BACTERIAL – PATHOGENIC FUNGAL INTERACTIONS: FINDING BACTERIAL COMPONENTS TO INHIBIT RICE BLAST

by

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ABSTRACT

Rice is an extremely important food source for billions of people around the world. According to the Food and Agriculture Organization (FAO) of the United Nations, the rice supply must double to meet the global demand by the year 2050. A huge barrier to this goal is crop loss due to rice blast. The rice blast is caused by a deleterious fungal pathogen, *Magnaporthe oryzae*, that accounts for about 30% loss of global rice yields. *Pseudomonas chlororaphis* EA105 (hereafter EA105), which was isolated from the rhizospheric soil of California rice cultivar M104, has been shown to significantly diminish the size of rice blast lesions by inducing a systemic response in the plants. *Magnaporthe oryzae*'s specialized infection cells called appressoria are also inhibited by EA105. Because of these abilities, the bacterium *P. chlororaphis* EA105 could be the key to solving the rice blast epidemic.

This research was aimed towards finding the components of EA105 which work in preventing *M. oryzae* from infecting rice plants. EA105 was grown together with *M. oryzae* in standard media as well as media containing various rice plant extracts. Characteristics of the interactions involved in the inhibition of rice blast were able to be hypothesized with the data obtained. However, this research is still in progress and much more has yet to be determined about the components of EA105 which inhibit rice blast.

Chapter 1

INTRODUCTION

Billions of people around the world rely on Rice (*Oryza sativa*) for their source of caloric intake. To meet the global demands of rice by the year 2050, the current world supply must double (FAO). A huge barrier to this goal is crop loss due to rice blast pathogens. Rice blast is caused by a deleterious fungal pathogen, *Magnaporthe oryzae*, which has been labeled the number one fungal pathogen in molecular plant pathology (1). *Magnaporthe oryzae, (aka M. grisea)* accounts for up to 42% loss of global rice yields, or 157 million tons of rice per year (2,3). Researchers have attempted generating host resistance in rice plants; however the fungus is known to quickly overcome the resistance within two to three growing seasons (4). Furthermore, inducing host resistance in plants reduces the variety and leads to rice plants which are less fit for survival, causing a lower crop yield potential. An environmentally sustainable strategy to control Rice Blast is desperately needed.

The airborne pathogen *Magnaporthe oryzae* is most known for infecting rice plants. However, *M. oryzae* also infects a wide range of other Gramineae (grass family) members. *M. oryzae* recently demonstrated its ability to adapt to selective pressures when it was observed switching hosts from rice plants to wheat in Brazil (18). *M. oryzae* infects rice by forming an infection structure called the appressorium, that penetrates the cuticle of rice plants through producing immense turgor pressure (19). *M. oryzae* then secretes effector proteins which carry out infection and may also suppress the host's immune system (20).

In recent years, it has been found that some bacteria living in the rhizosphere of the rice plants can protect the plants from the aerial-pathogen, *M. oryzae*. One of these species of bacteria, which was isolated from California rice rhizoshperic soil and is particularly successful in inhibiting rice blast, is *Pseudomonas chlororaphis* EA105 (5). It has been shown that this bacterium when associated with rice roots, can significantly diminish the size of rice blast lesions by inducing a systemic immune response in the plants, priming them for additional stress. *M. oryzae's* specialized infection cells called appressoria are also inhibited by EA105. Because of these abilities, the bacterium *P. chlororaphis* EA105 could be the key to solving the rice blast epidemic. In addition, application of a microbial inoculum strategy to reduce rice blast infections may also mitigate the resistance pressure caused by increased use of synthetic fungicides.

The rhizosphere is a community in the soil where bacteria and plants interact, and it plays a crucial role in plant health and survival (6). Microbes benefit plant health by providing nutrients, defending against pathogens, degrading toxins, and improving soil fertility (7). In return, the process of plant roots releasing organic molecules into the soil is the key energy supplier the microbial community (8). Roots play an active role in interacting with the microbial community by excreting compounds, which can act on microbes as either attractants or repellents. Past studies have even shown that there are distinct microbial communities within different distances from the roots of rice plants (9).

Taking advantage of natural rhizospheric microbes to reduce plant disease and increase crop production is not a new idea. There is already evidence that microbes can diminish both bacterial and fungal pathogens and promote growth. Pseudomonas

species are currently being used in marketed products such as Howler™, D7®, and many more (10).

Pseudomonas chlororaphis is a rod shaped, gram-negative bacteria that can be found universally, and especially in water and soil (11). In addition, *P. chlororaphis* EA105 is non-pathogenic and was naturally isolated from a rice paddy in California. Previously, it was shown that EA105 significantly down-regulated the expression of about half of *M. oryzae's* genes, reduced lesion sizes on pre-treated plants, and directly inhibited the growth *of M. oryzae* in vitro (5).

