IDENTIFICATION AND CHARACTERIZATION OF BENEFICAL BACTETRIA TO CONTROL PEA ASCOCHYTA BLIGHT DISEASE.

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

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in

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DEDICATION

I dedicate my work to my loving husband Kambwiri Banda for your endless support and encouragements and to my amazing children, Lusungu Banda, Maya Banda and Wanzi Banda who are my greatest inspiration. To my advisor, Dr. Qing Yan, for your invaluable guidance and wisdom. Your work and dedication in Plant Pathology have been a constant source of motivation for me and dedication to my success has been truly inspiring. To my committee members Dr. Li Huang and Dr. Kevin McPhee, for your insightful feedback and continuous support. Your expertise has greatly enriched this work.

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ABSTRACT

Ascochyta blight is one of the major diseases threating pea production worldwide. It is caused by a complex of fungal pathogens including *Ascochyta pisi*, *Ascochyta pinodes*, *Ascochyta pinodella* and *Phoma koolunga*. Among these pathogens, *A. pinodes* alone can cause yield loses of up to 50% and was the focus of this study. Our goal was to identify and characterize beneficial bacteria that can reduce the disease severity under greenhouse conditions. By screening 350 bacterial strains isolated from pea roots and soils, we found ten isolates, including *Pseudomonas* spp., *Bacillus* spp., *Peribacillus* spp., and *Penibacillus* spp., showed effective control of Ascochyta blight in greenhouse assays. All the 10 isolates inhibited *A. pinodes* on culture plates, indicating bacteria-derived antibiotics likely play a role in the disease control. To identify the antibiotics, we used a soil bacterium *Pseudomonas protegens* Pf-5 as a model because of its known production of a broad spectrum of antibiotics that are toxic to many fungal pathogens. Antibiotic production of Pf-5 requires a global regulator GacA/GacS. We found that wild type Pf-5, but not its Δ*gacA* mutant, inhibited *A. pinodes* on culture plates and reduced disease severity in greenhouse, supporting that antibiotic production contributes to disease control. Using a set of eight Pf-5 mutants, each lack one specific antibiotic, we found that a mutant lacking rhizoxin production showed decreased pathogen inhibition and reduced disease control. These results suggest that rhizoxin of Pf-5 contributes to the growth inhibition of *A. pinodes* and the disease control of Ascochyta blight in greenhouse conditions.

INTRODUCTION AND LITERATURE REVIEW

Introduction

Biological control, employing beneficial bacteria, is a promising eco-friendly strategy for managing foliar plant diseases (Liu et al., 2016). This approach presents appealing alternatives to conventional methods and used in integrated pest management (IPM) as the future paradigm for crop protection (Stenberg, 2017). This thesis comprehensively explores the potential of biological control in mitigating Ascochyta blight, a significant threat to field pea production globally. Field pea (*Pisum sativum* L.) is a self-pollinated dicotyledonous cool season legume that is grown for its edible seeds and seed pods (Dahl et al., 2012). Montana leads the production of dry pea in the United States; however, its production is affected by a foliar disease called Ascochyta blight (Owati et al., 2020). Ascochyta blight is caused by a complex of closely related necrotic pathogen namely *Ascochyta pinodes*, *Ascochyta pisi*, *Ascochyta pinodella* and *Phoma koolunga*. Among these, *A. pinodes* is the most devastating in causing the disease (Bretag et al., 2006). Therefore, this study focuses on beneficial bacteria to control Ascochyta blight of pea caused by *A. pinodes*.

Objectives:

1. Identifying and characterizing bacterial isolates that can reduce disease severity of Ascochyta blight of pea caused by *A. pinodes* under greenhouse conditions.
2. Investigating the mechanisms of how beneficial bacteria control Ascochyta blight of pea.

Literature Review

Key Strategies for Enhanced Biocontrol

One of the major reasons that hinder the implementation of biocontrol on a large scale is the variability and inconsistency of biocontrol activity due to fluctuating environment conditions such temperature, and relative humidity. Biocontrol is also affected by other practices of the farmer. Attempts being made to overcome the sensitivity of the biocontrol agents to the fluctuating environment include selecting biocontrol agents whose ecological requirements encompass those of the pathogen or by applying the biocontrol agents only when they are expected to be effective (Köhl & Molhoek, 2001). Another approach to overcome the variability of biocontrol is to use biocontrol agent(s) with several different modes of actions. (Guetsky et al., 2002) found that using two biocontrol's namely *Pichia guilemondii* (yeast) and *Bacillus mycoides* with different modes of action to control *Botrytis cinerea* on strawberry leaves and plants was more effective than their control individually. *Pichia guilemondii* was found to compete with *Botrytis cinera* for glucose, sucrose, adenine, histidine and folic acid and was found to produce compounds that activates the plants defense systems. It was also able to dissolve the extracellular matrix by the production of cell wall degrading enzymes chitinase and glucanase. Whereas *Bacillus mycoides* was found to secrete volatile and non-volatile inhibitory compounds that had both acropetal and basipetal effects. It was also found to systematically activate the defense system of the host.

Mechanisms of Biocontrol

Mechanism of biocontrol of beneficial bacteria include competition for resources such as nutrients and spaces, antibiosis, parasitism, cell wall degrading enzymes (CWDEs) and induced

systemic resistance (ISR) (Dahl et al., 2012; Stein, 2005). ISR may be induced locally or may be systemic and caused by various micro-organisms can protect plants against soil or foliar pathogens. Inoculation of first expanded leaves of pea seedlings with an avirulent strain of *Pseudomonas syringae* pv. *pisi*, decreased the susceptibility of subsequent leaves 7 or 14 days later to challenge inoculation with *A. pinodes* (Dann & Deverall, 2000). ISR is a complex biocontrol process involving various mechanisms. It can be triggered by elicitors molecules or by certain nonpathogenic microorganisms(Guetsky et al., 2002).

Activation of defense mechanisms can be rapid; salicylic acid was detected in phloem exudates of a cucumber leaf eight hours after inoculation with *Pseudomonas syringae pv. syringae*, and in the phloem of a leaf above the inoculation site 12 hours after inoculation(Zangerl, 2003). However, the efficacy of control achieved through this mechanism is highly variable.

Antibiosis is another important mechanism of biocontrol. Many microorganisms produce and excrete biologically active compounds that can be toxic or inhibitory to other organisms. Microorganisms often involves the production and release of antimicrobial metabolites or cell-wall degrading enzymes(Köhl et al., 2019). When beneficial organisms in situ produce biologically active substances that directly negatively affect pests, this mechanism serves as a foundation for biological control. The ultimate proof for the role of these antibiotics in biocontrol is the loss of activity in non-producing mutants of the beneficial microorganisms(Guetsky et al., 2002). Bacteria produces many antibiotics that offer competitive advantages for them to thrive in nearly every environment on Earth and often compete for limited resources like space and nutrients(Raaijmakers & Mazzola, 2012; Traxler & Kolter, 2015). The biosynthesis of various

antibiotics by a microorganism is often coordinated (Cui et al., 2016). *Pseudomonas protegens* strain Pf-5, a soil bacterium which is well-known for its wide-spectrum antimicrobial activity due to the production of many antibiotics including pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol (2,4-DAPG), orfamide A, rhizoxin, hydrogen cyanide and polyynes (Henkels et al., 2014; Raaijmakers et al., 1999; Yan et al., 2021) was used in this study. Metabolic co-regulation between biosynthesis pathways for antibiotics in common in microbes. (Brodhagen et al., 2004; Quecine et al., 2016) found that a mutation in pyoluteorin biosynthetic genes result in loss of pyoluteorin production along with overproduction of DAPG in *P. protegen* strain Pf-5. Whereas as (Yan et al., 2017) found that an intermediate in DAPG biosynthesis, phloroglucinol, is transformed by a halogenase encoded in the pyoluteorin gene cluster into mono-and di-chlorinated phloroglucinols. The chlorinated phloroglucinols function as intra-and inter-cellular signals that induce the expression of pyoluteorin biosynthetic genes.

Rhizoxin Antibiotic

Rhizoxin is a natural compound with notable antifungal and antitumor properties ((Partida-Martinez & Hertweck, 2005). Rhizoxin binds to tubulin, a protein that is crucial for cell division. This binding disrupts microtubule formation, leading to the inhibition of mitosis (cell division). Due to its potent antimitotic activity in most eukaryotic cells, including various human cancer cell lines, rhizoxin has garnered significant interest as a potential antitumor drug (Partida-Martinez & Hertweck, 2007).

In addition to the biocontrol traits produced by the biocontrol microbes, the effectiveness of biocontrol also depends on how pathogens behave during the pre-penetration phase of their growth on a healthy plant surface and the significance of necrotic tissues within the crop as a

source of inoculum. Unlike necrotrophy, which rely on external nutrients and are vulnerable to competition, biotrophic pathogens like rusts can initiate infection even on nutrient-depleted surfaces, as they do not depend on external nutrients during germination and penetration. However, on the plant surface, the conidia or germ tubes of biotrophs encounter antibiotics and lytic enzymes from microorganisms, which can hinder germination and disrupt germ tubes mainly bacteria such as *Bacillus* spp. and *Pseudomonas* spp. that can inhibit germination and lyse germ tubes(Elad & Freeman, 2002). Understanding how beneficial bacteria suppress plant diseases will help us increase the efficacy and consistency of biological control (Lai et al., 2022).

Importance of Peas

Peas are a nutritious vegetable and a good source of several important nutrients. They are relatively higher in protein compared to many other vegetables. They contain about 5-6 grams of protein per 100 grams, which is around 10-12% of the daily recommended intake. Hydrolyzed pea protein can produce peptides with bioactive properties, including angiotensin I-converting enzyme inhibition and antioxidant effects (Roy et al., 2010). The protein content of peas is influenced by a combination of environmental conditions and genetic factors with their seed dry matter containing about 13·7 to 33 % (Hacisalihoglu et al., 2020; Hood-Niefer et al., 2012; Walter et al., 2022) . Most pea proteins are storage proteins, known as globulins, and their amino acid profile determines their nutritional value. Due to their rich protein content, peas are cultivated for hay, pasture, or silage production, either on their own or mixed with cereals (Chen et al., 2012). Research has demonstrated that field peas are an excellent protein supplement for poultry rations feeder calves, cows, feeder calves, dairy cattle, and swine. In addition to protein, peas provide carbohydrates of around 14-16 grams per 100 grams serving which accounts for

about 5-6% of the daily recommended intake. The intermediate amylose content of pea starch contributes to a lower glycemic index and further reduces starch digestibility (Hoover et al., 2010).They are rich in dietary fiber, which is important for digestive health. They contain about 5-6 grams of fiber per 100 grams, contributing to around 20-24% of the daily recommended intake. They contain beta-carotene, which the body converts into vitamin A of which they provide approximately 33-36% of the daily recommended intake of vitamin A per 100 grams. Peas are a good source of vitamin C and K, providing around 20-24% and 24-28% respectively of the daily recommended intake per 100 grams. Vitamin K is important for blood clotting and bone health. Peas are a decent source of folate, providing about 16-20% of the daily recommended intake per 100 grams. It also contains iron, an essential mineral for transporting oxygen in the blood. They offer around 8-10% of the daily recommended intake per 100 grams. Peas are a good source of potassium, which is important for maintaining healthy blood pressure levels and proper muscle function. They provide approximately 4-6% of the daily recommended intake per 100 grams. They contain magnesium, which is essential for various biochemical reactions in the body. They offer about 6-8% of the daily recommended intake per 100 grams. These percentages can vary slightly depending on factors such as the variety of pea and how they are prepared/cooked. However, peas are generally recognized as a nutritious vegetable packed with essential vitamins, minerals, and fiber(Gawalko et al., 2009).

