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Utilizing Plant-Microbial Interactions in Controlling Rice Major Diseases and Increasing Rice Yields

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Biological control is an effective and powerful alternative to synthetic chemicals in controlling rice diseases. The rich diversity of the microbial world provides a seemingly endless resource for this purpose. Generally, the study aimed to control major rice pests and diseases using the benefits of plant-microbial interactions. This is through the identification and isolation of beneficial microorganisms, evaluating and determining the antagonistic effect of these microorganisms to major rice diseases, likewise identify their benefits to the growth and yield of the rice plant. The study was conducted in the screenhouse and laboratory at PhilRice Isabela station with four three fungal treatments arranged in a Randomized Complete Block Design (RCBD) with (3) replications. The fungal treatments identified included *Vesicular Arbuscular Mycorrhiza* (VAM), *Trichoderma harzianum,* and *Metarhizium anisopliae* with BLB stopper as positive control. Varieties used in the study included Mestizo 1, NSIC Rc222, IR24 (susceptible Check), and IRBB21 (resistant check). In in-vitro test, the growth of inhibition zones in the positive control and in the three (3) fungal treatments was not observed due to the incompatibility of the culture media. Thus, the result was considered inconclusive. Likewise, in-vivo test was also conducted. Statistical analysis of the disease severity (DS) and disease incidence (DI) of the plants treated separately with the spore suspensions of *T. harzianum, M. anisopliae,* and VAM were determined to be significantly different to the DS and DI obtained for the negative control. Results imply that the effectiveness of the three fungal treatments is relatively similar to the effect of the commercial BLB stopper when it comes to the inhibition and antagonism of BLB in rice. Furthermore, calculation of percent reduction of DS showed that there is a 5.34% reduction of BLB disease in plants treated with *T. harzianum*, 1.06% in plants treated with *M. anisopliae*, and 0.293% in plants treated with VAM. Therefore, the fungi used were confirmed to be effective biocontrol agents against BLB in rice. They are environment-friendly and cheaper substitutes for chemically-based bactericides against BLB, thus limiting the use of harmful chemicals.

Keywords: Biological control, antagonistic, *Trichoderma harzianum, Metarhizium anisopliae, Vesicular Arbuscular Mycorrhiza* (VAM)

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Introduction

Plant diseases cause a nearly 10-20% decrease in total world food production annually which lead to loss of billions of dollars. Agriculture is facing problems regarding the destructive activities of various pests and pathogens even during the early times. This leads to the reduction of seed germination and seed quality thus causing a limitation in the potential yield of crops, loss of large amount of money, and reduction of aesthetic value (Bhattachargee and Dey, 2013). Bacterial Leaf Blight (BLB) is one of the most prominent and destructive diseases that affect different species of rice in Asian countries, especially during the heavy rains of monsoon season (Ashrafuzzman, 1987). In the Philippines, current losses or reduction in yields due to BLB are of the order of 22.5% in wet seasons to 7.2% in dry seasons in vulnerable crops and 9.5-1.8%, correspondingly, in resistant crops (Exconde, 1973).

BLB is caused by *Xanthomonas oryzae* and was first reported in Japan over a century ago (Mew *et al.*, 1993). *X. oryzae* enters the plant through wounds or hydathodes, multiplies in the epitheme, and migrates to the xylem vessels where multiplication causes blight in leaves (European and Mediterranean Plant Protection Organization, 2007). *X. oryzae* interacts with the plant by secreting proteins to the host cells. These proteins, called effectors, are injected inside the host cells through type III secretion system, and play essential roles in pathogenicity in plants. The type III effectors have either enzymatic or transcription activator-like activities which tend to modify or degrade host proteins or regulate the gene expression of the host (Kay and Bonas, 2009). Warm temperatures with high humidity and deep water in rice fields due to irrigation favour the spread of the disease from infected fields to adjacent healthy plots (Saleem, 2012). Likewise, hurricanes, storms, and severe winds can spread the bacteria over many miles in water droplets (British Society for Plant Pathology, 2014). In addition, over fertilization with nitrogen and mechanical contact also favour the attack of disease on rice and other plants (Saleem, 2012).

