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# Evaluation of Biological and Chemical Seed Treatments for Management of Rice Diseases

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Evaluation of Biological and Chemical Seed Treatments for Management of Rice Diseases

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## Abstract

Rice is the primary staple for more than half of the global population and is the second most important cereal worldwide. In the US, rice is primarily grown in the southern states, with Arkansas leading production and responsible for 47% of the total rice production in the country. Diseases cause significant yield losses in rice. Sheath blight, rice blast and bacterial panicle blight are the main diseases in rice and cause significant yield losses. Sheath blight alone could cause up 50% yield loss in heavily damaged fields with highly susceptible cultivars. Additionally, rice seed and seedling diseases primarily caused by *Pythium* species and *Rhizoctonia solani* result in reduced germination, poor seedling development or seedling damping off, reducing plant stand.

The research conducted focused on a two-prong approach, by evaluating biological control and chemical control to manage seed and seedling diseases on rice, with the aim of providing different solutions for control of fungal pathogens. First, two *Pseudomonas* and two *Burkholderia* spp. were evaluated as potential biological control against three isolates of *Rhizoctonia solani* and one isolate of *Fusarium graminearum* using dual culture plate and as seed treatments in controlled environments. Results obtained 48- and 96-hours post-inoculation show both *P. fluorescens* and *B. cepacia* caused significant reductions in the mycelial growth of the *R. solani* and *F. graminearum* isolates. Further evaluation of these bacteria in seed plate assay indicated that all four bacterial have varying degrees of antagonism against *R. solani* and *F. graminearum.* Both *P. fluorescens* and *B. cepacia* remained active improving emergence as seed treatments. Further studies are needed to investigate the mechanisms through which the bacteria inhibit fungal growth and their effectiveness of foliar fungal diseases.

The second approach evaluated eight different chemical seed treatments containing azoxystrobin, fludioxonil and sedaxane and the combinations of these active ingredients. These seed treatments were evaluated against *R. solani* and *Pythium* spp. using seed plate and seedling cup assays in controlled environments with the rice cultivar 'Diamond'. To determine the efficacy and impact on plant health, emergence at 7-days post inoculation and planting. Plant stand, total plant weight and root weight were measured. Statistical analysis of the results showed a seed treatment of sedaxane or fludioxonil alone was as effective as two or three-way combinations of these active ingredients in improving emergence, plant stand, total plant weight and root weight. A single application of azoxystrobin improved the above parameters but it was significantly less effective than fludioxonil or sedaxane. Despite the effectiveness of most seed treatments, it is necessary to assess the reduced efficacy of single application of azoxystrobin by determining fungicide resistance and the impact on disease management.

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# Chapter I – Literature Review

## 1. Introduction

### *1.1. Rice production and importance in Arkansas*

Rice (*Oryza sativa L.*) is one of the world's three major staple food crops along with wheat and maize, feeding more than half of the global population of over 7 billion people (Mulaw et al. 2018). Approximately, 500 million metric tons of rice are produced annually worldwide, providing about 19% of the global dietary energy (Muthayya et al. 2014). Globally, over 90% of rice is produced and consumed in Asia, with India and China producing over 50% of global total (Bandumula 2018). The remaining production comes from South and North America primarily in the states of Texas, Louisiana, Mississippi, Missouri and California (Childs 2022).

Rice is an important crop in the United States, where it is primarily produced in the southern part of the country (Singh et al. 2019a). Despite representing only 2% of global rice production, the U.S. is the 4$^{th}$ largest exporter of rice in the global market (Espe et al. 2016). In the US, Arkansas is the leading rice producing state, representing about 47% of total US rice production (Hardke, 2020). Rice in Arkansas is produced primarily in the eastern half of the state, with the Arkansas Delta region leading in rice production (Mulaw et al. 2018). In 2020, the total rice production in Arkansas reached 591,245 hectares, which accounted for 48.1% of the total US rice acres (Hardke, 2021). Rice contributes more than 4 billion dollars to the state's economy, accounting for approximately 25,000 jobs that impact rural communities (Hardke, 2020).

*1.2 Impact of diseases on rice*

Despite the technological advancement and increased understanding of our cropping systems, diseases continue to cause significant losses in rice, reducing quality and quantity of yield in rice (Wamishe, Y, Cartwright and Lee 2013). Major diseases in rice include sheath blight, rice blast, stem rot, crown (black) rot, bacterial panicle blight, false smut, and kernel smut. The use of high-yielding cultivars that are susceptible to the major diseases, combined with higher nitrogen use and shorter rotation or no rotations, have promoted disease development and subsequent epidemics (Wamishe, Y, Cartwright and Lee 2013).

*1.2.a. Seed and seedling pathogens of rice*

Seed and seedling diseases are particularly important because they cause reduced germination, poor seedling development or seedling death, reducing plant stand and impacting yield (Toda et al. 2015). Seed and seedling pathogens cause different symptoms on rice depending on the genotype of the cultivar used and environmental factors including temperature and relative humidity (Salmaninezhad and Mostowfizadeh-Ghalamfarsa 2019a). Cool and wet weather is most favorable for seedling disease development (Groth and Hollier 2021).

A number of pathogens cause seedling blight and seedling damping off germination include *Alternaria* spp., *Cochliobolus* spp., *Curvularia* spp., *Pyricularia* spp., *Pythium* spp., *Fusarium spp.* and *Rhizoctonia* spp. (Toda et al. 2015). Several species of *Fusarium, Pythium,* and different isolates of *Rhizoctonia solani* also cause seed rot during germination and blights in rice seedlings (Verma et al. 2018). This can ultimately result in yield loss by reducing germination and ultimately reducing plant stand, depending on the level of damage. *Pythium*

species associated with root rot and damping-off in rice include *P. irregulare, P. ultimum, P. graminicola and P. arrhenomanes* (Chun and Schneider 1998). *Rhizoctonia solani* AG 4 and AG 11 can cause seedling diseases such as damping-off and seed or root rot (Gaire et al. 2020a).

Symptoms of seed rot and damping-off can occur in the pre-emergence and post-emergence phases. In the pre-emergence phase, some seeds become soft and fail to germinate, and those that germinate have stems affected with water-soaked lesions below the soil line, that eventually wilt and die before emergence (Lamichhane et al. 2017). In the post-emergence phase, emerged seedlings collapse and die and surviving seedlings that emerge above the soil line show water-soaked lesions, leaf spotting and stunting. Seedlings with those levels of damage show a complete loss of plant stand or reduced and uneven growth (Gaire 2021). Affected seeds may have spotted or discolored hulls and surviving seedling generally lack vigor and become yellow or pale, affecting the ability of the seedling actively compete with healthy seedlings (Lamichhane et al. 2017).

*1.2.b. Rhizoctonia solani*

*Rhizoctonia solani* is a ubiquitous necrotrophic soilborne fungus that causes damage on a wide range of economically important crops. As described by Ajayi-Oyetunde and Bradley (2018), *Rhizoctonia* isolates have a great morphological and genetic diversity. *Rhizoctonia solani* is classified into 14 groups known as anastomosis groups (AG) based on compatibility of hyphal fusion reaction (Abdelghany et al. 2022). Hyphae of the same AGs can fuse with each other and expand their genetic diversity. There are binucleate and multinucleate AGs on the basis of the number of nuclei in the fungal cell. Several of the AGs are further divided into subgroups based on pathogenicity and genetic characteristics (Muzhinji et al. 2015).

*1.2.c. Rhizoctonia solani* and *Sheath blight*

Sheath blight, caused by *Rhizoctonia solani* AG 1-IA is one of the most devastating diseases of rice worldwide. This disease has the potential to cause to up to 50% yield loss in heavily damaged fields with highly susceptible cultivars (Wamishe, Y, Cartwright and Lee 2013). This pathogen survives primarily between crops as sclerotia or mycelium. Sclerotia are compact masses of hardened mycelium that can be dormant in the soil for up to 2-3 years. Sclerotia can float and therefore can be dispersed by irrigation water and can serve as primary inoculum. Other sources of primary inoculum include asexual propagules, which can also live in the soil for several years. *R. solani* AG 1-IA also causes aerial blight in soybean, which regularly rotated with rice (Ajayi-Oyetunde and Bradley 2018). Hence, rice rotation with soybean may not reduce disease incidence.

The environmental conditions conducive to sheath blight include high nitrogen rates (>150 pounds N per acre), and hot and humid weather with temperatures between 80 to 92°F during the day and 74°F and above at night, with dense canopy cover (Wamishe, Y, Cartwright and Lee 2013). Under these favorable conditions, sclerotia germinate and the mycelia begin to grow. *Rhizoctonia solani* enters the rice host tissue using an infection cushion (aggregates of hyphae or an appressorium), and through stomata or wounds caused by mechanical damage (Li et al. 2021). The disease usually appears at the tillering stage.

*1.2.d. Rhizoctonia solani as seed and seedling pathogens*

While there are limited studies focused on the impact of seedling diseases on yield, it is known that reduced germination, poor stand establishment, reduction in biomass and uneven plant height can have negative impact on overall yield. A few *Rhizoctonia solani* anastomosis groups

(AGs) cause seed rot and seedling diseases in rice (Gaire 2021). *R. solani* AGs known to be seedling pathogens of rice include AG 4 (Gaire et al. 2020a), AG 7 (Rothrock et al. 1993) and AG 9 (Wamishe et al. 2019). *R. solani* AG-11 was also observed and reported in Texas (Jones and Carling 1999; Spurlock et al. 2016). *R. solani* AG 4 causes seed rot and damp-off in rice, resulting in yield losses (Gaire 2021). In recent field trials, *R. solani* AG 9 showed significant reduction in seedling stand and it is frequently used to test the efficacy of different chemical seed treatments (Wamishe et al. 2019). *Rhizoctonia solani* isolates recovered from infected seeds or seedlings can be used to test efficacy of chemical and biological products.

*1.2.e. Pythium spp. as seedling pathogens*

*Pythium* are an ecologically diverse, necrotrophic soilborne group of organisms (Urrea et al., 2013*).* The genus *Pythium* is described as a heterogenous group that comprises more than 230 identified species, several of which are found to be pathogenic in a wide range of plants, causing serious economic losses worldwide (Salmaninezhad and Mostowfizadeh-Ghalamfarsa 2019a). *Pythium spp.* survive in the soil as thick-walled oospores in the absence of host or host residue, which allows them to survive in the environment particularly long (Okubara et al. 2014). This makes *Pythium* spp. difficult to manage as seedling pathogens. Pathogen growth and the infection process are favored by high soil moisture which promotes spore germination and mobility of the motile spores, zoospores, in the soil (Syed et al. 2020). Rice-infecting *Pythium species* include P. *graminicola, P. irregulare, P. arrhenomanes and P. ultimum*. These species pre- and post-emergence damping-off and root and seed rot by forming necrotic lesions on roots, stolons and underground stems in cereal crops including rice (Wamishe, Y, Cartwright and Lee 2013). If plants infected with *Pythium* survive, the affected roots absorb less water and nutrients, leading to wilting

and the ultimate death of the plants (Syed et al. 2020). Infection of the host by *Pythium* spp. results in symptoms such as plant stunting, water-soak root rot, damping-off and leaf blight can occur, which all can result in host death (Wu et al. 2020).

*Pythium* seedling diseases decrease the development of plant over a great range of temperatures, but at cooler temperatures, *Pythium* spp. can cause significant reduction in plant stand and replanting may be necessary in these fields (Rothrock et al. 2003). *Pythium spp.* play an important role in determining stand establishment, and can result in an uneven plant heights (Rothrock et al. 2003). Seeds and young roots of germinating plants are susceptible to attack by different species of *Pythium*, as they release host root exudates that enable the adhesion of *Pythium* zoospores through pathogen-derived glycoproteins (Okubara et al. 2014).

*1.3. Management of diseases on rice*

Currently, foliar rice diseases are managed primarily by chemical control in the form of fungicide application and effective cultural practices, as there are no completely resistant cultivars (Wamishe et al. 2007). Cultural practices used in the management of rice diseases include use of disease-free seeds of less susceptible and more tolerant cultivars, avoiding dense plant populations by using spacing, using no more than recommended rate of nitrogen fertilizer, and use of clean equipment for all field management practices (Wamishe, Y, Cartwright and Lee 2013). Additionally, early planting date or planting in warm soils can reduce the impact of seedling diseases (Rothrock et al. 2003). Destruction of stubbles and weeds in and around the field, avoidance of field to field irrigation, and in some cases, early planting can be used to manage diseases in rice systems (Singh et al. 2019a). While crop rotation helps manage certain

diseases, crops rotated with rice such as soybean, corn, cotton, and sorghum are susceptible to different *R. solani* AGs (Wamishe et al. 2007)

Seed treatment is often used to protect seeds and emerging seedling from pathogens (Cardarelli et al. 2022). Fungicide seed treatments often used to manage various rice seedling pathogens are not species-specific (Verma et al. 2018). Fungicide seed treatments used to manage seedling pathogens include but not are limited to Dynasty (azoxystrobin), Maxim 4 FS (Fludioxonil), Vibrance (sedaxane) and Apron XL (mefenoxam) or the combination of 2 or more of these chemical pesticides (Faske et al. 2021). Whereas mefenoxam is used for the management of oomycetes, azoxystrobin, fludioxonil and sedaxane provide a broad-spectrum disease control (da Silva et al. 2017; Ko et al. 2015; Song et al. 2022). The application of these active ingredients or blends of active ingredients could be used as seed treatment to manage soilborne diseases.

An alternative to chemical control is the use of biological control agents (BCAs). Biological control refers to suppression pest population with microbial agents through parasitoid, predator, pathogen, antagonism or competition (Gnanamanickam et al. 2002). By suppressing the pathogen population, the surviving pathogen populations causes less damage. Although the development of biological products has been slow due to the variability in performance of the BCAs under different environmental conditions, BCAs have shown to be effective in managing rice diseases (Heydari and Pessarakli 2010). BCAs have been reported to reduce sheath blight and other foliar and seedling diseases rice include *Burkholderia cepacia* (Nicolaisen et al. 2018a), *Bacillus spp.* (Zhu et al. 2021), *Trichoderma* and *Pseudomonas spp.*(Heydari and Pessarakli 2010).

*1.3.a. Management of Rhizoctonia solani on rice*

Fungicide application to manage sheath blight is recommended when field scouting "indicates than 35% positive stops in susceptible to very susceptible varieties, or more than 50% positive stops in moderately susceptible varieties between panicle differentiation and early heading" (Faske et al., 2021). Fungicide recommended for and used in managing sheath blight include but are not limited to Quadris (azoxystrobin), Stratego (trifloxystrobin + propiconazole), and Amistar Top (azoxystrobin + difenoconazole) (Wamishe, Y, Cartwright and Lee 2013). However, among those azoxystrobin, a QoI fungicide, is widely recommended for control of sheath blight. Unfortunately, the emergence of *R. solani* isolates resistant to fungicides, has limited their use in the field (Hollier 2014).

Biological control agents (BCAs) including different *Burkholderia spp.* have shown to be effective in controlling sheath blight in controlled environments, but persistence of these BCAs can be lost within a short time in the field (Nicolaisen et al. 2018a).