Additionally, peas contain various phytochemicals, once considered antinutritive, which offer health benefits. These include polyphenolics, especially in colored seed coats, which may have antioxidant and anticarcinogenic properties; saponins, which can exhibit hypocholesterolemia and anticarcinogenic effects; and galactose oligosaccharides, which may

promote beneficial prebiotic effects in the large intestine(Dahl et al., 2012). This crop is usually used as a rotational crop, as its roots can fix atmospheric nitrogen through a symbiotic relationship with rhizobial (Annan et al., 2023). Peas are also beneficial in crop rotations with cereals as they disrupt disease and pest cycles, enrich the soil with nitrogen, enhance soil microbe diversity and activity, improve soil aggregation, conserve soil water, and offer economic diversity.

Dry peas can be cultivated in a variety of soil types, ranging from sandy to clay soils. However, they have low tolerance for saline and waterlogged conditions. Prolonged exposure to waterlogged soils, exceeding 24 hours, can damage or even kill the plants. Integrating dry peas into a cropping system can help manage troublesome grassy weeds and may also mitigate disease and insect pests. Planting requires row spacing from 6 to 12 inches is optimal. Dry peas tolerate cold soil temperatures but are sensitive to heat during flowering which can reduce pod and seed set. Field pea seed will germinate at a soil temperature of 40°F. Uncovered pea plants can tolerate temperatures as low as 14°F, and if covered with snow, they can withstand temperatures down to -22°F (Elzebroek and Wind, 2008). Peas are most productive at temperatures between 55 and 64°F. Therefore, in the northern Plains, they should be planted from late March to early May so flowering will occur during cooler weather in June and early July. Seeding peas after mid-May will cause the crop to begin flowering in mid-July, increasing the risk of heat stress and diseases like powdery mildew, which can reduce yields. Despite the benefits of peas, they are susceptible to various plant pathogens that can negatively impact their productivity (Mudryj et al., 2014).

Pea Production

Dry pea is mainly cultivated in the Northern Plains of the United States of America. It is grown in Idaho, Montana, Nebraska, North Dakota, South Dakota, and Washington. Among these, Montana leads the production of dry pea in the United States of America(Owati et al., 2020). Pea plants may be infected by various diseases, including Ascochyta blight, Bacterial blight, Root rot, Damping off, Downy mildew, Powdery mildew, Fusarium wilt, and several viruses.

Ascochyta Blight Disease of Field Peas

Ascochyta blight disease is one of the major diseases affecting field pea production (Annan et al., 2023; Liu et al., 2016; Owati et al., 2020). It is caused by a complex of fungal pathogens of the genus Ascochyta namely *Ascochyta pinodes*, *Ascochyta pisi, Ascochyta pinodella* and *Phoma koolunga* (Tivoli & Banniza, 2007). Among these, *A. pinodes* is the most aggressive in causing the disease (Annan et al., 2023; Sivachandra Kumar & Banniza, 2017). There are regional variations in the pathogens causing Ascochyta blight disease of field peas around the world (Annan et al., 2023). In Montana, *A. pisi* was identified as the prevailing species and traces amount of *A. pinodes* (Owati et al., 2020). Nonetheless, the detection of *A. pinodes* in Montana is a cause for concern as it is the most aggressive in causing the disease. Therefore, Ascochyta blight caused by *A. pinodes* will be the focus of this study because it is the most aggressive among the three pathogens.

A. pinodes processes sexual and asexual reproductions that generate different types of fruiting bodies. Sexual reproduction in *A. pinodes* leads to the formation of pseudothecia, releasing ascospores that are hyaline and exhibit constriction at the septum. The rupture of asci

within pseudothecia and subsequent ascospore release is triggered by rainfall, high humidity, or heavy dew. These spores serve as the primary source of inoculum (Gossen et al., 2011). Wind serves as the primary mode of transmission for these ascospores. Asexual reproduction leads to the formation of pycnidia, within which pycnidiospores develop and can be easily dispersed through rain splash (Sivachandra Kumar & Banniza, 2017). Pycnidia, characteristic of *A. pinodes*, exhibit a globose or pear-shaped morphology, featuring a dark brown hue with an aperture at the apex (ostiole), serving as the release point for pycnidiospores. Pycnidiospores are generated throughout the growing season under favorable field conditions. Both the ascospore and pycnidiospores can serve as secondary inoculum which occur many times in the field throughout the growing season (Sivachandra Kumar & Banniza, 2017). Furthermore, *A. pinodes* produces chlamydospores that are dark brown, spherical, or irregular in shape, occurring singly or in chains (Bretag et al., 2001). The survival of *A. pinodes* in the soil is due to the production of chlamydospores as they produce thick protective cell walls which enable them to overwinter and are partial saprophytes. The pathogen can overwinter in seed, infested crop residues and in the soil.

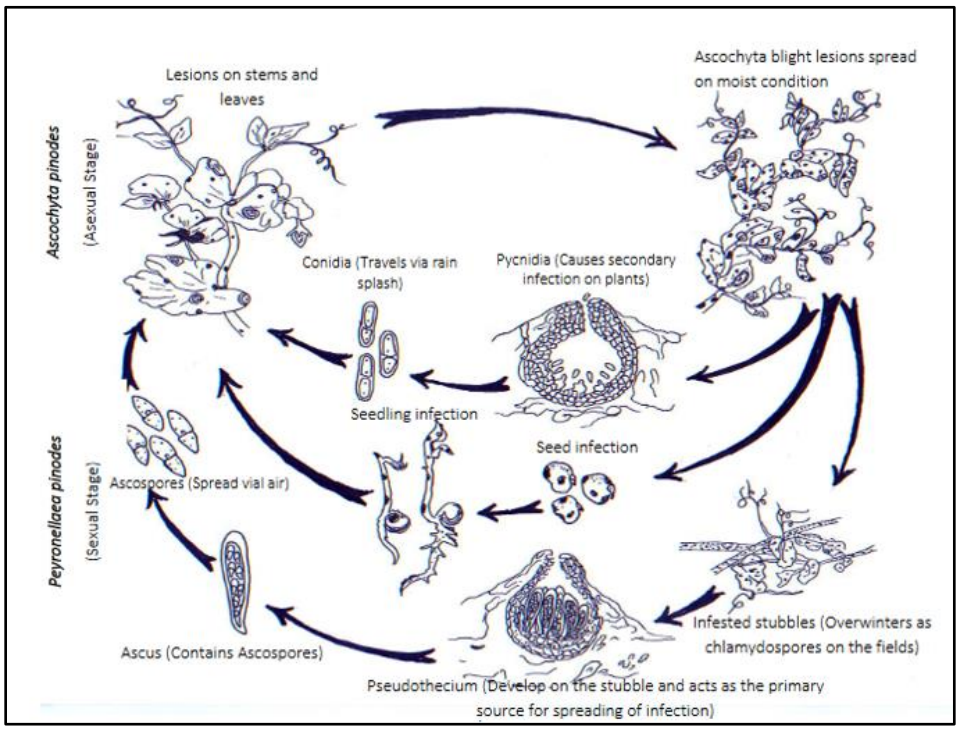


Figure 1: Life cycle of *Ascochyta pinodes*. The figure shows both the sexual and asexual reproduction of *A. pinodes*. Source: (Sivachandra Kumar, 2016)

Penetration of the plant surface by *A. pinodes* is accomplished either by direct penetration through leaf cuticle or through natural openings or wounds (Bretag et al., 2001). Ascochyta blight caused by *A. pinodes* development is favored by temperatures between 20 to 21˚C and high relative humidity. *A. pinodes* can infect seedlings and all aerial parts of pea plants, causing necrotic leaf spots, stem lesions and dark-brown discoloration of seeds. Infection process occurs when the spores germinate to produce germ tubes after 6 hours of inoculation and penetration and rapid colonization occurs within 24 hours of inoculation. *A. pinodes* produces several phytotoxins including pinolidoxin. Purified pinolidoxin caused necrotic lesions on pea leaves(Evidente, et al., 1993). It interferes with plant cell functions, which is essential for the fungus to colonize and spread within the host plant (Agudo-Jurado et al., 2023; Evidente, et al., 1993).

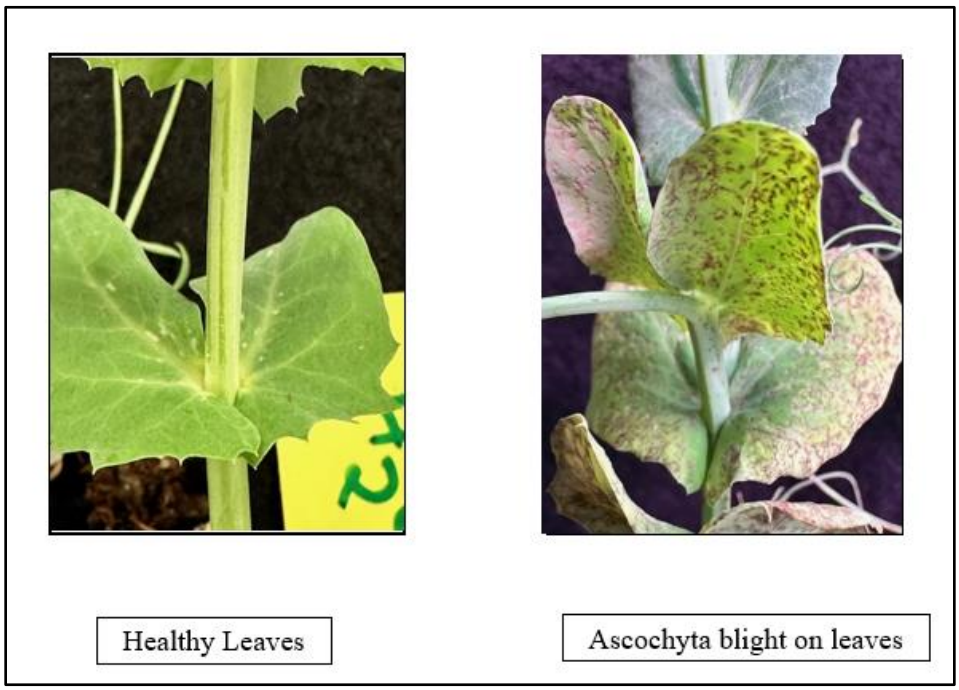


Figure 2: Symptoms of Ascochyta blight in field pea. On the left-hand side is a photo showing a health plant, on the right is a plant showing Ascochyta blight symptoms 10 days after pathogen inoculation. Symptoms include some necrotic leaf and stem lesions.

Biological Control

Biological control (or biocontrol) involves the use of living organisms, including viruses, to combat pests and pathogens directly or indirectly for human benefit. This approach leverages protective antagonists to manage pathogen populations (Jordan et al., 2014). This definition has been extended to biologically derived agents and products as well (Gray et al., 2018). Biocontrol, in several cases, has proven to be as effective as chemical control (Elad & Freeman, 2002). However, its efficacy can sometimes be inadequate, with significant variability. Understanding the mechanisms behind biological control could enhance its effectiveness and reduce inconsistency. Known mechanisms of biocontrol of plant diseases by beneficial bacteria include induced systemic resistance (ISR), competition for nutrients and/or spaces, antibiosis,

parasitism, production of volatile organic compounds (VOCs), the secretion of inhibitory compounds, and others.

Current Management Practices of Ascochyta Blight caused by *A. pinodes*

Management includes foliar fungicide application which are effective in controlling the disease, but these chemical control measures are not economical and eco-friendly. Crop rotation of at least 3 years (Zhang et al., 2005). Tillage is also one of the major practices for control of Ascochyta blight as this buries infected stubble after harvest of peas (Bailey et al., 2001). Cultural practices such as sowing dates help alleviate the problem as the ascospore would have been released and landed on the soil (Bailey et al., 2001; Salam et al., 2011).

