON) is of

great concern as it is associated with *Fusarium* Head Blight (FHB), and is known for causing outbreaks of feed refusal and emesis in animals, especially pigs, and is thought to effect humans as well. The United States Food and Drug Administration recommends DON levels not exceed levels of 1 ppm in food (Lilleboe 2019). An economically important loss of cereal grains can be attributed to FHB.

Fusarium head blight refers to the disease caused by a variety of fungi in the genus *Fusarium*. Infection of wheat by *Fusarium* causes spoilage of the wheat kernel by fungal production of sesquiterpenoid trichothecene toxins, such as deoxynivalenol (Miller 1994). The three most common causal agents of FHB are *Fusarium culmorum, Fusarium graminearum,* and *Fusarium avenaceum*, however there are many other less pathogenic *Fusarium* species which can still cause an opportunistic infection and are also toxigenic (Bottalico & Perrone 2002). An estimated direct economic loss of $870 million across several varieties of cereal grain during 1998-2000, and an estimated secondary loss of $2.3 billion was attributed to FHB in the northern United States during those years (Nganje et al., 2004). There has been a long systematic effort to combat FHB, including selection of FHB resistant cultivars, crop rotation regimes, and the field application of fungicides. These efforts have ranged from being somewhat effective to being very unsuccessful in preventing major crop loss/spoilage to FHB (Siranidou et al., 2002). *Fusarium* remains an economically significant cause of crop loss.

Not all *Fusarium* species are known for their pathogenicity. There have been many studies which have isolated *Fusarium* from soils and plants which are not plant pathogens and do not cause crop spoilage. Instead these nonpathogenic *Fusarium* have potential application as microbial biological control agents. Nonpathogenic *Fusarium* have been documented as

pathogen antagonists and competitors, inducers of plant systemic resistance, and plant growth promoters, preventing crop loss and increasing yield (Patil & Sriram 2020).

Nonpathogenic strains of *F. oxysporum* have been associated with a reduction of pathogenic infection of *F. oxysporum* and panama wilt disease in Banana (*Mesa acuminate)* cultivars. This association is likely due to non-pathogenic strains antagonizing and competing with pathogenic *Fusarium* (Kumari & Kumar 2015). Another way in which nonpathogenic *Fusarium* may be beneficial is by the elicitation of an induced systemic response (ISR) in host plants, providing resistance to infection by pathogenic fungi. Eggplants seedlings vaccinated by injection with conidial suspension of a nonpathogenic *Fusarium* isolate showed significant reduction in severity of verticillium wilt, attributed to an induced systemic response (Gizi et al., 2011). Another study demonstrated treatment of hydroponically grown lettuce with either the culture filtrate or spore suspension from nonpathogenic *F. oxysporum f.sp. lactucae* was able to significantly reduce lettuce root rot from several pathogenic fungi, and additionally to act as a strong plant growth promoter to the lettuce (Thongamngam & Jaenaksorn 2017).

While many species of *Fusarium* are medically and agriculturally significant due to their ability to cause disease and crop loss, there are also many strains which can be beneficial as biocontrol agents. These may prove to be useful as an alternative to other conventional agricultural pest control practices such as application of metal dusts, sulfur, naturally occurring chemical pesticides, or synthesized pesticides.

Fungicides and Conventional Pest Control

Many different strategies to combat fungal infection in crops can be employed. These include regulatory measures such as quarantine and seed inspection, cultural methods such as crop rotation, sanitation, and improved growing conditions, biological methods such as breeding of resistant varieties and use of microbials, and chemical controls which require seed, soil, foliar and post-harvest application of fungicides or chemical preservatives and cleaners to eliminate mycotoxins or presence of fungal contamination (Morton & Staub 2008).

The use of chemical treatment is widespread for pest control. The year 2014 was a recent peak in total world-wide pesticide use, with Asia having used two million tonnes, America used one million tonnes, Europe used 477 hundred thousand tonnes, Africa used 95 thousand tonnes, and Oceania used 55 thousand tonnes (Food and Agriculture Organization 2022). The global fungicide market in 2020 was estimated at 17 Billion USD, and is projected to be over 25 billion USD by 2028 (Fortune Business Insights 2022). The use of chemical fungicides in-situ by either foliar application or soil drench pose significant environmental risks.

The environmental risks associated with chemical fungicide use have undergone far less research than the risks of insecticides and herbicides. It is estimated that less than 13% of studies on the effects of pesticides between 1991 and 2013 were on fungicide use, a small fraction compared to 62% for insecticides and 24% for herbicides (Köhler & Triebskorn 2013). In a national survey of agrochemical residues in surface and ground waters conducted in 2006 by the United States Geological Survey, only one out of 75 agrochemicals in the analytical screens was a fungicide (Gillom et al., 2006). These proportions seem to fall short of addressing the scale of world-wide fungicide use, and is not indicative for the potential risks. The widespread use of

pesticides like DDT before proper research and consideration of health risks and environmental damage provides a cautionary tale that should not be repeated with fungicides.

Fungicides are designed to inhibit biosynthesis, energy production, cell division, and generally the growth of fungus, however the mechanisms targeted by fungicides are often universal in other domains of living organisms. Residual fungicide accumulation and run off into ground water and aquatic systems can have adverse effects on humans exposed to these chemicals, as well as native fungi, plants, and animals (Zubrod et al., 2019). Both synthesized organic and inorganic fungicides pose risks. Copper (Cu) is a popular inorganic fungicide, especially among vineyards. Elevated Cu levels in ground water beyond the EU legislative limits pose a risk for the microbial and macro-invertebrate populations in soils, and are potentially hazardous to human populations surrounding vineyards due to elevated Cu levels in well water (Wighwick et al., 2010; Komarek et al., 2010). Aquatic systems are especially vulnerable to adverse effects from organic synthesized fungicides. Fungicides have been demonstrated to be toxic to aquatic non-target fungi, aquatic invertebrates, aquatic plants, and fish (Elskus 2014; van Wijngaarden et al., 2014; Richter et al., 2013; Dijksterheuis et al., 2011). Beyond the environmental risks of organically synthesized fungicides, there is also the risk that repeated exposure of these fungicides will result in resistance arising in the targeted pathogens.

Developed resistance to fungicides by plant pathogens after continuous and exclusive use of inorganic or organically synthesized fungicides poses a major problem for agricultural systems worldwide. The problem of developed resistance to fungicides in agricultural is analogous to the problem of antibiotic resistance in the medical field. New active compound development can be costly and is growing increasingly more difficult, leaving farmers with less options. Fungicide resistance has been documented in isolates from fields which had previously

been exposed to organically synthesized fungicides (Deising 2008). Becher et al., (2010) demonstrated that *F. graminearum* can develop fungicide resistance by repeated exposure. There have been several strains of *Fusarium* isolated from field crops that have shown resistance to fungicides previously used to treat those crop sites (Yerkovich et al., 2020; Yuan & Zhao 2005). A study of the inhibitory effect of azole fungicides on isolates of *F. graminearum* taken from infected crops in fungicide treated field sites in Germany between 1987 and 2004 showed that the level of inhibitory effect of those azole fungicides on *F. graminearum* fell yearly (Klix et al., 2007).

Despite the effectiveness of many fungicides in reducing crop loss, fungal pathogens are estimated to contribute 7-24% of commercial crop loss (Oerke 2006). This may be due in part to fungal pathogens developing resistance to commercially used fungicides. This resistance, along with human health concerns and environmental damage from fungicides, makes alternative methods for reduction of crop loss desirable. One promising alternative to fungicides for reducing crop loss and generating larger yields is the use of microbial biological control agents and plant growth promoting microbes.

Microbial Biological Control Agents and Plant Growth Promoting Microbes

While there are many microbes which can be considered the bane of the gardener or farmer, there are also many microbes which can provide protection from pests and diseases, as well as promote plant growth and improve yield. Application of plant growth promoting microbes (PGPM) can improve crop growth rate, lower nutrient input requirements and reduce dependence on chemical fertilizers, as well as improve abiotic stress tolerance (Lopes et al.,

2021). Microbes can also be a powerful tool for increasing crop resistance to pests and disease. Microbial biological control agents (MBCA) are an attractive alternative to inorganic and naturally occurring or organically synthesized pesticides.

Research on the application of MBCAs in agriculture has been rising over the last 50 years, and largely in the last 20 years, growing to over 5000 articles, and over 400 patents by 2020 (Bejarano & Puopolo 2020). There are two main ways in which MCBAs have been shown to be effective in reducing disease prevalence and reducing crop loss. These are through antagonism of pathogens and pests, as well by inducing plant systemic resistance. Induced systemic resistance (ISR) refers to an induced state of resistance in plants which is triggered by chemical or biological inducers, which protects the plant against pathogenic microbes or insects (Kuć 1982).

The exposure of plants to nonpathogenic strains may act as a sort of immunization against future attacks or infection by pathogenic strains. This is thought to be activated by the plant's immune system, in which pattern-recognition receptors, specifically pathogen-associated or microbe-associated molecular patterns, as well as endogenous plant signals from tissue damage caused by pest invasion will trigger a plant defense response (Pieterse et al., 2014). Accumulation of plant endogenous antibiotic phytoalexins were observed in carnations previously inoculated with nonpathogenic *Pseudomonas* strain WCS417r, and prevalence of fusarium wilt was lower in those plants upon exposure to *F. oxysporum* as compared with carnations which had not been exposed to the *Pseudomonas* (van Peer et al., 1991).

Microbial antagonism of plant pathogens occurs by several modes of action. These include parasitism, competition for space, food, and water, as well as through the production of secondary metabolites which harm the target pathogen (Nega 2014). One of the first patented

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biocontrol agents is the fungus *Phlebiopsis gigantea;* this species is a registered biocontrol product used as an additive for chainsaw oils. By the addition to chainsaw oil, the *P. gigantea* would colonize pine tree stumps, resulting in protection by competition to the pathogenic fungi *Heterobasidion annosum,* which is capable of killing live trees and is a major concern for lumber sources (Pratt et al., 1999). There are many other products currently on the market utilizing strains from many genera of microbes such as *Bacillus, Pseudomonas, Serratia, Tichoderma,* and *Streptomyces* to protect from crop loss (Berg 2009).

Streptomyces

Streptomyces are a genus of Gram-positive aerobic bacteria in the Actinomycete family. *Streptomyces* generate vegetative hyphae typically 0.4 to 1.2 µm thick in diameter. These hyphae form long spore chains at maturity, at which point a smooth colony appearance turns to a granular, powdery, or velvety appearance as the aerial mycelium sporulate (Li et al., 2016). These bacteria are known for their production of complex secondary metabolites, and undergo a complex life-cycle. *Streptomyces* exhibit temporal and spatial control of gene expression, morphogenesis, and metabolism (Kämpfer 2006). Many antibiotics used in modern medical treatment come from *Streptomyces*. It has been estimated that the number of different antibiotics and antimicrobials produced by *Streptomyces* is likely over 100,000; however, most remain undiscovered (Watve et al., 2001).

In addition to producing antibiotics, *Streptomyces* produce amylase, chitinase, cellulase, invertase, lipase, keratinase, peroxidase, pectinase, protease, phytase, and xylanase. These enzymes are capable of both damaging plant pests and also of breaking down complex molecules

into simpler forms that plants can use for nutrients (Vurukonda et. al. 2018). *Streptomyces* are considered soil saprophytes that play a critical role in nutrient cycling (Kinkel et al., 2012). Since *Streptomyces* are ubiquitous soil microbes with a rich ability to produce complex primary and secondary metabolites, it is no surprise that they play a role in plant health, and can be utilized for crop protection and yield improvement.

Streptomyces as MBCA and PGPM

There are several commercially available biofertilizer products which utilize *Streptomyces* as MBCA or PGPM to improve yield and protect ornamental and edible plants (Vurukonda et al., 2018). One of the more well-established products, Mycostop™, utilizes isolate *S. griseoviridis* K61, and has been researched for several decades. This isolate has been shown to be antagonistic to plant pathogens, and capable of significantly decreasing crop loss due to fungal pathogens (Suleman 2002). Research by Tahvonen (1988) demonstrated that Mycostop™ dusting was nearly as effective as harsh chemical treatments such as mercury dusting or application of traditional fungicides in fighting damping off of cereal grains caused by *Rhizoctonia solani* or *Alternaria* spp.

Other studies have demonstrated *S. griseoviridis* to be directly antagonistic to fungal pathogens such as *Sclerotinia sclerotiorum* and various *Fusarium* strains *in vitro* as well as in field settings (Suleman 2002; Alsum 2017)*.* In addition to inhibiting plant pathogens, *S. griseoviridis* has been shown to release plant growth promoting factors leading to increased plant yield. (Gilardi et al., 2015). Other isolates of *Streptomyces* have also been proven to be antagonistic to plant pathogens. Repeated application of *Streptomyces* strain 272 was shown to

reduce incidence and severity of potato scab disease in field grown potatoes (Hiltunen et al.,

2017). Two other *Streptomyces, S. diastatochromegenes* strain PonSSII and *S. scabies* strain

PonR have also been demonstrated as being able to decrease potato scab in field tests (Liu et al.,

1995).

Some isolates have been shown to be effective in fighting *Fusarium* infection.

Streptomyces griseorubiginosus isolated from banana roots were shown to be antagonistic to

Fusarium oxysporum f. sp. *cubense in vitro* (Cao et al., 2005). When cultured and applied as a

MBCA, application of *Fusarium*-antagonistic *Streptomyces* can reduce *Fusarium* disease

severity in banana plants (Getha et al., 2005). Presence of *Streptomyces* bacteria in microbial soil

communities is usually considered beneficial to soil and plant health, and has been associated

with disease suppressive soils (Viaene et al., 2016).

Disease Suppressive Soils

Disease suppressive soils (DSS) are a desirable and naturally occurring phenomenon.

The occurrence of DSS are observable by low incidence of crop disease despite the presence of

susceptible host plant, climatic conditions favorable for disease outbreak, and opportunity for

infection (Alabouvette 1999). Reviews of a great number of studies on DSS have documented

the suppression of many plant pathogens such as *Fusarium*, *Streptomyces scabies*, *Phytophthora,*

and nematodes on a variety of plant hosts (Kinkel et al., 2011). Although it is thought that DSS

require physical and chemical soil properties that are advantageous to crop protection, much of

the research around DSS attribute the ability to provide a natural resistance to plant pathogens to

microflora of the soils (Jayaraman et al., 2021; Weller et al., 2002). The presence of microbial

species such as *Pseudomonas, Trichoderma, Flavobacterium,* and *Streptomyces* has been associated with DSS, and DSS has been differentiated from disease conducive soils by population levels of these species (Kopecky et al., 2019; Hoitink et al., 1997; Liu et al., 1995). A greater richness in fungal diversity has also been associated with DSS (Penton et al., 2014).

The mechanisms by which soil microbes contribute to disease suppression in DSS is likely very similar to the mechanisms of MBCAs, and DSS may be a potentially rich source for microbial species with potential to function as MBCAs. The naturally occurring microbiota in DSS act in a way to prime the plants natural immune response to infection, similar to MBCAs which act to activate a plant's ISR (Liu et al., 2021). Other studies have shown microbes isolated from DSS are capable of disease suppression when introduced into new soils (Kwak et al., 2018). *Streptomyces* isolated from DSS have also shown to be capable of inhibiting sympatric isolated plant pathogens (Becker et al., 1997).

Although the contribution to disease suppression may be through many different means, much of the research surrounding *Streptomyces* role in DSS focuses on antibiotic-mediated inhibition of pathogens (Kinkel et al., 2012). *Streptomyces* isolated from DSS have also been shown to produce many different antibiotics (Cordovez et al., 2015). Complex metabolic products enable *Streptomyces* to out compete soil pathogens; however, the production of complex molecules such as antifungals requires energy and nutrients. These may be conserved via quorum sensing.