*1.3.b. Management of Pythium pathogens*

*Pythium* species are widespread in soil and water (Wu et al. 2020). Different methods such as chemical, biological and cultural controls, as well as host resistance have been deployed for the management of diseases caused by *Pythium* spp. on hosts including rice (Syed et al. 2020). The integrated approach of two or more of those methods is generally used to gain the best management of diseases caused by pathogenic *Pythium* spp. (Syed et al. 2020). Cultural control practices such as crop rotation with non-host, use of cover crops, soil solarization, mulching, sanitation, and pasteurization, use of healthy seeds, fertilizer and manure may promote plant growth which could subsequently help reduce *Pythium* diseases (Syed et al. 2020). Due to

the wide host range and survival of *Pythium* in the soil, crop rotation cannot control *Pythium* effectively (Wu et al. 2020).

The use of fungicides for managing *Pythium spp.* is one of the effective and most reliable controls (Syed et al. 2020). However, control of *Pythium spp.* is difficult due to high prevalence, wide host range, and longevity of these species that can increase the cost of fungicide application (Wu et al. 2020). There are many chemical fungicides that are effective against *Pythium spp.* including metalaxyl, azoxystrobin, fosetyl-Al, pyraclostrobin, and trifloxystrobin (Wu et al., 2020). The recommended chemical fungicides are generally applied as seed treatment, side-dressing, soil treatment or chemigation (Syed et al. 2020).

Biological control is also considered as a promising way to control or manage *Pythium* diseases, by exploiting the antagonism between the biological control agents (BCAs) and the *Pythium* pathogens (Wu et al. 2020). No single biological control is effective against all *Pythium* spp. (Syed et al., 2020). *Trichoderma harzianum,* and *Pseudomonas fluorescen*s have been shown to be significantly antagonistic against *Pythium spp.* that include *P. ultimum* and *P. aphanidermatum,* decreasing disease incidence and increasing germination (Parveen and Sharma 2015). In different crops, *Pseudomonas fluorescens* is also shown to effectively control different *Pythium* species and to improve yield in various crops (Syed et al. 2020). *Pseudomonas* spp. have also been shown as a biological control to reduce symptoms of both *Rhizoctonia solani* and *Pythium ultimum* while promoting white root and shoot development (Wu et al. 2020).

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## Chapter II: Evaluation of potential bacterial biological control agents (BCAs) for the control of rice diseases


### Abstract

Management of plant diseases often relies in the use of chemical products that are effective for limited time and have a detrimental impact to the environment. An alternative to chemical control is the use of beneficial living organisms that have antagonistic effects on pathogens, also known as biological control agents (BCAs). Although the development of biological control strategies has been slow due to the variability in performance of the BCAs under different environmental conditions, BCAs have shown to be effective in managing rice disease. BCAs that have been shown to manage or control rice diseases include *Burkholderia* spp, *Bacillus sp*, *Trichoderma* and *Pseudomonas spp.* The overall goal of this study was to evaluate four bacterial strains: *Burkholderia cepacia*, *Pseudomonas protegens*, *Pseudomonas fluorescens* PBL and another *Burkholderia* spp. as BCAs against *Rhizoctonia solani* AG 1-IA, *R. solani* AG-4, *R. solani* AG-7, and *Fusarium graminearum:* Fg 4 in controlled environments.

In 48- and 96-hours post-inoculation in the dual plate assay, *Pseudomonas fluorescens* and *Burkholderia cepacia* have the highest control against *R. solani* and *F. graminearum*. *Pseudomonas protegens* showed reduced efficacy against these fungal pathogens but there was no significant difference among the three bacterial strains at 48 hours post-inoculation in most cases. However, *P. fluorescens* and *B. cepacia* showed significantly higher reduction against all the fungal isolates compared to the other two bacterial strains used. Evaluation of the BCAs in the seed plate assay as a seed treatment shows that all four bacterial strains had a degree of control against the four fungal isolates used in this study. but there was significant difference among the biocontrol agents in reducing the impacting of fungal pathogen.

## 2.1. Introduction

Fungicide application is the most widely used means of controlling diseases in rice due to efficacy and immediate availability (Singh et al. 2019b). Most of the known fungicides are classified into two main groups: contact and systemic. are vital to the effective control of plant diseases (Petit et al. 2012). When using fungicides, it is important to consider fungicides with or fungicides different target sites to reduce development of fungicide resistance (Hollomon 2016). Despite the diversity in the use of fungicides, resistance in plant pathogens to chemical pesticides (agrochemicals) used in agriculture is not uncommon. and major cause of concern when managing aggressive pathogens as *Rhizoctonia solani*. In addition, the growing public concern of the negative impact that these agrochemicals have on the environment, coupled with human health concern, has led to interest in environmentally-friendly options such as biological control (Nicolaisen et al. 2018a). Biological control relies on the use of microbial antagonists, known as biological control agents (BCA) as well as plant-incorporated products to inhibit growth, infection, or reproduction of pathogens (Heydari and Pessarakli 2010). BCAs do this through predator, infection of the pathogen, antagonism or competition to suppress the pathogen, making it less abundant and thus less damaging than it otherwise would be (Gnanamanickam et al. 2002). The mode of action of biological control organisms for managing fungal diseases of plants include hyperparasitism, predation, antibiosis, cross-protection, competition for site and nutrients and induced resistance which is activated upon infection (Heydari and Pessarakli 2010). Potential biological control agents have drawn the attention of researchers because of production of secondary metabolites such as siderophores, antibiotics, volatile compounds, hydrogen cyanide (HCN), enzymes and phytohormones that can have antagonistic effect against fungal pathogens (Kazempour 2004).

Some known biological control agents as in the bacterial genus *Pseudomonas* and the fungal genus *Trichoderma* produce metabolites and proteins with antimicrobial properties. The use of those isolated antimicrobials has been shown to directly suppress pathogen growth or disrupt the pathogenicity process. In some cases, the use of these isolated antimicrobials have been more effective than the intact organisms from which they are derived (Okubara et al. 2014). The secondary metabolite phenezine-1-carboxylic acid (PCA) produced by *Pseudomonas, Burkholderia, Myxococcus spp.* is an example among the best known naturally occurring antifungal metabolites that has activity against *R. solani* and *Pythium* (Okubara et al. 2014).

Common BCAs that have been successful in the past with potential biological control effects against fungal pathogens include *Pseudomonas, Trichoderma* and *Burkholderia* species. Spray treatment of field plots with a *Burkholderia* sp. resulted in significant suppression of sheath blight disease, decreasing disease incidence by 39% and disease severity by 56% in rice (Nicolaisen et al. 2018a). *Pseudomonas* spp. have been studied mainly because of their widespread distribution in the soil and their ability to produce a wide range of compounds with inhibitory activity against several serious plant pathogens (Rangarajan et al. 2003).

Biological control has not been fully tapped due constant failure of BCAs isolated in the laboratory to perform consistently in the e field , (Nicolaisen et al. 2018a). Additionally, successful biological control requires deep understanding of the complex regulation of disease suppression by the antagonists in response to both biotic and abiotic factors, as well as knowledge of the dynamics and composition of microbial community associated with plants, and what triggers colonization of the plants. As a result, BCAs isolated from a specific field may work well in controlling fungal pathogens in that field but could perform poorly or not perform at all in another field (Nicolaisen et al. 2018b).

To minimize the risk of fungicide resistance with chemical application and to provide an alternative to chemical fungicide, this study was conducted to investigate the biological control activity of two *Pseudomonas* spp. and two *Burkholderia* spp. strains against three isolates of *Rhizoctonia solani* anastomosis groups, AG 1-IA, AG-4, and AG-7 and one isolate of *Fusarium graminearum* (Fg 4), which causes Fusarium head blight (FHB) in wheat and other cereal crops.

Seedling pathogens including species of *Fusarium, Rhizoctonia solani, Cochliobolus* and other soilborne pathogens can result in low plant stand or seedling death by causing damping-off or seedling blight of rice (Groth and Hollier 2021). There are several *Fusarium* species including *F. graminearum* that infect rice kernels are capable of producing a wide range of mycotoxins that are harmful to human and animal health (Moreira et al. 2020). Additionally, *F. graminearum* causes Fusarium seedling blight in cereals, which can result in significant reduction in plant establishment due to sowing of seeds infected with *F. graminearum* and other *Fusarium* spp. (Yang et al. 2011).

## 2.2. Materials and Methods

### *2.2.a. Isolate collection and plant material*

Several potential biocontrol bacteria were originally isolated from wheat roots in Arkansas in 1991 and 1992 (Milus and Rothrock 1997) and were maintained in Dr. Rothrock's and Dr. Rojas' culture collections at the University of Arkansas. The bacterial strains used include *Pseudomonas protegens* PBL 3, *Pseudomonas fluorescens* PBL 24, *Burkholderia cepacia* PBL 18 and *Burkholderia spp.* PBL 33. Bacterial strains were preserved in 30% sterile glycerol (Research Products International Corp) in long-term storage at -80 ˚C freezer (New Brunswick Scientific Co., NJ). For the pathogens, *R. solani* AG-1-IA, AG-4 and AG-7, and *F. graminearum* Fg 4 were used in this study. *R. solani* AG-1-IA and AG-4 were collected from

rice fields in Arkansas. *R. solani* AG 7 was isolated from a cotton field and Fg-4 was isolate from a wheat field, both in Arkansas. All isolates were maintained on Potato Dextrose Agar (PDA) (BD Difco) and stored long term as plugs of mycelia stored in vials with sterile water or in 30% glycerol at -80ºC.

This study used the rice cultivar 'Diamond', developed at the University of Arkansas Division of Agriculture's Rice Research and Extension Center in Stuttgart, Arkansas (Hardke 2020). 'Diamond' is a high-yielding, short-season, long-grain rice cultivar. 'Diamond' is susceptible to some of the major diseases in rice including sheath blight, blast, stem rot and kernel smut. It is moderately susceptible to bacterial panicle blight and lodging.

*2.2.b. Dual culture plate assay*

To examine the inhibitory effect of the bacterial *Pseudomonas fluorescens, Pseudomonas protegens, Burkholderia cepacia* and *Burkholderia spp.* against the fungal pathogens, each strain was grown on Luria-Bertani (LB) media. Single colonies of each of the four strains were transferred into 15 mL centrifuge tubes (VWR Chemicals) containing 5 mL liquid LB media and incubated overnight at 27 ˚C using a gravity incubator (VWR Chemicals). The bacterial suspension, 1.5 mL of each strain was transferred into a 2 mL microcentrifuge tubes (USA Scientific) and vortexed at 6,000 rpm for 10 minutes using Eppendorf Centrifuge 5417R (Eppendorf North America Inc.). The supernatant solution was discarded, and the bacterial pellet was washed twice using dissolved 0.5x Phosphate-buffered saline (PBS, VWR Chemicals) by vortexing as described above. The pellet was finally resuspended in 1.5 mL of 0.5x PBS and mixed thoroughly. Optical density reading ($OD_{600}$) was taken for each bacterial suspension using

Synergy HT microplate reader (BioTek) and the concentration was adjusted to ($2.9 \times 10^7$ to $5.3 \times 10^8$ CFU/mL).

To determine the antagonistic effect of each strain against *R. solani* and *F. graminearum*, a sterile 10 μL disposable inoculation loops (Thermo Fisher Scientific, NH) into the bacterial suspension and streaking the middle of a PDA plate using a premade template. Two small plugs (about 5 mm in diameter) of *R.* solani (AG 1-IA, AG-4 and AG-7) and *Fusarium graminearum* (Fg 4) were placed equidistant from the bacterial streak at either side of the streak: 30 cm away from the streak for the *R. solani* plugs and 20 mm away for the *F. graminearum* plugs. Plugs on the plate were from the same isolate. There were five replicate plates per bacterial strain per fungal isolate. Controls were done a plug of the fungal isolate placed alone on a PDA plate. Five replicates per isolate were also used for the control. The plates were sealed using Parafilm M (Amcor, Zurich, Switzerland) and incubated at 27 ˚C using a gravity incubator (VWR Chemicals). Measurement of the fungal growth was taken using an electronic caliper by measuring the mycelial growth of the fungus from the plug toward the bacterial streak (*iGaging* electronic caliper) at 48 and 96 hours after inoculation. Values were restricted to the maximum radius determined by the distance of the streak to the plug, 30 mm for *R. solani* and 20 mm for *F. graminearum*. Cultures were regularly checked for contamination.

*2.2.c. Seed plate assay using biocontrol bacteria as a seed treatment*

Seeds of the Rice cultivar Diamond were first sterilized using 70% ethanol and air-dried. To coat the seeds with the bacterial strains, a suspension was prepared as described ($2.9 \times 10^7$ to $5.3 \times 10^8$ CFUs/mL) was mixed with an equal volume of 1% carboxymethylcellulose solution (CMC) (Sigma Chemical Company, St. Louis, MO) and gently shaken for 30 seconds then left

undisturbed for 20 minutes. Treated seeds were air-dried on sterile paper towels under a laminar flow hood. Isolates of *R. solani* AG 1-IA, AG-4, AG-7, and *F. graminearum* Fg4 were grown on solid PDA media for 5-7 days at room temperature. 8 g of autoclaved high-grade vermiculite (Palmetto Vermiculite Co., SC) was evenly spread on top of the fully colonized plate using a sterile metal forceps. The treated seeds, 15 per plate, were evenly spread on top of the thin layer of sterile vermiculite. In this experiment, two controls were used. The first control comprised of the bacterial treated seeds placed on the sterile vermiculite without the plant pathogenic fungus. The second control placed untreated seed on the sterile vermiculite without the plant pathogenic fungus. There were five replicates per treatment per pathogen. The plates were fully covered with aluminum foil (Reynolds Wrap, Richmond, VA) and kept at room temperature. After seven days, germination was determined using the Association of Official Seed Analyst (AOSA) protocol in which a seedling is considered healthy when 50 percent of its cotyledonary tissue remains attached to the seed or free of decay. Data was analyzed using JMP Pro 16.0 and SAS 9.4 software.

*2.2.d. Statistical analysis*

*Dual culture assay.* The average radii (AvgRad) at 48- and 96-hours post-inoculation were analyzed using the SAS GLIMMIX procedure with Kenward-Roger degrees of freedom using the logarithmic function with gamma response distribution. The analysis was done using combined results of three independent experiments using experiment as block factor. A total of 600 observations were used in this analysis for each incubation time (48h and 96h).

*Seed plate assay.* The experiment was carried using random complete block design (RCBD). The seed plate assay data were analyzed using the SAS GLIMMIX procedure with

Kenward-Roger degrees of freedom. Analysis of variance was done using combined data of three independent experiments with a total of 600 observations. Germination was the response variable with binomial distribution, with experiments as blocks using the logit function.

## 2.3. Results

### *2.3.a. Evaluation of BCAs in dual culture assay – 48 hours*

At 48 hours post -inoculation, there was a significant pathogen by biocontrol agent (BCA) interaction (Table 2.1). The results from the three experiments were combined for analysis since correlation tests of the results from the three experiments are not significantly different (data not shown). Radial growth of all the pathogens was significantly reduced by each of the BCAs. (Table 2.2). The average growth radius of *R. solani* AG 1-IA at 48 hours without a BCA was 28.9 mm. However, when this fungus was co-cultured with BCAs, its average growth radius ranged from 19.5 to 23.1 mm. The largest reduction in the average radius of the fungus was observed with *P. fluorescens* PBL 24 (Figure 2.1 a). For *R. solani* AG-4 control, the average growth radius was 28.0 mm, but co-cultured with BCAs the average growth radius ranged from 17.0 to 20.0 mm. With both AG—IA and AG-4, the largest growth reduction was observed with three *B. cepacia* PBL18, *P. fluorescens* PBL24 and *Burkholderia spp* PBL33 (Figure 2.1 b). *R. solani* AG-7 grown by itself showed an average radius of 28.0 mm, and a large reduction in growth was observed after co-culturing with the 4 BCAs, with an average growth radius ranging from 18.0 to 25.0 mm and with the least growth observed when co-culturing *R. solani* AG-7 with *P. fluorescens* PBL 24. (Figure 2.1 c). With *F. graminearum* Fg 4, the mean radius of the control was 13.5 mm but was significantly less (7.0 to 9.5 mm) when paired with any of the BCA's (Figure 2.1 d).