Objectives: Generally, it aimed to evaluate the potential of plant fungi as biocontrol agents against bacterial leaf blight (BLB) in rice. Specifically, the study aimed (1) to determine the antagonistic effects of *Trichoderma harzianum, Metarhizium anisopliae,* and Vesicular Arbuscular Mychorrizae (VAM) against the pathogen *Xanthomonas oryzae* and (2) to evaluate the effectiveness of the *T. harzianum, M. anisopliae,* and VAM against *X. oryzae in-vivo.*

Materials and methods

Bacterial Culture

The isolates of the fungi *Trichoderma harzianum*(in Potato Dextrose Agar plates stored at 30^{o}C), *Metarhizium anisopliae* (in PDA plates stored at 30^{o}C)and of the pathogen *Xanthomonas oryzae* (in Nutrient Agar slants stored at 37^{o}C) were obtained from Philippine Rice Research Institute (PhilRice), Nueva Ecija. Potato Dextrose Agar (PDA) was used for the mass production of the plant fungi while Soybean-Casein Digest Agar was used for the mass production of the pathogen.

Pathogenicity test

Four varieties of rice grains which include the susceptible (IR24), resistant (IRBB21) and intermediate (NSIC Rc 222 and M1) varieties was obtained from Philippine Rice Research Institute San Mateo, Isabela and was grown for 21 days under greenhouse conditions. After 21 days, the seedlings were transplanted in buckets. They were sprayed with a spore suspension of *Xanthomonas oryzae* at 5 x 10^5 cfu/mL obtained from the isolates of *X. oryzae* 35 days after the transplantation. The spraying was done prior to the application of biocontrol agents. Three replicates were used for each isolate. Disease severity (DS) and disease incidence (DI) were observed during the vegetative and reproductive phase of the samples. DI was estimated according to the disease index established by Rafi and company (2013) by measuring the percentage of infected plants out of the total plants examined.

In vitro experiments

Three hundred μL of a 24-hour bacterial suspension (12 x 10^8 cfu/ml) was spread plated in Nutrient Agar (NA) plate and was incubated for two days. After incubation, 5 mm diameter of a 5-day-old culture of *T. harzianum* (T1) was obtained using a sterilized cork borer and was placed at the center of the NA plate. The same steps were done for *M. anisopliae* (T2). For the positive control, 100 μL of bacteriacide (12 x 10^8 cfu/ml) was placed at the center of NA plate while the uninoculated NA plate with bacterial suspension was used as the negative control. Three replicates were made for each treatment. The plates were incubated at a temperature of 37℃ for 2 days and the inward linear growth was measured. The measurement of the size of inhibition zone and amount of overgrowth of *T. harzianum, M. anisopliae* on*X. oryzae* was used to

evaluate the interaction between the fungi and the bacteria. Larger inhibition zone indicated a higher biocontrol activity and vice versa.

In-vivo Experiments

Setting-up of plots

Twelve plots were set-up for the three treatments (*T. harzianum*, *M. anisopliae*, and VAM) for the screen house evaluation. The plots were ploughed and the soil was levelled. Nitrogen fertilizer was added at the rate of 357 kg ha^{-1} at two doses. After the disease infection (BLB), the first dose was integrated into the top 15 cm of the soil at day 1 and the second dose was integrated 30 days after the transplanting of the 21-day-old rice seedlings infected with BLB. Phosphorus was also be added at the rate of 238 kg ha^{-1} into the top 15 cm of the soil at day 1(Abdel- Fattah *et al*., 2007).

Another four other plots were set-up and were sprayed with a commercial bactericide which served as the positive control. For all treatments and control set-ups, one species of rice was used which is *O. sativa*. Each plot consisted of five seedlings. In order to prevent cross contamination, there was a physical barrier between plots while they were being sprayed with different treatments.

Randomized Complete Block design was used to set out the plots. (Abdel- Fattah *et. al.*, 2007).

Spraying of spore suspensions

Spore suspensions of A (*T. harzianum*), B (*M. anisopliae*) and C (VAM) were sprayed at varying concentrations in 10 $x10^{2}$, cfu/ml for A and 1.2 x 10^{11} for B and C. (Abdel- Fattah *et. al.*, 2007).The spraying of the spore suspensions was done two times at seven day intervals, beginning 14 days after infection of BLB.

Measuring of disease severity and disease incidence

Disease severity (DS) was measured as percentage of tissue area infected out of total leaf area examined. For each plot, all leaves were examined visually to determine the average lesion area percentage and measure the disease severity in each plot. The following scale was used to score the severity of BLB (Chaudhry, 1996).