However, the bacterial components of EA105 which help it to counter *Magnaporthe oryzae* have yet to be determined. Furthermore, different cultivars of rice may alter the interaction between EA105, *M. oryzae*, and rice plants differently, because different varieties of rice have unique characteristics (12). To test if this is true, *P. chlororaphis* EA105 and *M. oryzae* were grown together in a challenge assay, and radial growth of *M. oryzae* was observed.

Additionally, the interactions may also change when rice plants are under abiotic or biotic stresses. Mechanical wounding and foliar application of salicylic acid have both been shown to induce resistance in plants (13,14). To test if these stresses alter bacterial-fungal interactions, rice plant leaf extracts were used in the media of the same assays after experiencing stress from either wounding or salicylic acid (hereafter SA) application. Understanding the interactions between pathogenic fungi, bacteria, and the host plants they occupy, are crucial to develop efficient solutions to fight diseases.

Chapter 2

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Oryza sativa 'Nipponbare', 'Jasmine', 'Seraceltik', and 'BR16' were planted from seeds and kept in growth chambers with 80% humidity and 14 hours of light. The temperature was kept at 28°C during the day and 24°C during the nighttime.

Microbial Materials

Pseudomonas chlororaphis EA105 was naturally isolated in California rice paddy soil, from the rice cultivar M-104. *Magnaporthe oryzae* (strain 70-15) used in this study was obtained from Dr. Nicole Donofrio's lab.

In Vitro Challenge Assay

Pseudomonas chlororaphis EA105 and *M. oryzae* 70-15 were grown together on complete media agar as described in Carla Spence et al. (10g sucrose, 6g yeast extract, 6g casaminoacids, 15g agar, 1mL *Apergillus nidulans* trace elements, all in 1L water). Plant leaf extracts were added to the media to equal 5% of the media's volume. EA105 was cultured on Lysogeny broth (LB) agar in a 28°C incubator for 24 hours, followed by growth in liquid LB media on a 28°C and 200 RPM shaker for 12 hours. 5µl of 1 × 106/mL bacterial cells were then placed 4cm away from a 4mm plug of *M. oryzae*. The Petri dishes were sealed with parafilm and kept in the dark at room temperature. After 10 days of growth, the percentage of inhibition by EA105 on *M. oryzae* was calculated using the following formula: % inhibition = ([C-T) × 100]/C), where "C" is the fungal radius in the control Petri dish, and "T" is the fungal radius in the Pitri dishes containing EA105.

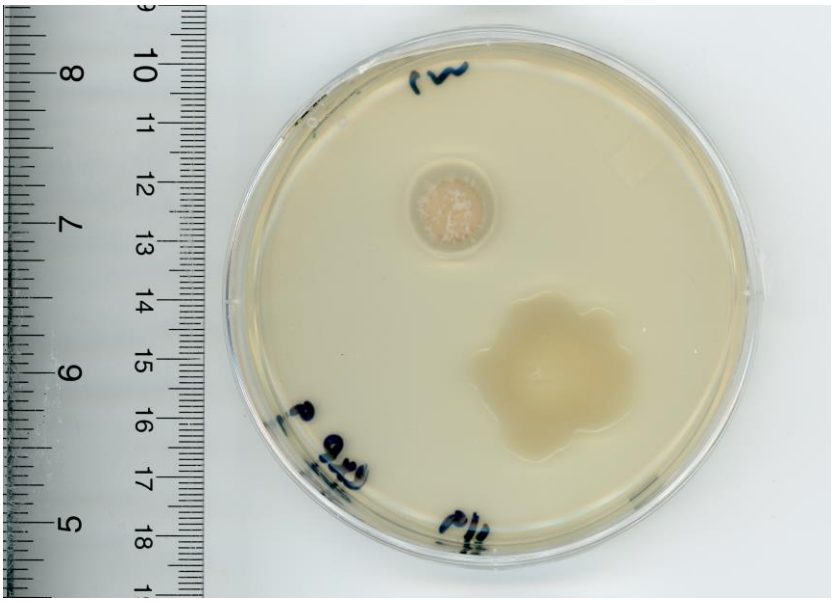

Figure 1 Figure of the challenge assay, containing EA105 on the bottom and *M. oryzae* 70-15 at the top

Plant Leaf Extracts

Rice plant leaves were harvested once the plants reached the adult stage of development, and were frozen at -80°C. They were then pulverized into a fine powder and added to water (2% by mass). The water extraction solution sat for 24 hours before filter sterilization and addition to the complete media.

Salicylic Acid (SA) Application and Mechanical Wounding of Rice Plants

Rice plants were either subjected to a 1mM SA foliar application or a mechanical wounding. A plastic bag was used to cover the plant leaves and a spray bottle was used to apply 1mM concentration of salicylic acid. Both the directly sprayed leaves and systemic leaves were harvested 24 hours afterwards. Scissors were used to cut the top of rice plant leaves and both the directly wounded leaves and systemic leaves were harvested after 24 hours.