*2.3.b Evaluation of BCAs in dual culture assay – 96 hours*

After 96 h, there were significant differences in growth between pathogens and between pathogens exposed to BCAs, but there was not a pathogen by BCA interaction (Table 2.3) With all pathogens, the radial was significantly less with BCAs PBL 18 and PBL24 than with PBL-3 or PBL-33 (Fig. 2.2). For *R. solani* AG 1-IA used as control, the average radius was 30.10 mm, whereas the average radius of *R. solani* AG1-IA after co-culturing with each one of the bacterial strains ranged from 21.9 to 28.1 mm (Figure 2.2 a). At this time point, both *B. cepacia* PBL 18 and *P. fluorescens* PBL 24 showed the highest reduction in the growth of *R. solani* AG 1-IA in comparison with the control, while *P. protegens* PBL3 and *Burkholderia* spp PBL33 showed minor effect but still statistically significant (Figure 2.2 a). In *R. solani* AG-4, the average radius of the control was 30.1 mm, but the average growth radius of *R. solani* AG-4 with each one of the four bacterial strains ranged from 20.9 to 26.5 mm (Figure 2.2 b). Similar to what was observed with *R. solani* AG-1-1A, *B. cepacia* PLB 18 and *P. fluorescens* PBL 24 showed the highest reduction in the growth of *R. solani* AG 4 and less effect was observed with *Burkholderia* spp PBL 33 and even less effect with *P. protegens* PBL3 (Figure 2.2 b). For *R. solani* AG-7, the average radius in the control was 30.1 mm. In the dual-culture assay, for the same isolate, the average growth radius after co-culturing with the four ranged from 21.5 to 28.1 mm (Figure 2.2 c). Interestingly, the patterns of growth inhibition were similar to those of *R. solani* AG1-1A, with *B. cepacia* PBL 18 and *P. fluorescens* PBL 24 showing equivalent growth inhibition, whereas *P. protegens* PBL 3 and *Burkholderia* spp PBL 33 showing equivalent but less inhibition that *B. cepacia* PBL18 and *P. fluorescens* PBL24 (Figure 2.2 c). In *F. graminearum* Fg 4, the mean radius for the control was 19.6 mm, but the growth of Fg 4 in the presence of each one of the four bacterial strains ranged from 12.5 to 18.0 mm (Figure 2.2 d). *B.*

*cepacia* PBL 18 and *P. fluorescens* PBL 24 have statistically higher reduction in the growth of *F. graminearum* than *P. protegens* PBL 3 and *Burkholderia* spp PBL 33 in comparison with the control (Figure 2.2 d). Although *P. fluorescens* and *B. cepacia* have the highest reduction in the growth for all four fungal isolates used, *Burkholderia* spp. PBL 33 was able to reduce the growth of all four pathogens significantly less in comparison to PBL 18 and 24 resulting inhibition (Table 2.3 and 2.4). No statistical difference was observed between *P. fluorescens* and *B. cepacia* in reducing the growth of all four fungal isolates used in this study (Table 2.1).

*2.3.c. Evaluating the effect of potential BCAs as seed treatments*

There was a significant pathogen effect and a significant pathogen by BCA interaction for seedling emergence (Table 2.3). In the absence of a pathogen, emergence was not significantly different between the CMC control and any of the BCA's (Fig. 2.2a). This was also the case in the presence of R. solani AG 1-IA (Fig. 2.2 b). In order to test the four bacterial strains as seed treatments, a control experiment was designed wherein seeds from the rice cultivar 'Diamond' were treated with the four BCAs to evaluate germination rates. In the control experiment, the results showed that non-BCA treated controls (CMC) had a germination rate of 75%, whereas seeds treated with each one of the bacterial strains had a variable germination rate that ranged from 78.0 to 82.0% but was not significantly different from the germination of the controls (Figure 2.3 a). When evaluating the effect of pretreating seeds with the bacterial strains on fungal infections, the results obtained were highly dependent on the fungus used. In *R. solani* AG 1-1A there were not significant differences between seeds non-treated with each one of the bacterial strains versus seeds treated with each one of the bacterial strains and the germination rates ranged from 71.0 to 82.0% (Figure 2.3 b). For *R. solani* AG-7, the seed germination was

similar to the germination with *R. solani* AG 1-1A. Unexpectedly, for *R. solani* AG-7, seed germination was affected by the bacterial strains and seeds pretreated with *P. protegens* PBL3 and *Burkholderia* spp PBL33 showed the lowest germination rates (Figure 2.3 d). Seed germination after inoculation with R. *solani* AG-4 was ~43% overall and pre-treatment with the bacterial strains caused a slight increase in germination rate across treatments, but only pretreatment with *Burkholderia cepacia* PBL18 and *P. fluorescens* PBL 24 showed significantly higher germination than CMC control, but still below 60% (Figure 2.3 c). Interestingly, germination rate for seeds inoculated with *F. graminearum* Fg 4 was 48%, but that germination rate increased from 59 to 66% when pre-treated with each one of the bacterial strains (Figure 2.3 e). When the effect of four BCAs was evaluated as a seed treatment against four fungal pathogens, variable results were observed (Table 2.5).

## 2.4 Discussion

Management practices to control rice diseases have relied on cultural practices and chemical control in the form of fungicide application. There are no cultivars completely resistant to diseases (Wamishe et al. 2007). However, the indiscriminate use of chemicals in agricultural systems has a negative effect on the environment and the health of humans and animals (Anith et al. 2021). In addition, the widespread use of fungicides can lead to fungicide resistance by pathogens, rendering these methods ineffective. The use of biological control agents (BCAs) represents an environmentally friendlier and more sustainable approach to control plant diseases in cases where the biological products work. However, since the implementation of these

methods in the field has been inconsistent and laborious, more research is needed to facilitate further implementation. This study assessed direct antagonism of four bacterial strains, two *Pseudomonas* spp. and two *Burkholderia spp.*, as potential BCAs against fungal pathogens in controlled environments and using a dual culture and seed plate assays against four important fungal pathogens that occur on rice: *R. solani* anastomosis groups (AGs) and *F. graminearum*.

A previous study using the dual culture method, evaluated the antagonistic effects of *P. fluorescens* isolates against *R. solani* and demonstrated that seed treatments with *P. fluorescens* were effective to control sheath blight (Kazempour 2004). In this study, *B. cepacia* and *P. fluorescens* were identified as the most effective in reducing fungal growth and promoting emergence in the presence of fungal pathogens tested. The dual culture plate assay demonstrated higher inhibition of fungal growth compared to the uninoculated control, and the effect was consistent for all the fungal isolates tested. *P. fluorescens* is known to have biocontrol activity against plant pathogens. Previous evaluation of *P. fluorescens* for its antagonistic effect against *Rhizoctonia solani* AG 1-IA in vitro showed *P. fluorescens* was effective in inhibiting mycelial growth of *R. solani* AG 1-IA (Nagarajkumar et al. 2005). Interestingly, *P. fluorescens* has also been reported to have a positive effect on seed germination and seedling vigor on young maize plantlets (Cardarelli et al. 2022). Hence not surprisingly, this study showed that P. *fluorescens* increases emergence even in the absence of a pathogen. In another study, *Burkholderia spp.* evaluated against fungal pathogen *Magnaporthe oryzae* showed that *Burkholderia* also strongly inhibited hyphal growth of *M. oryzae* by producing low molecular weight secondary metabolites with efficient antifungal activity (Xue et al. 2022). Previous studies has shown *Pseudomonas* spp. had the ability to colonize roots of crop plants and produce antifungal metabolites provide a strong and a sustainable alternative to the application of chemical fungicide (Walsh et al. 2001).

Prominent *Pseudomonas spp.* that produce antifungal or antimicrobial compounds and can potentially be used as BCAs in managing diseases include *P. protegens* (Zhang et al. 2020) and *P. fluorescens* (Walsh et al. 2001). *Burkholderia* spp. including *B. cepacia* also have garnered interest as BCAs due to secretion of diverse antimicrobial compounds such as siderophores and the promising effect in reducing incidence of rice blast and sheath caused by *Magnaporthe oryzae* and *R. solani* AG 1-IA at the field level (Yang et al. 2007). Previous studies have shown with *In vitro* analysis indicates that *Pseudomonas fluorescens* produces diffusible antifungal and volatile antifungal compounds including hydrogen cyanide (Choi et al. 2006). These volatile and diffusible compounds could play a role in inhibiting fungal growth. While we did not measure the production of volatile and diffusible compounds in the bacteria we used in this study, a previous study has provided evidence that *Pseudomonas protegens* has also been shown produce secondary antifungal metabolites such as siderophores in addition to been a plant growth-promoting (PGP) organism (Ramette et al. 2011).

In addition to the effect of the potential biocontrol agents on fungal growth inhibition, all four bacterial strains also promoted higher seed germination, although that effect did not appear to be as drastic as the in vitro assay (Table 2.2). There was no statistical difference observed among the four bacteria in promoting emergence in the presence of *R. solani* AG 1-IA, AG 4 and AG 7 and *F. graminearum* Fg 4. More work is needed to define specific conditions that would improve the effect of these strains in reducing the detrimental effects of these pathogens in rice.

It is not uncommon that laboratory-isolated BCAs that are consistently effective suppressing fungal growth in controlled environments, produce inconsistent results in the field (Walsh et al. 2001). It is therefore necessary not only to evaluate potential BCAs *in vitro* assays

but to include *in planta* assays at early-stage testing to incorporate different environmental conditions. BCAs fail to perform consistently under greenhouse and field conditions, but more field-compatible BCAs can be obtained at a "lower cost by focusing on an ecologically relevant isolation strategy" coupled with an "early stage *in planta* bioassays can have a high translational towards field conditions" (Nicolaisen et al. 2018a). BCAs that perform that perform well in controlled environments may not necessarily perform similarly in the field.

*Pseudomonas fluorescens* reduced *R. solani* AG 1-IA by 7 mm compared to the growth of *R. solani* AG 1-IA in the absence of the biocontrol agent. The fact that *Pseudomonas fluorescens* showed an increased reduction in the growth of these fungal pathogens is consistent with previous findings that *Pseudomonas* spp. are known to colonize roots of crop plants and produce siderophores and other antifungal metabolites to antagonize fungal phytopathogens by reducing mycelial growth, lysis or sclerotia or other mechanisms (Walsh et al. 2001). Although *Pseudomonas fluorescens* has shown to be the most effective in reducing the growth of fungal pathogens, reduction was not significantly different from *Burkholderia cepacia* (Figure 2.1 and Table 2.1). In a previous *in vivo* study, *Pseudomonas fluorescens* has been shown to exhibit strong disease control activity against rice sheath blight caused by *R. solani* AG 1-IA and rice blast caused by *Magnaporthe grisea* (Choi et al. 2006).

In the control where seeds treated with bacteria and/or CMC were incubated at room temperature, emergence rate in CMC was 75% and ranged from 78 to 82% for the four biocontrol agents, no significant difference was observed among biocontrol agents in the control (Figure 2.3 a). Similar to the control, emergence rate for all four biocontrol and CMC ranged from 78 to 82% in *R. solani* AG 1-IA (Figure 2.3 b). Although *R. solani* AG 1-IA is the causal agent of sheath blight of rice, it is not reported to be a seedling pathogen in rice. Seeds with at

least 80% germination are considered “good” seed (Gummert, G 2010). The biocontrol agents used in this study may not have negative impact on emergence. In *R. solani* AG-4, which is the most aggressive pathogen used in this study, emergence rate was 38% with CMC whereas emergence rates ranged from 43 to 53% for all four biocontrol agents evaluated (Figure 2.3 c). PBL 18 (*Pseudomonas fluorescens)* is the most effective biocontrol agent in reducing the impact of *R. solani* AG-4, but it is significantly different from the other biocontrol agents used (Figure 2.3 c). *R. solani* AG-4 was reported to be most aggressive as a seedling pathogen when compared with other AGs (Gaire et al. 2020b). Interestingly in *R. solani* AG-7, emergence rate of 85% in CMC was higher than emergence rates where biocontrol agents were used (Figure 2.3 d). The application of CMC may have promoted germination by providing a moist surrounding to the seed. Emergence rate for all four biocontrol agents ranged from 71 to 77% for *R. solani* 7 (Figure 2.3 d). In contrast, CMC produced lower emergence compared to the BCAs when the treated seeds were grown in the absence of the fungal pathogens *(*Figure 2.3 a), suggesting minor effects on seed quality. *R. solani AG* 7 was reported to be pathogenic on soybean (Yung-Cheng et al. 2021) and on cotton (Baird et al 1996). However, when *R. solani* AG-7 isolated from cotton was tested on rice in a controlled environment, the results show that this isolate is not pathogenic on rice when assessed in controlled environments. In *F. graminearum,* emergence rate was just below 50% CMC in the absence of the biocontrol agents, (Figure 2.3 e). However, emergence rates ranged from 59 to 66% (Figure 2.3 e). Contrary to the dual culture plate assay results, the *Burkholderia* spp. (PBL 33) was the most effective in reducing the impact of *F. graminearum* Fg 4 in seed plate assay but was not statistically different from the other three bacterial strains (Figure 2.3 d).

Overall, all four bacterial strains used in this study showed considerable reduction in the growth of the pathogens used. However, *Pseudomonas fluorescens* and *Burkholderia cepacia* consistently demonstrated significantly higher reduction in the growth of *Fusarium graminearum* and *Rhizoctonia solani* isolates in the dual culture plate assay. When the two strains of *Pseudomonas* and two strains of Burkholderia were challenged as seed treatment against fungal pathogens in seed plate assay, *P. pseudomonas* and *B. cepacia* generally showed improved germination although these two bacteria are not significantly different from the two bacteria in many cases. This study explored and determined the efficacy of various potential BCAs as a seed treatment against important fungal pathogens. This paved a way for future studies to explore for further testing *P. pseudomonas* and *B. cepacia* and the mechanisms of antagonism as these two species showed to be more effective in reducing the growth of fungal pathogens and the impact these pathogens have on seedling emergence. There was generally a positive correlation between the results of the dual culture and seed plate assays. Also, to curb the potential human and animal health issue that may rise from application of live bacterial cells, the use of cell-free molecules and compound of the bacteria used in this study is proposed.

Extensive *in vivo* and *in vitro* evaluation of these potential BCAs in different conditions will give us a great insight into the future of biocontrol as a more robust alternative to the application of chemical fungicide. Also, further studies are needed to investigate the mechanisms through which the biocontrol agents inhibit fungal growth.

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**Tables and Figures**

**Table 2.1.** Type III test of fixed effects of dual culture experiments conducted showing the response variable (average radius) and the significance of the effects tested at 48 hours post-inoculation.

| **Effect** | ***Num DF*[a]** | ***Den DF*[b]** | ***F Value*** | ***Pr > F*[c]** |
|---|---|---|---|---|
| Biocontrol agent (BCA) | 4 | 278 | 59.28 | <.0001 |
| Pathogen | 3 | 278 | 429.48 | <.0001 |
| BCA x Pathogen | 12 | 278 | 2.55 | 0.0033 |

[a] Num DF is the number of degrees of freedom in the model
[b] Den DF is the number of degrees of freedom associated with the model error
[c] Probability of greater F value, P values were considered significant with $\alpha$=0.05.