The growing interest in utilizing beneficial bacteria for disease control of Ascochyta blight disease caused by *A. pinodes* can be attributed to the lack of resistant pea cultivars and concerns about potential fungicide resistance in pathogens. For example, *Ascochyta rabiei* isolates, a pathogen of chickpea Ascochyta blight increased a 100-fold resistance to strobilurin fungicides. There is also rising demand for environmentally friendly management solutions (Liu et al., 2016). Fungicides are highly effective in controlling the disease; however, they can result in environmental contamination and presence of the chemicals in food such as dichlorodiphenyltrichloroethane (DDT) which was extensively used in agriculture between 1950 and 1980 and was found genotoxic to humans and responsible for endocrine disorder (Gilden et al., 2010). Advantages of using biocontrol's include that they decompose more quickly in the environment and are generally less toxic towards non-target species (Thakore, 2006). Several beneficial bacteria, including strains of *Pseudomonas* spp., *Bacillus* spp, *Pantoea agglomerans*, *Serratia* spp, significantly reduced the severity of Ascochyta blight in peas under greenhouse

conditions (Liu et al., 2016; Wang et al., 2003; Stein, 2005). However, their mechanisms of biocontrol have not been investigated.

Although antibiosis is suggested as a significant mechanism for pathogen suppression, a more detailed understanding of the specific antibiotics by which the beneficial bacteria inhibit the growth or activity of *Ascochyta* spp is needed. Despite these previous efforts in controlling Ascochyta blight of pea, pathogens evolve over time, leading to the emergence of new strains that may be more virulent or resistant to existing antibiotics. Therefore, identifying more bacterial-derived antibiotics offer a diverse array of compounds that could potentially target these new strains. Additionally, understanding of regulation of production of antibiotics production is important to enhance the effectiveness of disease control through antibiotic-producing beneficial bacteria. In understanding the mechanisms of biocontrol, Green fluorescent protein (GFP) was used as a marker to study gene expression in this study. In emits green fluorescence when excited by blue light at 395 nm (Zimmer, 2002). It can be monitored in vivo as GFP function without the need for exogenous proteins, substrates, or cofactors. The GFP gene encodes a protein consisting of 238 amino acids, forming a cylindrical shape known as a beta-barrel. Inside this barrel lies the chromophore, the part of the protein responsible for fluorescence(Ormö et al., 1996; Prasher et al., 1992; Yang et al., 1996). This protein undergoes a unique post-translational modification that forms the chromophore through the cyclization of the tripeptide serine, tyrosine, and glycine at positions 65-67, followed by the oxidation of the tyrosine residue by atmospheric oxygen. The mechanism in fluorescence involves an excitation of the chromophore when exposed to light of a specific wavelength (395 nm for blue light in the case of wild type. The chromophore then releases this energy by emitting light at a longer

wavelength, which is green (around 509 nm). This emitted light is what is observed as green fluorescence (Zimmer, 2002).

Interest in utilizing beneficial bacteria for disease control of Ascochyta blight disease has been growing due to the advantages of using biocontrol's include that they decompose more quickly in the environment and are generally less toxic towards non-target species (Thakore, 2006). Several beneficial bacteria, including strains of *Pseudomonas* spp*., Bacillus* spp*, Pantoea agglomerans, Serratia* spp, significantly reduced the severity of Ascochyta blight in peas under greenhouse conditions (Liu et al., 2016; Wang et al., 2003). These reports suggested that antibiosis is likely an important mechanism used by the beneficial bacteria to inhibit pathogen's growth and suppress disease although the direct evidence is still missing. Identifying new biocontrol agents is a crucial step in developing commercial biocontrol products. This process requires rapid and robust screening methods capable of evaluating large numbers of candidate agents efficiently Additionally, understanding of regulation of production of antibiotics production is an important aspect in increasing the efficacy of biocontrol.

The goals of this study were to identify beneficial bacteria that inhibit the growth of *A. pinodes* and investigate the mechanisms of biological control of Ascochyta blight disease caused by *A. pinodes*. Antagonistic bacterial isolates that belong to different genera, including *Pseudomonas* spp., *Paenibacillus* spp., *Bacillus* spp. and *Peribacillus* sp. were identified. We focus on species of the *Pseudomonas* genus because of their production of many antibiotics. *Pseudomonas protegens* strain Pf-5, a soil bacterium which is well-known for its wide-spectrum antimicrobial activity due to the production of many antibiotics including pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol (2,4-DAPG), orfamide A, rhizoxin,

hydrogen cyanide and polyynes (Henkels et al., 2014; Raaijmakers et al., 1999; Yan et al., 2021) was used in this study. The antibiotic required by *P. protegens* Pf-5 to inhibit *A. pinodes* was identified and the mechanisms of biocontrol of beneficial bacteria were investigated.

MATERIALS AND METHODS

Isolation of Bacterial Isolates Against *Ascochyta pinodes*

To identify bacterial isolates that can inhibit the growth of *A. pinodes*, bacterial isolates were recovered from soils were pea/camelina were grown in Montana, pea leaves and stems. Each sample was homogenized with a sterilized mortar and pestle to access bacteria in the tissue (Liu et al., 2016). Macerated samples were diluted with sterile water, and resulting suspensions were plated onto R2A, PDA and TSA media to capture different kinds of bacterial isolates. Single colonies were randomly picked according to colony morphology from plates after 48 hours incubation at 28°C, purified and stored at −80°C freezer for further investigation.

Plant Materials and Growth Conditions

Pea cv. Fallon was used in all biocontrol experiments with different isolates in this study as it is susceptible to *A. pinodes* and resistant to *A. pisi* (Annan et al., 2023). In addition to Fallon, three other cultivars Agassiz, CDC Treasure, and Greenwood were used in evaluating the efficacy of *P. protegens* strain Pf-5 biocontrol effects on different varieties. Pea plants were grown in a greenhouse with a day/night temperature of 30.2/18 °C, 22% humidity and 16 hours of light at the Plant Growth Centre of Montana State University (MSU). The seeds were sown in soils sunshine mix 1 (Sun Gro Horticulture, Agawam, MA, USA). Plants were ready for inoculation after 2 weeks from sowing.

Pathogens and Growth Conditions

A. pinodes isolate "C6" fungal species were used for the study. The pathogens were isolated from Ascochyta blight contaminated seed lots submitted by growers to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) at MSU (Annan et al., 2023). The fungal species was purified and confirmed as a pure culture of the species by PCR using species-specific primers (Nyamesorto., et al 2023) and sequencing. The isolate was cultured on a pea meal agar (PMA), consisting of 25 g of blended dry pea and 15 g of agar in 1 L of double-distilled water (ddH2O), at room temperature on the laboratory lab bench for 14 days to reproduce conidia.

Inhibition of the *A. pinodes* Pathogens by *P. protegens* Pf-5

Using Pf-5 mutants, the inhibition assay was done by spreading 200 µL of spore's concentration of 5.0 x 10^5 spores/mL on PDA plates and a 3 µL of bacterial concentration of 1.0 x 10^9 cells/mL was inoculated on top of the pathogen. Plates were kept at 28˚C for 6 days before the results of the inhibition were recorded. The zones of inhibition were obtained by measuring the clear zone distance.

Screening Antagonistic Bacteria on Culture Plates

A 5 mm agar plug from a 2-week-old culture of the fungal pathogens was placed onto the center of the PMA plates and allowed to grow for seven days before bacterial inoculation. Bacterial cells were washed three times using sterilized water and made into 1 mL suspension with a concentration of 1.0 x 10^9 cells/mL. A drop of 3 µL of the bacterial cell suspension was placed on the plate about 2.5 cm away from the center of the fungal plug. Isolates were tested in quadruplicate on plate and the plates were air-dried and incubated at 28°C without light. The

inhibition zone was measured at 6 days after inoculation. Isolates that showed an inhibition zone on plate qualified for biocontrol experiments in greenhouse condition.

Inoculum Preparation and Inoculation of Bacterium

To prepare the bacterial inoculum, different bacterial isolates including Pf-5 were cultured overnight on nutrient agar and King's B medium plates respectively at 28°C. The cells were harvested from the plates and washed three times using sterilized water into 15 mL suspension with a cell density of 1.0×10^9 cells/mL. The bacterial suspension was spray-inoculated onto pea leaves until the leaf surfaces were coated and appeared uniformly wet. The inoculated plants were kept at a humid growth chamber (28°C) for 24 hours before inoculation of the fungal pathogens as described above.

Inoculum Preparation and Inoculation of *A. pinodes*

The inoculum of *A. pinodes*, was prepared by flooding the cultured surface with 30 mL sterilized ddH2O and gently scraping the flooded culture surface with a glass rod for 10 min to dislodge conidia. Conidia spores were then collected after filtration through one layer of cheesecloth and used as stocks. The spore density of the stocks was counted with 10 µL pipetted onto a hemacytometer under a light microscope. The inoculum was prepared by diluting a calculated amount of each stock with sterilized ddH2O + Tween 20 (two drops per 200 mL) to achieve a spore density of 1.0×10^5 spores/mL. Inoculum was applied to plants by spraying and ensured every part of a plant was covered with the inoculum. The inoculated plants were kept in a dew chamber with an air temperature of 22°C for 24 hours in the dark and then transferred to a

greenhouse and kept inside a plastic tent with mists produced by humidifiers for at least 12 hours.

Evaluation of the Biocontrol Effect

Evaluation of biocontrol effects was done 7-10 days after pathogen inoculation. It was done by employing a standardized disease severity scale to quantify the extent of infection. The scale used below was based on the percentage of lesions on the shoots, leaves and stems:

1 = no infection on any part of plant

3 = lesions on 10-25% leaves and stems surface

5 = lesions on 26-50% of leaves and stems surface

7 = lesions on 51-75% of leaves and stems surface

9 = lesions on more than 75% of leaves and stems surface

Taxonomic Identification via 16s DNA Analysis

Ten isolates were identified for Sanger sequencing, and we performed DNA extractions using the Qiagen PowerLyzer Microbial DNA Kit (Germantown, MD, USA). We then amplified the 16S rRNA region with primers 27F and 1492R, which amplify portions of the gene. Amplicons were prepared with 2 µL of template, 39.75 µL of molecular grade water, 5 µL of DreamTaq buffer, 1 µL of 10xDNTP mix, 1 µL forward primer (27F), 1 µL reverse primer (1492R), and 0.25 µL DreamTaq. Thermocycler conditions for bacterial PCR amplification were 98°C for 5 minutes, followed by 33 cycles of 98°C for 30 seconds, 53°C for 30 seconds, 72°C for 2 minutes, with a final extension of 72°C for 3 minutes. PCR products were purified (Thermo Fisher Scientific, Waltham, MA, USA) and eluted in elution buffer. Samples were submitted to

the Genewiz Laboratory for Biotechnology and Bioanalysis (LBB) for Sanger sequencing using the ABI 3730 DNA Analyzer (ThermoFisher Scientific, Waltham, MA, USA).

For taxonomic identification, sequences were used as BLAST queries to the NCBI 16S rRNA database with the following settings: "low complexity filter, max e-value = 0.05, word size = 28, gap cost = linear, program = mega BLAST." The species with the lowest E value and highest percentage identity were selected as the identity of the isolate. A phylogenetic tree based on 16S ribosomal RNA genes. Alignments of 16S sequence regions were performed in MUSCLE (Edgar, 2004) and phylogenetic trees were constructed via maximum likelihood analysis and Tamura-Nei model ("Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial DNA in Humans and Chimpanzees.," 1993).The percentage of trees in which the associated taxa clustered together is shown next to the branches Each DNA alignment and a concatenated alignment were run in MEGA with 1000 bootstrap replicates. Evolutionary analyses were conducted in MEGA X(Kumar et al., 2018)

Construction of Rhizoxin (*rzxB*) Gene Expression Reporter

GFP reporter constructs were made by using primers P286 and P287 (Table 1) and the PCR product was 684bp from wildtype *P. protegens* genome. Digested by EcoRI and HindIII and ligated into pPROBE-NT. This construct was later transformed using electroporation in wildtype (WT) and the mutants which lacks pyoluteorin (*pltA*), 2,4-diacetyl-phloroglucinol (*phlA*), 2,4-diacetyl-phloroglucinol (*phlD*) & GacA/GacS system (*gacA*).