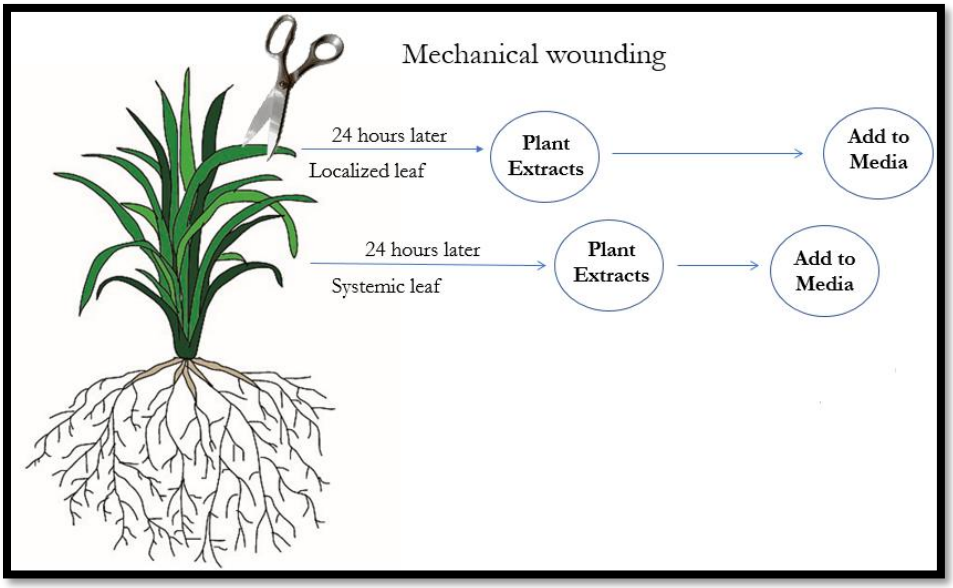

Figure 2 Schematic of mechanical wounding procedure

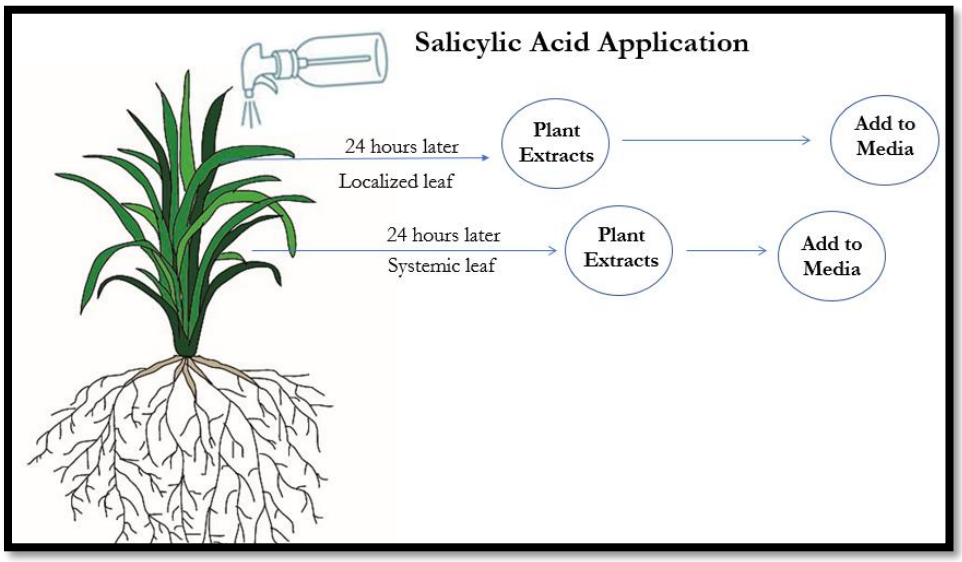

Figure 3 Schematic of salicylic acid application procedure

Chapter 3

RESULTS

Growth of EA105 in media supplemented with rice leaf extracts: As can be seen in figure 4, *P. chlororaphis* EA105 began to lose motility as concentration of plant extracts increased. It was also found that fungal growth appears to be reduced at the highest concentration tested (figure 5). When both EA105 and 70-15 are together on the same plate, percent inhibition by EA105 is different depending on what plant extract is being used (figure 3).