**Table 2.2.** Pathogen colony mean radius (mm) when challenged with biocontrol agents (BCAs) or control at 48 h post-inoculation. Means comparisons for BCA x Pathogen interaction in dual culture assay. Values followed by the same letter are not significantly different at $\alpha$=0.05.

| | **Pathogen mean radius (mm)** | | | |
|---|---|---|---|---|
| **Treatment** | ***R. solani* AG 1-IA** | ***R. solani* AG-4** | ***R. solani* AG-7** | ***F. graminearum* Fg 4** |
| **Control** | 28.87 A[a] | 26.99 A | 28.26 A | 13.33 K |
| **PBL 3** | 21.99 D-F | 19.33 F-H | 24.83 BC | 9.72 L |
| **PBL 18** | 21.28 E-G | 16.38 J | 21.30 E-G | 8.59 M |
| **PBL 24** | 19.51 GH | 16.86 IJ | 18.73 HI | 9.24 M |
| **PBL 33** | 23.10 C-E | 16.25 L | 24.01 B-D | 9.56 ML |

[a] Means across columns with the same letter are not significantly different based on Fisher's protected LSD ($\alpha$=0.05)

**Table 2.3.** Type III test of fixed effects of dual culture experiments conducted showing the response variable (average radius) and the significance of the effects tested at 96 hours post-inoculation.

| **Effect** | ***Num DF*** [a] | ***Den DF*** [b] | ***F Value*** | ***Pr > F*** [c] |
|---|---|---|---|---|
| Biocontrol agent (BCA) | 4 | 278 | 95.76 | <.0001 |
| Pathogen | 3 | 278 | 263.57 | <.0001 |
| BCA x Pathogen | 12 | 278 | 1.22 | 0.2719 |

[a] Num DF is the number of degrees of freedom in the model
[b] Den DF is the number of degrees of freedom associated with the model error
[c] Probability of greater F value, P values were considered significant with $\alpha$=0.05.

**Table 2.4.** Pathogen colony radius (mm) when challenged with biocontrol agents (BCAs) or control at 96 h post-inoculation. Means comparisons for BCA by Pathogen interaction in dual culture assay. Values followed by the same letter are not significantly different at $\alpha$=0.05.

| **Treatment** | **Pathogen mean radius (mm)** | | | |
|---|---|---|---|---|
| | ***R. solani*** **AG 1-IA** | ***R. solani*** **AG-4** | ***R. solani*** **AG-7** | ***F. graminearum*** **Fg 4** |
| Control | 32.47 A | 32.47.10 A | 32.47 A | 20.93 EF |
| PBL 3 | 28.12 AB | 26.50 B | 28.13 AB | 18.01 F |
| PBL 18 | 21.92 DC | 21.05 DE | 22.42 CD | 12.46 H |
| PBL 24 | 21.92 CD | 20.93 IJ | 21.45 HI | 12.98 H |
| PBL 33 | 27.46 B | 23.04 C | 26.49 B | 16.14 G |

[a] Means across columns with the same letter are not significantly different based on Fisher's protected LSD ($\alpha$=0.05).

**Table 2.5.** Type III Tests of Fixed Effects for the seed plate assay (SPA) between Biocontrol agent (BCA) and pathogen with regard to the response variable (emergence).

| ***Effect*** | ***Num DF*** [a] | ***Den DF*** [b] | ***F Value*** | ***Pr > F*** [c] |
|---|---|---|---|---|
| Biocontrol agent (BCA) | 4 | 340 | 0.73 | 0.5689 |
| Pathogen | 4 | 340 | 99.49 | <.0001 |
| BCA x Pathogen | 16 | 340 | 2.62 | 0.0007 |

[a] Num DF is the number of degrees of freedom in the model
[b] Den DF is the number of degrees of freedom associated with the model error
[c] Probability of greater F value, P values were considered significant with $\alpha$=0.05.

**Table 2.6.** Rice cv. 'Diamond' mean emergence at 7-days post inoculation when treated with biocontrol agent (BCA) or binding agent (CMC) and challenged with pathogen or control. Experiment was conducted as a seed plate assay and the interaction biocontrol agent x Pathogen is shown. Values followed by the same letter are not significantly different at $\alpha$=0.05 for comparisons of mean rice emergence among biocontrol agents across fungal pathogens and control treatment combinations.

| | **Mean emergence proportions** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | **Control** [a] | | ***R. solani*** **AG 1-IA** | | ***R. solani*** **AG-4** | | ***R. solani*** **AG-7** | | ***F. graminearum*** **Fg 4** | |
| **CMC** | 0.75 | B-D | 0.81 | AB | 0.38 | I | 0.85 | A | 0.48 | GH |
| **PBL 3** | 0.81 | BC | 0.82 | AB | 0.43 | HI | 0.71 | DC | 0.60 | EF |
| **PBL 18** | 0.82 | BC | 0.82 | BC | 0.53 | FG | 0.77 | A-C | 0.59 | EF |
| **PBL 24** | 0.78 | A-C | 0.78 | BC | 0.49 | GH | 0.77 | A-C | 0.65 | DE |
| **PBL 33** | 0.78 | A-C | 0.78 | BC | 0.47 | HI | 0.77 | BC | 0.66 | DE |

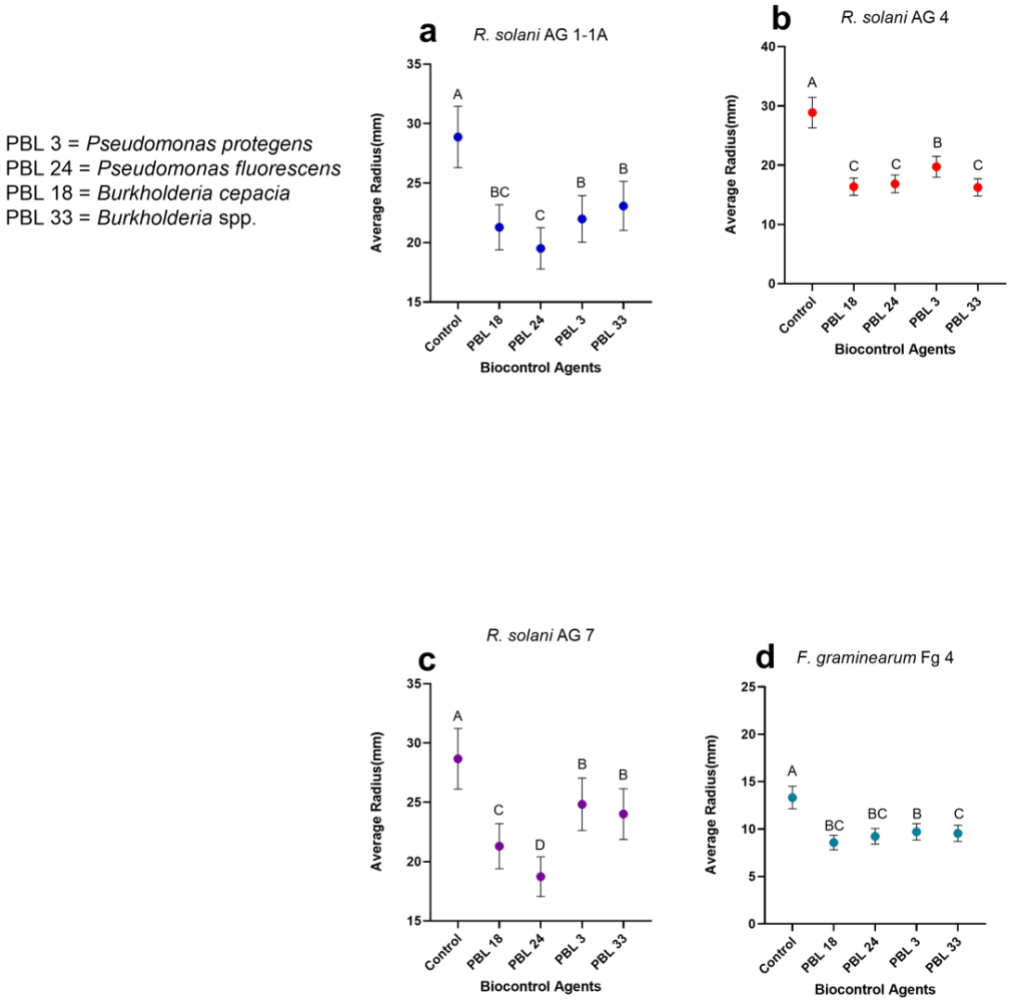


**Figure 2.1. Evaluating antagonistic effect of bacterial strains against fungal pathogens *R. solani* and *F. graminearum* in a dual culture assay measured at 48 hours post-inoculation:** Average radius in millimeters (mm) of three *R. solani* anastomosis groups (a) *R. solani* AG1-1A, (b) *R. solani* AG-4, (c) *R. solani* AG-7 and (d) *F. graminearum* strain Fg 4 challenged with four potential biological control agents (BCAs): PBL 3, PBL 18, PBL 24 and PBL 33. Two plugs of each fungal pathogen were placed equidistant from the bacterial streak of each strain on the same agar plate. Radius was measured 48 hours post-inoculation. Data presented here is the combination of three independent experiments. Data was analyzed by pathogen. The error bars represent standard errors of the mean radius. Letters above the error bars represents difference among biocontrol agents across pathogens. Values followed by the same letter are not significantly different at $\alpha$=0.05 for comparisons of mean radius among treatments based on Fisher's protected LSD.

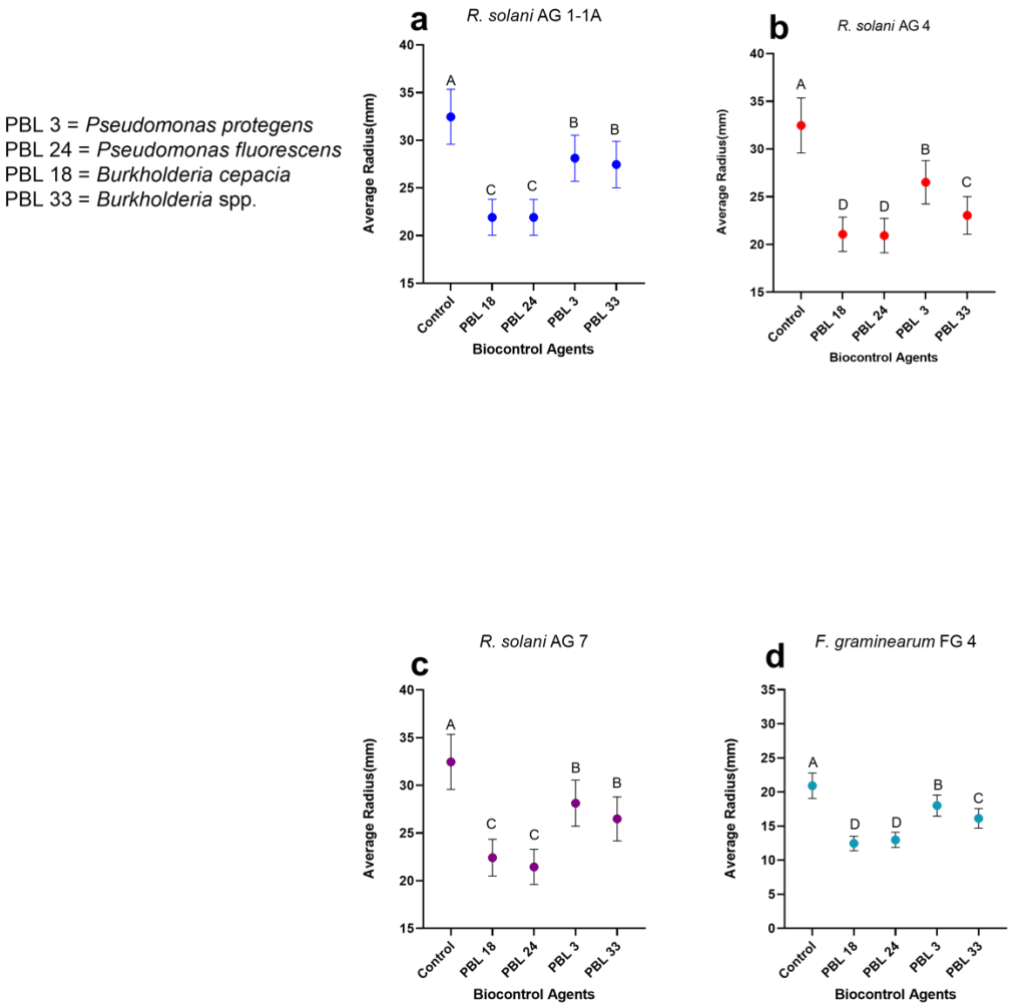


**Figure 2.2. Evaluating the antagonistic effect of bacterial strains against fungal *R. solani* and *F. graminearum* dual culture assay measured 96 hours post-inoculation** Average radius in millimeters (mm) of three *R. solani* anastomosis groups (a) *R. solani* AG 1-IA, (b) *R. solani* AG-4, (c) *R. solani* AG-7 and (d) F. *graminearum* Fg 4 challenged with four potential biological control agents (BCAs): PBL 3, PBL 18, PBL 24 and PBL 33. Data presented is a combination of three independent experiments. The error bars represent standard errors of the mean radius. Letters above the error bars represents difference among biocontrol agents across pathogens. Values followed by the same letter are not significantly different at $\alpha$=0.05 for comparisons of mean radius among treatments at 96 hours post-inoculation based on Fisher's protected LSD.

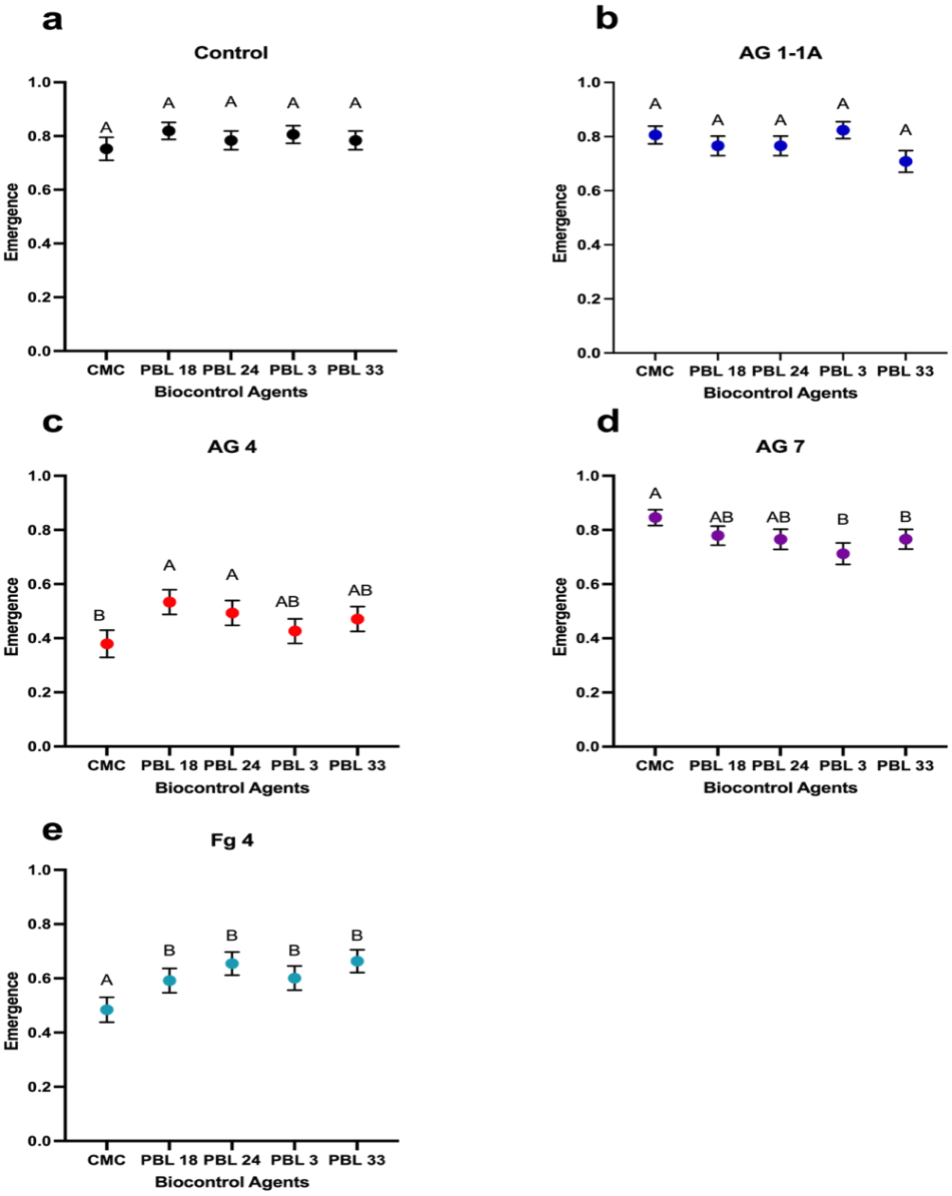


**Figure 2.3.** Proportion emergence of rice cv. 'Diamond' seven days post-inoculation in the presence of fungal pathogens and control (no pathogen). Seeds were treated with the four bacterial strains used as biocontrol agents and a control for the binding agent used (CMC – carboxyl methyl cellulose). The error bars represent standard errors of the mean emergence. Letters above the error bars represent differences. Values followed by the same letter are not significantly different at $\alpha$=0.05 for comparisons of mean rice emergence among biocontrol agents by fungal pathogen based on Fisher's protected LSD.