Quorum Sensing

Production of biofilms, toxins, and antibiotics are not useful or effective in small bacterial populations where energy is better spent on immediate reproduction or survival. Bacteria are known to regulate metabolism and production of complex molecules based on which, and how many, other bacteria are present. This regulation is a process known as quorum sensing. Quorum sensing relies on continuous release of chemical signals, called autoinducers, which accumulate as cell density increases (Miller & Bassler 2001). These chemical signals are commonly N-acyl homoserine lactones in gram-negative bacteria, and secreted peptides in Gram-positive bacteria, although there are some notable exceptions (de Kievit & Iglewski 2000; Kleerebezem et al., 1997). This behavior allows bacteria to perform a population census and population density-dependent regulation of gene expression.

One of the most well studied examples of bacteria using quorum sensing is the regulation of genes coding for the bioluminescence enzyme luciferase in *Vibrio fischeri* by two regulatory proteins called LuxI and LuxR in the *luxICDABE* operon. LuxI is an autoinducer synthase enzyme which is required for production of the autoinducer N-(3-oxohexanoyl)-homoserine lactone, a homoserine-lactone (HSL) signaling molecule which is freely diffusible inter and intracellularly (Hanzelk & Greenberg 1995). LuxR acts to bind the HSL autoinducer. At low cell densities *luxICDABE* operon is always transcribed at a low level, but this is not enough expression to produce sufficient luciferase for bioluminescence. As cell density increases, the levels of the HSL signaling molecule increases. Once the HSL autoinducer accumulates to a threshold level, LuxR acts to bind the HSL autoinducer, and subsequently bind the *luxICDABE* promoter, exponentially increasing transcription of *luxICDABE,* which leads to enough luciferase

production for bioluminescence (Stevens & Greenberg 1997). By this mechanism, *V. fischeri* are able to regulate bioluminescence based on reaching a critical population level.

Quorum sensing has been documented in species of the genus *Streptomyces* through a two-component signal transduction mechanism. A group of small signaling molecules called γ-butyrolactones (GBLs) are able to freely diffuse out through cell membranes and, upon reaching a stimulatory level, act upon surface receptor proteins (sensor kinases) that induce regulatory activity (through phosphorylation of a response regulator protein) of antibiotic production and morphological differentiation (Bhukya et al., 2014; Polkade et al., 2016). It is interesting to note the chemically structural similarities between the HSL chemical signals found in Gram-negative bacteria and the GBL chemical signals of *Streptomyces*, however GBL and HSL receptors are distinct enough that activity of HSL on GBL receptors has not been demonstrated (Santos et al., 2012).

The GBL signal 2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone, called A-factor, is a critical trigger in production of secondary metabolites and aerial mycelium formation in *S. griseus* (Wiley & Gaskell 2011). In a study by Ohnishi et al., (2005) building upon previous work on A-factor signaling, a model was outlined for the mechanisms of A-factor triggering the conformational change of the ArpA transcriptional repressor into the transcriptional activator shape, which activates the A-factor regulatory cascade, and leads to the morphological development of aerial mycelium, as well as production of streptomycin, grixazone, and polyketide. This would occur typically in *S. griseus* cultures after the A-factor signal molecule crossed the critical accumulation point of around 25 ng/ml during growth, usually observed during the exponential growth phase.

The presence of diffusible chemical signaling and regulatory molecules is likely to affect intragenic species which have similar or same quorum sensing pathways. In a study by Becker et al., (1997) it was shown that the presence of pathogenic *Streptomyces* triggered an earlier synthesis of antibiotics by a nonpathogenic *Streptomyces* strain. This sort of interspecies communication may have a key role in the ecology of disease suppressive soils. There is evidence for microbial sensing for intergenic communities.

Co-Culture

Microbial species, while often studied as individual entities, are often found in communities of other microflora, where they are subject to interactions of mutualism, commensalism, amensalism, and antagonism (O'Brien et al., 2013; Essarioui et al., 2017). There has been a growing interest in studying microbial species under conditions where they are grown in co-culture with other species. The influence on the production of secondary metabolites by the co-culture of microbes is a relatively new topic in research, and has led to the discovery of new antibiotics (Abdalla et al., 2017). Genetic analysis of microbes can reveal putative biosynthesis gene clusters which greatly outnumber the known secondary metabolites we have discovered from those species. Silent gene clusters have been activated by both the presence of diffusible signaling chemicals from, and the physical interaction between, two intergenic species of microbes, leading to the synthesis of new compounds which were not present in monocultures of either organism (Schroeckh et al., 2009; König et al., 2013).

During co-culture of *Streptomyces coelicolor* with other actinomycetes, Traxler et al., (2013) discovered a previously unidentified family of secondary compounds triggered by

siderophores from neighboring strains, as well as an influence on level of expression of secondary metabolites which varied between strain interactions. A study by Li et al., (2022) showed an increase in antibiotic activity against *Fusarium* when two *Streptomyces* strains were co-cultured in the same broth, as compared to either *Streptomyces* cultured separately. There have been several studies on the effects of co-culture of *Streptomyces* and *Fusarium,* although the literature on the topic is very limited.

On same-day inoculation of wheat grain by *Streptomyces* strains and pathogenic *F. graminearum*, Colombo et al., (2020) recorded an inhibition of up to 99% of DON contamination, and fungal biomass inhibition of up to 71%. A study of the interaction between *F. verticilliodes* and *Streptomyces* strain AV05 was conducted by Nguyen et al., (2020) and showed that when grown in confrontation on dual culture plates, where both organisms were inoculated simultaneously, the endometabolome of *F. verticilloides* was significantly altered, and the *Streptomyces* AV05 underwent an overproduction of many metabolites. Moussa et al., (2019) induced the production of several new dimeric naphthoquinones and dihydrolaterpyrones from *F. tricinctum* by the addition of live *S. lividans* culture to flasks containing solid rice medium three days prior to inoculation with *Fusarium,* followed by several days of co-culture until the fungus had completely covered the culture media.

One of the most recent studies on co-culture between *Fusarium* and *Streptomyces* tested the antibiotic activity of ethyl acetate extracts from co-cultures of isolates from both species against *Staphylococcus aureus* and a *Micrococcus* species as test organisms. Antibiotic activity of extracts from 14-day old co-cultures of *Streptomyces* and *Fusarium,* where either organism was introduced into broth 7 days following culturing of the first organism, were universally

higher than those from cultures of either species grown alone, and contained novel metabolites not found in the individual cultures (Zawawi et al., 2022).

Aims & Goals

The aim of this project is to explore the interactions between *Streptomyces* isolated from disease suppressive soils and *Fusarium* isolated from the same soils, and to identify *Streptomyces* isolates which show strong inhibition of *Fusarium*. These *Fusarium* isolates are non-pathogenic, but are closely related to virulent strains, making them good study organisms for identifying *Streptomyces* which may contribute to disease suppression in DSS by antagonism toward plant pathogenic fungi. Isolates of *Streptomyces* which show strong antifungal activity against *Fusarium* may prove to be useful as more safe or cost-effective MBCA alternatives to environmentally damaging conventional fungicide treatment, and may be sources of potent antifungal compounds useful for treating or preventing fungal infection.

The goals of this project were to 1) Identify *Streptomyces* isolates with antifungal activity against *Fusarium*, and *Fusarium* isolates with antibacterial activity against *Streptomyces* 2) Identify if *Streptomyces* antifungal chemical production is population density dependent, and if quorum sensing may play a role in production of fungal inhibitory compounds 3) Identify if *Streptomyces* antifungal chemical production is affected by the presence of *Fusarium*, and 4) to identify antifungal compounds produced by *Streptomyces*.

METHODS

Research Organisms

All research organisms were provided by Dr. Linda Kinkel from the University of Minnesota. This included 10 *Fusarium* and 10 *Streptomyces* isolates. *Fusarium* isolates were designated the reference names F2-1, F2-2, F2-4, F2-6, F2-7, F10-3, F10-4, F10-7, F10-8, and F10-10. *Streptomyces* isolates were designated reference names S2-2, S2-4, S2-5, S2-8, S2-9, S10-1, S10-2, S10-7, S10-8, and S10-9. These isolates were all sympatric species isolated from prairie soils in Minnesota.

Master plates were maintained by re-plating every isolate every 3-4 months, sourcing from initial master cultures, until initial master cultures became unviable, after which the most recent re-plating from master culture was used as the new master culture. This practice was followed for both *Fusarium* and for *Streptomyces* isolates in order to minimize number of generations and reduce risk of genetic mutation across generations grown in lab conditions during the course of this experiment.

Fusarium was sourced from plates by removing three, 5 mm diameter discs of mycelium and adding them onto fresh potato dextrose agar plates. *Streptomyces* isolates were sourced from master plates using a metal inoculating loop, and streaked in a three-quadrant streak onto fresh growth media.

Growth Media

Cultures were maintained and grown in and on a variety of media.

Oatmeal Agar (OA)

Oatmeal Agar (OA) was chosen as the preferred media for cultivating *Streptomyces* on solid agar medium for the duration of these experiments. This complex medium was decided upon after growing *Streptomyces* isolates on several types of agar. *Streptomyces* growth on OA was found to be vigorous, and result in consistent sporulation and development of pigments. *Fusarium* isolates were also able to grow on OA, although they appeared to have reduced vigor.

Oatmeal agar was prepared by heating 1 L of deionized water (dH_2O) in a 2 L Erlenmeyer flask on an electric heat plate with a magnetic stir bar, and adding 20.0 grams of Gerber© Oatmeal ground oatmeal, 1.0 gram casamino acids, and 15 grams of granulated agar. The mixture was allowed to come to a brief boil, at which point it was removed and then autoclaved.

Starch Casein Agar (SCA)

Initial cultures of *Streptomyces* strains were provided on starch casein agar (SCA), and initial master cultures of *Streptomyces* isolates were maintained on SCA, however the use of this agar was later discontinued due to slower growth and less rapid sporulation and pigment production as compared to cultures on OA. *Fusarium* isolates appeared to have poor growth on SCA as compared to Potato Dextrose Agar.

Starch casein agar was prepared by dissolving 10.0 g of starch, 0.30 g of casein, 0.02 g $CaCO_3$, 15 g granulated agar, and dH_2O to bring a total volume of 800 mL. This was combined

with 100 mL of Solution A (20.0 g KNO_3 20.0 g NaCl, and 20.0 g K_2HPO_4 dissolved in 1.0 L dH_2O) and 100 mL of Solution B (0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, and 0.01 g $ZnCl_2$ dissolved in 1.0 L dH_2O) brought to a boil, then immediately removed and autoclaved.

Potato Dextrose Agar (PDA)

Initial cultures of *Fusarium* isolates were provided on Potato Dextrose Agar (PDA), and all culturing of *Fusarium* isolates for master cultures utilized PDA due to the vigorous growth on this media as compared to other agar medias.

Potato Dextrose agar was prepared by adding 39 g/L of Neogen© Potato Dextrose Agar (4.0 g Potato Extract 20.0 g Dextrose 15.0 g Agar) to dH_2O, heating while stirring until dissolved, and then autoclaved. During antifungal assays, PDA was prepared with an additional 5.0 g/L of agar to help add rigidness to the media plates for ease of agar well removal.

Potato Dextrose Broth (PDB)

Liquid culture of *Fusarium* Isolates for antibiotic assays and filtrate collection utilized Potato Dextrose Broth (PDB). This broth was prepared by dissolving 24.0 g/L of HIMEDIA© Potato Dextrose Broth powder (Infusion from 200.00 g potatoes, 20.00 Dextrose) in dH_2O, then autoclaving.

*2/3 Strength Tryptic Soy Broth (*TSB*)*

Two-thirds strength tryptic soy broth (TSB) was chosen for its optical clarity, and a nutrient profile that was adequate for *Streptomyces* antibiotic production. Two-thirds strength tryptic soy broth was prepared by dissolving 20.0 g/L of Bacto™ Tryptic Soy Broth (17.0 g Pancreatic Digest of Casein, 3.0 g Enzymatic Digest of Soybean meal, 2.5 g Dextrose, 5.0 g

NaCl, 2.5 g K_2HPO_4) in dH_2O, heating while stirring until dissolved, and then dispensed into 500

mL Erlenmeyer flasks which contained a circle of stainless steel spring coil and stoppered with

glass wool and covered with aluminum foil, and then autoclaved.

2/3 Strength Tryptic Soy Agar (TSA)

      Streptomyces isolate S2-2 and *Fusarium* isolate F10-8 were grown on 2/3 strength tryptic

soy agar (TSA) to see how the switch from their preferred media, OA and PDA respectively,

would impact growth vigor, and again on dual layer TSA and PDA plates to test the ability of

S2-2 grown on TSA to inhibit F10-8. The inhibition of F10-8 by S2-2 when grown on TSA:PDA

dual-layer medium was nearly identical to when using OA.

      Two-thirds strength tryptic soy agar was prepared following the recipe for TSB with the

addition of 15 g of granulated agar per liter dH_2O.

Streptomyces **High Concentration Spore Suspension**

      Streptomyces strains were inoculated via crosshatch streaking on OA and incubated at 28

°C for 4 days. After incubation and sporulation, *Streptomyces* spores were removed by scraping

with sterile cotton swabs, swabs were then dipped in 10 mL of sterile 20% glycerol solution in

30 mL test tubes, which were then vortexed and stored at 4 °C. This resulted in a high

concentration spore suspension, which was then diluted using additional sterile 20% glycerol to

achieve an optical density of ABS ~1.00 at 650 nm (Thermo Scientific© Spectronic 200) (11 x

10^8 CFU/mL). Spore suspension was always vortexed for 30 seconds prior to using as inoculum.

Dual layer Inhibition Assay

Dual layer inhibition assay was used to test for inhibitory activity of *Streptomyces* against *Fusarium* and *Fusarium* against *Streptomyces* (Essarioui et al., 2019)

Fusarium Spread
Potato Dextrose Agar Overlay
Streptomyces Triple Spot
Oatmeal Agar

Streptomyces Spread
Oatmeal Agar Overlay
Fusarium Triple Disc
Potato Dextrose Agar

Figure 1. **Depiction of Dual Layer Inhibition Assay**. Depiction of method used to assay inhibitory activity of *Streptomyces* isolates against *Fusarium* isolates, and *Fusarium* isolates against *Streptomyces* isolates.

Streptomyces inhibition of *Fusarium*

For each *Streptomyces* strain, OA plates were triple spot inoculated with 10 µL aliquots of *Streptomyces* high concentration spore suspension and incubated for 4 days at 28 °C. Following incubation, these cultures were sterilized by inversion over watch glasses containing 1 mL of chloroform for 30 minutes. The plates were then left open in a positive pressure sterile flow hood for 30 minutes to allow any residual chloroform to dissipate. Sterilization of chloroform exposed *Streptomyces* cultures was verified by streaking onto fresh OA then incubating as before. This was done with extra plates, and the experimental plates were not used for verification. There was no growth following 4 days of incubation of verification plates.
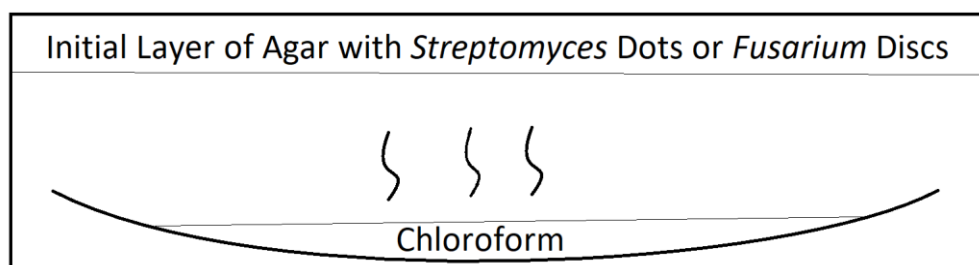
Figure 2. **Depiction of Chloroform Sterilization of Dual Layer Inhibition Assay Plate**.
Depiction of culture plate with either OA agar triple dotted with *Streptomyces* spore suspension,
or PDA with three *Fusarium* disks, inverted over a watch glass containing 1.0 mL of chloroform.