Assays for Monitoring GFP Activity of Reporter Constructs

Pf-5 strains containing *rzxB* reporter constructs were cultured overnight King's B medium plus kanamycin (50µg/mL) cultural plate at 28˚C. The cells were washed once into 1 mL fresh AB Minimal media which is composed of 20X salt (made by adding 20g of NH_4Cl, 6g of M_gSO_4 $7H_2O$, 3g of KCL, 0.2g of $CaCl_2$ $2H_2O$ and 0.05g of $FeSO_4 7H_2O$ in 1 liter), 20X buffer (made by adding 60g of K_2HPO_4 and 23g of NaH_2PO_4 to 1 liter water) and 10% carbon source (Glucose was used in this study) with an optical density at 600 nm (OD_{600}) of 1.0, and then used to inoculate 200µL AB minimal media plus kanamycin to obtain a start OD_{600} of 0.01. Each strain was grown in three wells of 96-well plate, which was incubated in a Tecan spark microplates reader at 28˚C with shaking at approximately 180 rpm. Bacterial growth was monitored by measuring the OD_{600}. The green fluorescence of bacteria was monitored by measuring emission at 535nm with an excitation at 485nm and corrected for background by subtracting fluorescence emitted by control strain which has an empty vector of the reporter construct.

Duration of Biocontrol of *P. peoriae* and *P. protegens* Against *A. pinodes*

To check how long the model strain *P. protegens* and the best performing isolate *P. peoriae* from Montana provides biocontrol effects against *A. pinodes*, some 3 weeks old pea plants were inoculated with bacterial suspension of concentration 1.0×10^9 cells/mL and put in the growth chamber for 24 hours for bacterial to establish. The inoculated plants were later inoculated with a spore suspension of 1.0×10^6 spores/mL on day 3, 7, 10, & 17 post bacterial

inoculation and their disease severity evaluated 7 days post pathogen inoculation. Plants were kept in the mist chamber for 24 hours after pathogen inoculation.

Statistical Analysis

Statistical analysis was performed using R version 4.4.0. All the experiments were conducted in controlled conditions with two factors, cultivar, and inoculum. Disease severity was documented as percentage of leaf and stem lesions on the plants. Statistical differences between mean values were determined by general liner model because the data was nonparametric and Tukey's HSD.

RESULTS

Ten Bacterial Isolates Showed Biocontrol Effect to Pea Ascochyta Blight Caused by *A. pinodes*

Ascochyta pinodes is known to cause significant damage to pea crops, leading to reduced yields of up to 50% and economic losses. Only partial resistance exists against *A. pinodes*, therefore, the methods of controlling such diseases often rely on chemical fungicides. These chemicals can have adverse environmental impacts and lead to the development of resistant pathogen strains. Hence, there is a growing interest in finding sustainable and environmentally friendly alternatives, such as biological control using beneficial microorganisms. Fallon has been found to be resistant to *A. pisi,* which implies finding biocontrol's that can control *A. pinodes* would lessen the disease pressure of the Ascochyta blight complex in integrated pests' management. By isolating and identifying bacterial strains from soils where peas are grown and testing their biocontrol potential against *A. pinodes*, this experiment aims to identify locally adapted candidates for sustainable disease management in pea crops. This knowledge can contribute to developing new biocontrol products that are both effective and locally adapted to Montana States.

This experiment was done by isolating bacterial strains from soils from fields in Montana where peas, camelina and other crops were grown. Suspension was plated on different media to capture the different isolates from the samples. Single colonies were picked, purified, and stored for use in biocontrol experiments. For biocontrol experiments, the isolates were inoculated together with *A. pinodes* and the disease severity employing a standardized scale recorded 7-10 days post inoculation.

Results from this experiment indicate that ten bacterial isolates out of the 350 isolates screened showed biocontrol effects against *A. pinodes* (Figure 3). This experiment has identified ten promising bacterial isolates from Montana soils that exhibit biocontrol activity against *A. pinodes*. These isolates, adapted to local conditions, represent potential biocontrol agents for managing Ascochyta blight in pea crops, offering a sustainable alternative to chemical fungicides or can be incorporated in integrated pest management (IPM).

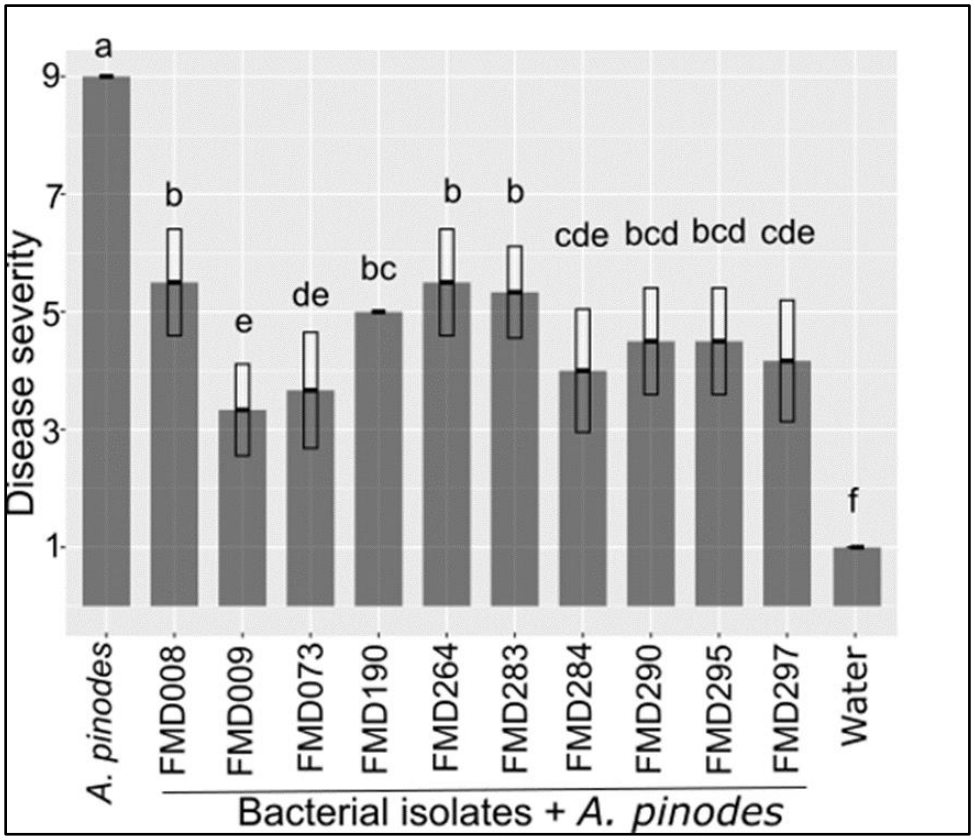


Figure 3: Ten bacterial isolates reduced disease severity and inhibits the growth of pathogen *A. pinodes.* The inoculum of *A. pinodes*, was prepared by flooding the cultured surface with sterilized ddH2O and gently scraping conidia spore. Spore density was brought to 1.0×10^5 spores/mL. Inoculum was applied to plants inoculated with bacterial suspension of concentration 1.0×10^9 cells/ml 24 hours before pathogen inoculation by spraying every part of a plant. The inoculated plants were kept in a dew chamber with an air temperature of 22°C for 24 hours in the dark and then transferred to a greenhouse and kept inside a plastic tent. Disease severity was recorded following a scale of 1,3,5,7,9 on the 10^{th} day after pathogen inoculation.

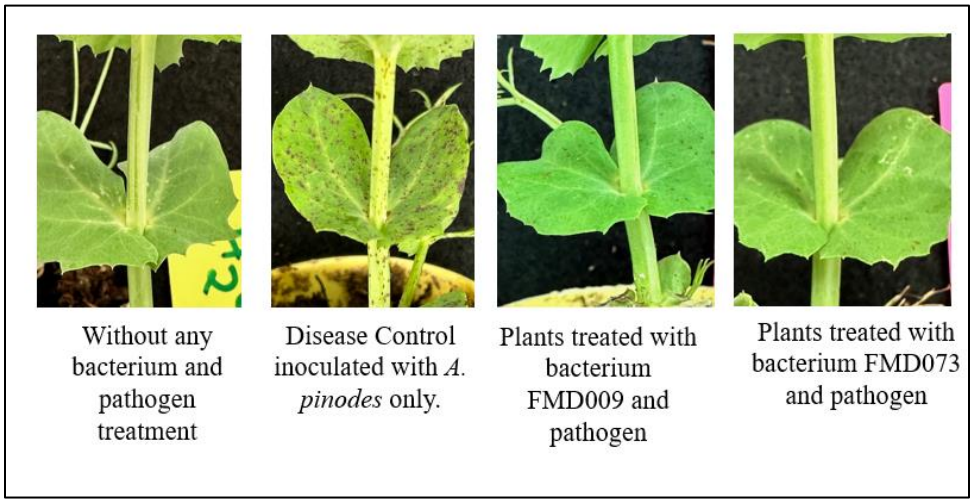


Figure 4: Greenhouse assay photos of best performing isolates. The experiment was conducted as outlined in Figure 3.

Bacterial Isolates Inhibited Growth of *A. pinodes* on Cultural Plate

To further characterize the top ten isolates, a plate inhibition assay against *A. pinodes* was done by plugging a 5mm agar containing the pathogen in the middle of pea meal agar (PMA) and allowing the pathogen to grown for 7 days. A drop of 3 µL bacterial cells suspension was later placed 2.5cm from the center of the fungal plug. Isolates were tested in quadruplicate on plate and the plates were air-dried and incubated at 28°C without light. The inhibition zone was measured at 6 days after inoculation.

Results showed that all the ten isolates inhibited *A. pinodes* on culture plates (Figure 5) as evidenced by the formation of *A. pinodes* inhibition zones. The formation of inhibition zones in plate assays indicates that these isolates produce antibiotics or other antimicrobial compounds, making them promising candidates for biocontrol applications. This information forms the basis to study the mechanisms of their biocontrol.

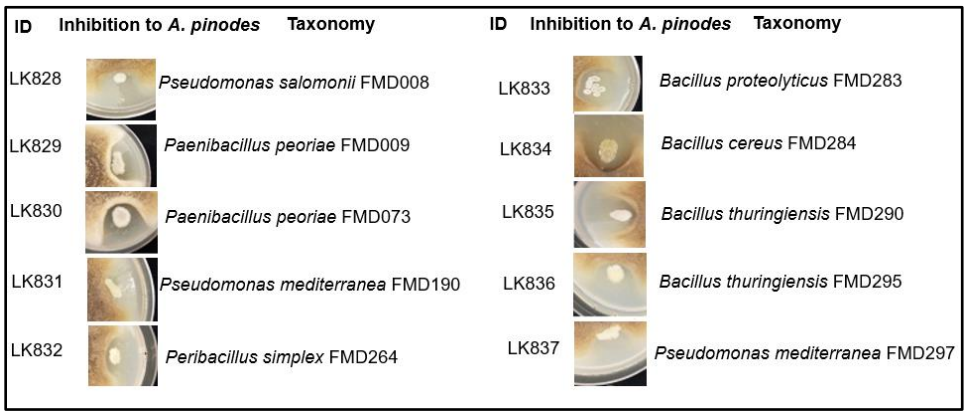


Figure 5: Inhibition zones of the ten isolates. A 5 mm agar plug from a 2-week-old culture of the fungal pathogens was placed onto the center of the PMA plates and allowed to grow for seven days before bacterial inoculation. Bacterial cells were washed three times using sterilized water and made into 1 mL suspension with a concentration of 1.0 x 10^9 cells/mL. A drop of 3 µL of the bacterial cell suspension was placed on the plate about 2.5 cm away from the center of the fungal plug. Isolates were tested in quadruplicate on plate and the plates were air-dried and incubated at 28°C without light. The inhibition zone was measured 6 days after inoculation.