## Chapter III: Evaluation of Chemical Seed Treatments against *Rhizoctonia solani* and *Pythium spp.*


### Abstract

Rice diseases are managed primarily by chemical control in the form of fungicide application and effective cultural practices as there may not be completely resistant cultivars to all the major foliar and seedling diseases. Fungicide seed treatments often used to manage various rice seedling pathogens are not species-specific. Fungicide seed treatments used to manage seedling pathogens include but are not limited to Dynasty (azoxystrobin), Maxim 4 FS (Fludioxonil), Vibrance (sedaxane) and Apron XL (mefenoxam), or the combination of two or more of these chemical. In the current study, seed treatments with different fungicides were evaluated against *Rhizoctonia* and *Pythium* on a seed plate assay and greenhouse bioassay. In this work, eight different chemical seed treatments, containing a single active ingredients or blends of two or three active ingredients, were evaluated against *Rhizoctonia* and *Pythium* and using the rice cultivar 'Diamond.' The results showed that sedaxane and azoxystrobin in single or combined treatment was the most effective treatment to control *Pythium* species in seed plate assay, whereas fludioxonil, sedaxane and azoxystrobin or the combination of these chemistries were the most effective treatment to control *R. solani* in both seed plate and seedling cup assays.


### 3.1 Introduction

Seedling diseases cause significant yield losses in rice by reducing emergence, plant stand and by reducing quality of grains. Although an integrated pest management (IPM) approach is recommended and promoted in managing diseases, chemical control plays a major role as a control tactic deployed worldwide for different cropping systems (Hollier 2014). Application of synthetic fungicides has greatly contributed to sustaining quality food production

by protecting crops from diseases (Ishii 2006). The application of fungicides with different modes of action and the combination of chemistries with different modes or site of action is recommended in battling fungicide resistance development.

Typically, seed treatments for various crops include a mixture of active ingredients that combine fungicides with oomyceticide activity such as phenylamines as well fungal activity such as such as demethylation inhibitors, quinone outside inhibitors (QoIs) and succinate dehydrogenase (SDHIs) (Matthiesen and Robertson 2021). Fungicides belonging to different Resistance Action Committee group (FRAC) groups are used to control or manage the impact of diseases. FRAC assigns letters and numbers to different groups of pesticides based on the mode of action and chemical structures of the active ingredients (Hollier 2014) Active ingredients frequently used in seed treatments include metalaxyl, propiconazole, azoxystrobin, sedaxane and fludioxonil. The QoI fungicides (strobilurin fungicides), which belong to the Fungicide group 11 are used for control of a broad range of fungal and oomycete pathogens (Sierotzki and Scalliet 2013). QoIs, which inhibit mitochondrial respiration at the quinol oxidation site of the cytochrome bc1 enzyme complex, are the most important class of current fungicides (Ishii 2006). By inhibiting mitochondrial respiration, energy production for various biological functions of the fungal pathogen is disrupted. An analogue of strobilurin, azoxystrobin is one of the most widely used fungicides because of its high efficacy in various crops including rice and a broad spectrum of control against a large number of pathogens on various crops (Singh et al. 2019a).

Succinate Dehydrogenase Inhibitors (SDHIs FRAC group 7) are a broad spectrum fungicide used primarily as seed coating to control seed and seedling-borne fungal pathogens (Dal Cortivo et al. 2017). SDHI, in the fungicide sedaxane, also acts in the fungal mitochondrial respiration inhibiting the succinate dehydrogenase activity by binding to this enzyme in complex

II (Sierotzki and Scalliet 2013). Fludioxonil which belongs to the phenylpyrrole class is a contact fungicide that does not translocate into the plant system, but has a broad spectrum of control and it is registered for use on major food crops such as maize, sorghum, potato and rice (Duan et al. 2013). The precise mode of action of fludioxonil is not clear. However, a study of fludioxonil on morphological and physiological characteristics of *Sclerotina sclerotium* shows that fludioxonil seems to interfere with osmotic stress in the fungal cells (Duan et al. 2013).

The objective of this study was to investigate the effect of three synthetic fungicides/oomyceticide as seed treatments in controlling rice diseases. Azoxystrobin, fludioxonil and sedaxane alone and in combination, were evaluated in rice using seed plate assay seedling cup assay to collectively assess emergence, plant stand, total plant weight and root weight.

## 3.2 Materials and Methods

### *3.2.a. Isolate collection and storage*

The fungal pathogen *Rhizoctonia solani* AG 1-IA, AG 4 and AG 7 and the oomycetes *Pythium graminicola* and *Pythium irregulare* were isolated from rice production systems in Arkansas. The isolates were preserved in the collection of Dr. Craig Rothrock at the University of Arkansas. *Fusarium graminearum* isolate (Fg 4) was collected from wheat fields in Arkansas by David Moon in the Crop, Soil, and Environmental Sciences (CSES) Department at the University of Arkansas. All isolates were grown on Potato Dextrose Agar (PDA, Criterion, Santa Maria, CA). These isolates were stored at room temperature in 30% glycerol (Research Products International Corp, Mount Prospect, IL) in the ultra-freezer (-80 ˚C).

*3.2.b. Chemical seed treatment*

Eight different chemical seed treatments were evaluated against soil isolates of *Rhizoctonia solani* and *Pythium* species, using untreated seeds as controls. The seed treatments contained a base treatment (mefenoxam and thiamethoxam). To this base treatment were added Azoxystrobin + Sedaxane + Fludioxonil either as a single active ingredient or as a blend of two or three of these active ingredients with respective concentrations as shown in Table 3.1. Seed treatments were provided by Syngenta (Syngenta Crop Protection, Greensboro, NC). For proprietary purposes, the treatment of the seeds was not provided.

*3.2.c. Seed plate assay using chemically treated seeds*

Eight chemical seed treatments listed above and an untreated control on rice cultivar 'Diamond' were evaluated against the three *Rhizoctonia solani* isolates (AG 1-IA, AG 4 and AG 7), *Pythium graminicola* and *Pythium irregulare*. Isolates of *R. solani* AG 1-IA, AG 4, AG 7, *Pythium graminicola* and *Pythium irregulare* were grown on solid PDA media for 5-7 days at room temperature. Once the fungus or oomycete covered the surface of the agar, eight grams of sterile high-grade vermiculite (Palmetto Vermiculite Co., SC) was evenly spread on top of the fully colonized PDA media on each plate. Solid PDA without fungi was used in all eight treatments as untreated control. For every treatment, fifteen treated seeds were place per plate on top of the thin layer of sterile vermiculite. Five replicates were done for every treatment combination per pathogen or control and the experiment was repeated three times. The plates were complete wrapped in aluminum foil and incubated at room temperature for 7 days. Emergence was recorded at 7 days post-inoculation. Germination was assessed using the Association of Official Seed Analyst (AOSA) germination protocol in which a seedling is

considered healthy when 50 percent of its cotyledonary tissue remains attached or free of decay. Percent germination was determined using the emergence data recorded after one week of incubation at room temperature.

The seed plate assay was conducted using randomized block design (RCD) with 2-factorial with seed treatment and fungal pathogens as the factors, with each pathogen tested separately. Seed plate assay data was analyzed using PROC GLIMMIX procedure as described below.

*3.2.d. Seedling cup assay using chemically treated seeds*

3.2.d.1 Inoculum preparation

For a greenhouse seedling cup assay, one hundred grams of prosso millet were soaked overnight in a 100 mL of deionized water on a 500 mL Erlenmeyer flask. The soaked millet was autoclaved at 121ºC and 15 psi for 40 minutes. After cooling down, the millet was autoclaved again the following day under the same conditions. About 5-7 small plugs (5 mm in diameter) of the isolates of interest grown on solid PDA were transferred into different 500 mL Erlenmeyer flasks containing the sterile millet, sealed with aluminum foil, and kept at room temperature for 7 – 10 days. The flasks containing the inoculated millet were periodically shaken to ensure even colonization of the millet. Fully colonized millet was checked for contamination from environmental or opportunistic fungi or bacteria by plating a gram of the colonized millet onto solid PDA and plates were incubated at room temperature for 5-7 days. Contaminated inoculum was discarded. The inoculum was stored in sterile brown paper bags in the refrigerator to be used for the seedling assays.

*3.2.d.2. Seedling assay in the greenhouse using chemically treated seeds*

By assessing these four *R. solani* and two *Pythium* spp. using seed plate assay, *R. solani* AG 4 and AG 9 were selected for further greenhouse screening based on pathogenicity and aggressiveness of the pathogens. In order to assess the efficacy of seed treatment against *Rhizoctonia solani* and *Pythium* spp., a seedling cup assay was conducted in the greenhouse using the prepared inoculum. Sixteen-ounce (16 oz) foam cups (Genuine Joe) perforated with 4 holes, about 10 mm in diameter at the base for draining were used in this experiment. Sterile vermiculite was mixed with Promix MP (Pro-mix, Quakertown, PA) in equal ratios. Eight grams of the prepared inoculum were thoroughly mixed with 100 g of the soil medium and used to fill the perforated foam. Ten filled cups were placed on plastic greenhouse trays (1020 Trays, Greenhouse Megastore, Danville, IL). Next, five Diamond rice seeds per cup of each chemical treatment and control were sown about 5 mm below the soil medium containing the inoculum or sterile millet in the control and watered regularly through the trays. A Watchdog data logger (Spectrum Technologies Inc.) was used to record temperature and relative humidity every 30 minutes in the greenhouse. Germination was recorded at 7 dpi. For the experiments, seedling emergence was recorded at seven days post planting, and plants were harvested four weeks post-inoculation and planting. At harvesting, plant stand (NPlants) was recorded as the number of plants per cup. Roots were thoroughly washed to remove residual soil. Excess water was drained from the roots using sterile paper towels. Whole plants were weighed followed by weighing the roots.

### *3.2.f. Statistical analysis*

*Seed plate assay.* Seed plate assay experiments were carried using randomized block design (RBD). The seed plate assay data were analyzed using the SAS GLIMMIX procedure

with Kenward-Roger degrees of freedom with treatment and pathogen as experimental factors and emergence as response variable. Correlation between experiments was evaluated to determine if experiments could be combined for analysis. Analysis of variance was done using combined data of three independent experiments with a total of 1080 observations. Germination was the response variable with binomial distribution, with experiments as blocks using the logit function.

*Seedling cup assay.* The seedling plate assay data analysis of *R. solani* AG 4 and AG 9 was done using the GLIMMIX procedure with Kenward-Roger degrees of freedom of SAS 9.4 software. Correlation between experiments was evaluated to determine if experiments could be combined for analysis. A combine dataset of three independent experiments with 540 observations was used for the data analysis using gamma distribution for the response variables. Number of plants, total plant weight, total root weight, average plant weight and average root weight were the response variables. All these factors were analyzed with the same distributions.

## 3.3 Results

### *3.3.a. Chemical Seed Treatment – seed plate assay*

In the seed plate assay, efficacy of the chemical seed treatments against the *R. solani* isolates was determined by emergence rate recorded seven days post inoculation. Results of three independent experiments were combined for analysis. The mean seed emergence for all eight treatments and the untreated control without pathogen inoculation ranged from 87.6 to 92.2%. Fludioxonil and Sedaxane (FS) and Fludioxonil (F) showed the highest emergence, but it was not significantly different from the other treatments (Figure 3.1 a). There was a significant pathogen by treatment interaction in the seed plate assay data when analyzed (Table 3.2). In the presence

of *Pythium irregulare,* emergence rate ranged from 88.3 to 93.8% for all treatments including the untreated control. A blend of Azoxystrobin + Sedaxane (AS) showed the highest emergence and the untreated control showing the lowest emergence. Mefenoxam + Thiamethoxam (MT), Azoxystrobin + Fludioxonil and Azoxystrobin + Fludioxonil + Sedaxane (AFS) did not show statistically significance difference (Figure 3.1 b). Inoculation with P. *graminicola* caused an emergence rate of 67.1% in the untreated control, but the emergence rate increased from 81.3 to 87.1% with all fungicide treatments (Figure 3.1 c). The highest emergence was observed when a blend of Azoxystrobin + Fludioxonil + Sedaxane (AFS) was used, whereas the lowest emergence was observed with Fludioxonil (F), but those differences were not significant in comparison with the other treatments (Figure 3.1 c). *R. solani* AG 1-IA did not have any effect on seed emergence, and the emergence rate in the untreated control and all the treatments ranged from 84.5 to 87.6% with no statistical difference among them (Figure 3.1 d). In contrast, inoculation with *R. solani* AG 4 caused an emergence rate of 16.0% in the untreated control, but treatments increased emergence rate from 21.7 to 84.5% for all treatments (Figure 3.1 e). Although Azoxystrobin (A) significantly improved emergence compared to the untreated control. However, emergence was significantly in Azoxystrobin (A) compared to the Fludioxonil (F), Sedaxane (S) or the blend of these active ingredients ((Figure 3.1 e). The emergence rate was 81.4% in the untreated control for *R. solani* AG 7 and ranged from 83.6 to 88.9% for all eight treatments (Figure 3.1 f). A blend of Azoxystrobin + Fludioxonil (AF) had the highest emergence rate, whereas Azoxystrobin(A) showed the lowest emergence rate (Figure 3.1 f). In *R. solani* AG 9, emergence rate was 36.9% for the untreated control, but ranged from 53.8 to 94.42% for all eight treatments (Figure 3.1 g). Mefenoxam (M) showed the lowest emergence and Sedaxane + Fludioxonil (SF) and Azoxystrobin + Fludioxonil (AF) had the highest

emergence but these two treatments were not significantly different from treatments Sedaxane (S), Azoxystrobin + Sedaxane (AS) or Azoxystrobin + Fludioxonil + Sedaxane (AFS), which all of them contained Sedaxane mixed with other active ingredients (Figure 3.1 g). For all the pathogens evaluated, *R. solani* AG 4 and AG 9 had the strongest negative effect on seed, for that reason the following experiments were only conducted with those strains.