Each of the 10 *Fusarium* strains were inoculated onto PDA plates by adding 0.5 cm disks
from master cultures, and then incubated for 7 days. From each of the 7-day old cultures, a 1 cm
diameter disk of mycelium was removed from the edge of actively growing *Fusarium* and placed
into 15 mL of PDB in a 30 mL test tube. The test tube was then incubated on a New Brunswick
C24 Benchtop Shaker at 60 rpm and 28 °C for 4 days. Ten milliliters of 4-day old *Fusarium*
broth culture was added to 90 mL of sterile molten PDA in a 250 mL bottle, which was then
allowed to mix by returning to the shaking incubator for several minutes before pouring 10.0 mL
over the 4-day old triple spot inoculated and chloroform exposed OA *Streptomyces* plates. The
dual layer OA:PDA plate was then incubated at 28 °C for 7 days.

Zones of total inhibition or partial inhibition of *Fusarium* growth surrounding the sites of
Streptomyces spot inoculation were measured using a metric ruler across the greatest length of
total or partial inhibition. Control plates followed the spot inoculation procedure using deionized
sterile water (dsH$_2$O) as a control, and were also exposed to chloroform and incubated for 4 days.
This was performed for each combination of *Streptomyces* isolate and *Fusarium* isolate. This
method was performed in triplicate.

Fusarium Inhibition of *Streptomyces*

For each *Fusarium* strain, three 1 cm disks of mycelium were removed from the outer actively growing edges of a 7-day old *Fusarium* culture on PDA and placed onto fresh PDA plates. The plates were then sterilized by exposure to chloroform by inverting over a watch glass containing 1 mL of chloroform for 30 minutes. The plates were then left open in a positive pressure sterile flow hood for 30 minutes to allow any residual chloroform to dissipate. Sterilization of chloroform exposed *Fusarium* cultures was verified by removing disks and placing onto fresh PDA, then incubating for 7 days. This was done with extra plates, and the experimental plates were not used for verification. There was no growth on verification plates following 7 days of incubation.

The chloroform exposed plates were then overlaid with 10.0 mL of molten OA and allowed to cool till agar was fully solid. The dual layer PDA:OA plates were then refrigerated at 4 °C overnight to allow for *Fusarium* antimicrobial compounds to diffuse. The following day the plates were inoculated by dispensing 100 µL of high concentration spore solution of a single *Streptomyces* strain, which was spread evenly across the agar surface using a sterile bent glass spreader. The dual layer plates were then incubated at 28 °C for 4 days, and zones of inhibition of *Streptomyces* growth surrounding the mycelium plugs was measured using a metric ruler; the greatest diameter in the zone of inhibition was recorded. This was performed for each combination of *Streptomyces* isolate and *Fusarium* isolate. This method was performed in triplicate.

Generating Growth Curve of *Streptomyces*

Streptomyces isolate S2-2 was cultured in 500 mL Erlenmeyer flasks containing 200 mL TSB and a coiled stainless-steel spring to improve aeration. High concentration spore solution was used to inoculate broth at 1:100 (v:v) spore solution to liquid broth. The culture was then placed in a shaker and incubated at 250 rpm and 28 °C for up to 38 hours. After 8 hours of incubation 2.0 mL of broth sample was removed, 1.0 mL of broth sample was placed in a cuvette and absorbance was read at 650 nm, while the other 1.0 mL of broth sample was put in a syringe and expelled through a 0.22 µm Polyether sulfone (PES) syringe filter into a 10 mL glass test tube. This process was repeated every 2 hours for the duration of the incubation. Filtrate was stored at 4 °C for later use. This process was performed in quadruplicate.

Agar Well Diffusion Antifungal Assay

A modified agar well diffusion method was used as the antifungal assay for testing the inhibitory activity of filtrates and later extracts (Harikrishnan & Shanmugaiah 2012). Antifungal assay plates (AAP) were prepared by pouring 10.0 mL PDA prepared with an additional 5.0 grams agar granules for extra solidity into 100mm x 15mm plastic petri plates. A 1 cm disk of agar was removed from slightly off of center of each plate to form a well. These wells were able to fit 50 µL of filtrate or aqueous extract.

Inhibitory activity of filtrate or extract solution was tested by adding 50 µL of *Streptomyces* filtrate or extract solution into the empty well, then placing a 1 cm mycelium disk of *Fusarium* removed from the actively growing edge of a 7-day old *Fusarium* culture grown on

PDA onto the AAP at a distance of 10 mm from the agar well. Plates were then incubated at 28 °C for 7 days. Control plates used either dsH$_2$O or extract solvent in equal volumes to the treatment plates. Radial growth of the *Fusarium* disc toward the well on the AAP was measured on the seventh day. Percent inhibition of radial growth (PIRG) was calculated using the following equation:

$$\mathrm{PIRG} = \left\{\frac{(R1 - R2)}{R1}\right\} \times 100$$

Where R1 is the radial growth towards well on control plates, and R2 is the radial growth towards well on treatment plates. Measurements of PIRG from filtrate is referred to as filtrate percent inhibition (FPI).

Effect of Late Log-phase Filtrate on *Streptomyces* Growth-rate and Antibiotic Production

Late log-phase growth filtrate, referred to as conditioned media (CM) collected from *Streptomyces* isolate S2-2 grown in TSB was added to S2-2 TSB cultures at either 1:10 or 1:100 v:v at hour 8 of incubation at 28 °C and 250 rpm. Two milliliters were removed from 8-hour old S2-2 culture prior to and immediately following addition of CM for absorbance measurement (A650) and filtrate collection. The absorbance measurement and filtrate collections were repeated every 2 hours for 16 more hours. Uninoculated TSB flasks which received the same CM treatment, and S2-2 cultures which did not receive CM, were also included in sampling. Filtrate samples from each treatment and control flasks were stored at 4 °C. This was performed in

triplicate. Filtrate was tested for inhibition of F10-8 using Agar well diffusion method.

Effect of *Fusarium* Exposure on *Streptomyces* Growth-rate and Antibiotic Production

Two treatments were used to test the effect of exposure to *Fusarium* on growth-rate and antibiotic production on S2-2 broth cultures. These were exposure to *Fusarium* filtrate, and exposure by inclusion of *Fusarium* F10-8 disc for co-culture.

Fusarium isolate F10-8 was cultured at 28 °C and 250 rpm in 500 mL Erlenmeyer flasks containing 200 mL PDB and a coiled stainless-steel spring to improve aeration. *Fusarium* filtrate was collected from 24-hour old PDB cultures by expelling through a 0.22 µm PES syringe filter.

Fusarium filtrate was added to S2-2 TSB cultures at hour 8 of incubation at 28 °C and 250 rpm. Two milliliters were removed from 8-hour old S2-2 culture prior to, and again immediately following, addition of F10-8 filtrate for absorbance measurement (A650) and filtrate collection. The absorbance measurement and filtrate collection were repeated every 2 hours for 16 more hours.

A co-culture method was performed simultaneously. Immediately following inoculation of S2-2 broth cultures, a 1 cm disc from 7-day old PDA *Fusarium* F10-8 was added to the broth as well. The absorbance and filtrate collection from broths treated with discs followed the same procedure as those treated with F10-8 filtrate. Co-culture broths also were checked for presence of *Fusarium* by 1.0 ml broth samples at hours 0, 8, and 24 which were spread-plated from 10^{-3} to 10^{-7} serial dilution on OA and PDA.

Uninoculated TSB flasks which received the F10-8 filtrate, and S2-2 cultures which did not receive F10-8 Filtrate or simultaneous F10-08 inoculation were also included in sampling.

Filtrate samples from each treatment and control flasks were stored at 4 °C. This was performed in triplicate. Filtrate was tested for inhibition of F10-8 using agar well diffusion method.

Chemical Extraction

Solvent Testing

Several solvents were tested in initial small batches for their ability to extract antibiotic from *Streptomyces* S2-2 broth culture filtrate. Filtrate was collected from TSB broth cultures of S2-2 during late log-phase growth at hour 30. Ten milliliters of filtrate were combined in equal parts by volume with solvent, and vortexed three times for 30 seconds each. This mixture was left unagitated for one hour to allow separation of the solvent phase and the aqueous liquid phase. Solvents chosen included ethyl acetate (EtOAc), chloroform, and mixed hexanes. Following separation, the solvent layer was pipetted off and stored in test tubes, which were stored at 4 °C. The solvent layer was then poured into 3 cm watch glass dishes, and allowed to evaporate in a fume hood. The watch glasses were then scraped and the crude extract was re-dissolved in 100 µL dsH$_2$O. The re-dissolved crude extract was tested for antifungal activity by agar well diffusion.

Large-scale Extraction

Ethyl acetate was used for a large-scale extraction. Following 30 hours incubation at 28 °C and 250 rpm, ten 200 mL broth cultures of S2-2 were removed and combined in a 2800 mL flask, which was swirled vigorously for homogeneity. The broth was then put into centrifuge

bottles and centrifuged on a Beckman Coulter J2-HS Centrifuge at 10,000x g-force for 20 minutes. The supernatant was then removed and filtered by vacuum filtration through Whatman® #1 filters to remove any pellet which had been agitated during pouring off supernatant.

The filtered supernatant was then combined to a total volume of 1500 mL. This was separated into two 1 L flasks, and combined with 1:3 volume EtOAc to supernatant, and vigorously shaken for 20 minutes. The flasks were then put in a BRANSON model 2210 ultrasonic cleaner for 10 minutes to break emulsion layer. Solvent layer was removed by pipetting, placed into 500 mL separatory funnels, and allowed to settle. Any settled aqueous layer was allowed to drain prior to collecting the extraction solvent layer. Aqueous layer was recombined and then extracted with 1:3 volume solvent two more times. All solvent layers were combined, and refrigerated at 4 °C.

Analysis of Extract

Agar Well Diffusion for Extraction Solution

Following recombination of all EtOAc, 100 mL of extraction solution was removed and dried on watch glasses in a fume hood. The crude extraction was scraped off of watch glasses and into test tubes. Addition of dsH_2O created a 10% (w:v) crude extract solution. The test tube was vortexed for 30 seconds. The solution was then tested for antifungal activity using the agar well diffusion method.

Concentration

A 500 mL extraction solution volume was concentrated on a IKA RV8 ROTOVAP rotary evaporator. Condensing column was cooled by running -20 °C ethylene glycol and water solution. The extraction solution was added to a 1 L flask and lowered into a 40 °C water bath, and rotation was set to 107 RPM to achieve a thin film on the flasks inner surface. A vacuum was applied to reach -850 mBAR, at which point the solvent started to be reclaimed on the condensation column. The extraction solution was evaporated down under these conditions for approximately 30 minutes until precipitate started to form in the concentrated solution.

Fractioning

A silica gel fractioning column was prepared by combining 20.0 g silica powder with 20.0 mL of a 1:1 EtOAc:hexane solution and mixing until a slurry was formed. Slurry was then added to glass column and allowed to settle to form a 20 cm x 2 cm silica gel column. Once visibly settled, 40 mL of 1:1 EtOAc:hexane solution was allowed to run through the column to ensure everything was completely settled and no air pockets remained.

Some of the EtOAc evaporate while stored in a glass vial, 3.8 mL remained. This was combined with 3.8 mL of hexane to equalize polarity with the column solution, and added on top of the column. Fraction collection was immediately started following dispensing the concentrated solution on top of the column. Elution was collected using a BIO-RAD model 2110 Fraction Collector set to collect each elution fraction for 1.65 minutes for collection of 2 mL fractions. The column was allowed to elute until the concentrated solution had loaded nearly completely into the silica gel column. Afterwards the running solvent was added to the column. The running solvent was made increasingly polar throughout the course of the fraction collection

by increasing the ratio of EtOAc to hexane until the solution was entirely EtOAc, and then finishing with an EtOAc:Methanol solution. The sequence of solvent additions were 12.4 mL 1:1, 60 mL 3:1, 40 mL 5:1, and 20 mL 9:1 of EtOAc:Hexane, followed by 10 mL of EtOAc, and finally 10.0 mL of 9:1 EtOAc:Methanol solution. Fractions were stored at 4 °C for further analysis

UV-Vis Data

Concentrated extraction solution and fractions were analyzed in a Shimadzu Model 1800 UV-Vis spectrophotometer. An absorption spectra was generated for the concentrated extraction solution. The absorption values for peaks in the absorption spectra for the concentrated extraction solution (280 nm, 330 nm, 500 nm) were measured for all 80 fractions.

Agar Well Diffusion for Fractions

Fractions were dried in a Thermo Scientific© Savant SpeedVac SPD120 at ambient room temperature (20-24 °C) for 40-60 minutes. The dry fractions were observed for pellet volume, consistency, and color. Each fraction was vortexed for 20 seconds following addition of 100 µL of dsH_2O. This solution was then tested for antifungal activity using the agar well diffusion method.

Statistical Analysis

Data for comparing treatments of S2-2 broths with conditioned media, *Fusarium* filtrate, and simultaneous inoculation with F10-8 was analyzed using R-studio running either fully saturated ANOVA test with Tukey's HSD for comparing means, or an ANOVA model with just the main effects. Results of ANOVA models were analyzed using Emmeans data package.

RESULTS

Dual Layer Assay

Inhibitory activity of *Streptomyces* isolates against sympatric *Fusarium* isolates was observed on dual layer assay plates. Zones of total inhibition and zones of partial inhibition were observed on many pairwise combinations, although many combinations did not show any inhibition. Zones of total inhibition, which had no *Fusarium* growth on the PDA layer in an area surrounding the location of *Streptomyces* spot inoculation on the OA layer below, varied in size between 10-42 mm. Several combinations did not result in a zone of total clearing of fungal growth, but did result in a zone of partial inhibition, where *Fusarium* grew with a significant reduction in density and major reduction of aerial hyphae. Some *Streptomyces* isolates, notably S10-2 and S10-7, caused both a zone of total inhibition, as well as a greater area of partial inhibition. The length of total or partial inhibition from all three spots was averaged to get a mean zone per plate, and the average from all three replicate plates was used to categorize the level of inhibition into several designations due to the nonuniformity of zones of total clearing or partial inhibition (Table 1).

Streptomyces isolates S2-2, S10-7, and S10-9 showed zones of total inhibition against all *Fusarium* isolates. Isolate S10-2 showed zones of total or partial inhibition against all but F2-6. Isolate S2-2 had the largest zones of total inhibition against F10-8 out of all combinations.

Inhibitory activity of *Fusarium* isolates against sympatric *Streptomyces* isolates was observed on dual layer assay plates. Zones of total inhibition were observed on all pairwise combinations. The length of total inhibition surrounding each site of *Fusarium* disc was averaged

to get one number per plate, and the average from all three replicate plates was used to categorize various levels of inhibition (Table 2).

Streptomyces **Growth Curve and Inhibitory Activity**

Filtrate and absorbance readings at 650 nm were collected every other hour for growth of *Streptomyces* isolate S2-2 in TSB. Filtrate was used for agar well diffusion to assay antifungal activity against *Fusarium* isolate F10-8. Absorbance increased across the duration of sampling, with a sharp increase during log-growth stage, followed by a reduced rate of increase in the stationary phase. The antifungal activity of filtrate, as indicated by the filtrate percent growth inhibition (FPI) of F10-8 on agar well diffusion, increased across the growth cycle as well. Antifungal activity appeared several hours into log-growth phase, and continued to increase throughout log-growth phase following a similar trend as the growth curve (Figure 3). It is interesting to note that another sharp rise in FPI occurred several hours into the stationary growth phase.