Table 1.Disease severity scale for evaluation of Bacterial Blight of rice in the field

Disease Rating	Lesion size (% of leaf length)
0	0
1	>1-10 %
3	>11-30 %
5	>31-50 %
7	>51-75 %
9	>76-100 %

Disease incidence (DI) was measured as percentage of infected plants out of total plants examined. The formula that was used is outlined below (Rafi *et. al.*, 2013):

$$\text{Disease Incidence \%} = \frac{\text{Number of bacterial blight infected plants}}{\text{total number of plants examined}} \text{ x } 100\%$$

Measuring of DS and DI was done prior to the infection of BLB, and two times at seven day intervals beginning seven days after the initial spraying of spore suspensions on the rice seedlings.

Statistical analyses

Data was analyzed using statistical analysis software (SPSS). Data was subjected to analysis of variance (ANOVA), and the means was compared using Duncan's multiple range test at P=0.05.

Results and Discussion

Morphological Observation

According to Central Rice Research Institute (CRRI), the bacteria that causes BLB is rod-shaped, with typically 1.2 x 0.3- 0.5 um in dimension. They are usually single but sometimes in pairs, and never in chains. The bacteria do not possess endospores and capsules. They are considered Gram-negative and aerobic (Central Rice Research Institute, 2011). Because of these descriptions, different staining methods were done to confirm if the *X. oryzae* used satisfies all these morphological descriptions.

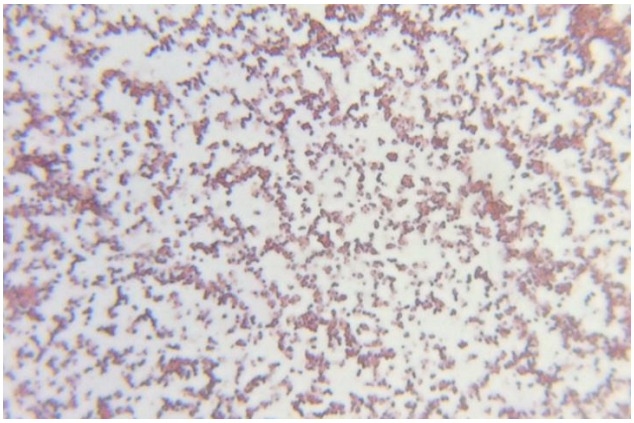

Figure 1.*Xanthomonas oryzae* subjected to Gram staining procedure. The bacterial cells are reddish to pinkish in color (Gram-negative) as they retained safranin instead of crystal violet. Magnification: 400x (under HPO of Compound Light Microscope- OPTIKA)

Using Gram Staining Method, *X. oryzae* was observed to be a gram-negative bacterium (see Figure 1). Its cells do not retain the crystal violet dye. They appear red or pink as a result of safranin (counterstain).

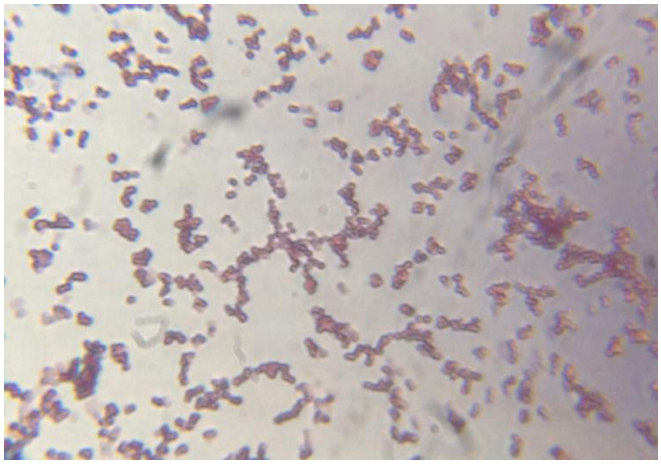

Figure 2.*Xanthomonas oryzae* subjected to differential staining procedure. The bacterial vegetative cells are reddish to pinkish in color without traces of green coloration (endospore) within the cells. Magnification: 1000x (under OIO of Compound Light Microscope-OPTIKA)

Differential staining (endospore staining technique) was used to distinguish between the vegetative cells and the endospores. It was determined in the results that *X. oryzae* do not possess endospores as the entire cell was

pink in color with no dark green stains within the cell that would signify the presence of endospores (see Figure 2).