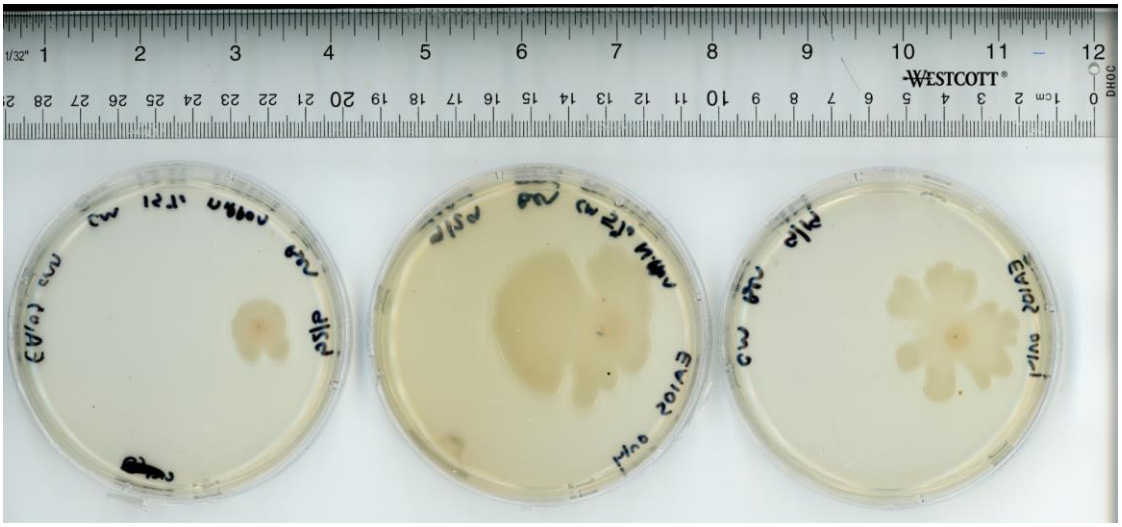

Figure 4 EA105 grown by itself in 15%, 5%, and 0% (from left to right) Nipponbare plant extracts.

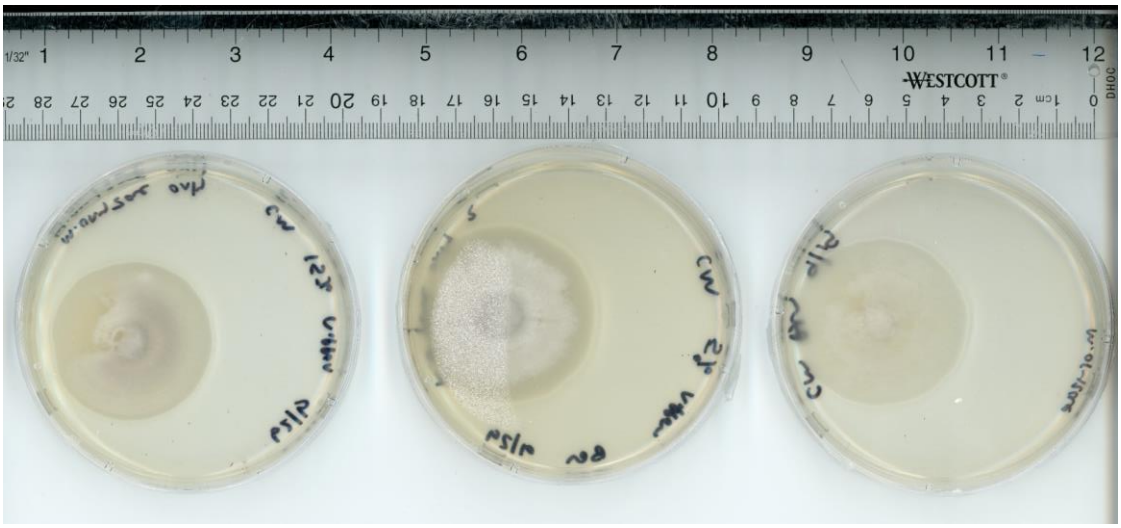

Figure 5 *M. oryzae* grown by itself in 15%, 5%, and 0% (from left to right) Nipponbare plant extracts.

Without plant extracts, there is a percent inhibition by EA105 on *M. oryzae* of about 43%. On media with Jasmine extracts the percent inhibition is about 39%, on BR16 it is about 42%, and on Seraceltik it is about 30% (figure 6). P-values calculated by an ANOVA test were all less than 10^-5, showing significance.