Effect of Conditioned Media on Inhibition Activity of *Streptomyces* **S2-2**

Addition of 1% or 10% CM by volume to broth cultures of *Streptomyces* isolate S2-2 did not have a significant effect on population density as measured by absorbance at 650 nm. Addition of CM did cause an initial increase in absorbance after addition to broth cultures, however absorbance values of all cultures prior to addition of CM were similar, and the change in A650 was noted as being proportional to the rise in A650 of sterile broth after addition of CM (Figure 4). An ANOVA test with Tukey's HSD also revealed there was a significant interaction

between time and treatment (F=2.99; df=6,24; p=0.0249) indicating a variable effect of CM treatment over time.

Addition of CM generally increased mean FPI values compared to S2-2 culture grown under the same conditions without the addition of CM (Figure 5). There was a significant effect of treatment on FPI (F=30.5; df=2; p=2.566e-07). The addition of either 1% CM or 10% CM had a significant effect on mean FPI at hours 22 and 24 according to a saturated ANOVA test with Tukey adjustments (SE=0.053; df=24; p ≤0.05). Treatment with either 1% or 10% increased mean FPI during those hours (Figure 6). Percent inhibition from filtrate collected from sterile control broth flasks with addition of 10% CM showed a rapid decrease in inhibitory activity after 4 hours of incubation under the same condition of culture broths (Figure 5).

Effect of *Fusarium* Filtrate or *Fusarium* Co-culture on the Growth Curve and Inhibition Activity of *Streptomyces* S2-2

Addition of 5% filtrate from *Fusarium* isolate F10-8 by volume to broth culture of *Streptomyces* isolate S2-2 at hour 8, and simultaneous inoculation with F10-8 into S2-2 broth culture did not have a significant effect on growth rates (Figure 7). Serial dilution plates were able to detect some viable F10-8 immediately following inoculation, however no live F10-8 was observed on serial dilution spread plates from any other sample time.

Addition of 5% F10-8 filtrate and simultaneous inoculation with F10-8 had only some observable differences in FPI compared to S2-2 culture grown under the same conditions without the addition of *Fusarium* filtrate or co-culture (Figure 8). Mean FPI from co-culture filtrate was lower for all sample times. A saturated ANOVA did not show significant interaction between

factors of time and treatment. Running a simplified ANOVA test, which only included the main effects of treatment and time, did show a significant difference in mean FPI among the S2-2 + F10-8 co-culture treatment and untreated S2-2 filtrates *(F=12.741; df=2,22; p≤0.05)* at hours 16-20, during log-phase growth (Figure 9). There was no significant difference between S2-2 and treatment with 5% F10-8 filtrate. Filtrate collected from sterile broth control flasks with addition of 5% filtrate from *Fusarium* isolate F10-8 showed no inhibitory activity.

Antifungal Extraction

Extractions of S2-2 broth culture with EtOAc, chloroform, and mixed hexanes were compared for inhibitory activity against F10-8 with agar well diffusion method. Mixed hexane extracts did not show inhibition, and when dried only yielded a very small amount of oily substance. Chloroform extracts did result in inhibition. Chloroform also melted some plastics during pipetting, and extraction, and so when chloroform extracts were dried on watch glasses there were, what appeared to be, also plastic impurities. Ethyl acetate extracts also showed inhibition, and yielded a brown pink tar crude.

The large-scale EtOAc extraction was a bright pink color, and when concentrated turned to an even deeper shade. When dried, 100 ml of extract solution yielded 11.4 mg of pink crude extract product. Both 1% and 10% (w:v) crude extract in dsH_2O showed antifungal activity. Both 1% and 10% crude extract solution caused percent radial growth inhibition of 35% compared to 50 µl dsH_2O controls, and a greater area of partial inhibition (Figure 10). Post-extraction filtrate was also tested for antifungal activity after large scale EtOAc extraction. This percent radial growth inhibition of post-extraction filtrate was 82%.

Fractioning and Analysis

The absorption values for peaks in the absorption spectra for the concentrated extraction solution (280 nm, 330 nm, 500 nm) were measured for all 80 fractions (Appendix). Fractions 16-34, as well as other groups were noted for having higher absorbance at these wavelengths, and fraction 20 was noted for having the greatest A500 of any fraction. A strong odor was also noted for fraction 19.

All fractions were speed vacuumed down and re-suspended in 100 µl dsH_2O. All even numbered fractions were tested for antifungal activity by agar well diffusion assay (Appendix). Fractions 18 and 26 showed the most antifungal activity, with PIRG of 38% and 31% respectively. Odd numbered fractions between 16 and 30 were also tested. The strongest observed PIRG values observed were from fractions 20 at 28% and fraction 25 at 34%.

Table 1. ***Streptomyces*** **Inhibition of** *Fusarium* **on Dual Layer Inhibition Assay**. Level of inhibition was designated based on averages from measurements from zones of total or partial inhibition of *Fusarium* growth surrounding the location of the spot inoculation of *Streptomyces* (n=9). Level of inhibition is designated using the following categories: "-" for no observable inhibition, "(P)" for a partial inhibition of up to 20 mm, "(P+)" for partial inhibition exceeding 20 mm, "+" for total inhibition up to 20 mm, "++" for total inhibition exceeding 20 mm. Certain plates displayed both a zone of total clearing, as well as greater zones of partial inhibition.

Fusarium Isolates	*Streptomyces Isolates*									
	S2-2	S2-4	S2-5	S2-8	S2-9	S10-1	S10-2	S10-7	S10-8	S10-9
F2-1	++	-	-	-	-	-	+	+ (P+)	-	+
F2-2	+	-	-	-	-	-	(P)	+ (P+)	(P)	++
F2-4	+	-	-	-	-	-	(P)	+	-	+
F2-6	++	-	-	-	-	-	-	+	-	++
F2-7	+	-	-	-	-	-	+ (P+)	+ (P+)	(P+)	+
F10-3	++	-	-	(P)	-	-	+	+ (P+)	-	++
F10-4	++	-	(P)	-	-	-	(P)	+	-	++
F10-7	++	-	-	-	(P)	-	(P)	+	-	++
F10-8	++	-	(P)	(P)	(P)	-	(P+)	+ (P+)	-	++
F10-10	++	(P+)	(P)	+	+	+	+	+	-	++

Table 2. **Fusarium Inhibition of *Streptomyces* on Dual Layer Inhibition Assay**. Zones of inhibition were measured across the greatest diameter of zone of total clearing, and averaged from three zones per plate, and again averaged from three replicate plates to give the average inhibition in centimeters (n=9). Level of inhibition is designated using the following categories: "+" for total inhibition up to 3 cm, "++" for total inhibition exceeding 3 cm.

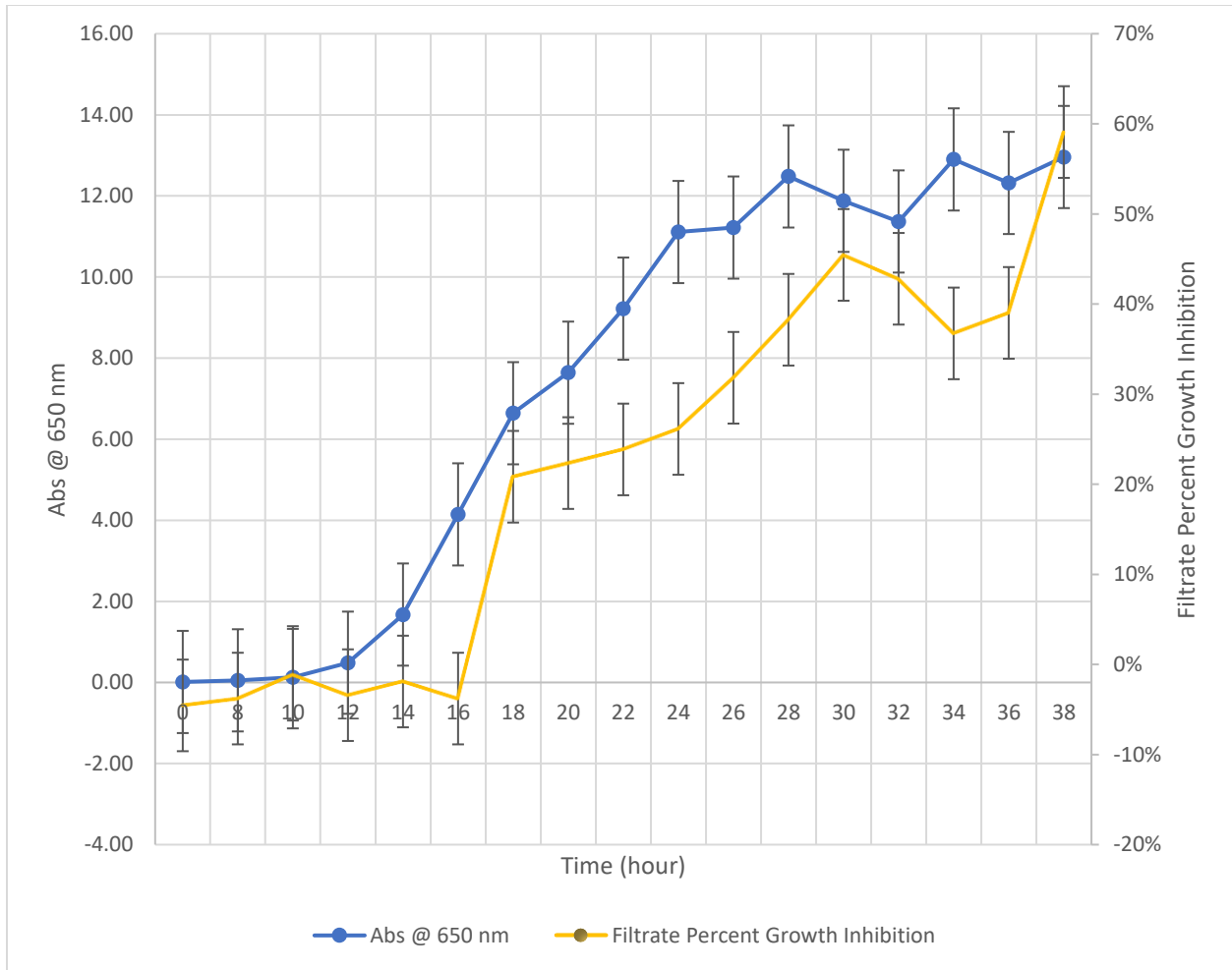| *Streptomyces* Isolates	*Fusarium* Isolates									
	F2-1	F2-2	F2-4	F2-6	F2-7	F10-3	F10-4	F10-7	F10-8	F10-10
S2-2	+	+	+	++	++	+	+	+	+	+
S2-4	++	++	++	++	++	++	+	++	++	++
S2-5	+	++	++	++	++	++	++	++	++	++
S2-8	++	++	++	++	++	++	++	++	++	++
S2-9	++	++	++	++	++	++	++	++	++	++
S10-1	+	++	+	++	++	++	+	++	++	++
S10-2	+	+	+	++	+	+	+	+	+	+
S10-7	+	+	+	++	+	+	+	+	+	+
S10-8	+	+	++	++	++	++	+	+	+	+
S10-9	+	+	+	++	++	+	+	+	+	+

Figure 3. **Optical Density and Filtrate Percent Inhibition of S2-2 Broth Culture.**
Streptomyces isolate S2-2 TSB broth mean culture absorbance level at 650 nm and filtrate percent growth inhibition of F10-8 on agar well diffusion plates (n=4). Sampling was conducted at Hour 0, 8, and every 2 hours until hour 38. Standard error bars are included.
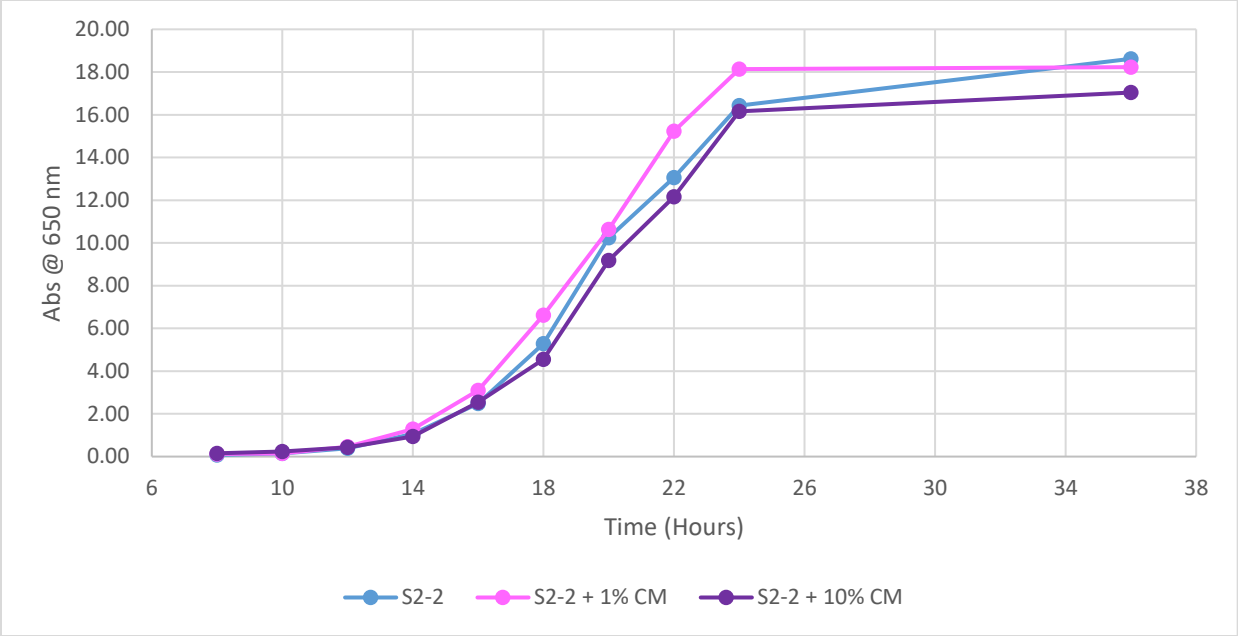
40