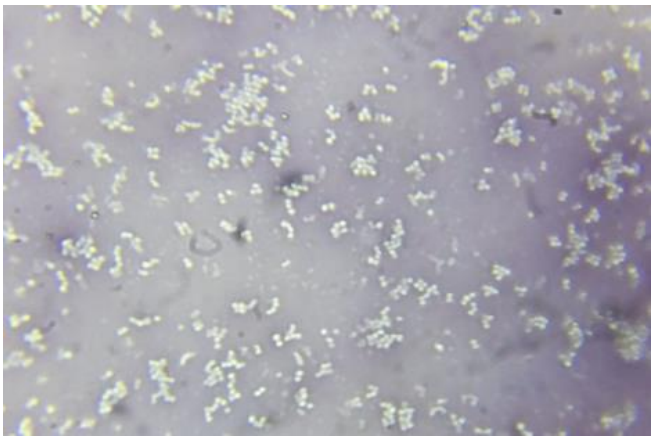

Figure 3.*Xanthomonas oryzae* subjected to negative staining procedure. The bacterial cells are clear in contrast with the darker background. No capsules encapsulate the bacterial cells. Magnification: 1000x (under OIO of Compound Light Microscope-OPTIKA)

Negative staining was used to determine the presence of capsule in *X. oryzae.* Microscopic observations showed that *X. oryzae* do not possess capsule due to the absence of a clear layer that circumferentially envelopes each cell (see Figure 3).

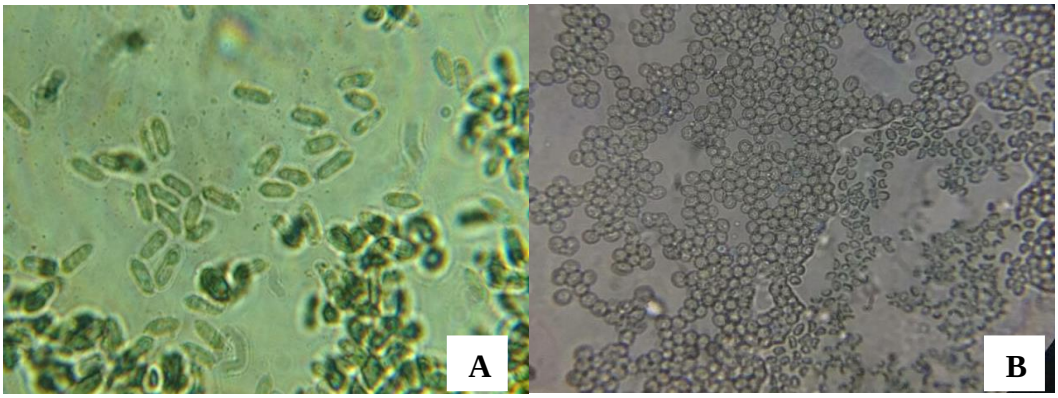

Figure 4.Conidia of *Trichoderma harzianum* (A) and *Metarhizium anisopliae* (B) after being subjected to Adhesive tape technique. The conidia exhibit pigmentation making them green in color. Magnification: 1000x (under OIO of Compound Light Microscope-OPTIKA)

As for two fungi (*T. harzianum* and *M. anisopliae*), Adhesive Tape Technique was done to observe the structure of conidia of each of the fungi. Figure 4 shows the conidia of *T. harzianum* and *M. anisopliae*. They were heat-fixed on a slide prior to microscopic observation. The *T. harzianum* is circular in shape while the *M. anisopliae* is ovular. These conidia are asexually produced spores which are externally borne to the cells that synthesize them. Conidia also function for dispersal ("Fungi reproducing asexually by means of conidia," N.d.). In addition, the conidia are also involved in the propagation of the fungi and their infection to the organisms they antagonize. In *M. anisopliae*, the proteins found in conidia are those that are involved in protective processes, appressorium formation, as well as degradation of the cuticle of their host (Su *et al.*, 2013). These proteins are unlike the proteins found in mycelia which includes those proteins participating in biosynthetic and energy metabolism, such as UTP-glucose-1-phosphate uridylyltransferase and heat shock protein 70 (Su *et al.*, 2013). Conversely, in *T. harzianum*, the proteins found in conidia are cellobiohydrolases I and II (CBH I and II) and endoglucanase I (EG I) degrade crystalline cellulose into glucose (Messner *et al.*, 1991) (Dong and Young, 2001). Cellobiohydrolases I and II (CBH I and II) hydrolyze from the chain ends and predominantly synthesize cellobiose, while endoglucanase I (EG I) hydrolyze the internal bonds in the cellulose chains (Dong and Young, 2001). The conidia from both organisms were green in color. This conidial pigmentation is significant for solar UV radiation tolerance in order not to delay or not to stop their germination process when exposed to the sun/ solar UV radiation, making them capable of doing their function (Braga *et al.*, 2006). These proteins are unlike the proteins found in mycelia which includes cellulases, which degrade cellulose and chitinases, which degrade chitin (Volk, 2004).