Identification of the Bacterial Isolate by 16S rRNA Analysis

The bacterial isolates that showed biocontrol effects in greenhouse assay were identified using 16S rDNA sequencing with primers 27F and 1492R, which amplify portions of the gene. This is a molecular technique used to identify and classify bacteria based on their genetic sequences. Phylogenetic analysis was performed to determine the relationships between the isolated strains and to identify them at the genus and species levels. Phylogenetic trees were constructed via maximum likelihood analysis. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Each DNA alignment and a concatenated alignment were run in MEGA with 1000 bootstrap replicates. Evolutionary analyses were conducted in MEGA.

This analysis reveals that among the ten isolates, three are *Pseudomonas* spp which are grouped together., two are *Paenibacillus* spp which cluster together, distinct from other genera,

highlighting their specific lineage., one is *Peribacillus* spp., and the remaining four are *Bacillus* spp which cluster closely together, indicating a shared evolutionary lineage (Figure 4). The clustering of biocontrol-effective isolates within certain branches suggests that genetic relatedness might correlate with biocontrol capabilities. Overall, the isolates of the *Paenibacillus* spp. showed greater biocontrol effects than strains of the other groups. The phylogenetic analysis of the ten bacterial isolates reveals significant genetic diversity and robust evolutionary relationships. The clustering patterns indicate that these isolates, especially those closely related to known biocontrol species such as *P. protegens* Pf-5, hold strong potential as biocontrol agents against *A. pinodes*. These findings provide a solid foundation for further research into their biocontrol mechanisms.

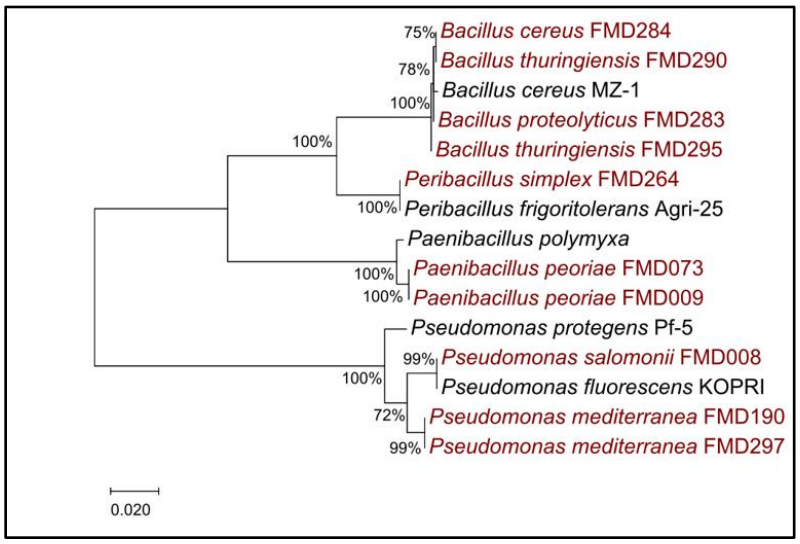


Figure 6: A phylogenetic tree based on 16S ribosomal RNA genes of the ten bacteria isolates highlighted in red. Alignments of 16S sequence regions were performed in MUSCLE (Edgar, 2004) and phylogenetic trees were constructed via maximum likelihood analysis and Tamura-Nei model (Tamura K. and Nei M, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Each DNA alignment and a concatenated alignment were run in MEGA with 1000 bootstrap replicates. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Pseudomonas protegens Pf-5 Reduced the Disease Severity of Ascochyta Blight in Pot Assay and Inhibited *A. pinodes* Growth on Culture Plate

Pf-5 is well known for its wide-spectrum antimicrobial activity due to the production of many antibiotics. The antibiotics produced by Pf-5 include 2,4-diacetyl-phloroglucinol, pyoluteorin, pyrrolnitrin, hydrogen cyanide, rhizoxin, orfamide A, polyyne and toxiflavin. The plate inhibition assay was done by spreading 200 µL of spore' concentration of 5.0×10^5 spores/ml on PDA plates and a 3 µL of bacterial concentration of 1.0×10^9 cells/mL was inoculated on top of the pathogen. Plates were kept at 28°C for six days before the results of the inhibition were recorded. The zones of inhibition were obtained by measuring the clear zone distance. The greenhouse experiment was done similarly to the testing of the isolates described above.

Results of these assays demonstrated strong biocontrol against *A. pinodes*, both in reducing disease severity in pot assays and inhibiting pathogen growth on culture plates (Figure 7). The production of a wide range of antibiotics is likely responsible for these effects.

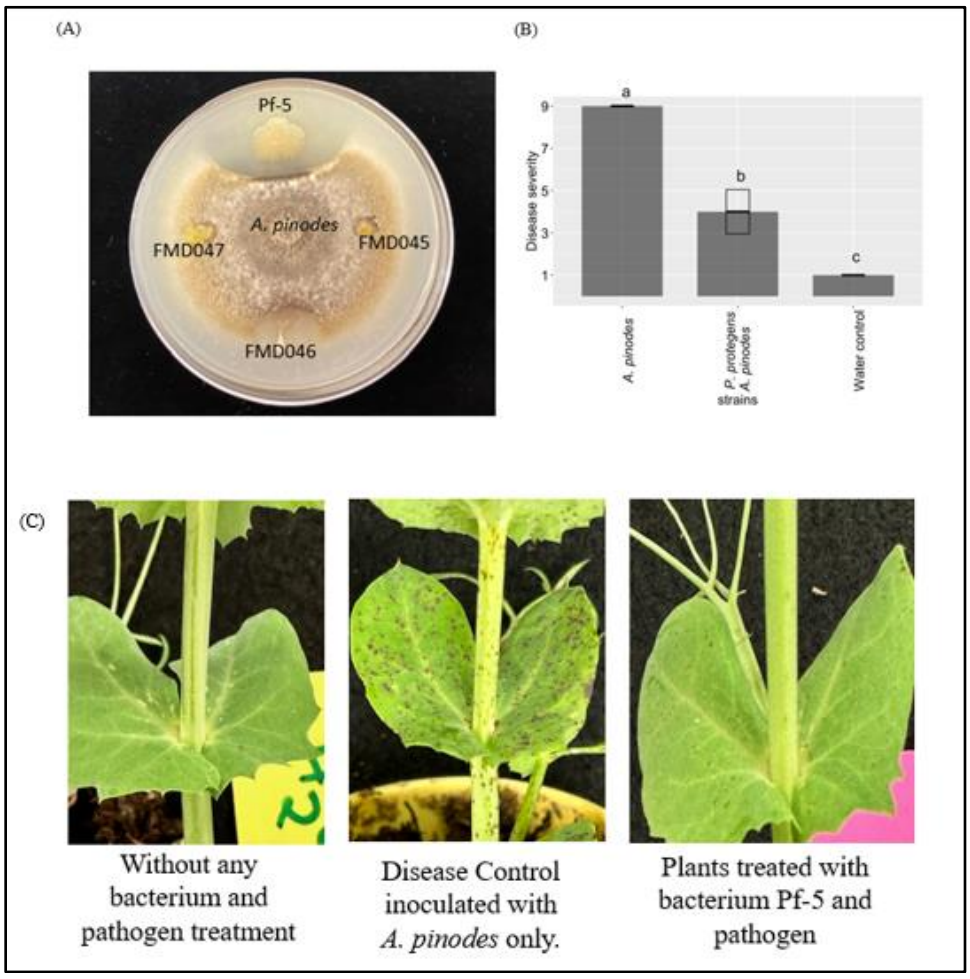


Figure 7*: Pseudomonas protegen* strain Pf-5 inhibits pathogen growth on culture plates and biocontrol. Green house assay was done as outlined in figure 3 and. A) Inhibition zones of Pf-5. B) Disease severity of Pf-5 in greenhouse assay. C) Greenhouse photos of Pf-5.

Rhizoxin is Required for Pf-5 to Inhibit Growth of *A. pinodes* and Control the Disease

To find the antibiotics responsible for the biocontrol effects, multiple and single mutants were used in the inhibition assay. Plate inhibition assay was done by spreading 200 µL of spore's concentration of 5.0 x 10^5 spores/mL on PDA plates and a drop (3 µL) of bacterial concentration of 1.0 x 10^9 cells/mL was inoculated on top of the pathogen. Plates were kept at 28˚C for six days before the results of the inhibition were recorded. The zones of inhibition were obtained by

measuring the clear zone distance. Results reviewed that GacA/GacS system mutant does not inhibit the pathogen implying that antibiotics that are controlled by the GacA/GacS system are required for pathogen inhibition (Figure 8). A mutation in all the eight antibiotics does not inhibit the pathogen confirming that antibiotics are required for pathogen inhibition. To pinpoint the antibiotics, we used a 6-fold mutant which lacks production of six antibiotics including 2,4-diacetyl-phloroglucinol, rhizoxin, pyoluteorin, hydrogen cyanide, orfamide A and pyrrolnitrin. Result shows the 6-fold mutant did not inhibit *A. pinodes*, indicating that at least one of these six antibiotics are required for Pf-5 to inhibit *A. pinodes*. We then used six mutants which lack only one of these six antibiotics in the inhibition assay. A mutation in rhizoxin significantly reduced the inhibition zone as compared to the wildtype unlike other antibiotics. This result implies that rhizoxin plays a major role in the inhibition of *A. pinodes*.

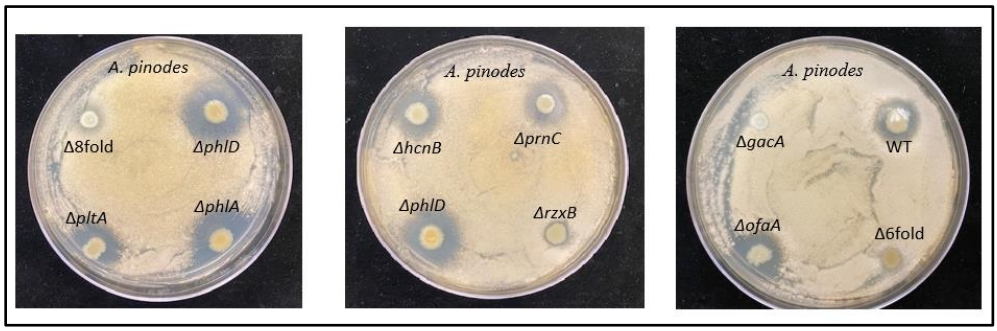


Figure 8: Rhizoxin antibiotics plays a role in growth inhibition and biocontrol of *A. pinodes*. Plate inhibition assay were done by spreading 200 µL of spore's concentration of 5.0 x 10^5 spores/ml on PDA plates and a drop of 3 µL of bacterial concentration of 1.0 x 10^9 cells/ml was inoculated on top of the pathogen. Plates were kept at 28˚C for six days before the results of the inhibition were recorded.

Table1: Bacterial strains used in the study.