Figure 4. **Optical Density of S2-2 Culture with or without 1% or 10% Conditioned Media.** Absorbance values for *Streptomyces* isolate S2-2 TSB broth culture, with or without addition of 1% or 10% conditioned media from 30-hour old S2-2 culture (n=3). Sampling was conducted at Hour 0, 8, and every 2 hours until hour 24, with one more sample at hour 36. The Figure shows individual growth curves. Standard error bars are included in the appendix.
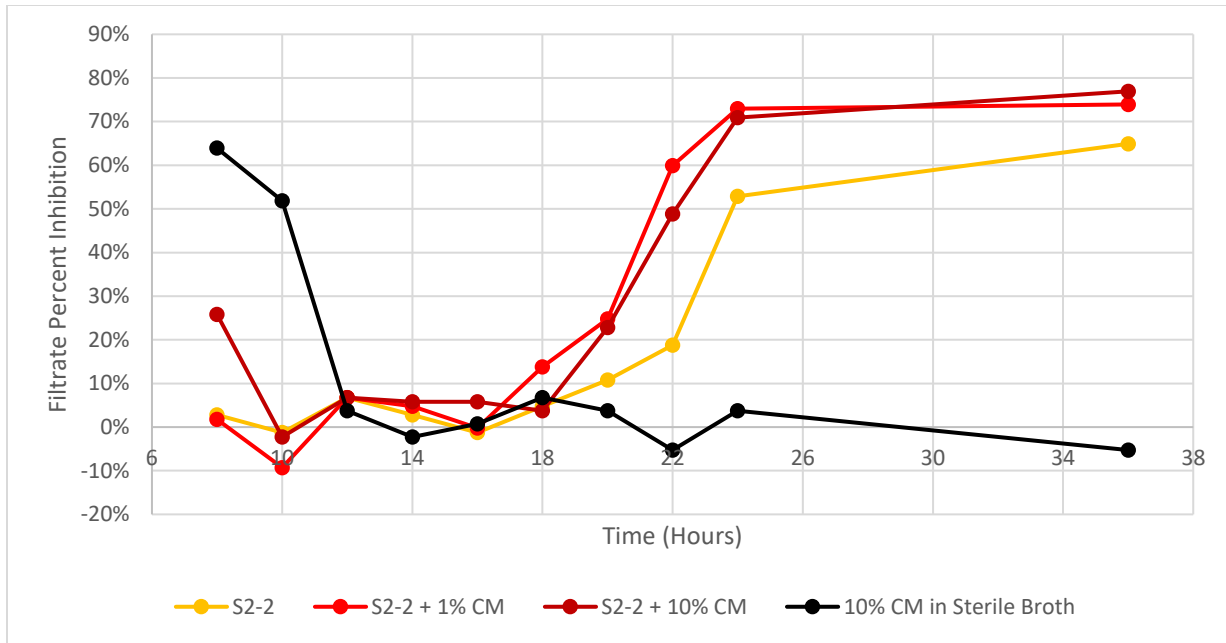
Figure 5. **Filtrate Percent Inhibition of S2-2 with or without 1% or 10% Conditioned Media**. Filtrate percent inhibition from filtrate collected from *Streptomyces* isolate S2-2 TSB broth culture, with or without addition of 1% or 10% conditioned media from 30-hour old S2-2 culture at hour 8 across 36 hours (n=3). Sampling was conducted at hour 8 after addition of CM, and every 2 hours after until hour 24, with one more sampling at hour 36. FPI from filtrate collected from a sterile broth flask with 10% CM added also at hour 8 is included (n=1). Figure shows individual FPI. Standard error bars are included in the appendix.
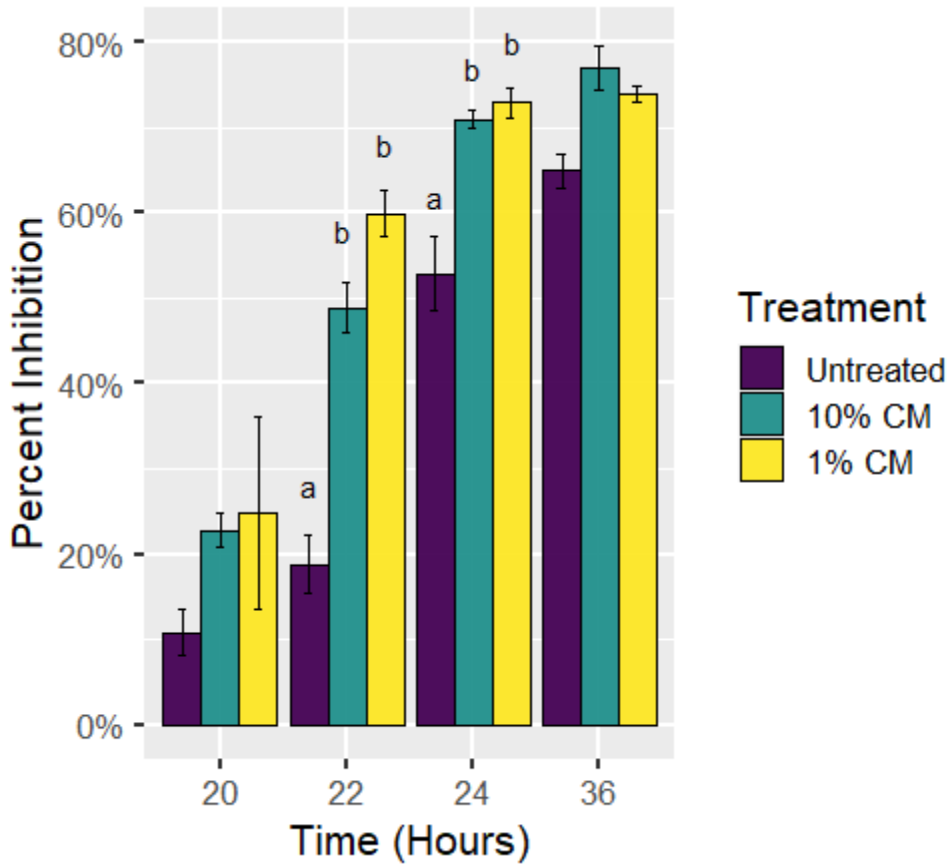
Figure 6. **Filtrate Percent Inhibition with or without 1% or 10% Conditioned Media Hours 20-36.** Filtrate Percent Inhibition of filtrate S2-2, S2-2 with 1% or 10% CM treatment at hour 8 of incubation, for hours 20, 22, 24, and 36 (n=3). Mean FPI was higher in broth treated with either 1% or 10% CM at each sample time, with significant difference during hours 22 and 24 (SE=0.053; df=24; p ≤0.05). Groups labeled with "a" differed significantly from groups labelled "b" during those sampling times. Standard error bars are included.
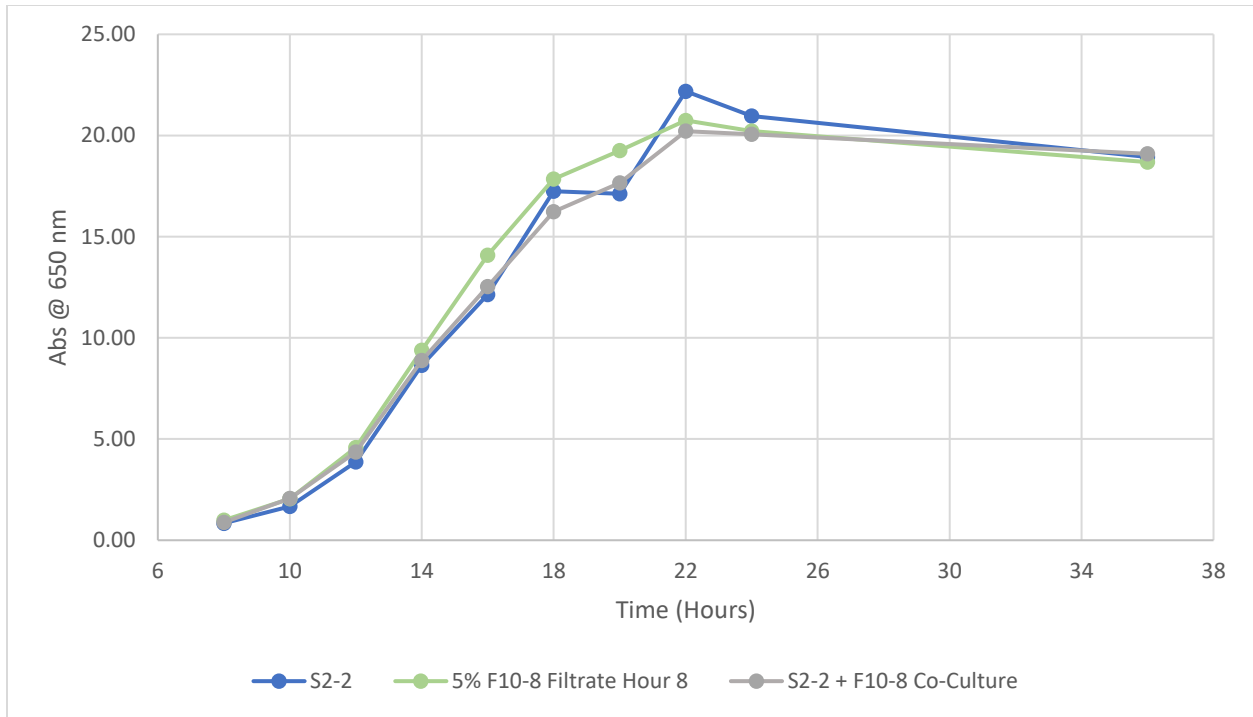
Figure 7. **Optical Density of S2-2 with or without F10-8 Filtrate or F10-8 Co-culture.**
Absorbance level at 650 nm of *Streptomyces* isolate S2-2 TSB broth culture, with or without
addition 5% F10-8 filtrate at hour 8 or simultaneous inoculation with F10-8, across 36 hours
(n=3). Sampling was conducted at Hour 0, 8, and every 2 hours until hour 24, with one more
sample at hour 36. Figures showing individual growth curves with standard error bars are
included in appendix.

Figure 8. **Filtrate Percent Inhibition with or without F10-8 Filtrate or F10-8 Co-culture**.
Filtrate percent inhibition at hours 22, 24, and 36 from filtrate of untreated *Streptomyces* isolate
S2-2 TSB broth culture, S2-2 culture with addition of 5% F10-8 filtrate at hour 8, or S2-2 and
co-culture with F10-8 (n=3). Sampling was conducted at hour 8 after addition of CM, and every
2 hours after until hour 24, with one more sampling at hour 36. Figures showing individual FPI
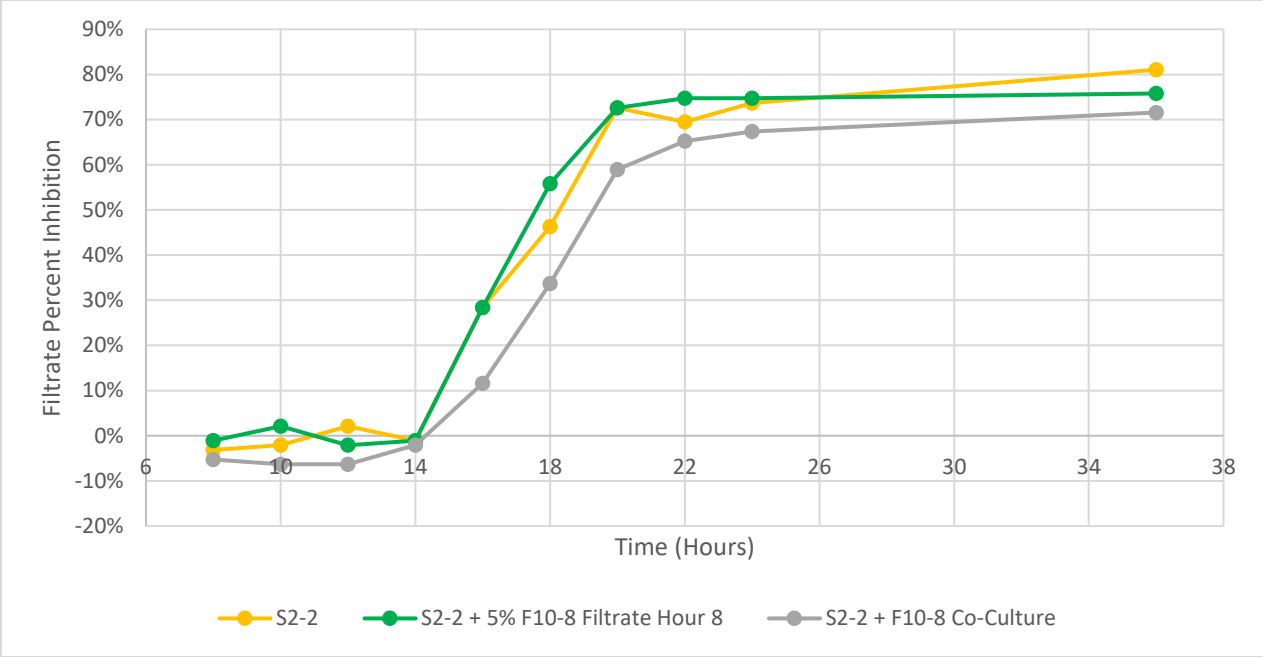with standard error bars are included in appendix.
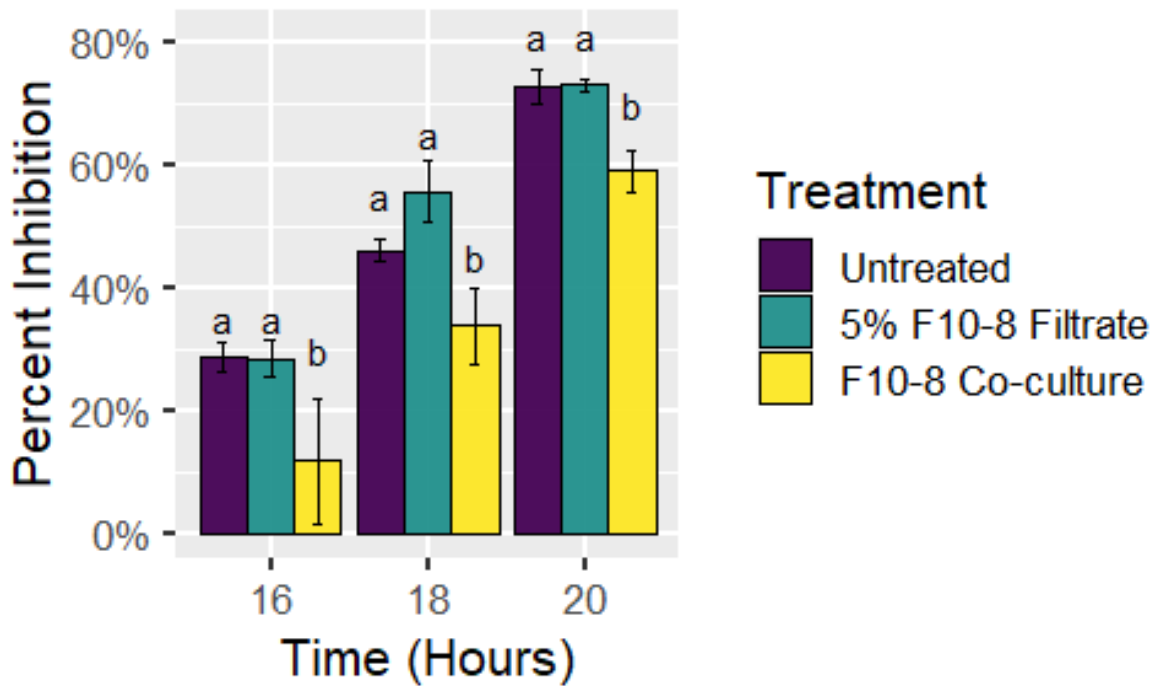
Figure 9. **Filtrate Percent Inhibition with or without F10-8 Filtrate or F10-8 Co-culture Hours 16-20**. Filtrate percent inhibition at hours 16-20 (log-growth phase) from filtrate of untreated *Streptomyces* isolate S2-2 TSB broth culture, S2-2 culture with addition of 5% F10-8 filtrate at hour 8, or S2-2 and co-culture with F10-8 (n=3). A simplified ANOVA test compared the effect of treatments, and revealed significant difference between treatment with F10-8 co-culture and untreated S2-2 filtrate percent inhibition *(F=12.741; df=2,22; p≤0.05)*. Bars labeled with "a" did not differ significantly, but did differ significantly from bars labeled "b" at those sampling times. Standard error bars are shown for each treatment.
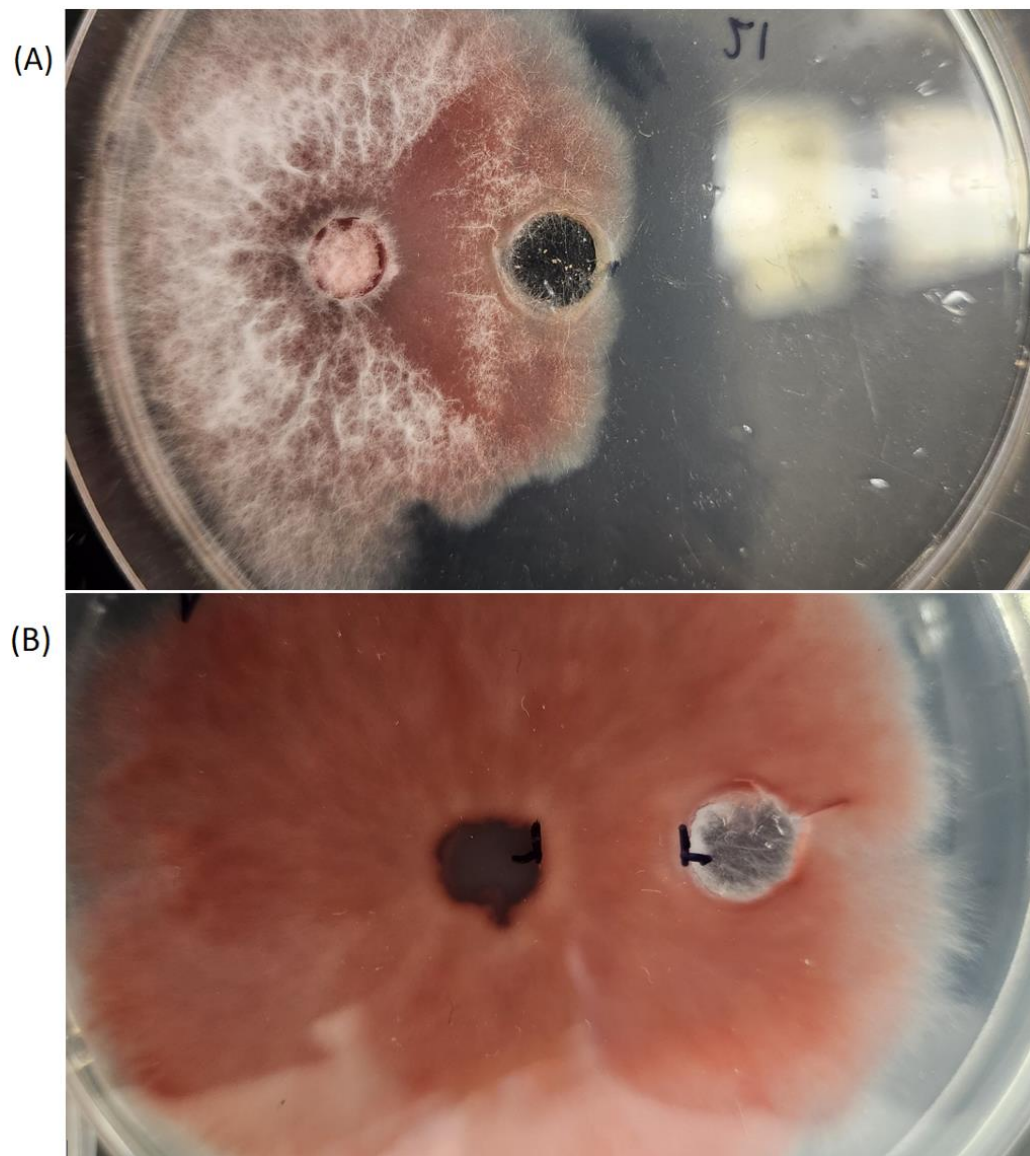
Figure 10. **Agar Well Diffusion Assay with 10% S2-2 Ethyl Acetate Extraction or Water.**
Agar well diffusion antifungal assay with either (A) 50 µl of 10% (w:v) dried ethyl acetate crude
extract placed in well, or (B) 50 µl of dsH$_2$O . *Fusarium* isolate F10-8 was placed 1 cm away
from the well and the plate was incubated for 7 days. Treatment with 10% Extract solution (A)
shows a zone of partial inhibition with less aerial mycelium, and a reduced directional radial
growth towards agar well compared to control plate (B).