Macroscopic observations (Colony characterization)

Trichoderma harzianum

T. harzianum was inoculated on Potato Dextrose Agar. After four days of incubation, radial growth of the fungus, as manifested by white colour spreading all throughout the plate, was observed. Based on the colony characterization of *T. harzianum* in several journals (Bhattacharjee *et al.*, 2014; Jahan, *et al.*, 2013), the observed characteristics were similar in terms of the margin, texture and hypahal thickness. The colony grown on PDA had a compact texture, thick hyphae, and had a regular margin. The conidia were observed to be ovoid in shape, and the conidiophore was found to be highly

branched. After 8 days of incubation, the colonies were observed to be mostly white and greenish in color (Figure 5).

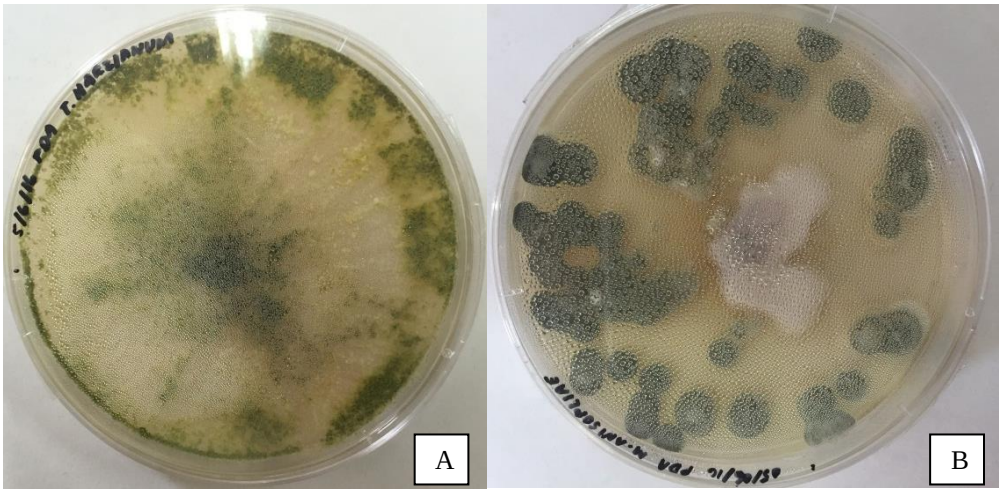

Figure 5. A five (5) mm diameter plug of *Trichoderma harzianum* (A) and *M. anisopliae* (B)transferred from a pure cultures into the center of two freshly-prepared Potato Dextrose Agar media plates using cork borer. The plates were stored in a 30^{o}C-incubator.

Metarhizium anisopliae

Colonies of *M. anisopliae* were observed to be largely branched and cylindrical in shape (Figure 5). The conidia were also observed to be white to light green in color and ovoid in shape, which are similar with the characteristics described by Brady (1979). The branching and change in color of the conidiophore were observed as the spores began to develop, which is similar to the characterization published by Ghayedi (2013). Moreover, the margins of the mycelia were white in color.

Xanthomonas oryzae

The colonies were observed to be yellow in color and were circular in shape. The margin was also observed to be even, which is similar to the description published by Arshad *et al*.(2015). Moreover, the colonies become thicker and become more yellowish upon longer days of incubation.