Strain	**Genotype, Relevant Characteristics**	**Reference**
LK099	Wild-type strain Pf-5. Isolated from soils from cotton field in Taxes. Produces multiple antibiotics and shows biocontrol effect to several plant diseases.	(Howell and Stipanovic, 1979)
JL4805	Pf-5 derivative strain contains an in-frame deletion of *pltA* in the chromosome for production of pyoluteorin antibiotic	(Henkels et al., 2014)
LK023	Pf-5 derivative strain contains an in-frame deletion *phlA* of in the chromosome for production of 2,4-diacetyl-phloroglucinol antibiotic	(Henkels et al., 2014; Kidarsa et al., 2011; Quecine et al., 2015)
LK024	Pf-5 derivative strain contains an in-frame deletion *phlD* of in the chromosome for production of 2,4-diacetyl-phloroglucinol antibiotic	(Kidarsa et al., 2011)
JL4975	Pf-5 derivative strain contains an in-frame deletion *gacA* of in the chromosome	(Henkels et al., 2014)
JL4809	Pf-5 derivative strain contains an in-frame deletion of *hcnB* in the chromosome for production of hydrogen cyanide	(Loper et al., 2012)
JL4950	Pf-5 derivative strain contains an in-frame deletion of *prnC, phlD, rzxB, pltA, hcnB* and *ofaA*	(Kidarsa et al., 2011)
LK237	The Pf-5 derivative strain, with deletions *prnC, phlD, rzxB, pltA, hcnB, ofaA, phlA, PFL_0261-8,* and *PFL_1028-29,* produces antibiotics like 2,4-diacetyl-phloroglucinol, rhizoxin D1, polyyne, pyoluteorin, hydrogen cyanide, toxoflavin, orfamide A, and pyrrolnitrin.	(Philmus et al., 2015)
JL4776	Pf-5 derivative strain contains an in-frame deletion of *ofaA* in the chromosome for production of orfamide A	(Gross et al., 2007)
JL4793	Pf-5 derivative strain contains an in-frame deletion of *prnC* in the chromosome for production of pyrrolnitrin	(Henkels et al., 2014)
JL4901	Pf-5 derivative strain contains an in-frame deletion of *phlD rzxB* in the chromosome for production of 2,4-diacetyl-phloroglucinol and rhizoxin D1	(Kidarsa et al., 2011)
CM011	GFP reporter constructs were made by primer P286 and P287 and the PCR product was 684bp. Digested by EcoRI and HindIII and ligated into pPROBE-NT.	This study
CM009	LK099 containing a *rzxB* promoter GFP reporter constructs	This study
CM007	JL4975containing a *rzxB* promoter GFP reporter constructs	This study
CM001	JL4805 containing a *rzxB* promoter GFP reporter constructs	This study
CM003	LK023 containing a *rzxB* promoter GFP reporter constructs	This study
CM005	LK024 containing a *rzxB* promoter GFP reporter constructs	This study

Table 2: Primers used in the study.

Primers	**Sequence**	**Reference**
P286	atagaaTTCCAGCCGGCAATGAGC	This study
P287	tataaGCTTTCGATCCGCTGAAACC	This study

A greenhouse biocontrol experiment which was done as described above against *A. pinodes* using the wildtype and different Pf-5 mutants confirmed that the rhizoxin is important for the biocontrol effects against *A. pinodes* as they were no significant differences between the pathogen treatment (*A. pinodes*) and the mutant which lacks rhizoxin antibiotics (Figure 9). The in-planta expression study showed that unlike the wild type, the *gacA* mutant exhibited significantly lower expression of the rhizoxin biosynthesis gene promoter activity in pea plants. This indicates that rhizoxin biosynthesis is regulated by *gacA* and is actively expressed in pea plants (Figure 10B). The *phlA* and *phlD* mutants of Pf-5 showed a larger inhibition zone against *A. pinodes* on culture plates (Figure 8) and had a greater biocontrol effect in the greenhouse compared to the wild type (Figure 9). This suggests that these mutations may enhance the bacterium's ability to inhibit the pathogen and reduce disease severity. The experiment highlights the crucial role of rhizoxin in the biocontrol activity of *P. protegens* Pf-5 against *A. pinodes*. These findings provide a strong foundation for developing targeted biocontrol strategies and products that leverage the antimicrobial properties of Pf-5, with a focus on enhancing and utilizing rhizoxin production to control Ascochyta blight in pea crops. This shows potential of specific genetic modifications to improve the biocontrol efficacy of *P. protegens* Pf-5 and the significance of rhizoxin in managing plant diseases was highlighted in this experiment.

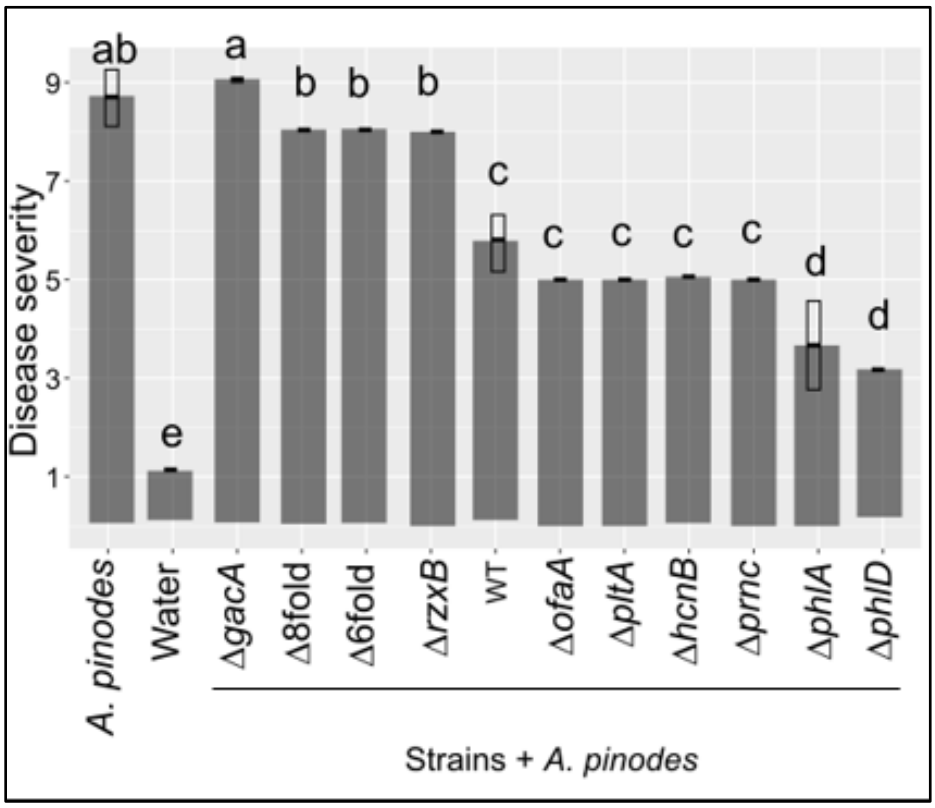


Figure 9: Greenhouse assay of Pf-5 and its mutant against *A. pinodes*. The experiment was carried out following Figure 3.

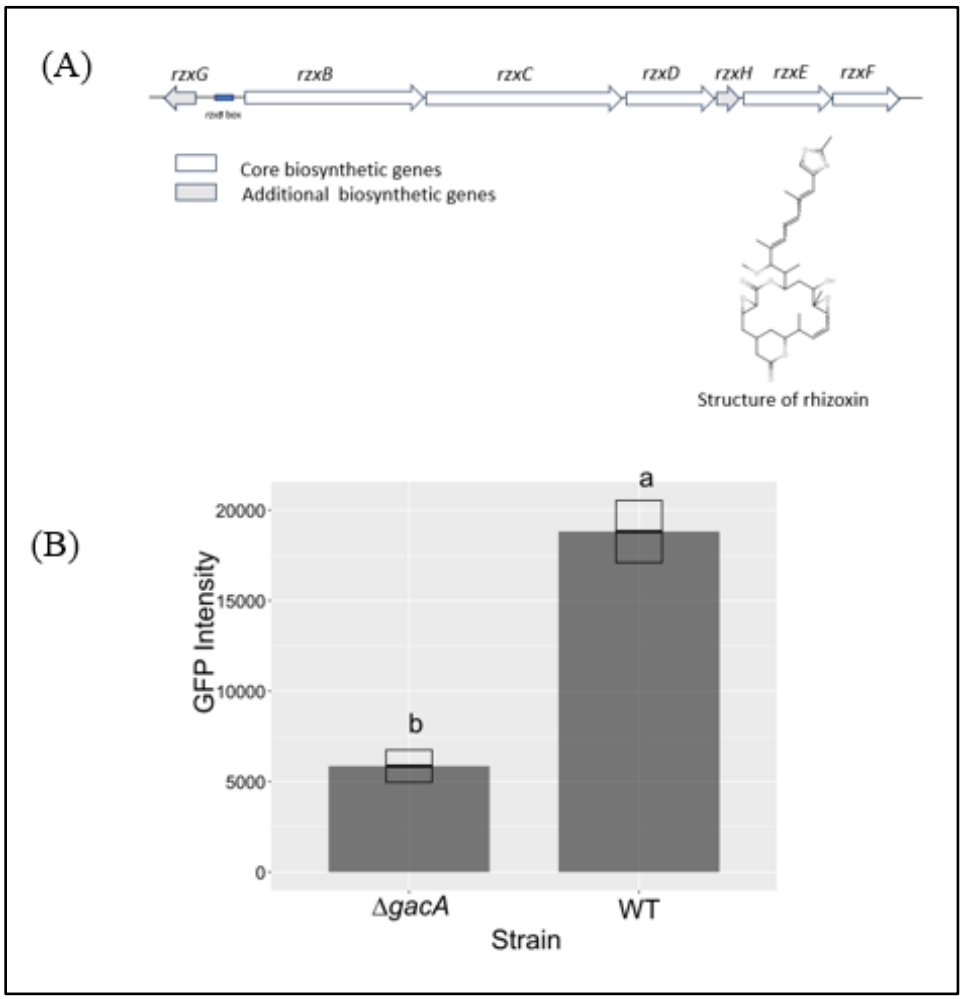


Figure 10: Production of rhizoxin antibiotics on pea leaves. The experiment was done by inoculating 2 weeks pea plants with Pf-5 bacterial suspension of Concentration 1.0 x 10^9 cells/ml. After 16 hours, piece of leaf 5mm in diameter was cut and submerged in 200 µL of water in a 96 well plate and its Green fluorescent protein (GFP) measured using Tecan spark microplates reader. The green fluorescence of bacteria was monitored by measuring emission at 535nm with an excitation at 485nm. (A) Rhizoxin biosynthesis gene cluster and its structure. (B) Expression of rhizoxin biosynthesis gene cluster on pea leaves.