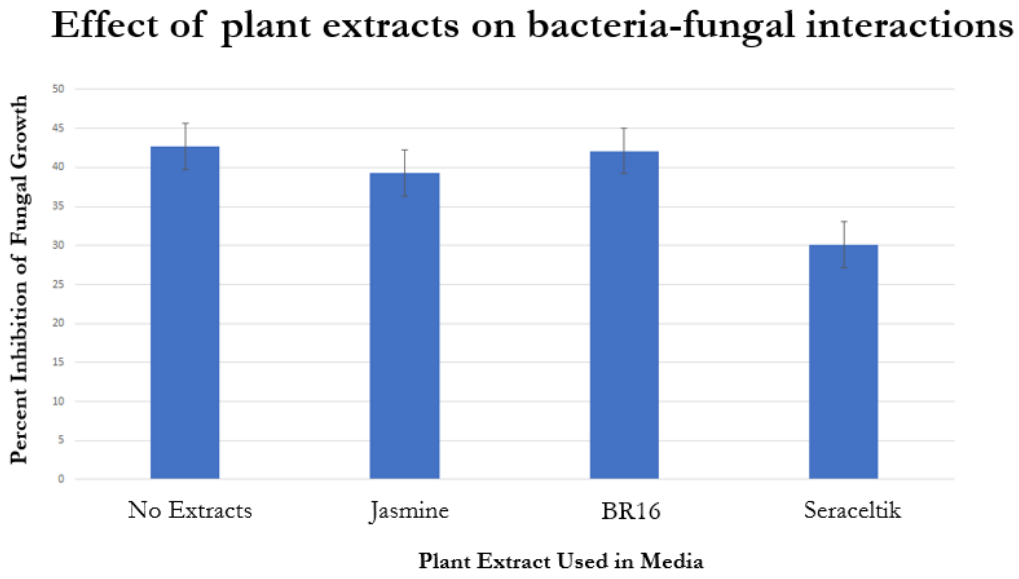

Figure 6 The percent inhibition by EA105 on *M. oryzae* on media containing differing plant extracts

M. oryzae was shown to be inhibited by media containing plant extracts from mechanically wounded rice plants (figures 7,8). On media with plant extracts from wounded BR16 leaves (localized) and systemic leaves, *M. oryzae* was inhibited by about 30%. *M. oryzae* was inhibited by about 20% and 25% on media containing wounded Jasmine localized and systemic leaves respectively. Finally, media with Seraceltik plant extracts from localized and systemic leaves each inhibited fungal growth by 13.5%. P-values calculated by an ANOVA test were less than .01 except for data from BR16, which showed P-values of about .2.

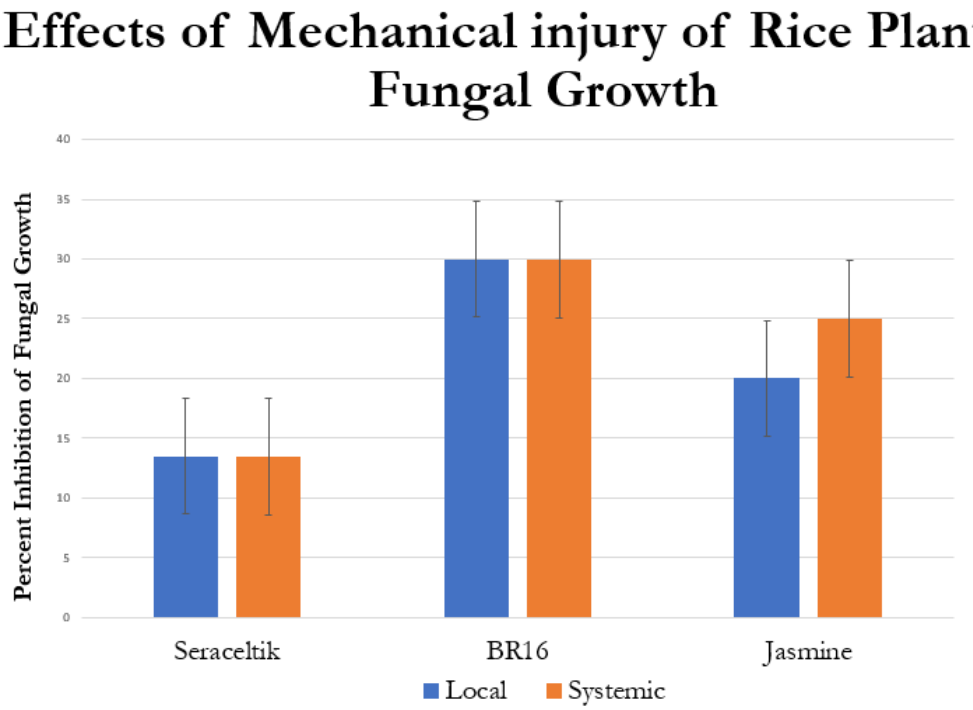

Figure 7 Percent inhibition by media alone on *M. oryzae*, without EA105

Figure 8 *M. oryzae* growing by itself in media containing extracts from Jasmine rice plants which have been mechanically wounded

When EA105 was added to the Petri dishes with *M. oryzae* on media containing plant extracts from mechanically wounded rice plants, the percent inhibition on fungal growth increased (figure 9). In media containing wounded BR16 leaf extracts, the percent inhibition of fungal growth was about 46% for localized leaves and 48% for systemic leaves. *M. oryzae* was inhibited by 56% and 52% by media containing wounded Jasmine localized and systemic leaves respectively. On media containing Jasmine and BR16 extracts that were not affected by mechanical wounding, EA105 inhibited the growth of *M. oryzae* by 36% and 46%. Media with Seraceltik plant extracts from localized and systemic leaves each inhibited fungal growth by 33%. P-values calculated by an ANOVA test were all less than 10^-6, showing significance.