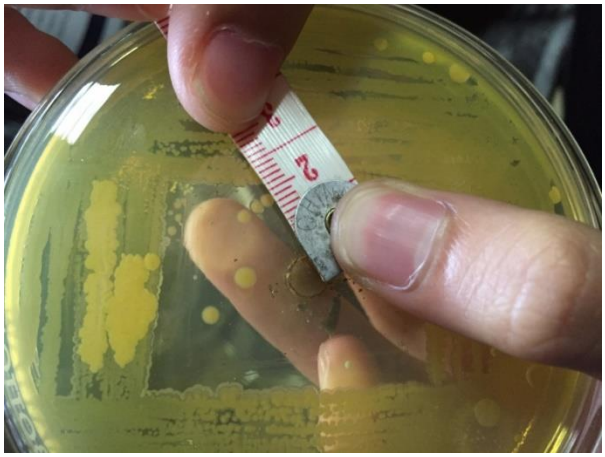

Figure 6. Isolates *of X. oryzae* in soybean agar after 48 hours of incubation

In Vivo

The Disease Severity (DS) and Disease incidence (DI) were measured on the first and second week after the spraying of spore suspension. One-way ANOVA and Post-hoc/ Multiple Comparison Test (Tukey HSD) were used to analyze the DS and DI of the set-ups. The latter test was used to compare the fungal treatments with control set-ups (positive and negative controls) and/or other fungal treatments in order to determine if the fungal treatments were capable of inhibiting the growth of *X. oryzae*.

As a basis, the positive control was treated with commercial BLB stopper which surely inhibits BLB in rice, while the negative control is the which untreated set-up which shows no inhibition against BLB in rice.

While it is true that three varieties of rice (susceptible, resistant, and intermediate) were tested, statistical analysis showed that there is no significant difference among the results of the varieties.

The significance values of *T. harzianum* against the control set-ups (positive and negative controls) and/or other fungal treatments (*M. anisopliae* and VAM). On the first week (Week 1) after the spraying of spore suspension, comparison between *T. harzianum* and positive control showed significant difference which a p-value of 0.038 and 0.014, respectively (Table 2). However, a significant difference was also obtained for the comparison between *T. harzianum* and negative control for all the three replicates having the same p-value of 0.000. The comparison between *T. harzianum* and *M. anisopliae* showed a p-value higher than 0.05. This indicated that there is no significant difference on the measured DS and DI between *T. harzianum* and *M. anisopliae*. Since *M. anisopliae* was found to be capable to inhibit BLB in rice that no

significant difference with the positive control (Table 2). It can be inferred that *T. harzianum* and *M. anisopliae* were relatively similar effect on the inhibition of BLB in rice. Hence, *T. harzianum* was still capable to inhibit BLB in rice.

Table 2. Significance values obtained for the determination of the inhibiting ability of *Trichoderma harzianum* against BLB in rice versus the control set-ups and/or other fungal treatments

	Trichoderma harzianum	**Replicate 1**	**Replicate 2**	**Replicate 3**
Week 1	Vs. Positive Control	0.038*	0.003*	0.014*
	Vs. Negative Control	0.000*	0.000*	0.000*
	Vs. *M. Anisopliae*	0.963	1.000	0.836
	Vs. VAM	0.032*	0.004*	0.033*
Week 2	Vs. Positive Control	0.315	0.618	0.725
	Vs. Negative Control	0.000*	0.000*	0.000*
	Vs. *M. Anisopliae*	0.594	0.770	0.439
	Vs. VAM	0.014*	0.009*	0.020

Note: Values with (*) showed significant difference

The 2nd week after the spraying of spore suspension, comparison between *T. harzianum* and positive control showed no significant difference for all the three replicates. Replicate 1, Replicate 2, and Replicate 3 obtained p-values of 0.315, 0.618, and 0.725, respectively. Whereas comparison between *T. harzianum* and negative control showed significant difference for all the three replicates having a p-value of 0.000. Since higher p-values were obtained in VAM vs. positive control, the null hypothesis (H_O) is accepted. This indicates that the DS and DI measured a week after the second spraying of *T. harzianum* at 10 x10^2 cfu/ml have no significant difference to the DS and DI obtained for the positive control. This implies that *T. harzianum* is relatively similar to the positive control which can inhibit BLB in rice. Therefore, *T. harzianum* is capable of inhibiting BLB in rice as same results were obtained for all the three replicates.

In addition, the Disease Severity (D.S.) of the three replicates was reduced by an average of 5.34%by the first two weeks of spraying.