Mutation of *phlD* Overproduced Rhizoxin and Increased Disease Control Effect

A mutation in *phlD* which produces 2,4-diacetyl-phloroglucinol antibiotic and provide a signal to produce pyoluteorin reviewed a greater inhibition zone compared to the wildtype. Since rhizoxin was found to be the major antibiotics against the *A. pinodes*, we hypothesized that a

mutation in *phlD* caused overproduction of rhizoxin antibiotics. To test the hypothesis, a double mutant which lacks Δ*phlD* and Δ*rzxB* was used in the plate inhibition assay (Figure 11). The plate inhibition assay was done as described above. Results reviewed that there were significant differences between the single mutant of Δ*phlD* and the double mutant of Δ*phlD* and Δ*rzxB* indicating that importance of rhizoxin in causing the inhibition against the pathogen. This implies that the bigger inhibition zones were caused by overproduction of rhizoxin antibiotic. To further confirm this, we tagged the promoter region of the *rzxB* biosynthesis gene with a promotor less green fluorescent protein (GFP) to study the expression of rhizoxin biosynthesis genes overall in WT, Δ*pltA,* Δ*phlA,* Δ*phlD* and Δ*gacA*. The experiment was prepared by inoculating 200µL AB minimal media plus kanamycin with bacterial strains and the 96 well plates were incubated in Tecan plates reader at 28˚C with shaking at approximately 180 rpm. Bacterial growth was monitored by measuring the OD_{600} and the GFP of bacteria was monitored by measuring emission at 535nm with an excitation at 485nm. Results showed a higher GFP promotor activity of *rzxB* gene in Δ*phlD* and Δ*phlA* and compared to the other strains including the wildtype (Figure 12). These results also confirm which pathway causes overproduction as *phlD* affect both the production of 2,4-diacetyl-phloroglucinol antibiotic and provide a signal to produce pyoluteorin. The pathway which causes overproduction is the production of 2,4-diacetyl-phloroglucinol as there were a higher GFP promoter activity in Δ*phlA* which the first gene in biosynthesis gene of 2,4-diacetyl-phloroglucinol antibiotic. In summary, mutation of *phlD* in leads to overproduction of rhizoxin, significantly enhancing its biocontrol effect against *A. pinodes*. The double mutant *(ΔphlDΔrzxB*) showing reduced inhibition confirms the crucial role of rhizoxin in pathogen suppression. These results suggest to us that these genes Δ*phlD* and

Δ*phlA* as potential targets for genetic improvement of biocontrol strains to boost biocontrol efficacy by enhancing the production of key antimicrobial compounds like rhizoxin.

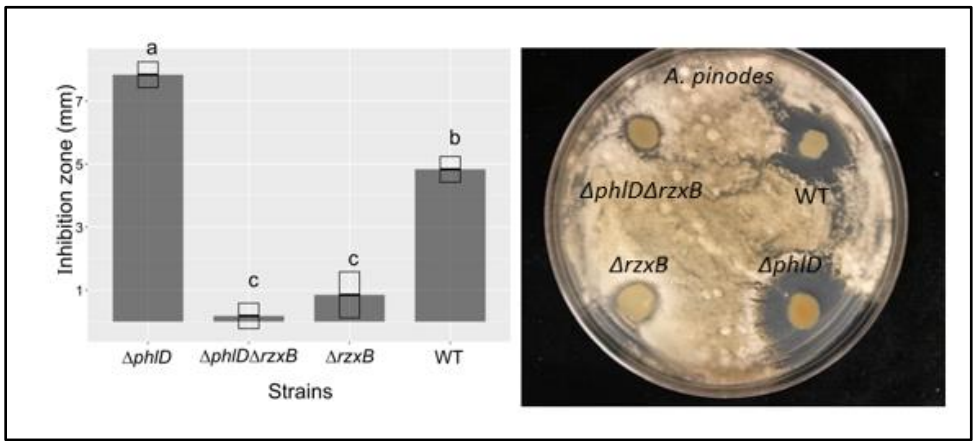


Figure 11: Plate inhibition assay of a double mutant in Rhizoxin and 2,4-Diacetyl-phloroglucinol antibiotics. The plate inhibition was done as described in Figure 8.

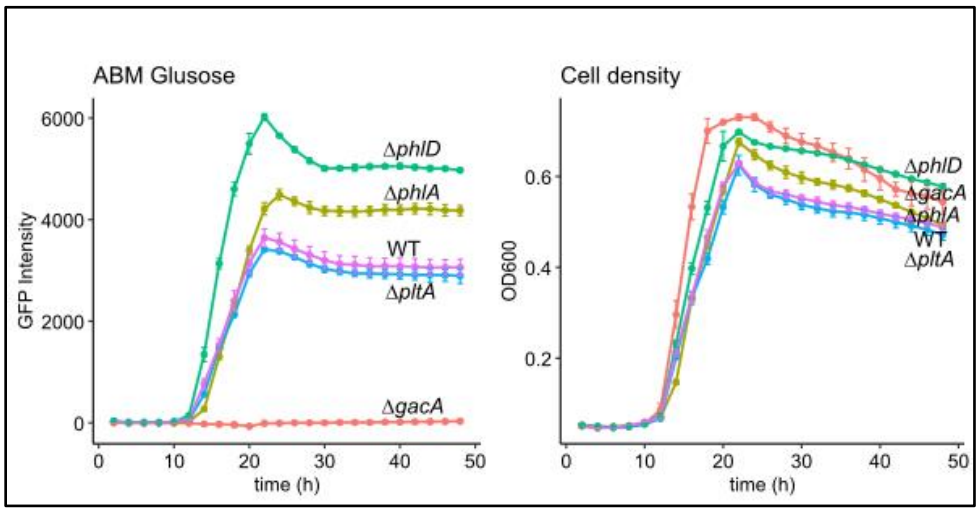


Figure 12: Expression of rhizoxin biosynthesis gene *rzxB* in wild type Pf-5 WT, *ΔpltA, ΔphlA, ΔphlD* and *ΔgacA* mutants using a GFP reporter construct. Pf-5 strains containing *rzxB* reporter constructs were cultured overnight King's B medium plus kanamycin (50µg/mL) cultural plate at 28 ◦C. The cells were washed once into 1 mL fresh AB minimal media which is composed of (1) 20X salt (made by adding 20g of NH_4Cl, 6g of $MgSO_4$ $7H_2O$, 3g of KCL, 0.2g of $CaCl_2$ $2H_2O$ and 0.05g of $FeSO_4$7H2O in 1 liter). (2) 20X buffer (made by adding 60g of K_2HPO_4 and 23g of NaH_2PO_4 to 1 liter water) and 10% carbon source (used Glucose in this study) plus kanamycin with an optical density at 600 nm (OD_{600}) of 1.0, and then used to inoculate 200 µL Minimal media plus kanamycin to obtain a start OD_{600} of 0.01. Each strain was grown in three wells of 96-well plate, which was incubated in a Tecan spark microplates reader at 28 ˚C with shaking at approximately 180 rpm. Bacterial growth was monitored by measuring the OD_{600}. The green fluorescence of bacteria was monitored by measuring emission at 535 nm with an excitation at 485nm and corrected for background by subtracting fluorescence emitted by control strain which has an empty vector of the reporter construct.

Biocontrol of *A. pinodes* on Different Pea Varieties by *P. protegens*

Four different varieties namely Fallon, Greenwood, Agassiz, and CDC Treasure were used in this biocontrol experiment in greenhouse assay as done above. The experiment demonstrates the biocontrol effectiveness of *P. protegens* Pf-5 against *A. pinodes* in different varieties. However, some variations were observed among varieties. Agassiz showed less biocontrol effects when inoculated with Pf-5 compared other varieties (Figure 13) whereas there was better disease control in cv. Greenwood.

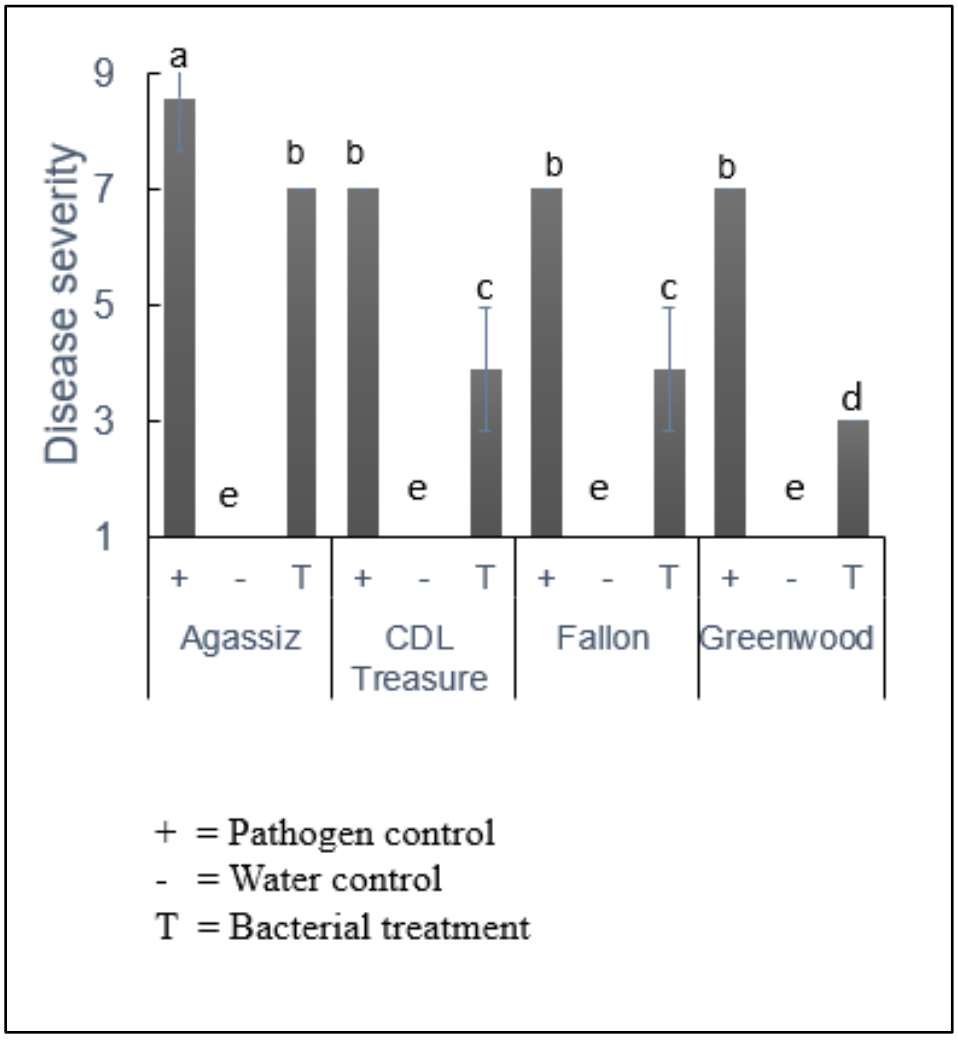


Figure 13: Disease severity on different varieties Pf-5 biocontrol effects of *A. pinodes* on different pea varieties and expression on rhizoxin antibiotics on peas leaves. Greenhouse assay was done as outlined in Figure 3.

Duration of Biocontrol of *P. peoriae* and *P. protegens* against *A. pinodes*

The study evaluated the biocontrol effects of *P. peoriae* (FMD009) and *P. protegens* (Pf-5) against *A. pinodes* in pea plants over a period of time post-bacterial inoculation (3, 7, 10, and 17 days post-inoculation (DPI). Disease severity was assessed 7 days after pathogen inoculation for each time point. The water control was included to measure baseline disease severity without

bacterial inoculation. *P. peoriae* (FMD009) showed a significant reduction in disease severity up to 17 DPI implying that it provided protection against the pathogen in this experiment. *P. protegens* (Pf-5) also showed significantly reduced disease severity the first seven days post bacterial inoculation. However, there was no significant reduction in disease severity after 7 days post bacterial inoculation (Figure14). *P. peoriae* (FMD009) showed prolonged and effective biocontrol activity against *A. pinodes* over the entire duration of the experiment, while *P. protegens* (Pf-5) exhibited effective but short-lived protection, highlighting differences in their effectiveness as biocontrol agents against this pathogen.