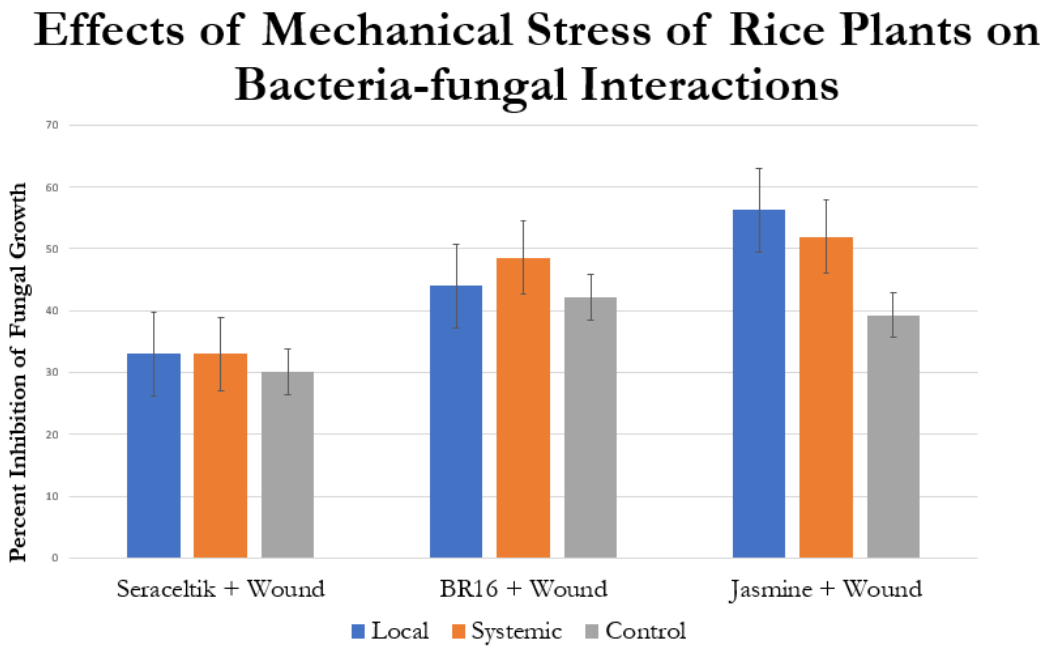

Figure 9 Percent Inhibition by EA105 on *M. oryzae* on media containing extracts from wounded rice plants

M. oryzae was inhibited by media containing plant extracts from rice plants that were affected by salicylic acid application (figures 10,11). On media with plant extracts from treated Jasmine localized leaves and systemic leaves, *M. oryzae* was inhibited by about 24% and 23%. *M. oryzae* was inhibited by about 30% on media containing plant extracts from treated BR16 systemic rice leaves, and about 31% from localized leaves. Media with Seraceltik plant extracts from localized and systemic leaves inhibited fungal growth by 15% and 14%. P-values calculated by an ANOVA test were all less than 10^-5, showing significance.

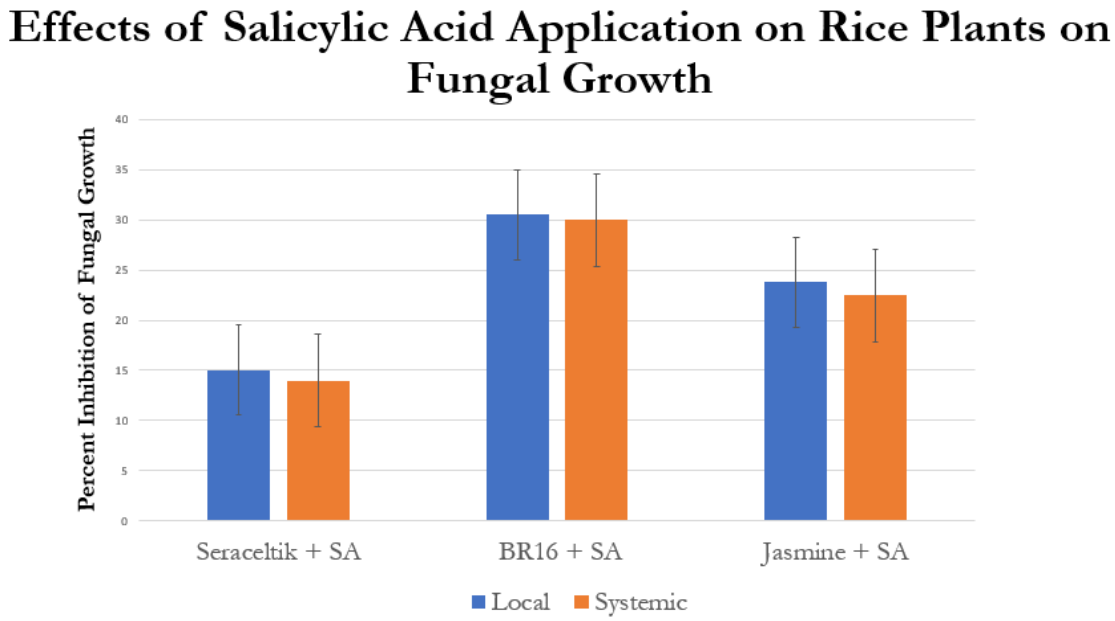

Figure 10 Percent Inhibition of *M. oryzae's* growth by Media containing extracts from plants treated with SA, without EA105

Figure 11 *M. oryzae* growing without EA105 on media containing extracts of rice plants treated with SA.