Table 3. Significance values obtained for the determination of the inhibiting ability of *Metarhizium anisopliae* against BLB in riceversus the control set-ups and/or other fungal treatments

	Metarhizium anisopliae	**Replicate 1**	**Replicate 2**	**Replicate 3**
Week 1	Vs. Positive Control	0.124	0.002*	0.082
	Vs. Negative Control	0.000*	0.000*	0.000*
	Vs. *T. harzianum*	0.963	1.000	0.836
	Vs. VAM	0.108	0.004*	0.199
Week 2	Vs. Positive Control	0.984	0.996	0.996
	Vs. Negative Control	0.001*	0.000*	0.000*
	Vs. *T. harzianum*	0.594	0.770	0.439
	Vs. VAM	0.209	0.077	0.382

Note: Values with (*) showed significant difference

Table 3 showed the significance values of *M. anisopliae* against the control set-ups (positive and negative controls) and/or other fungal treatments (*T. harzianum* and VAM). On the first week (Week 1) after the spraying of spore suspension, a value higher than the p-value of 0.05 was obtained for the comparison between *M. anisopliae* and positive control, and a value lower than 0.05 was obtained for the comparison between *M. anisopliae* and negative control. This is true for Replicate 1 having a value of 0.124 (*M. anisopliae* vs. positive control) and 0.000 (*M. anisopliae* vs. negative control), and Replicate 3 having a value of 0.082 (*M. anisopliae* vs. positive control) and 0.000 ((*M. anisopliae* vs. negative control). Since it was in the *M. anisopliae* vs. positive control where a higher value than the p-value of 0.05 was obtained, the null hypothesis (H_O) is accepted rather than the alternate hypothesis (H_A). This implies that the DS and DI measured a week after spraying *M. anisopliae* spore suspension at 1.2 x 10^{11} cfu/ml has no significant difference to the DS and DI obtained for the positive control. In other words, the effectiveness of the commercial BLB stopper when it comes to the inhibition of BLB in rice is relatively similar to the effect of *M. anisopliae* on the inhibition of the disease. This means that the *M. anisopliae* is capable of inhibiting BLB in rice. This result only applies for Replicate 1 and Replicate 3. Conversely, in Replicate 2, a value lower than the p-value of 0.05 was obtained for the comparison between *M. anisopliae* and both of the control set-ups. However, in order to tell where the effect or the disease severity obtained for *M. anisopliae* is nearer, it was compared to the two other fungal treatments (*T. harzianum* and VAM). Results in Table 2 showed that a value of 1.000, higher than the p-value of 0.05, was obtained when *M. anisopliae* was compared to *T. harzianum*. And, a value of 0.004, lower than the p-value of 0.05, was obtained when *M. anisopliae* was compared to VAM. This means that the DS and DI measured in the plants

treated with *M. anisopliae* had no significant difference to that of the plants treated with *T. harzianum*. The effectiveness of the *T. harzianum* when it comes to the inhibition of BLB in rice was relatively similar to the effect of *M. anisopliae* on the inhibition of the said disease. This means that for Replicate 2, *M. anisopliae* is still capable of inhibiting BLB in rice.

On the 2nd week after the spraying of spore suspension, a value higher than the p-value of 0.05 was obtained for the comparison of the effect of *M. anisopliae* and positive control, and a value lower than the p-value of 0.05 were obtained for the comparison of the effect of *M. anisopliae* and negative control. This is visible on all replicates (Table 3). Because it is in the comparison between *M. anisopliae* and positive control where the significance value was greater than the p-value of 0.05, the alternate hypothesis (H_A) was accepted and the null hypothesis (H_O) is rejected. Therefore, the DS and DI of the rice plants treated with *M. anisopliae* measured a week after the second spraying had no significant difference to the DS and DI measured in the positive control. The effectiveness of the commercial BLB stopper in terms of the growth of inhibition or eradication of BLB in rice was relatively similar to the effect of *M. anisopliae* when it come to the inhibition of BLB. This strongly implies that *M. anisopliae* is capable of inhibiting BLB in rice as same results were obtained.

Moreover, the Disease Severity (D.S.) of the three replicates was reduced by an average of 1.06% by the first two weeks of spraying.