DISCUSSION

The aim of this research was to explore the interactions between *Streptomyces* isolated from disease suppressive soils and *Fusarium* isolated from the same soils, and identify *Streptomyces* isolates which were able to inhibit *Fusarium,* as well as explore factors which may influence antifungal production in *Fusarium*-inhibiting *Streptomyces*. A modified dual layer plate assay method was used for assaying inhibition between pairwise combinations of isolates (Essarioui et al., 2017). This method allowed for the identification of *Streptomyces* isolates able to inhibit *Fusarium* growth, as well as *Fusarium* isolates able to inhibit *Streptomyces* growth. Understanding these pairwise interactions may be useful as part of larger models of interactions between these isolates, and similar species interactions in disease suppressive soils.

Every *Fusarium* isolate was able to inhibit *Streptomyces* isolate's growth on dual layer plates. While the *Fusarium* isolates were not tested for activity against plant pathogenic *Streptomyces*, it seems likely that these *Fusarium* isolates, which had inhibitory activity against all ten *Streptomyces* isolates in this research, could have inhibitory activity against *Streptomyces scabies* or other *Streptomyces* species responsible for potato scab disease (Ismail et al., 2020). Application of antagonistic fungi such as *Trichoderma* has been shown to be an effective tool in management of potato scab (Hassan et al., 2021). While *Trichoderma* is one of the most popular fungi utilized as a MBCA, in such products as Rootshield® and several others, nonpathogenic *Fusarium* have been documented as pathogen antagonists and competitors, inducers of plant systemic resistance, and plant growth promoters (Collinge et al., 2022; Patil & Sriram 2020). The future may be promising for *Fusarium* based MCBAs, however much more testing would be

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required, such as field tests and the verification of non-pathogenicity of the *Fusarium* isolates on a wide variety of host plants.

The prevalence of *Streptomyces* inhibition by these *Fusarium* isolates may also have implications for *Fusarium* disease management with *Streptomyces* based MCBAs, and in understanding the dynamic interactions in soils between these microbes. A field setting with a large presence of *Fusarium*, either in plant infection or in soil, may prove to be a difficult place for *Streptomyces* to become or stay established. Such a setting might see a reduction in the population of endogenous *Streptomyces*, or a decrease in the effectiveness of application of *Streptomyces* based MCBAs, since the *Fusarium* may prevent *Streptomyces* from establishing.

The dual layer plate assays showed a large variation in the ability of *Streptomyces* isolates to inhibit *Fusarium* isolates. Of the 100 pairwise combinations of *Streptomyces* and *Fusarium* isolates, 46 did not show any *Fusarium* inhibition in this study (46%). This is consistent with previous research which has shown a strong resistance of *Fusarium* to inhibition by sympatric *Streptomyces,* and a lesser resistance of *Streptomyces* to inhibition by sympatric *Fusarium* (Winter et al., 2019; Essarioui et al., 2020). The literature on interactions of sympatric and allopatric *Fusarium* and *Streptomyces* isolates, as well as interactions between sympatric and allopatric *Streptomyces* isolates, suggests a high correlation between inhibitory potential and nutrient use overlap, which is likely indicative of co-evolution strategies between these organisms; however, this research is limited (Kinkel et al., 2014; Winter et al., 2019).

There was a wide range in the level of *Fusarium* inhibition among the *Streptomyces* strains. *Streptomyces* isolates S2-4 and S2-5 showed only partial inhibition of *Fusarium* growth in some combinations. This partial reduction was observed as a reduction in aerial hyphae and fungal density, but there was no zone of total clearing. *Streptomyces* isolates S10-2 and S10-7

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were able to cause zones of total inhibition, but were also noted for having larger zones of partial inhibition. It is possible that these strains do produce potentially useful antifungal compounds; however, these compounds were either at too small a concentration to provide total fungal inhibition, or these compounds target mechanisms in fungi related to the production of aerial mycelium or other fungal metabolic pathways that are not essential for growth. Partial inhibition was also seen in agar well assays with certain extraction fractions.

Streptomyces isolates S2-2 and S10-9 had zones of total inhibition against all ten *Fusarium* isolates on dual layer plate assays. These *Streptomyces* had larger inhibition zones on average for most pairwise combinations than other *Streptomyces* isolates. These isolates may have potential application as a MCBA for combatting *Fusarium* pathogens, and likely other fungal diseases. The application of *Streptomyces* as a control agent for *Fusarium* plant pathogens such as *F. oxysporum, F. graminearum,* and *F. tricinctum* has been researched in several studies already, and *Fusarium* is listed as one of the diseases controlled by the commercially available *Streptomyces*-based products Mycostop® and Actinovate® (Cao et al., 2005; Colombo et al., 2020; Moussa et al 2019).

The largest zones of inhibition of *Fusarium* in dual layer assays appeared on plates with *Streptomyces* isolate S2-2 acting against the overlay of *Fusarium* isolate F10-8. These two isolates were used for the duration of further experiments in this study. *Streptomyces* isolate S2-2 was chosen for this strong inhibitory activity, its ability to strongly inhibit all nine other *Fusarium* isolates, and for having rapid sporulation and strong growth on Oatmeal Agar. The *Fusarium* isolate F10-8 was chosen due to its sensitivity to inhibition by S2-2, making it a good choice as an indicator of antifungal production by S2-2.

Further experiments aimed to identify factors which have an effect on antifungal production in *Streptomyces*. These factors were population density, exposure to conditioned media (which may contain autoinducers for antifungal production) and exposure of *Streptomyces* to *Fusarium* (by either filtrate from *Fusarium* broth culture, or by direct contact in a co-culture with *Fusarium*).

A growth curve experiment was performed by tracking S2-2 broth culture optical density by absorbance at 650 nm across a 38-hour period. The A650 was used as an indicator of population density level, as is common in bacterial research (Myers et al., 2013). The growth pattern for S2-2 broth culture was observed as including a lag-phase with little growth, a log-phase with rapid rise in population density, and then a tapering off of rise in population density during the stationary phase. This pattern of broth culture population growth has been well established in microbial research (Navarro Llorens et al., 2010; Finkel 2006). Filtrate was collected from broth cultures during optical sampling, and was used in agar well diffusion assays to test for antifungal activity based on the methods used in previous research (Phuakjaiphaeo et al., 2016; Singh et al., 2014).

The antifungal activity of filtrates collected during this experiment was indicated by the percent inhibition of radial growth of F10-8 on agar well diffusion plates, referred to as filtrate percent inhibition (FPI) in this study. This method was chosen due to its sensitivity being adequate for detecting small amounts of antifungal activity. Filtrate with even small activity was observed to cause a reduction in directional growth towards the agar well from A *Fusarium* disc placed 1 cm away from the well.

The antifungal activity of filtrate collected from the S2-2 broth showed a dramatic rise during log-phase growth (Figure 3). The antifungal activity continued a gradual rise afterwards,

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closely following the rate of rise in population density. This indicated that there was a certain population density which triggered antifungal production. There was another sharp increase in antifungal activity at hour 38 of this experiment. This may have been due to the presence of new or greater levels of antifungal production, although it may also be that this rise was due to older *Streptomyces* beginning to die off and undergo lysis, releasing intracellular enzymes which would have antifungal activity. This sort of pattern for secondary metabolite production during log-phase growth, where production of secondary metabolites only begins after reaching a critical population density, has been established in research, and has been documented as being governed by quorum sensing autoinducers (van Delden et al., 2001; Jones et al., 1993). It is important that we understand the effects of factors such as population density and quorum sensing mediated expression of secondary metabolites in order to optimally utilize these and other bacteria as MCBAs and sources for antimicrobial compounds.

The next experiment investigated the effect on S2-2 broth growth and antifungal production during treatment with conditioned media (CM) from older S2-2 broth samples. The influence of autoinducers such as PI factor and A-factor on antibiotic production in *Streptomyces* has been well established (Ohnishi et al., 2005; Recio et al., 2004). While the presence of these or other autoinducers was not tested for in CM, the presence of such autoinducers seems likely, and it has been shown previously that exposure to same species CM can have an effect on antibiotic production in *Streptomyces* broth cultures (Becker et al., 1997).

Addition of CM at either 1% or 10% volume of broth culture resulted in an earlier rise in FPI, and significantly increased antifungal activity of filtrate from S2-2 broths. This effect was most evident at hours 20 through 24, which was during the log-phase growth period, with the final sampling time at hour 36 still showing greater FPI (Figure 6). The bacterial population

density as indicated by optical density was not significantly different between untreated and CM treatments at those times (Figure 5). Addition of CM treatment caused an initial spike in FPI as compared to untreated S2-2 broth at hours 8 through 12, however that spike soon dissipated. Inclusion of sterile broth treated with the greater volume of CM treatments simultaneously to S2-2 treatment broths also showed a spike and dissipation of FPI during those times. It is likely that antifungal compounds in CM were responsible for this initial spike, and that those compounds were degraded during incubation. The sterile broths treated with 10% CM did not have antifungal activity after hour 12, which indicates the increase in activity in CM treated broths by hour 20 was not caused by the initial antifungal activity of the CM. The results of this experiment suggest the presence of some sort of autoinducer in the CM responsible for an earlier rise in antifungal production in CM treated S2-2 cultures.

A growing area of interest is the effect of intergenic cultures of bacteria and fungi, and the effects of intergenic and interkingdom signaling molecules, on antibiotic production (Abdalla et al., 2017; Matila et al., 2018). Co-culture experiments of interspecies and interkingdom bacteria and fungi have already led to the discovery of new metabolites which were not present in absence of co-culture (Schroeckh et al., 2009; Traxler et al., 2013; Zawawi et al., 2022). The production of known antibiotics by *Streptomyces* has been shown to increase during co-culture with specific other intergenic bacteria (Carlson et al., 2015).

The exposure of *Streptomyces* S2-2 to *Fusarium* was attempted in several ways, and methods were adjusted or discontinued. Addition of unfiltered 30-hour old PDB F10-8 culture at 10%, 5%, or 1% of the volume of S2-2 culture broth at hours 0 or 8 resulted in the S2-2 being outcompeted by F10-8 during sampling times. Exposure to 10% by volume F10-8 filtrate seemed to severely inhibit S2-2 growth rate, and so was not used for comparison (data not shown).

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Exposure of 5% by volume F10-8 filtrate at hour 8 did not seem to impact growth rate of S2-2 broth culture. There was no clear trend on the effect of treatment with 5% F10-8 filtrate on FPI, and there was no significant difference in FPI compared to untreated S2-2 culture.

There are a few explanations as to why this treatment did not seem to produce a significant effect. The F10-8 filtrate may not have contained anything which would alter production of antifungal compounds in *Streptomyces* S2-2. It is also possible that there was something which would, or did, have an effect on S2-2 antifungal production, but not at sufficient quantity or effect to be distinguished in this experiment. If *Fusarium* filtrate did elicit induction of novel antifungal compounds in S2-2, they were either in too small a quantity to be noticeable compared to other antifungals produced by S2-2, or not able to act in synergy with the antifungals already produced in S2-2 broth to a degree which was detectable by the antifungal assays in this experiment. One way this could be tested for would be through analysis and purification of the constituents present in fungal broth filtrate, followed by S2-2 broth treatment with the isolated components, and followed by analysis of the components for the S2-2 treated broth and comparison to untreated S2-2 metabolites. This would require extensive analysis and purification procedures which were not an option in this research.

There was also no significant difference in absorbance values for S2-2 co-culture, however the simultaneous inoculation of F10-8 by way of adding a 1 cm *Fusarium* disk at hour 0 did seem to reduce FPI from co-culture broths as compared to untreated S2-2. This effect was most evident during hours 16 to 20 where FPI was significantly lower. This occurred during log-growth phase. While S2-2 had outcompeted F10-8 by hour 8 of sampling time, the presence of the *Fusarium* disc did have an effect on antifungal production. The mechanisms for how the initial physical presence of *Fusarium* reduced antifungal activity, but did not have a significant

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effect on growth, is not clear. It has been shown that *Fusarium* species are capable of producing

quorum quenching molecules which disrupt quorum sensing in bacteria, and thus interfere with

secondary metabolite production (Kamath et al., 2023; Martín-Rodríguez et al., 2014; Rajesh &

Rai 2013). This may explain why growth of S2-2 with F10-8 did not differ significantly, but

antifungal activity did.