*3.3.b. Seedling cup assay – Emergence*

In the uninoculated control, mean emergence rate ranged from 84.69 to 93.4% for all 8 treatments and untreated control (Figure 3.2 a). Also, emergence was highest in treatment 6 (Sedaxane + Fludioxonil) and lowest in Fludioxonil (F) in the uninoculated control (Figure 3.2 a). The results of three independent experiments analyzed show that there was a significant pathogen by chemical treatment interaction (Table 3.3). For *R. solani* AG 4, the emergence rate recorded was 6.8% in the untreated control (Figure 3.2 b). Emergence rate was 9.5% in Mefenoxam + Thiamethoxam (MT) but ranged from 33.4 to 86.7% for treatments containing Azoxystrobin (A), Fludioxonil (F), Sedaxane (S) or the blend of these active ingredients in *R. solani* AG 4 with Azoxystrobin + Sedaxane (AS) having the highest emergence (Figure 3.2 b). In *R. solani* AG 9, emergence rate was 77.4% in the uninoculated control, but emergence rates ranged from 77.4 to 96.0% for all eight treatments (Figure 3.2 c). The highest emergence rate was observed in treatments containing Azoxystrobin (A) or Sedaxane + Fludioxonil, whereas the lowest emergence among the treatments was observed in Mefenoxam (M) (Figure 3.2 c).

*3.3.c. Seedling cup assay – average plant stand*

Plant stand (NPlants) was recorded four weeks post-inoculation. Data recorded show that rates of plant stand ranged from 79.3. to 89.1% in the uninoculated control for all eight treatments and the untreated control (Figure 3.3 a). The highest rate of plant stand was observed in Azoxystrobin + Fludioxonil (AF) and the lowest rate was observed with Mefenoxam (M), but there was no significant difference among treatments and the untreated control in the uninoculated control (Figure 3.3 a). Statistical analysis of combined results of three independent experiments shows that there was significant pathogen by treatment interaction (Table 3.3). When inoculated with *R. solani* AG 4, rate of plant stand was 5.7% in the untreated control, 9.3% in Mefenoxam (M), but ranged from 40.0 to 85.7% for treatments containing Azoxystrobin (A), Fludioxonil (F), Sedaxane (S) or treatments that combine these active ingredients (Figure 3.3 b). Azoxystrobin (A) + Sedaxane (S) had the highest rate of plant stand and Mefenoxam (M) showed the lowest plant stand, followed by Fludioxonil (F) (Figure 3.3 b). In *R. solani* AG 9, rate of plant stand in the untreated control was 66.2% and plant stand ranged from 60.47 to 91.1% among the eight treatments (Figure 3.3 c). In the presence of *R. solani* AG 9, plant stand was highest in Azoxystrobin + Fludioxonil (AF), but statistically different from Azoxystrobin (A), Sedaxane (S) or Azoxystrobin + Sedaxane (AS) (Figure 3.3 c). In the presence of the same isolate, plant stand was lowest in Mefenoxam (M), but it was statistically different from Fludioxonil(F) and the untreated control (Figure 3.3 c).

*3.3.d. Seedling cup assay – average plant weight and average root weight*

Plants were harvested four weeks post-inoculation. Average plant weight and root weight were recorded in grams. For average plant weight, in the uninoculated control, the mean plant weight ranged was 0.6 in the untreated control, but on the seed treatments average plant weight

ranged from 0.6 to 0.8 grams with no statistical difference among the eight treatments and the untreated control (Figure 3.4 a). Results indicate there was significant pathogen by treatment interaction (Table 3.3). When challenged with *R. solani* AG 4, the average plant weight in the untreated control was 0.02 g (Figure 3.4 b), but average plant weight for the seed treatments ranged from 0.03 to 0.3 grams for all eight treatments in the presence of *R. solani* AG 4 with Azoxystrobin + Sedaxane (AS) having the highest average plant weight and Mefenoxam (M) showing the lowest average plant weight (Figure 3.4 b). In *R. solani* AG 9, the average plant weight was 0.63 grams in the untreated control and ranged from 0.5 to. 0.9 grams for all eight treatments with significant difference Azoxystrobin (A), Fludioxonil (F), Sedaxane (S) or the blends of these active ingredients (Figure 3.4 c).

For root weight, the uninoculated control for all eight treatments and the untreated control ranged 0.3 to 0.4 grams with no statistical difference among the treatments and the untreated control (Figure 3.5 a). Likewise, the statistical analysis indicates there was significant pathogen by treatment interaction (Table 3.3). In the presence *R. solani* AG 4, the average root weight was 0.01 grams in the untreated control, but the average root weight ranged from 0.01 to 0.2 gram for all eight treatments (Figure 3.5 b). The lowest average root weight was recorded in Mefenoxam (M) for *R. solani,* and the highest average root weight was recorded with Azoxystrobin + Sedaxane (AS) (Figure 3.5 b). In the presence of *R. solani* 9, the average root weight in the untreated control was 0.3 grams and ranged from 0.3 to 0.4 gram for all eight treatments (Figure 3.5 c). The highest average root weight among the treatments was recorded with Azoxystrobin + Sedaxane (AS) but this was not statistically different from Fludioxonil (F), Sedaxane (S), Azoxystrobin (A) or the blend of these active ingredients (Figure 3.5 c). The lowest average root weight among the treatments was recorded in treatment Mefenoxam (M), but

this was not significantly different from the untreated control and Fludioxonil (F), Azoxystrobin (A) or the blend of these active ingredients (Figure 3.5 c).

### 3.4. Discussion

With no completely resistant rice cultivars available and limited availability of reliable biological products currently on the market, disease control in rice relies heavily on application of chemical fungicides (Gnanamanickam et al. 2002). Commercial fungicides containing metalaxyl (mefenoxam), fludioxonil, sedaxane, azoxystrobin, triticonazole and trifloxystrobin and combinations of these active ingredients are used generally to manage seedling pathogens in rice. Although there is a risk of fungicide resistance development in addition to the environmental concerns, chemical fungicides nonetheless are reliable in controlling or managing disease with longer lasting effect.

In this study, different chemical seed treatments shown in Table 3.1 were evaluated against four *Rhizoctonia solani (*AG 1-IA, AG 4, AG 7, and AG 9) and two *Pythium (P. graminicola* and *P. irregulare*) using seed plate assay using 'Diamond' rice cultivar in laboratory settings by assessing emergence. *R. solani* anastomosis groups (AGs) known or reported to be seed pathogens in rice include but not limited to AG 4 (Gaire 2021) and AG 9 (Wamishe et al. 2019). Although *R. solani* AG 1-IA is the causal agent of sheath blight in rice, it is not reported to be a seed or seedling pathogen in rice. *Rhizoctonia solani* AG 7 has been recovered and reported on cotton (Abd-Elsalam et al. 2010), soybean and corn (Ajayi-Oyetunde and Bradley 2017), and rice seedlings in Arkansas (Rothrock et al. 1993). *Pythium* spp. known to infect young plant tissues and cause pre- and post-emergence in rice and other crops include but not limited to *P. irregulare* which produces globose hyphal swellings to colonize emerging seedling

(Salmaninezhad and Mostowfizadeh-Ghalamfarsa 2019b) and *P. graminicola* which invades the rice rhizodermis via penetration hyphae (Van Buyten and Höfte 2013).

To follow up the evaluation, chemical seed treatments were evaluated against *R. solani* AG 4 and AG 9 using seedlings of 'Diamond' rice cultivar in a greenhouse setting. To achieve this goal, we assessed seedling emergence seven days post-inoculation and planting, and plants were harvested four weeks post-inoculation to evaluate plant stand, total plant weight and root weight. When emergence was assessed in the seed plate assay in the absence of any pathogen for the different chemical treatments, emergence rates ranged from 88.03 to 92.2% on potato dextrose agar (PDA) control, averaging just over 90% for all eight treatments and the in the untreated control (Figure 3.1 a). Emergence rate over 80% is considered "good" although commercial rice seeds have emergence rate higher than 90% (Gummert, G 2010). Fungicide from different classes have been reported to be toxic to plants at different growth stages (Petit et al. 2012). However, emergence rate of 88 to 93.8% demonstrates the chemical treatments used may not be toxic to rice seeds. When the eight chemical seed treatments and the untreated 'Diamond' rice seeds were challenged against *P. irregulare,* emergence rate in the untreated control was 81.3% but emergence rates ranged from 87.1 to 93.8 % for all eight treatments (Figure 3.1 b). Highest emergence rate was observed with Azoxystrobin + Sedaxane (AS) but this was not statistically different from treatments containing Azoxystrobin (A), Fludioxonil (F), Sedaxane (S) or the blend of these active ingredients in reducing the impact of *P. irregulare* (Figure 3.1 b). Mefenoxam is one of the recommended active ingredients to control oomycetes, however resistance is always an issue to be considered (Noel et al. 2020), while other chemistries could also have an effect against *Pythium*. Emergence rate was 67.1% in untreated control in the presence of *P. graminicola,* but emergence rates ranged from 81.4 to 87.1% with statistical

difference observed among treatments (Figure 3.1 c). In most cases, mefenoxam was sufficient to control *Pythium* seedling rot without affecting germination and final plant stand.

When challenged with *R. solani* AG 1-IA, emergence rate from the analyzed results of three independent experiments was 82.7% in the untreated control and ranged from 83.6 to 87.6% among the eight treatments, but there was no significant difference among the treatments and the untreated control (Figure 3.1 d). Even though *R. solani* AG 1-IA is the causal agent of sheath blight, a devastating disease in rice, this isolate is not reported to cause seedling damage. Hence, it is not surprising to observe high emergence rates with no statistical difference in the emergence rage between the treated and untreated rice seeds when these were challenged with *R. solani* AG 1-IA. However, when the same treatments and untreated control were challenged with *R. solani* AG 4, emergence rates in the untreated control and with Mefenoxam + Thiamethoxam (MT) were 16 %, 21.7% and 49.8% respectively (Figure 3.1 e). All eight treatments contain mefenoxam and thiamethoxam (base treatments), which do not target fungi, hence it is expected not to control *Rhizoctonia solani*. Emergence rates ranged from to 80.9 to 84.5% with Azoxystrobin + Sedaxane + Fludioxonil (ASF) with significant difference observed among these treatments (Figure 3.1 e). Mefenoxam (M). is primarily used manage or control seedling diseases caused by *Pythium* and *Phytophthora* spp. (Syngenta, Greensboro, NC). Hence, the low emergence rate of 21.7% with mefenoxam in the presence of *R. solani* AG 4 was not unexpected. The third treatment contains a blend of the active ingredients mefenoxam and fludioxonil. Fludioxonil is a contact fungicide that penetrates the surface and accumulates around the seed to provide long-lasting protection to young seedlings against a wide variety of fungal seed and seedling pathogens including *Rhizoctonia, Fusarium* and *Septoria* spp. (Ko et al. 2015). Hence, when fludioxonil was introduced (treatment 3), marked improvement in emergence rate

was observed when challenged with R. *solani* AG 4, improving emergence from 15.99% in the untreated control to 80.92% with fludioxonil. Azoxystrobin (treatment 4) some protection against *R. solani* AG 4. Azoxystrobin is a broad-spectrum fungicide used to control different species of fungal pathogens including *R. solani* (da Silva et al. 2017). Azoxystrobin provided significantly lower protection, with an emergence rate of just under 50% against the aggressive *R. solani* AG 4 isolate used (Figure 3.1 e). Although azoxystrobin is a broad-spectrum fungicide, it has a high risk for the development of fungicide resistance as fungicides in the FRAC group 11 are classified at-risk due to a single action site (Song et al. 2022). Therefore, it is necessary to carry resistance test to monitor resistance and establish a baseline for the sensitivity of pathogens to fungicides in this FRAC group. Treatment 5 which contains sedaxane provide high protection against *R. solani* AG 4 with an emergence rate of 81.81% (Figure 3.1 e). Treatments 6 through 9 are double and triple combinations of fludioxonil, azoxystrobin and sedaxane with the base treatment (mefenoxam + thiamethoxam). The double and triple combinations of these active ingredients provided high protection against *R. solani* AG 4 with average emergence rates ranging from 83.15 to 84.48%. However, the double and combinations of fludioxonil, azoxystrobin and sedaxane did not provide significantly different protection *R. solani* AG 4 compared to single use of fludioxonil or sedaxane (Figure 3.1 e). *Rhizoctonia solani* AG 7, which has been reported on maize, soybean and cotton average emergence rate was 81.37% (Figure 3.1 f), which is not statistically different from a majority of the treatments used in this study. The average emergence rates among the eight treatments ranged from 83.59 to 92.91% with no statistical difference among most treatments (Figure 3.1f). The same chemical treatments were evacuated against *R. solani* AG 9. There is limited literature on the impact of *R. solani* AG 9 on rice, but a field survey chemical seed treatment using two rice varieties; 'Diamond' and

'RT Gemini 214 CL' showed that *R. solani* AG 9 had significant reduction in seedling count, seedling height and yield (Wamishe et al. 2019). When challenged with *R. solani* AG 9, the average emergence rate was 36.9 % untreated control (Figure 3.1 g), showing *R. solani* AG 9 is an aggressive seedling pathogen of rice. Expectedly, Mefenoxam + Thiamethoxam (MT) did not have high protection *R. solani* AG 9, where the average emergence rate was 53.8% (Figure 3.1 g). However, the single application of fludioxonil and sedaxane as well as double and triple combinations of Fludioxonil (F), Sedaxane (S) and Azoxystrobin (A) provided an improved protection against *R. solani* AG 9 with average emergence rates ranging from 85.4 to 94.2% (Figure 3.1 g).

Following the analysis of data from the seed plate assay from three independent experiments, *R. solani* AG 4 and AG 9 were selected for further evaluation using seedling cup assay in the greenhouse. When average emergence was assessed seven days post-planting in the uninoculated control, highest emergence was recorded with Fludioxonil (F) + Sedaxane (S), and emergence in this treatment was not significantly different from emergence in the other treatments including the untreated control (Figure 3.2 a). In the same uninoculated control, average emergence rates ranged from 84.7 to 93.4% for all eight treatments and untreated control. However, when the eight seed treatments and the untreated control were challenged with *R. solani* AG 4, mean emergence rate was 6.8% in the untreated control, and 9.5% with Mefenoxam + Thiamethoxam (MT) (Figure 3.2 b). The drastic reduction in emergence in the untreated control signifies the aggressiveness of *R. solani* AG 4 as a seedling pathogen of rice. Mean emergence rate with Mefenoxam + Thiamethoxam (MT was not significantly different from emergence rate in the untreated control. Mefenoxam is primarily used for control or management of oomycete pathogen and thiamethoxam is deployed to manage or control early-

season insect. Therefore, Mefenoxam + Thiamethoxam (MT) did not show any impact against *R. solani* AG 4. When single application of Fludioxonil, Azoxystrobin and Sedaxane were introduced, mean emergence rates raised to 33.4, 45.4 and 42.7% respectively, but there was no significant difference among these treatments (Figure. 3.2 b). Highest mean emergence was recorded with Azoxystrobin + Sedaxane (AS), but it was statistically different from Azoxystrobin + Fludioxonil + Sedaxane (AFS) (Figure. 3.2 b). When the same treatments were challenged against *R. solani* AG 9, average emergence rate was 77.4% in the untreated control, but this was not statistically different Mefenoxam + Thiamethoxam (MT) or a single application of Fludioxonil (F) (Figure 3.2 c), however, this could be a result of reduced virulence in *R. solani* AG 9. Single application of Azoxystrobin(A) yielded the emergence rate of. 96 %, suggesting that Azoxystrobin (A) performs better in the presence of a less aggressive pathogens. Although, single application of Azoxystrobin (A) yielded the highest mean emergence rate, this was not statistically different from mean emergence of single application of Sedaxane or two-way application of Sedaxane + Fludioxonil (SF), Azoxystrobin + Fludioxonil (AF) or three-way application of Azoxystrobin + Fludioxonil + Sedaxane (AFS) (Figure 3.2 c).