EA105 was then added to Petri dishes with *M. oryzae* and media from salicylic acid treated rice plant extracts (figures 12,13). On media plates with BR16 systemic leaves added to, fungal growth was inhibited by 49%. Fungal growth was inhibited by 53% and 50% on plates with Jasmine localized and systemic leaf extracts respectively. On plates with extracts from Seraceltik localized and systemic leaves, the inhibition of fungal growth was 39% and 35% respectively. *M. oryzae* was inhibited by 36% on control plates with Jasmine extracts, and by 46% by control plates with BR16 extracts. P-values calculated by an ANOVA test were all less than 10^-6, showing significance.

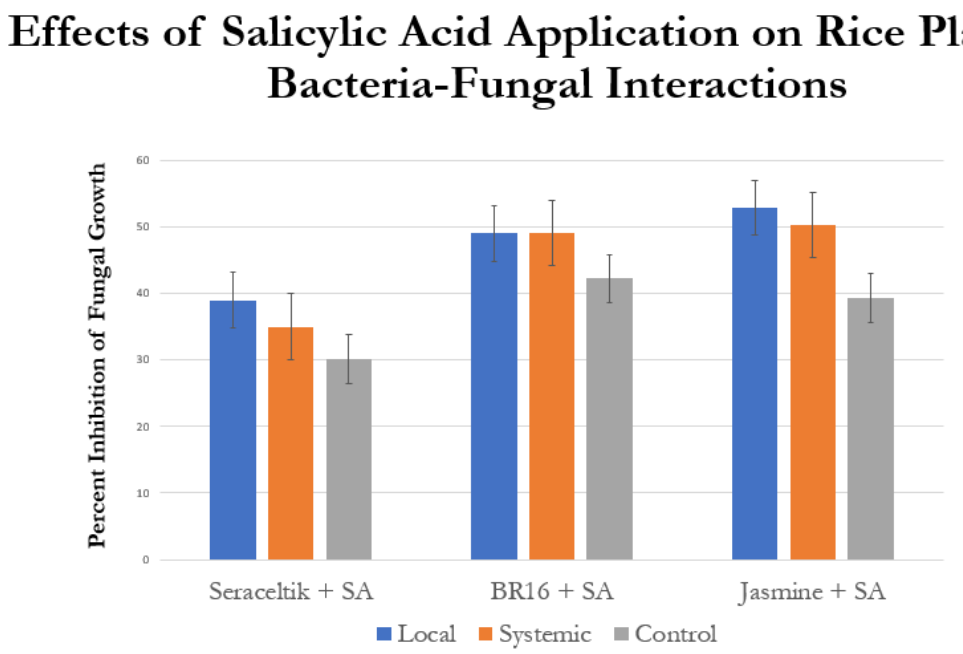

Figure 12 Percent Inhibition by EA105 on *M. oryzae* on media containing different extracts from plants treated with SA

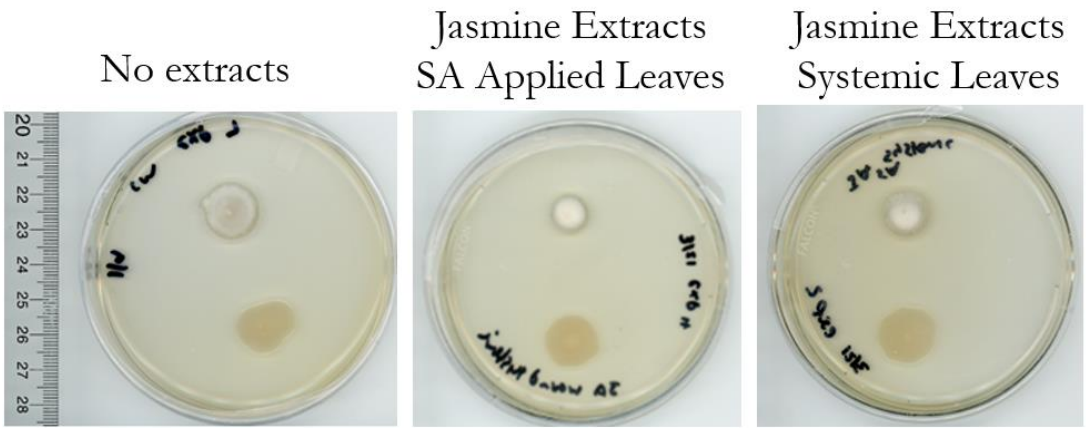

Figure 13 *M. oryzae* growing with EA105 on media containing extracts of Jasmine rice plants treated with SA.