Chemical extraction of S2-2 culture filtrate with ethyl acetate, chloroform, and mixed

hexanes yielded different results. These solvents were chosen for their varying levels of polarity

and hydrogen bonding. Extraction with mixed hexanes yielded a small amount of oily substance,

which did not show antifungal activity. Chloroform did yield a brown crude extract, which

showed some antifungal activity. Ethyl acetate crude extract yielded a pink crude extract, and

the antifungal activity was similar to chloroform crude extract. Ethyl acetate is a common

solvent used in *Streptomyces* broth extractions (Rajaram et al., 2020; Singh et al., 2014; Ilić et

al., 2005). Based on the prevalent use of ethyl acetate on *Streptomyces* extractions in the

literature and the verified antifungal activity in ethyl acetate crude extract, it was decided that

ethyl acetate would be used for larger extraction.

The antifungal activity of 1% and 10% (w:v) crude ethyl acetate extract did inhibit radial

growth on agar well assays by around 35%. This FPI was smaller than the FPI from the source

filtrate both pre and post-extraction, around 80%. This may have been due to a lack of

consistency in concentration of active compounds in filtrate compared to the known

concentration of ethyl acetate crude extract; however, we might expect with an extraction using

1:1 solvent to filtrate, that 50 µl of 10% solution from 100 ml dried solvent, a 5 ml filtrate

equivalent in an ideal scenario, would have an antifungal content much greater than 50 µl of

unextracted filtrate. These results indicate that the ethyl acetate extraction did not pick up the

compounds most responsible for the greater inhibition seen in unextracted and post-extraction filtrate.

What was interesting was that there was a very large zone of partial inhibition on assay plates treated with the 1% and 10% ethyl acetate crude extract. It is clear that ethyl acetate extraction did extract antifungally active compounds, however there were more, or other desirable compounds, left in filtrate. An alternative method of extracting and isolating the compound or compounds responsible for the majority of antifungal activity in S2-2 filtrate is desirable. Purifying the compounds from extraction, whether from filtrate directly, or from extraction with an alternative solvent would be ideal.

Fractionation of ethyl acetate extract was performed to purify and work towards identifying the compound(s) responsible for the antifungal activity observed in S2-2 filtrate. Analysis of the ethyl acetate extract fractions included antifungal assays and UV-Vis analysis. The UV-Vis analysis of a 10x concentrated ethyl acetate extraction solvent showed a spectrum with peaks around 280, 330, and 500 nm (Appendix). These wavelengths were monitored for absorbance for all 80 fractions as a way to gauge level of analytes in effluent, and for later comparison to antifungal activity. A notable rise in absorbance at 330 nm started around fraction 16 and lasted to a varying degree until fraction 34. Fraction 19 showed a brief spike in A280 and A330, and was also noted for having a distinct smell. Fraction 20 was also notable for having the largest A500 out of any sample. Antifungal assay showed antifungal activity that was most abundant around fractions 18-20 and 24-26. This may indicate there were multiple antifungal compounds which were able to be separated during this fractioning, however it is also possible that these fractions were similar in absorbance and activity due to similar contents, and the difference in retention time was due to tailing in the fractioning process.

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Further analysis of fractions which showed the greatest inhibitory activity was attempted using liquid-chromatography mass-spectroscopy (LCMS), however the broad range of chemical classes produced by *Streptomyces* makes this a complicated step in analysis. Metabolite identification in LCMS relies on ionization of the target compounds, and the ability of the antifungal analytes present in these fractions to be ionized is not guaranteed. Species identification of S2-2 may help narrow down the classes of compounds which are being searched for. Further purification via sub-fractioning, and other chemical analysis would also be ideal, and is a great next step if this research were to continue.

Both genera of isolates used in this research encompass a large diversity of microbes with complex physiology and metabolisms. *Fusarium* is often associated with plant disease, and for good reason; however, the antimicrobial activity observed in this study and in the literature suggests that *Fusarium* may be a promising source of antimicrobial compounds, and non-pathogenic species may have potential for use as microbial biological control agents (Patil & Sriram 2020). *Streptomyces* have a long-standing reputation as producers of useful antimicrobial secondary metabolites, as useful microbial biological control agents, and are often associated with disease suppressive soils (Bubici 2018; Wanner et al., 2014).

There is a growing need for new antifungal compounds in both agriculture and medicine. The rate of mortality from fungal infection, even with treatment of antifungal medicines, remains alarmingly high (Roemer & Krysan 2014). *Fusarium* has been documented as an opportunistic fungal pathogen which can infect humans, primarily when a patient is immunocompromised (Nucci & Anaisse 2007). Rising infection rates from opportunistic fungal pathogens, as well as the emergence of highly drug resistant fungal pathogens such as *Candida auris*, result in a growing necessity for the discovery of new antifungal compounds (Perfect & Schell 1996;

Chaabane et al., 2019). This same escalation of need for new antifungal agents is also seen in agriculture, where fungal plant pathogens are becoming increasingly resistant to current antifungal chemical treatments (Klix et al., 2007; Deising 2008).

The risks of conventional chemical and organically synthesized fungicide use are not well studied; however, the literature suggests significant risks (Köhler & Triebskorn 2013). Alternative strategies for reducing crop loss are highly desirable. The use of microbial biological control agents, and the fostering and utilization of disease suppressive soils are both promising strategies for reducing reliance on conventional fungicides. The factors which promote disease suppression in disease suppressive soils are not fully understood. By researching organisms isolated from these soils we may strengthen our understanding of the complex interactions which cause soils to become disease suppressive. While much research of soil microbes is performed on the individual microbes, there has been a growing trend of performing studies which investigate the effects of cultivation of multiple species in concert. Co-cultivation and exposure to interspecies, intergenic, or even interkingdom, extracellular signaling molecules is useful as a means of exploring organismal interactions, and is likely to provide insight into the complex ecological interactions of soil microbes which occur in nature.

This study focused on the inhibitory interactions of sympatric *Streptomyces* and *Fusarium* isolates, as well as the antifungal production of *Streptomyces* isolate S2-2. it was discovered that a large cohort of *Fusarium* were resistant to *Streptomyces* inhibition. It has been suggested in previous research that this greater ability of *Fusarium* to inhibit *Streptomyces* than of *Streptomyces* to inhibit *Fusarium* isolated from the same soils may be due to *Fusarium's* greater genome size, allowing for more adaptation to competitor species, while *Streptomyces* can rely on other mechanisms, such as spore formation, to avoid competition (Essarioui et al., 2020).

These interactions are likely to have been part of a product of the co-evolution of these species. Of the ten isolates from each genus, there were isolates which showed strong antimicrobial capabilities.

These organisms have potential for use as microbial biological control agents, and as sources for antimicrobial compounds which may be useful medically or in agriculture as an alternative to chemical or synthesized organic pesticides. Factors which influence antifungal production of *Streptomyces* isolate S2-2 were also investigated, and revealed antifungal production was population density dependent, a mechanism which is likely governed by quorum sensing mechanisms, as shown in trials exposing S2-2 to mature S2-2 culture filtrate, referred to as conditioned media. It was also revealed that physical interaction with *Fusarium* isolate F10-8 was able to lower antifungal production of S2-2, however the mechanism for this action is not understood. It may be that quorum quenching is involved. It is of critical importance to continue investigation into soil microbial interactions, as our understanding of such interactions further our ability to promote disease suppression in soils, to find useful microbial biological control agents, and to discover secondary metabolites valuable to both medicine and agriculture.

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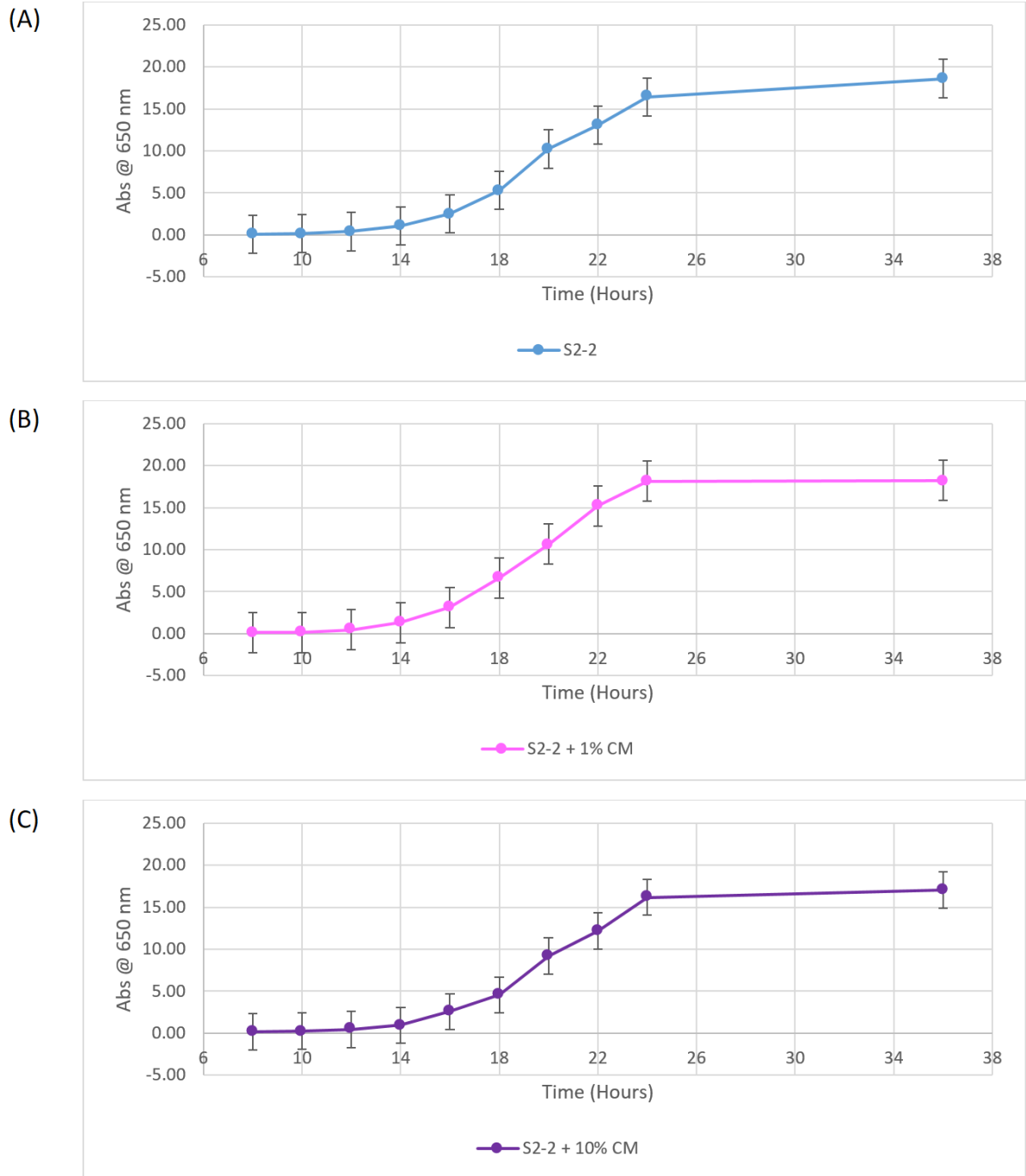
APPENDIX

Figure A1 Absorbance values for Streptomyces isolate S2-2 TSB broth culture, with or without addition of 1% or 10% conditioned media from 30-hour old S2-2 culture. Sampling was conducted at Hour 0, 8, and every 2 hours until hour 24, with one more sample at hour 36. Standard error bars are included (A) S2-2 (B) S2-2 treated with 1% CM (C) S2-2 Treated with 10% CM.

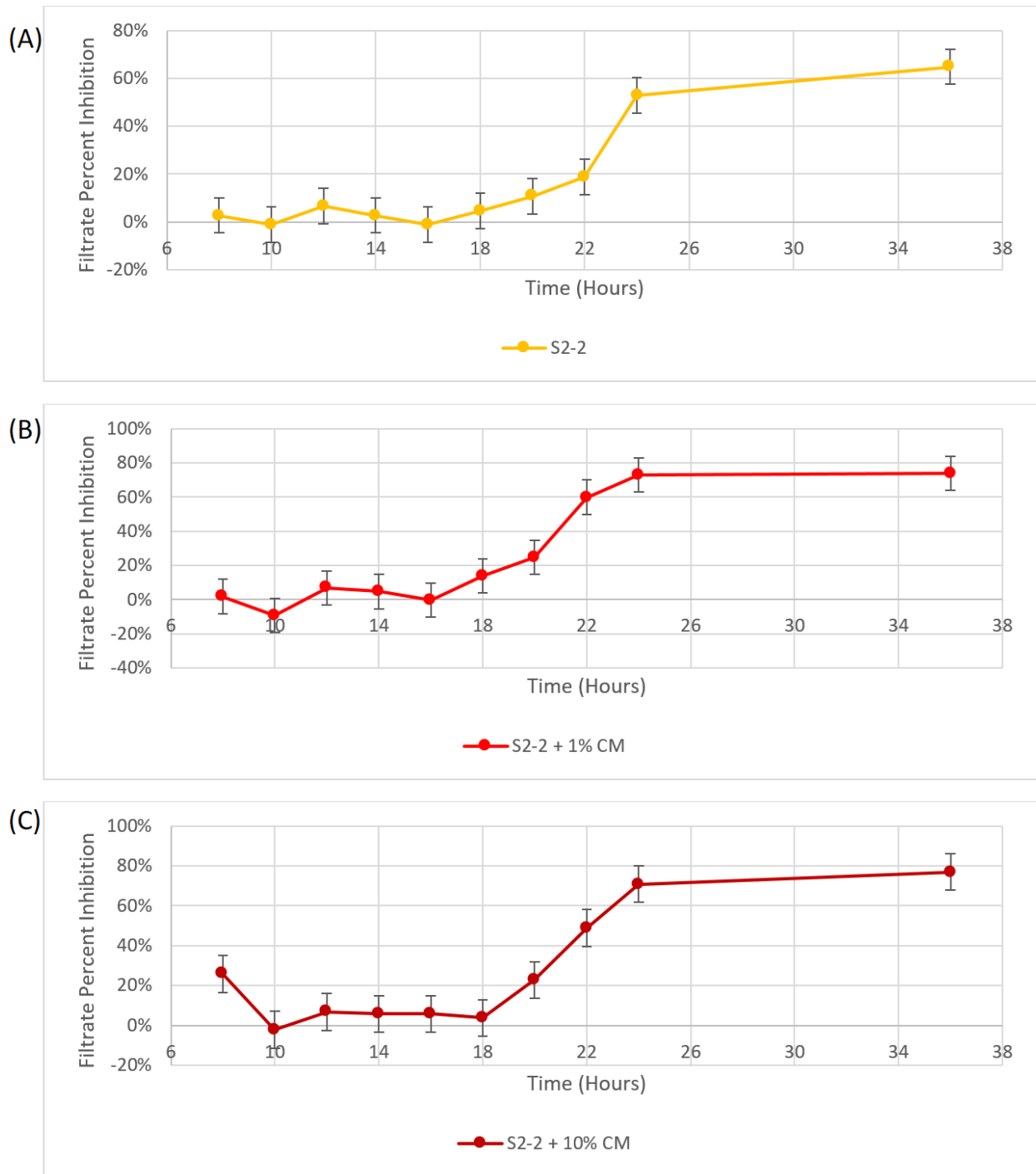*Figure A2.* Filtrate percent inhibition from filtrate collected from *Streptomyces* isolate S2-2 TSB broth culture, with or without addition of 1% or 10% conditioned media from 30-hour old S2-2 culture at hour 8 across 36 hours. Sampling was conducted at hour 8 after addition of CM, and every 2 hours after until hour 24, with one more sampling at hour 36. (A) S2-2 (B) S2-2 treated with 1% CM (C) S2-2 Treated with 10% CM.