The final plant stand data shows that Azoxystrobin + Fludioxonil (AF) resulted in the highest in the highest plant stand with Mefenoxam + Thiamethoxam (MT) yielded the lowest plant stand in the inoculated control (Figure 3.3 a). In the absence of any pathogen, plant stand was not significantly different among the eight treatments and untreated control, with average plant stand ranging from 79.3 to 89.1% (Figure 3.3 a). In contrast, when *R. solani* AG 4 was introduced, plant stand was reduced to 5.7% and 9.3% in the untreated control and in Mefenoxam + Thiamethoxam (MT) respectively (Figure 3.3 a). In the presence *R. solani* AG 4, mean plant stand was 47.9, 49.3 and 50.7% with Fludioxonil (F), Sedaxane (S) and Azoxystrobin

(A) alone respectively (Figure 3.3 b). The combination of Azoxystrobin + Sedaxane (AS) resulted in the highest control against *R. solani* AG 4, but this was not statistically different from combination of Azoxystrobin + Fludioxonil (AF) or Fludioxonil + Sedaxane (FS) or the combination of Azoxystrobin + Fludioxonil + Sedaxane (AFS) (Figure 3.3 b). In the presence of the AG 9, average plant stand was 66.2% in the untreated control, which was not statistically different from single application of Fludioxonil (F) or three-way application of Azoxystrobin + Fludioxonil + Sedaxane (AFS) (Figure 3.3 c). Two-way application of Azoxystrobin + Fludioxonil (AFS) gave the best control against *R. solani* AG 9 but the results are not statistically different from two-applications of Azoxystrobin + Sedaxane (AS) or Sedaxane and Fludioxonil (SF) or a three-way application of Azoxystrobin + Fludioxonil and Sedaxane (Figure 3.3 c). Although pathogenic, *R. solani* AG 9 was not as aggressive as AG 4 in reducing emergence or plant stand.

Furthermore, in this study, mean plant weight in grams was determined to examine the extent to which *R. solani* AG 4 and AG 9 impact rice plant growth. The average weight of the plants that were grown in the absence of any pathogen was 0.6 g in the untreated control and range from 0.6 to 0.8 g among the eight treatments (Figure 3.4 a). The two-way application of azoxystrobin and fludioxonil yielded the highest average plant weight, but the average plant weight in this treatment was statistically different from single use of azoxystrobin, two-way application of azoxystrobin and sedaxane or fludioxonil and sedaxane or a three-way application of azoxystrobin, fludioxonil and sedaxane (Figure 3.4 a). When *R. solani* AG 4 was introduced, mean plant weight, which is dependent on the number and size of plants was 0.05 grams in the untreated control (Figure 3.4 b). The introduction of azoxystrobin, fludioxonil and sedaxane increased average plant weight to 0.2, 0.2 and 0.1 g respectively (Figure 3.4 b). Despite the

increase in mean plant weight as a result of the chemical seed treatments, the mean plant weight in presence of *R. solani* was lower compared to the plant weight in the uninoculated control. The mean plant weight in the untreated control in the presence of *R. solani* AG 9 was 0.5 g (Figure 3.4 c). The mean plant weight with single application of azoxystrobin, fludioxonil and sedaxane or the combinations of these active ingredients ranged from 0.6 to 0.8 g in the presence of *R. solani* AG 9 (Figure 3.4c). The mean plant weight among the treatments was similar to the mean plant weight in the uninoculated control.

The results from this study have shown that single application of azoxystrobin, fludioxonil and sedaxane and two or three-way combinations of these active ingredients had significant control *R. solani* and *Pythium* spp. by improving emergence in seed plate assay. When the same *R. solani* AG 4 and AG 9 were challenged against these active ingredients in controlled, single application of sedaxane and fludioxonil had significant control similar to the two or three-way combinations of these active ingredients against both *R. solani* isolates by improving emergence, plant stand, total plant weight and root weight. Single application of azoxystrobin was less effective in improving emergence, plant stand, total plant weight and root weight compared to single application of fludioxonil or sedaxane. Further research needs to be done to evaluate efficacy of seed treatments with azoxystrobin, fludioxonil and sedaxane at the field level with variables factors and different cultivars.

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## Tables and Figures

**UC** = untreated control
**M** = Mefenoxam
**T** = Thiamethoxam
**F** = Fludioxonil
**A** = Azoxystrobin
**S** = Sedaxane

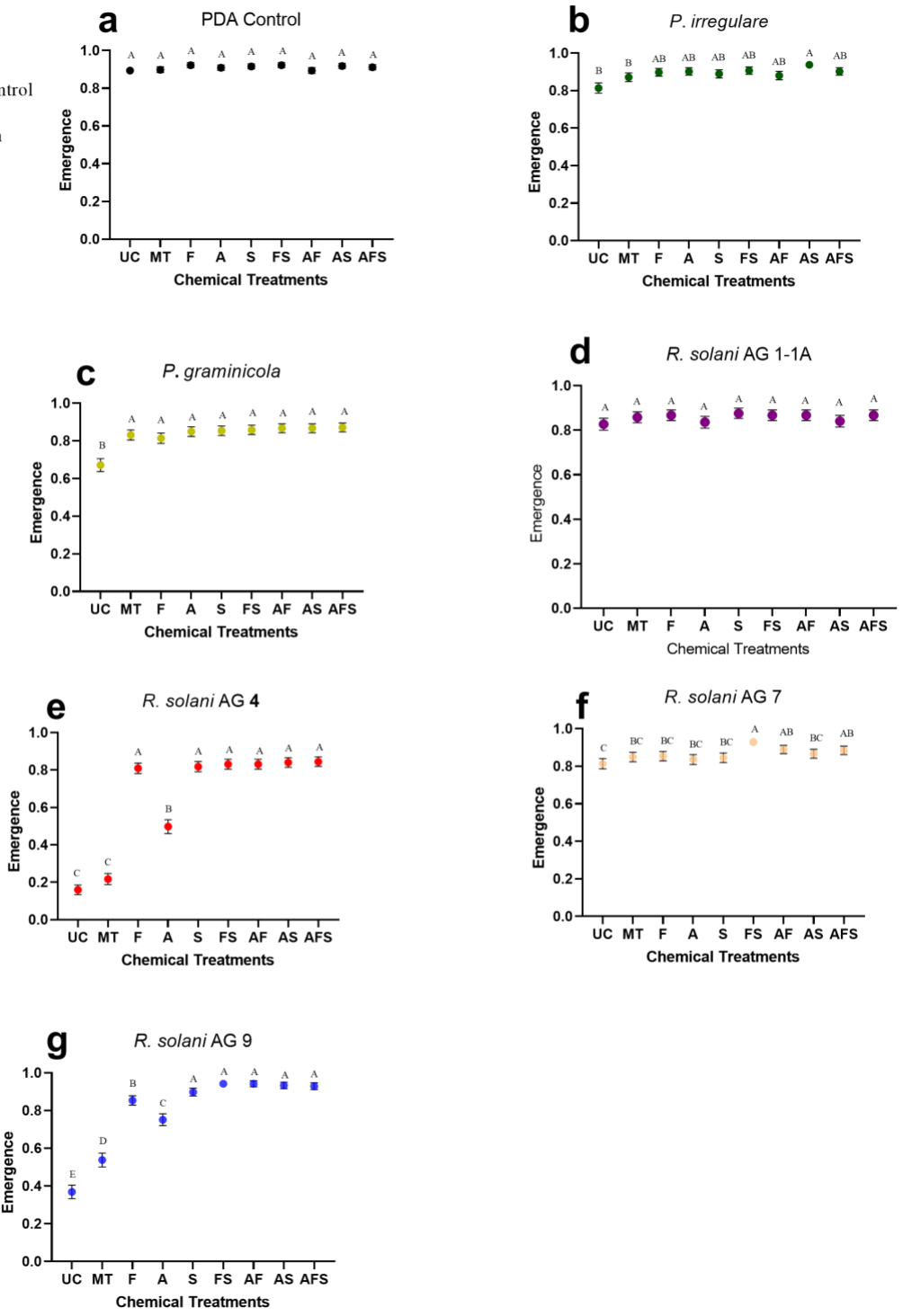


**Figure 3.1. Evaluating efficacy of chemical seed treatment against fungal pathogens; *R. solani* and *P. graminicola* and *P. irregulare* in a seed plate culture assay taken at 7 days**

**post-inoculation.** Average germination proportion of uninoculated control (a), *P. graminicola* (b), *P. irregulare* (c*) R. solani* anastomosis groups AG1-1A (d), AG 4 (e), AG 7 (f) and AG 9 (g) were challenged with 8 chemical seed treatments and a non-treated control in a seedling cup assay using 'Diamond' rice seeds at room temperature. Emergence was recorded seven days post planting. Combined data of three independent experiments were combined for analysis. The error bars represent standard errors of the mean emergence. Letters above the error bars represents difference among seed treatments across pathogens. Values followed by the same letter are not significantly different at $\alpha$=0.05 for comparisons of mean radius among treatments based on Fisher's protected LSD.

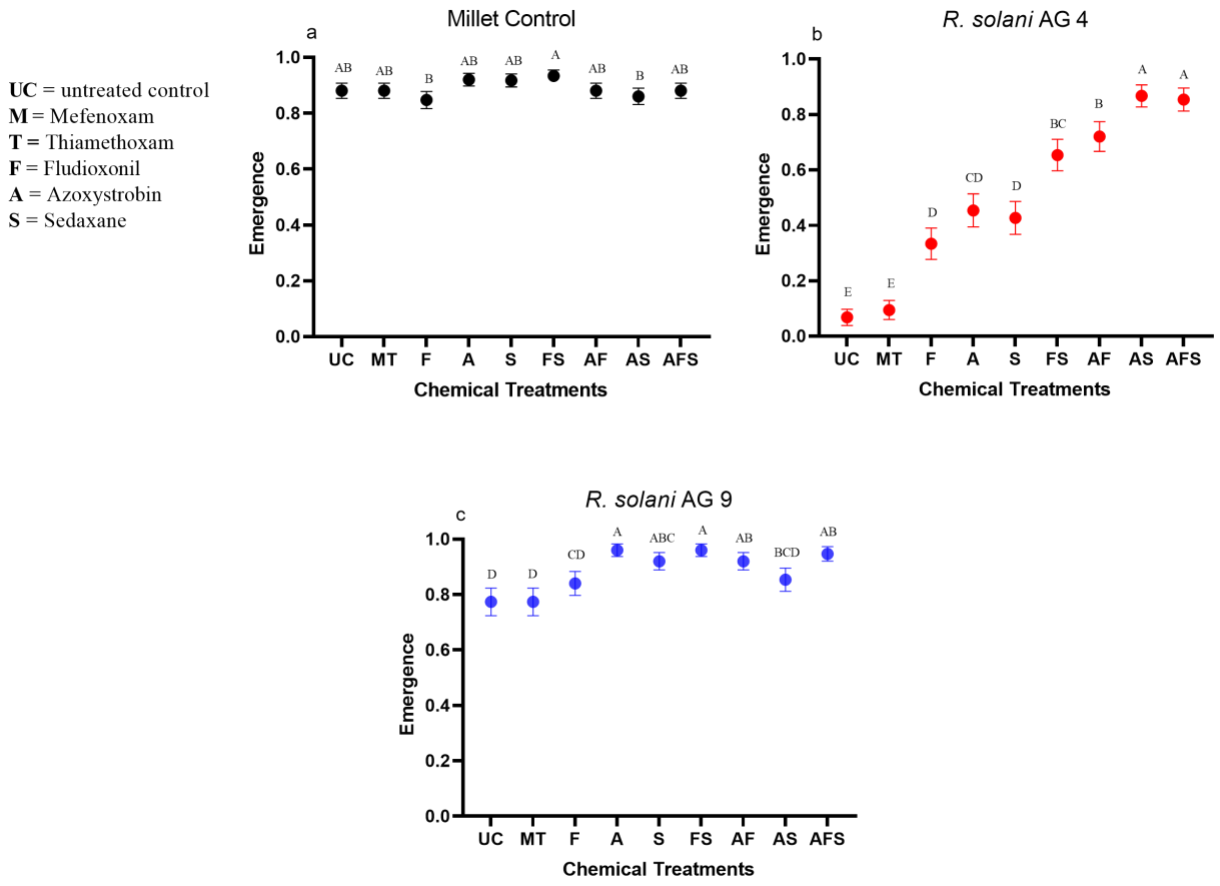


**Figure 3.2. Assessing efficacy of chemical seed treatment against two strains of *R. solani* through emergence in a seedling cup assay recorded seven days post-planting.** Mean emergence proportions of uninoculated control, *R. solani* AG 4 and AG 9 were challenged against eight chemical seed treatments and a non-treated 'Diamond' rice seeds in a controlled environment (greenhouse). Emergence was recorded seven days post planting. Data of three independent experiments were combined for analysis. The error bars represent standard errors of the mean emergence. Letters above the error bars represents difference among seed treatments across pathogens. Values followed by the same letter are not significantly different at $\alpha$=0.05 for comparisons of mean radius among treatments based on Fisher's protected LSD.