Chapter 4

DISCUSSION

The data from the in vitro assays reveal that plant extracts effect growth of *Pseudomonas chlororaphis* EA105 and *Magnaporthe oryzae* 70-15. The percent inhibition of *M. oryzae* decreased compared to the control when Jasmine plant extracts were added to the media and decreased even more so when Seraceltik plant extracts were added to the media. The percent inhibition did not significantly change when BR16 extracts were added. Jasmine is a variety of rice that is primarily grown in southeast Asia, while BR16 is a variety of Bangladeshi rice. The Seraceltik rice plant is a variety that is hyper-susceptible to infection by *Magnaporthe oryzae*. This is probably connected to *M. oryzae* being least inhibited in media containing Seraceltik rice plant extracts.

When plant extracts from mechanically wounded rice plants were added to the complete media, the growth of *Magnaporthe oryzae* was reduced regardless of which genotype of rice plant was used. This indicates that mechanically wounding plants can change the composition of molecules being expressed in the rice plant leaves. BR16 extracts showed higher levels of anti-fungal properties than Jasmine extracts, which correlates with the data from figure 6. Seraceltik extracts showed to inhibit fungal growth the least. When EA105 was added into the mix, percent inhibition increased in all of the Petri dishes (figure 9). Percent inhibition was more significantly elevated in the Jasmine extracts plates, than in the BR16 plant extracts plates, when compared to the controls. In Seraceltik extracts plates, the percent inhibition did not significantly change.

When plant extracts from salicylic acid treated rice plants were added to the complete media, the growth of *Magnaporthe oryzae* was also reduced regardless of the genotype used. Once again, BR16 extracts showed higher levels of anti-fungal properties than the Jasmine extracts, but when EA105 was added to the plates the Jasmine extracts plates inhibited fungal growth more than BR16 plates. The reasons these results occurred are unknown but must have something to do with differing interactions between EA105 and the plant extracts, depending on the genotype of the rice plants. Seraceltik extracts again did not increase the inhibition of fungal growth. This may connect to the fact that Seraceltik is a variety of rice which is hyper-susceptible to rice blast infection.

Both localized and systemic plant extracts from mechanically wounded and salicylic acid treated plants inhibited fungal growth. This demonstrates plants' ability to communicate and send signals from one leaf to the rest of the leaves. Mechanical wounding of plants is known to activate jasmonic acid (JA) induced pathways which leads to an induced systemic resistance in plants. The wounding has been discovered to release certain peptides or oligosaccharides from the cell wall or precursor proteins, which lead to a signaling cascade involving jasmonic acid (15). Salicylic acid (SA) is a signaling molecule in plants that can lead to defense responses, such as stomatal closure, transcriptional activation of defense molecules, and programmed cell death (16). Both SA and JA signaling cascades can lead to the generation of defense molecules called "pathogen related" proteins, which include antifungals and oxidative enzymes (17). These antifungal proteins, such as chitinases, could be present in the plant tissue which inhibit *Magnaporthe oryzae*.

Spence et al. (5) showed that when rice plant roots are inoculated with EA105 and challenged with *M. oryzae* 24 hours afterwards, blast lesions were significantly reduced in size. This demonstrates EA105's ability to produce an induced systemic resistance (ISR) in rice plants, priming them for future infections from pathogens. The in vitro assays demonstrate how the salicylic acid and jasmonic acid signaling may lead to expression of antifungal proteins in rice leaves. The extracts from plant leaves were able to significantly reduce fungal growth by simply being added to the complete media (figures 4,7). Because EA105 activates similar pathways using jasmonic acid a salicylic acid, the assays connect to its ability to aid in the defense against pathogens when present in the soil of the rice plants.

Chapter 5

CONCLUSION

In this study, it was found that different genotypes of rice plants have different effects on *M. oryzae's* growth and on the pathogen's interactions with *Pseudomonas chlororaphis* EA105. When plant extracts of different genotypes were added with *M. oryzae* and EA105, the percent inhibitions of fungal growth differed depending on the genotype. When rice plants were wounded prior to the extractions, fungal growth was inhibited by the media alone and even more so when EA105 was added to the plates. This also was the case for when plants were treated with salicylic acid prior to extractions. This demonstrated the power of plant signaling and how beneficial microbes can impact a plant's defense system.

Rice blast, caused by *Magnaporthe oryzae*, is the primary cause of rice crop failures globally, and accounts for about 30% of the loss in yields. Furthermore, as Earth's population continues to grow rapidly, the world supply of rice will need to double to meet the global demand. Using plant-beneficial microbes that already grow naturally in the soil to combat rice blast, is an effective, cost efficient, and environmentally sustainable long-term resolution which is already being deployed successfully. To further this success, it is important to understand the interactions between beneficial microbes, plants, and pathogens. This will help to successfully administrate this solution, identify additional and more beneficial microbes, and to react to new diseases more quickly in the future.

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