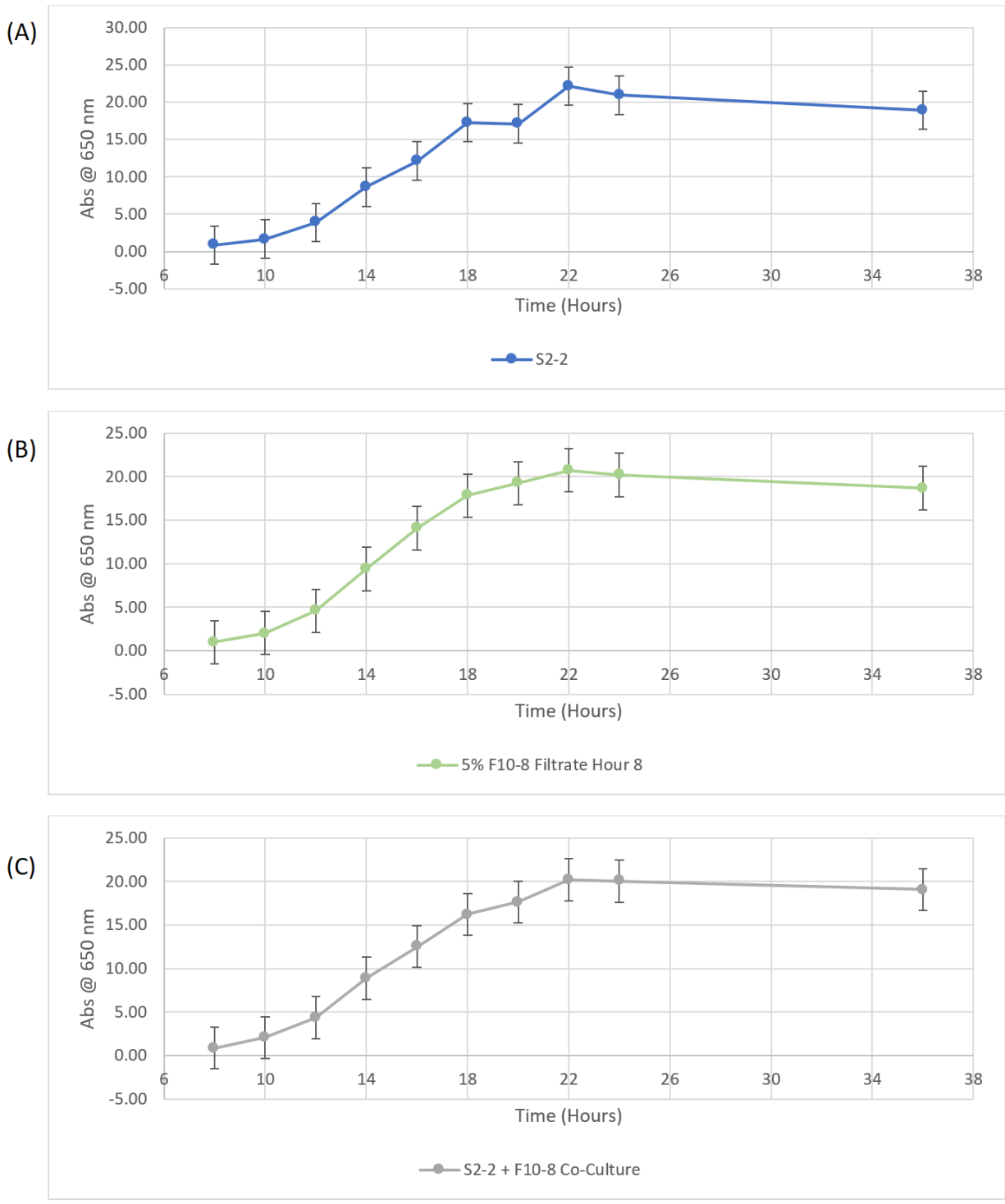*Figure A3.* Absorbance level at 650 nm of *Streptomyces* isolate S2-2 TSB broth culture, with or without addition 5% F10-8 filtrate at hour 8 or simultaneous inoculation with F10-8, across 36 hours. Sampling was conducted at Hour 0, 8, and every 2 hours until hour 24, with one more sample at hour 36. Standard error bars are included. (A) S2-2 (B) S2-2 with 5% F10-8 Filtrate (C) S2-2 + F10-8 co-culture.

Figure A4. Filtrate percent inhibition of *Streptomyces* isolate S2-2 TSB broth culture, with or without addition 5% F10-8 filtrate at hour 8 or simultaneous inoculation with F10-8, across 36 hours. Sampling was conducted at Hour 0, 8, and every 2 hours until hour 24, with one more sample at hour 36. Standard error bars are included. (A) S2-2 (B) S2-2 with 5% F10-8 Filtrate (C) S2-2 + F10-8 co-culture.
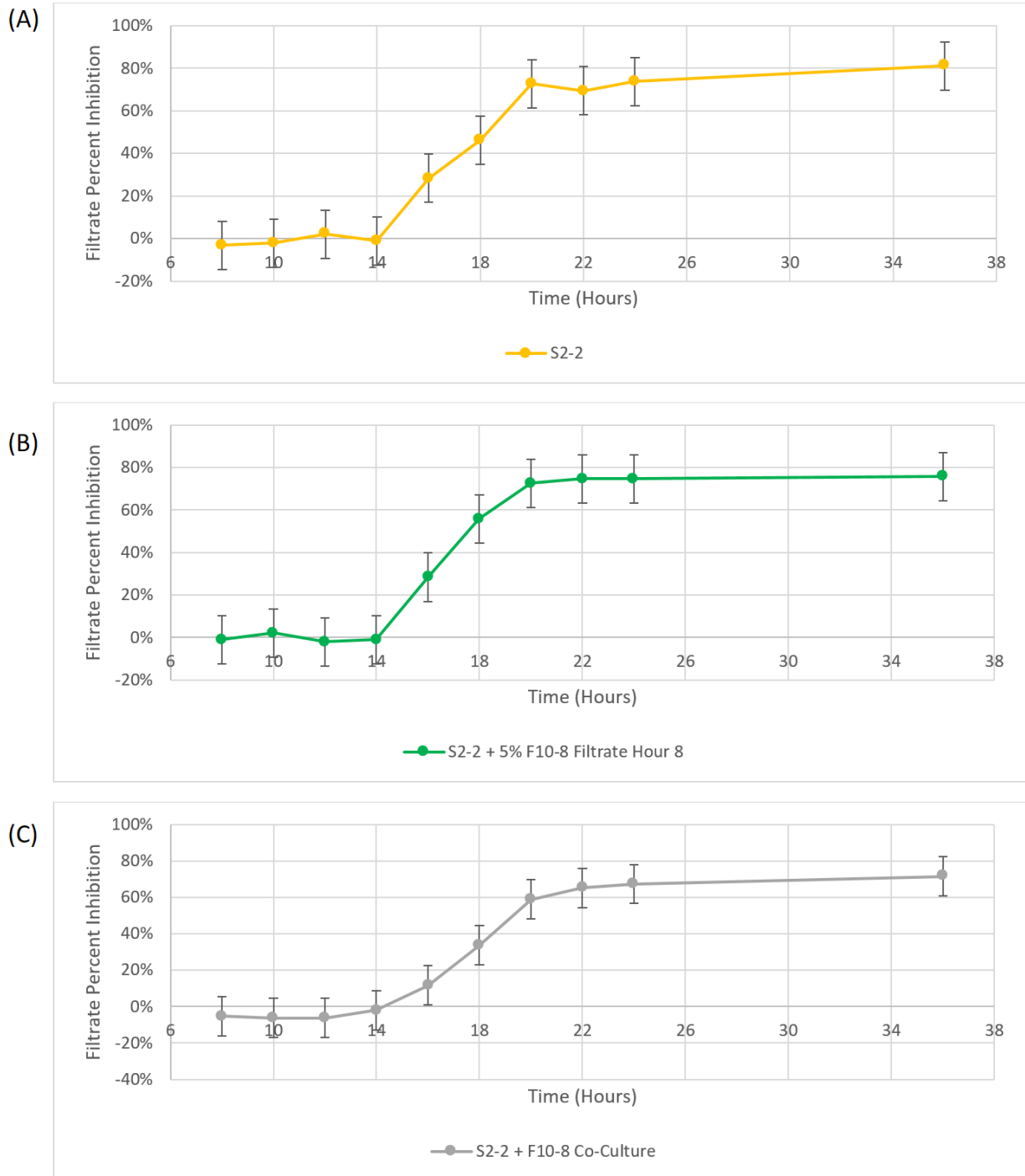
Table A1. Percent radial growth inhibition (PIRG) of *Fusarium* isolate F10-8 on agar well diffusion assay plates from S2-2 filtrate extraction fractions. Control plates averaged 32 mm of growth, consistent with previous experiments.

Fraction	PIRG	Radial Growth (mm)	Fraction	PIRG	Radial Growth (mm)
2	0%	32	36	3%	31
4	0%	32	38	3%	31
8	-3%	33	40	0%	32
10	-3%	33	42	-9%	35
12	9%	29	44	-3%	33
14	16%	27	46	0%	32
16	28%	23	48	0%	32
17	22%	25	50	-6%	34
18	38%	20	52	0%	32
19	28%	23	54	0%	32
20	28%	23	56	0%	32
21	25%	24	58	-3%	33
22	19%	26	60	0%	32
23	22%	25	62	9%	29
24	28%	23	64	0%	32
25	34%	21	66	0%	32
26	31%	22	68	9%	29
27	9%	29	70	3%	31
28	13%	28	72	3%	31
29	9%	29	74	3%	31
30	6%	30	76	13%	28
32	13%	28	78	3%	31
34	9%	29	80	0%	32
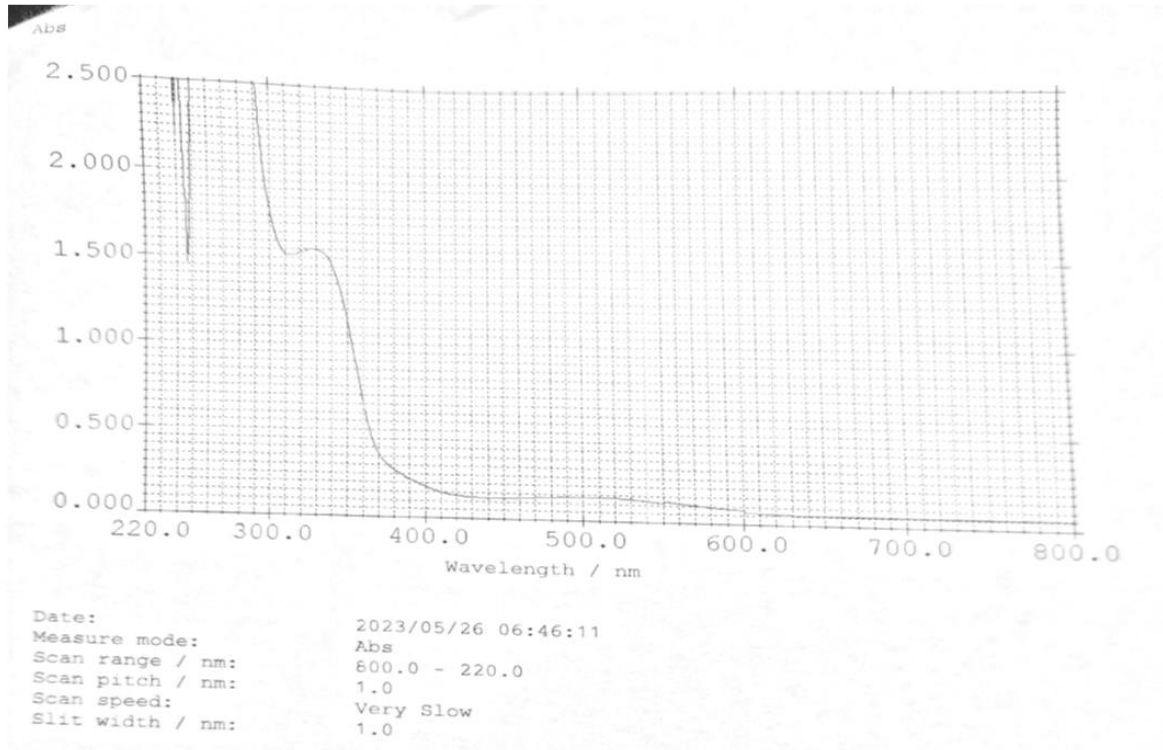
Figure A5. Scanned copy of Shimadzu Model 1800 UV-Vis spectrophotometer print out showing absorption spectra and peaks automatically detected for the concentrated ethyl acetate extraction from S2-2 filtrate.

Table A2. Scanned copy of Shimadzu Model 1800 UV-Vis spectrophotometer print out showing absorption values for 280, 330, and 500 nm for 80 fractions collected during fractioning of concentrated ethyl acetate extraction from S2-2 filtrate.

```
Date:
Measure mode:                        2023/05/26 07:34:06
Wavelength / nm:                     Abs
                                     280.0   330.0   500.0
Data accumulation / sec: 0.2
Slit width / nm:                     1.0
Equation:                            None
```

WL/nm No.	280.0 A1	330.0 A2	500.0 A3
1	0.399	0.022	0.016
2	0.250	0.007	0.013
3	0.221	0.007	0.014
4	0.256	0.008	0.013
5	0.271	0.008	0.013
6	0.271	0.009	0.014
7	0.289	0.010	0.013
8	0.286	0.012	0.013
9	0.329	0.021	0.015
10	0.374	0.038	0.015
11	0.701	0.057	0.016
12	1.159	0.064	0.015
13	1.326	0.077	0.015
14	1.396	0.094	0.014
15	1.162	0.122	0.015
16	0.762	0.152	0.015
17	0.575	0.166	0.014
18	0.718	0.224	0.016
19	1.983	3.171	0.019
20	2.243	3.281	0.024
21	2.053	2.950	0.022
22	2.180	1.866	0.020
23	2.405	0.872	0.018
24	2.420	0.412	0.019
25	2.003	0.245	0.019
26	1.938	0.186	0.019
27	2.258	0.179	0.018
28	1.729	0.168	0.021
29	1.241	0.144	0.021
30	0.946	0.120	0.020
31	0.980	0.157	0.022
32	0.698	0.142	0.019
33	0.508	0.125	0.018
34	0.484	0.146	0.020
35	0.295	0.100	0.016
36	0.267	0.082	0.016
37	0.344	0.081	0.017
38	0.382	0.073	0.017
39	0.390	0.074	0.018
40	0.396	0.099	0.019
41	0.298	0.068	0.017
42	0.284	0.072	0.017
43	0.247	0.072	0.017
44	0.242	0.075	0.016
45	0.234	0.072	0.016
46	0.247	0.074	0.017
47	0.407	0.078	0.017
48	0.828	0.077	0.017
49	1.015	0.072	0.019
50	0.812	0.060	0.016
51	0.580	0.049	0.016
52	0.475	0.045	0.016
53	0.433	0.045	0.017
54	0.414	0.040	0.017
55	0.417	0.037	0.017
56	0.456	0.036	0.017
57	0.523	0.036	0.017
58	0.597	0.036	0.017
59	0.620	0.036	0.017
60	0.612	0.035	0.017
61	0.567	0.034	0.017
62	0.519	0.032	0.018
63	0.456	0.032	0.018
64	0.403	0.030	0.017
65	0.362	0.033	0.017
66	0.340	0.039	0.017
67	0.329	0.046	0.018
68	0.322	0.052	0.018
69	0.298	0.052	0.018
70	0.276	0.051	0.017
71	0.249	0.049	0.017
72	0.225	0.046	0.017
73	0.211	0.046	0.017
74	0.198	0.046	0.017
75	0.186	0.045	0.017
76	0.171	0.042	0.017
77	0.172	0.043	0.017
78	0.171	0.040	0.017
79	0.162	0.037	0.018
80	0.162	0.037	0.017