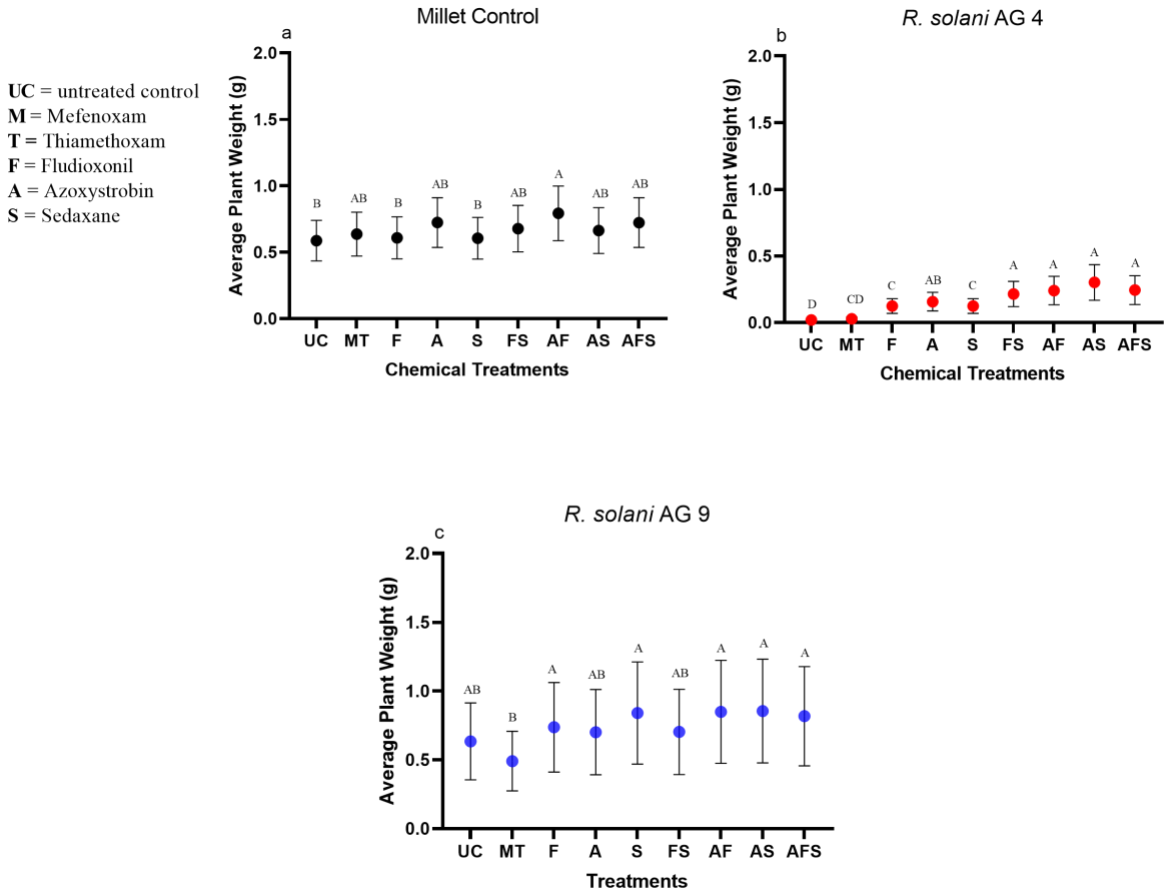


**Figure 3.3. Evaluating efficacy of chemical seed treatment against uninoculated control and two isolates of *R. solani* AG 4 and AG 9 by evaluating final plant stand (NPlants) four weeks post-inoculation in seedling cup assay.** Mean plant stands proportions in the uninoculated control and AG 4 and AG 9 were challenged against eight chemical treatments and an untreated control in the greenhouse. Plant stand was recorded four weeks post-inoculation and planting. Combined data of three independent experiments were combined for analysis. The error bars represent standard errors of the mean NPlants (number of plants). Letters above the error bars represents difference among seed treatments across pathogens. Values followed by the same letter are not significantly different at $\alpha$=0.05 for comparisons of mean radius among treatments based on Fisher's protected LSD.

**UC** = untreated control
**M** = Mefenoxam
**T =** Thiamethoxam
**F** = Fludioxonil
**A** = Azoxystrobin
**S** = Sedaxane

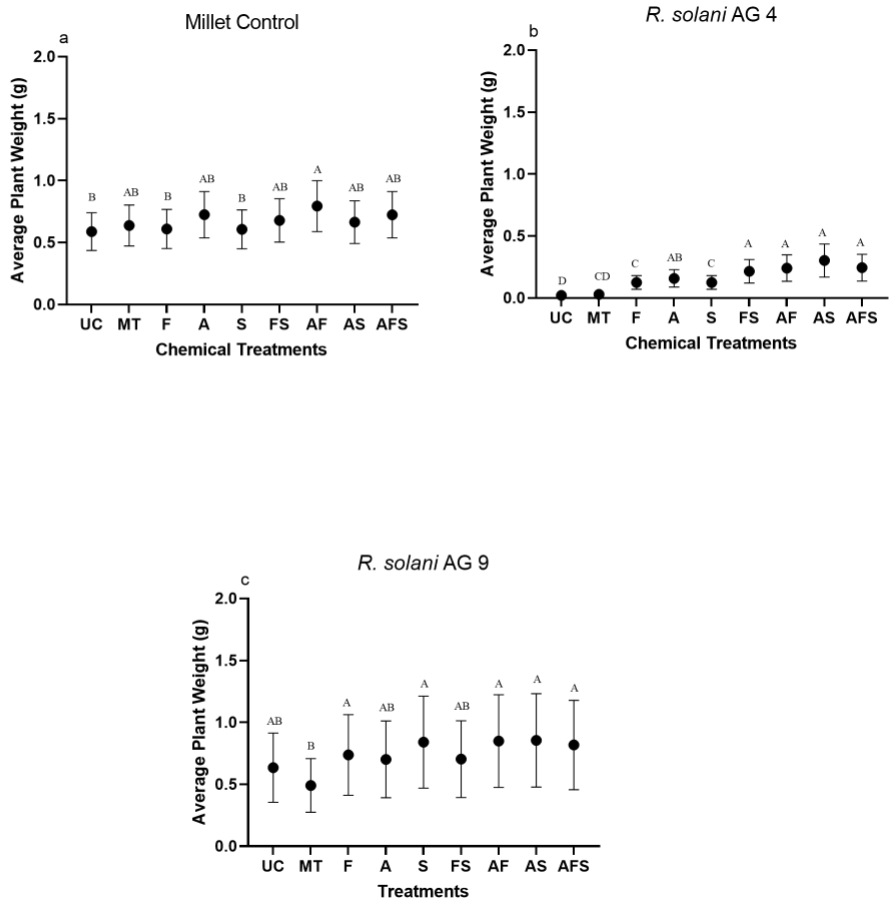


**Figure 3.4. Assessing the efficacy of chemical seed treatment against 2 strains of *R. solani* through average plant weight (in grams) in seedling cup assay recorded four weeks post-inoculation and planting.** Average plant weight (proportions) of uninoculated control (a) and AG 4 (b) and AG 9 (c) challenged against eight chemical treatments and a non-treated control of 'Diamond' rice seeds in the greenhouse. Average plant weight was recorded four weeks post inoculation and planting. Data of three independent experiments were combined for analysis. The error bars represent standard errors of the mean plant weight in grams. Letters above the error bars represents difference among seed treatments across pathogens. Values followed by the same letter are not significantly different at $\alpha$=0.05 for comparisons of mean radius among treatments based on Fisher's protected LSD.

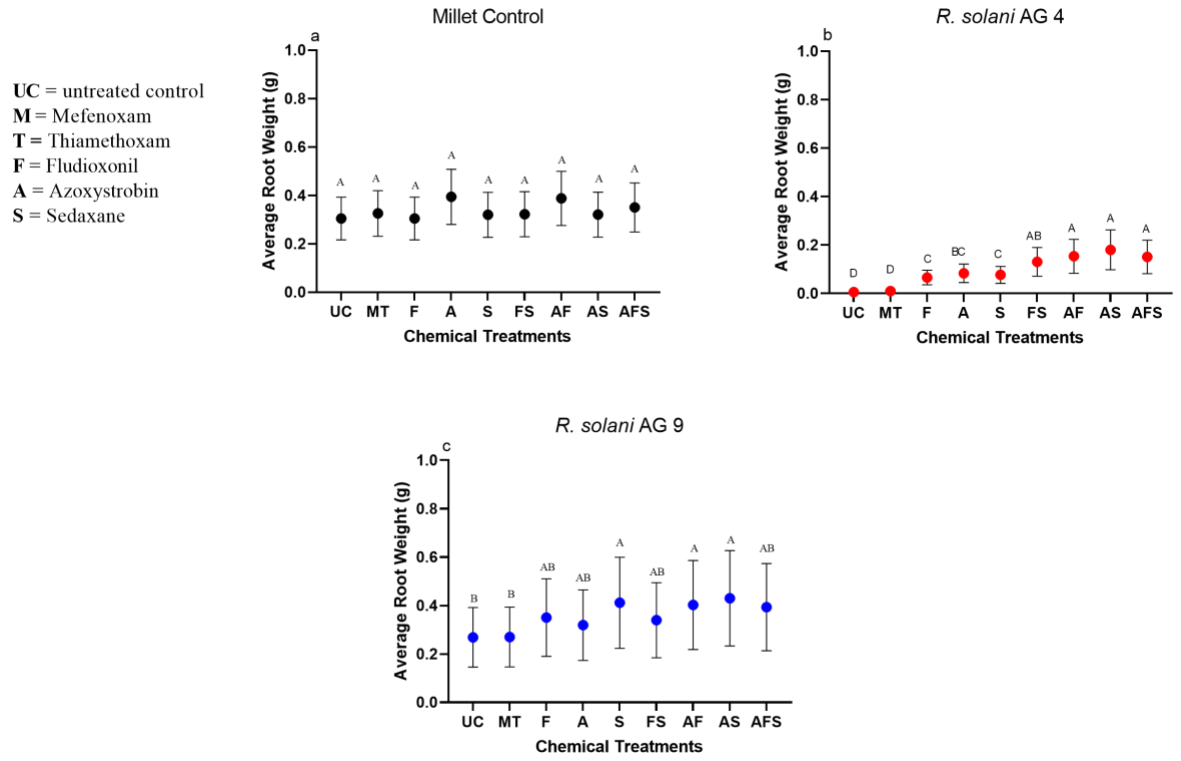


**Figure 3.5. Evaluating assessing the efficacy of chemical seed treatment against 2 strains of *R. solani* through average root weight (in grams) in seedling cup assay recorded four weeks post-inoculation and planting.** Average root weight (proportions) of uninoculated control (a)and AG 4 (b) and AG 9 (c) challenged against eight chemical treatments and a non-treated control of 'Diamond' rice seeds in the greenhouse. Average root weight was recorded four weeks post inoculation and planting. Data of three independent experiments were combined for analysis. The error bars represent standard errors of the mean root weight in grams. Letters above the error bars represents difference among seed treatments across pathogens. Values followed by the same letter are not significantly different at $\alpha$=0.05 for comparisons of mean radius among treatments based on Fisher's protected LSD.

**Table 3.1.** Active ingredients used and the rate at which the active ingredients are used

| Treatment | Formulation/Active Ingredients | Rate (g AI/ 100kg seed) | Commercial name |
|---|---|---|---|
| 1 | **Untreated control** | | |
| 2 | Thiamethoxam + Mefenoxam **(Base treatment)** | 8.323 | Apron XL, Cruiser 5FS |
| 3 | **Base treatment** + Fludioxonil | 8.323<br>1.389 | Apron XL, Cruiser 5FS , Maxim 4FS |
| 4 | **Base treatment** + Azoxystrobin | 8.323<br>8.323 | Apron XL, Cruiser 5FS, Dynasty |
| 5 | **Base treatment** + Sedaxane | 8.323<br>3.882 | Apron XL, Cruiser 5FS, Vibrance |
| 6 | **Base treatment** + Sedaxane + Fludioxonil | 8.323<br>3.882<br>1.389 | Apron XL, Cruiser 5FS, Vibrance, Maxim 4FS |
| 7 | **Base treatment** + Azoxystrobin + Fludioxonil | 8.323<br>8.323<br>1.289 | Apron XL, Cruiser 5FS, Dynasty, Maxim 4FS |
| 8 | **Base treatment** + Azoxystrobin + Sedaxane | 8.323<br>8.323<br>3.882 | Apron XL, Cruiser 5FS, Dynasty, Vibrance |
| 9 | **Base treatment** + Azoxystrobin + Sedaxane + Fludioxonil | 21.918 | Vibrance RST |

**Table 3.2.** Type III test of fixed effects of seed plate assay experiments conducted at room temperature showing the response variable (emergence) and the significance of the effects tested at seven days post-inoculation.

| Effect | [a]Num DF | [b]Den DF | F Value | Pr > F[c] |
|---|---|---|---|---|
| *Treatment* | 8 | 1017 | 55.14 | <.0001 |
| *Pathogen* | 6 | 1017 | 75.87 | <.0001 |
| *Treatment*Pathogen* | 48 | 1017 | 10.40 | <.0001 |

[a] Num DF is the number of degrees of freedom in the model
[b] Den DF is the number of degrees of freedom associated with the model error
[c] Probability of greater F value, P values were considered significant with $\alpha$=0.05.

**Table 3.3:** Type III test of fixed effects of seedling cup assay experiments conducted in a greenhouse showing the response variables (emergence, NPlants, plant weight and root weight) and the significance of the effects tested at four weeks post-inoculation and plantings

| Response Variable | [a]Num DF | [b]Den DF | F Value | Pr > F[c] |
|---|---|---|---|---|
| Emergence | | | | |
| *Treatment* | 8 | 512 | 14.95 | <.0001 |
| *Pathogen* | 2 | 512 | 150.63 | <.0001 |
| *Treatment*Pathogen* | 16 | 512 | 7.21 | <.0001 |
| Plants | | | | |
| *Pathogen* | 2 | 5.743 | 10.09 | 0.0132 |
| *Treatment* | 8 | 513 | 18.37 | <.0001 |
| *Pathogen*Treatment* | 16 | 513 | 6.11 | <.0001 |
| Plant weight | | | | |
| *Pathogen* | 2 | 3.583 | 5.08 | 0.0899 |
| *Treatment* | 8 | 432.3 | 8.87 | <.0001 |
| *Pathogen*Treatment* | 16 | 431 | 3.51 | <.0001 |
| Root weight | | | | |
| *Pathogen* | 2 | 5.393 | 5.71 | 0.0466 |
| *Treatment* | 8 | 456 | 22 | <.0001 |
| *Pathogen*Treatment* | 16 | 456 | 11.71 | <.0001 |

[a] Num DF is the number of degrees of freedom in the model
[b] Den DF is the number of degrees of freedom associated with the model error
[c] Probability of greater F value, P values were considered significant with $\alpha$=0.05.

**Table 3.4:** Shows *Rhiozoctonia solani* and *Pythium* spp. used in this study

| Pathogen | Designation | Host | Location | Collector |
|---|---|---|---|---|
| *R. solani* AG 1-IA | Rs AG 1-IA (Aggressive) | Rice | Arkansas | Yeshi Wamishe |
| *R. solani* AG 4 | R107-35P | Rice | Arkansas | Craig Rothrock |
| *R. solani* AG 7 | R88-34A | Rice | Arkansas | Craig Rothrock |
| *R. solani* AG 9 | Rs AG 9 #2 | Rice | Arkansas | Yeshi Wamishe |
| *F. graminearum* | *Fg 4* | Wheat | Arkansas | David Moon |
| *P. graminicola* | WS 2001 | | Arkansas | Craig Rothrock |
| *P. irregulare* | ME 2009 | | Arkansas | Craig Rothrock |

## Chapter IV: Conclusion

Rice (*Oryza sativa L.*) is one of the world's three major staple food crops along with wheat and maize, feeding more than half of the increasing global population. However, both. Foliar and seedling diseases continue to cause significant yield losses in rice. Management of these diseases relies heavily on chemical control and cultural practices as there are no completely resistant cultivars to most of the rice diseases. Although a number of biological control products are registered and available that are active on multiple rice diseases, the use of biological control is less prevalent in controlling rice diseases.

In this study, the use bacteria in the genera *Burkholderia* and *Pseudomonas* as biological control agents (BCAs) revealed that *B. cepacia* and *P. fluorescens* consistently had significant reduction in the impact of fungal pathogens in controlled environments by reducing mycelial growth of these pathogens and improving emergence in the presence of fungal and oomycete pathogens. Also in this study, Mefenoxam, Azoxystrobin, Fludioxonil and Sedaxane and blends of these active ingredients were evaluated as seed treatments against different *Rhizoctonia solani* anastomosis groups (AGs) and *Pythium* spp. Results indicate single application of Mefenoxam is enough in controlling *Pythium* spp. but single use or blends of Azoxystrobin, Fludioxonil and Sedaxane control *R. solani.* For future study, we propose evaluation of the BCAs and chemical seed treatments at the field level using different cultivars.