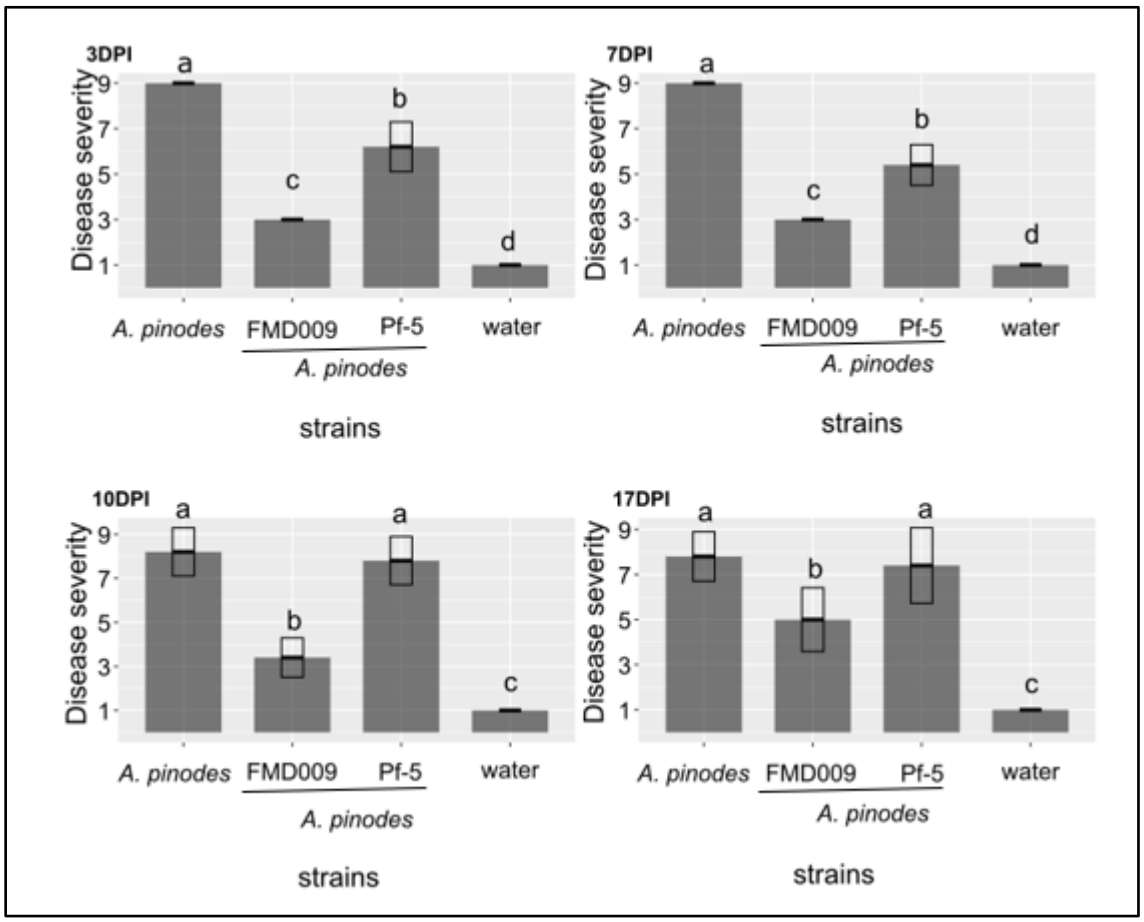
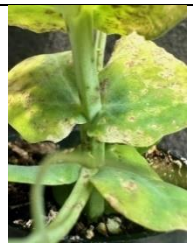
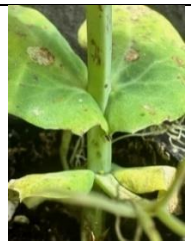


Figure 14: Biocontrol effects of *P. peoriae* (FMD009) and *P. protegens* (Pf-5) against *A. pinodes* over a period of 17 days post inoculation of bacteria. *P. protegens* and the best performing isolate *P. peoriae* from Montana provides biocontrol effects against *A. pinodes*, some 3 weeks old pea plants were inoculated with bacterial suspension of concentration 1.0 x 10^9 cells/mL and put in the growth chamber for 24 hours for bacterial to establish. The inoculated plants were later inoculated with a spore suspension of 1.0 x 10^6 spores/mL on day 3, 7, 10, & 17 post bacterial inoculation and their disease severity evaluated 7 days post pathogen inoculation. Plants were kept in the mist chamber for 24 hours after pathogen inoculation. Each treatment has 5 pea plant replicates at each time point. The experiment was done once.

Table 3: Photos of biocontrol effects of *P. peoriae* and *P. protegens* against *A. pinodes* over a period of 17 days post inoculation of bacteria

Days post Inoculation (DPI)	***A. pinodes***	***P. peoriae* + *A. pinodes***	***P. protegens* + *A. pinodes***
3DPI			
7DPI			
10DPI			
17DPI			

DISCUSSION

In this study, we found that rhizoxin antibiotics produced by beneficial bacteria of the *Pseudomonas* genus plays a major role in the growth inhibition of an Ascomycota pathogen *A. pinodes* on culture plates and the suppression of Ascochyta blight disease under greenhouse conditions. This is the first report that rhizoxin inhibits *A. pinodes* and contributes to the beneficial bacteria-mediated biocontrol of Ascochyta blight disease.

The significant role of rhizoxin in the inhibition of *A. pinodes* was clearly supported by the results that Pf-5 mutant that lacks rhizoxin exhibited greatly reduced inhibition on culture plates (Figure 8) and reduced disease control efficacy against *A. pinodes* compared to their wild type strains in greenhouse pot assays (Figure 9). We recognized that the culture conditions used in the inhibition assays may not be conducive for the tested bacterial strains to produce all their antibiotics. Therefore, antibiotics other than rhizoxin may contribute to the inhibition of *A. pinodes* by Pf-5 in different culture conditions. Our results are consistent with the previous reports that rhizoxin has antifungal activities to other pathogens (Iwasaki et al., 1984; Quecine et al., 2015). It was reported that rhizoxin can bind to tubulin, a protein that is crucial for cell division(Prota et al., 2014). This binding disrupts microtubule formation, leading to the inhibition of mitosis (cell division).

The overproduction of rhizoxin in *phlD* mutant, which lacks the antibiotics 2,4-diacetyl-phloroglucinol, resulted in larger inhibition zones, suggesting that manipulating antibiotic biosynthesis pathways could enhance biocontrol efficacy. This approach can be particularly useful in developing more potent biocontrol agents through genetic modifications. Metabolic co-regulation between biosynthetic pathways for secondary metabolites is common in microbes and

plays a significant role in microbial interaction. Similarly to our findings of metabolic co-regulation between biosynthesis pathways, (Brodhagen et al., 2004; Quecine et al., 2016) found that a mutation in pyoluteorin biosynthetic genes result in loss of pyoluteorin production along with overproduction of DAPG in *P protegen* strain Pf-5. Whereas (Yan et al., 2017) found that an intermediate in DAPG biosynthesis, phloroglucinol, is transformed by a halogenase encoded in the pyoluteorin gene cluster into mono-and di-chlorinated phloroglucinols. The chlorinated phloroglucinols function as intra-and inter-cellular signals that induce the expression of pyoluteorin biosynthetic genes. In this study, we found that the overproduction of rhizoxin in *phlD* mutants occurs in the presence of the pathogen and when minimal media is used. This could be because of the bacteria adapting to stressful environments. The mechanisms by which *phlD* interferes with the rhizoxin pathway is not yet understood.

The study successfully isolated and identified ten bacterial strains from Montana with biocontrol potential against *Ascochyta pinodes*. These isolates included *Pseudomonas* spp., *Paenibacillus* spp., *Peribacillus* spp., and *Bacillus* spp. Among them, *Paenibacillus* spp. showed the highest biocontrol efficacy, indicating its potential as a strong biocontrol agent. The ability of all isolates to inhibit *A. pinodes* on culture plates suggests that the production of antibiotics may be a mechanism of their biocontrol activity. The use of 16S rDNA sequencing and phylogenetic analysis provided taxonomic information of these isolates, which is critical for understanding their taxonomic relationships and potential functional capabilities. The possible mechanisms of biocontrol of this study are likely production of active compounds such as antibiotics as seen from the plate inhibition zones from some of the isolates in (Figure 5). The antibiotics produced by these strains were not characterized in this study. Future investigations, including whole

genome sequencing analysis and bacterial mutagenesis, of these isolates may identify new antibiotics for the disease management of *A. pinodes.* Several beneficial bacteria, including strains of *Pseudomonas* spp., *Bacillus subtilis, Bacillus amyloliquefaciens*, *Pantoea agglomerans*, *Serratia spp*, significantly reduced the severity of Ascochyta blight in peas under greenhouse conditions (Liu et al., 2016; Wang et al., 2003). The antagonistic bacteria identified in this study were isolated from fields in Montana and have adapted to the local climates and soil/plant environments. Thus, they can serve as useful microbial resources for the development of biocontrol agents to manage Ascochyta disease in Montana.

The study also evaluated the biocontrol efficacy of Pf-5 on different pea varieties, revealing variability in resistance to *A. pinodes*. Agassiz was the most susceptible variety, showing less biocontrol effect when treated with Pf-5 compared to Fallon, Greenwood, and CDC Treasure. This differential response indicates that the effectiveness of biocontrol agents can vary depending on the plant variety, which should be considered when developing integrated pest management strategies.

The confirmation of rhizoxin biosynthesis gene expression in pea plants, particularly in the wildtype but not in the *gacA* mutant, supports the role of rhizoxin in in planta biocontrol activity. This finding is crucial for understanding how biocontrol agents interact with plants and pathogens within the plant environment. The preliminary results of the duration of protection of *P. peoriae* and *P. protegens* showed that both *P. peoriae* FMD009 and *P. protegens* Pf-5 exhibited significant biocontrol effects against *A. pinodes,* reducing disease severity compared to the untreated control. *P. peoriae* FMD009 consistently showed a significant reduction in disease severity across all time points, maintaining a mean severity score around 4-5. This suggests that

P. peoriae FMD009 provided effective and sustained biocontrol over a period of up to 17 days post-inoculation of bacteria. The lower disease severity indicates a strong and possibly long-lasting protective effect against *A. pinodes. P. peoriae* offers a promising biocontrol alternative to chemical treatments, particularly given its consistent performance over 17 days. While it may not reduce disease severity as effectively as chemical controls initially, it provides significant and sustained disease management. It is known that pea plants are susceptible to Ascochyta blight throughout the whole growth season. Fungicides such Chlorothalonil, Mancozeb and others provide protection for 7-14days which implies that the *P. peoriae* as good as chemical control in providing protection as fungicides. This also implies that like chemical control, biocontrol also has to be applied several times during the growing season. This makes *P. peoriae* an attractive option, especially for integrated pest management (IPM) strategies aiming to reduce chemical inputs and mitigate resistance development. Although more repeats are needed to test the duration of the biocontrol effect of *P. peoriae* FMD009, this preliminary experiment provided data supporting that FMD009 may serve as a promising biocontrol agent to control pea Ascochyta blight disease. Additionally, future research should determine the longest duration of action of the top isolated identified in this work and focus on understanding the mechanisms behind the long-term efficacy of *P. peoriae* and exploring ways to enhance the duration of protection provided by *P. protegens.*

CONCLUSION

In Conclusion, ten promising bacterial isolates that showed biocontrol effects on pea Ascochyta blight disease in greenhouse conditions were identified in this study. These isolates may serve as potential biocontrol agents for managing Ascochyta blight in pea crops in Montana. The mechanism of their biocontrol is likely antibiosis. Rhizoxin antibiotics produced by *Pseudomonas protegens* Pf-5 plays a vital role in the growth inhibition of an Ascomycota pathogen *A. pinodes* on culture plates and suppression of Ascochyta blight under greenhouse conditions. Biocontrol effects against *A. pinodes* were significantly enhanced by a mutation in *phlD,* implying genetic improvements is possible to enhance the efficacy of disease controls. Preliminary data showed that the best performing isolate FMD009 from Montana, also provided a protection of up to 17days indicating that the biocontrol can be very effective in protecting the pea plants against *A. pinodes*.

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