Table 4 showed the significance values of VAM against the control set-ups (positive and negative controls) and/or other fungal treatments (*T. harzianum* and *M. anisopliae*). On the first week (Week 1), no significant difference was obtained for the comparison between VAM and positive control with a p-value higher than 0.05, while a significant difference was obtained for the comparison between VAM and negative control with a p-value lower than 0.05. This applies for the three replicates. Since p-values of 1.000, 0.970, and 0.953 for Replicate 1, Replicate 2 and Replicate 3, respectively, were obtained in VAM vs. positive control, the null hypothesis (H_O) is accepted. This indicates that the DS and DI measured a week after spraying spore suspension of VAM at 1.2 x 10^{11} cfu/mlhas no significant difference to the DS and DI obtained for the positive control. Thisfurther implies that VAM is relatively similar to the positive control which can inhibit BLB in rice. Therefore, VAM was capable of inhibiting BLB in rice as same results were obtained for all the three replicates.

Table 4. Significance values obtained for the determination of the inhibiting ability of Vesicular Arbuscular Mycorrhizae (VAM) against BLB in riceversus the control set-ups and/or other fungal treatments

	VAM	Replicate 1	Replicate 2	Replicate 3
Week 1	Vs. Positive Control	1.000	0.970	0.953
	Vs. Negative Control	0.024*	0.000*	0.000*
	Vs. *T. harzianum*	0.032*	0.004*	0.033*
	Vs. *M. Anisopliae*	0.108	0.004*	0.199
Week 2	Vs. Positive Control	0.438	0.200	0.278
	Vs. Negative Control	0.067	0.000*	0.000*
	Vs. *T. harzianum*	0.014	0.009*	0.020*
	Vs. *M. Anisopliae*	0.209	0.077	0.382

Note: Values with (*) showed significant difference

When compared with the other treatments, VAM showed significant difference with *T. harzianum* in the three replicates with p-values of 0.032, 0.004, and 0.033 for Replicate 1, Replicate 2 and Replicate 3, respectively. Whereas VAM showed no significant difference with *M. anisopliae* in Replicate 1 and Replicate 3 with p-values of 0.108 and 0.199, respectively. This indicates that both VAM and *M. anisopliae* generally have relatively similar effect in inhibiting the disease compared to *T. harzianum*, although VAM showed a significant difference with *M. anisopliae* in Replicate 2.

As for the 2nd week, it was revealed that VAM had no significant difference with the positive control with a p-value higher than 0.05. This is visible for the three replicates. On the other hand, VAM had a significant difference with the negative control with a p-value lower than 0.05. This applies for Replicate 2 and Replicate 3. A p-value higher than 0.05 was obtained in VAM vs. positive control, thus the null hypothesis (H_O) is accepted. This indicated that the DS and DI measured the week after the second spraying of spore suspension of VAM at 1.2 x 10^{11} cfu/ml has no significant difference to the DS and DI obtained for the positive control. This further implies that VAM was relatively similar to the positive control which was capable of inhibiting BLB in rice. Therefore, VAM was capable of inhibiting the disease despite having no significant difference with the negative control in Replicate 1. When compared with the other treatments, VAM showed significant difference with *T. harzianum* with p-values of 0.014, 0.009, and 0.020 for Replicate 1, Replicate 2 and Replicate 3, respectively, while it showed no significant difference with *M. anisopliae* with a p-value of 0.209, 0.077 and 0.382 for Replicate 1, Replicate 2 and Replicate 3, respectively. This implies that both VAM and *M. anisopliae* had relatively similar effect in inhibiting the disease compared to *T. harzianum* as same results were observed for all the three

replicates. The comparison between VAM and *M. anisopliae* obtained on the second week of spraying spore suspensions was consistent with the comparison which obtained in the first week of spraying the spore suspension.Furthermore, the Disease Severity (D.S.) of the three replicates was reduced by an average of 0.293% by the first two weeks of spraying.

In Vitro

For the antagonistic test, spread plate method was done to evenly distribute the bacteria all over the Nutrient agar plate. Three hundred microliter (300 µl) of broth inoculated with bacteria which has a similar turbidity as that of a 4.0 MacFarland standard was transferred to each of the plates. This turbidity of the broth is equivalent to an approximate bacterial density of 12 x 10^8 cfu/ml. Further trials on spread plating showed that 300-µl volume of broth is the most suitable volume that can occupy and cover the nutrient agar media without being too runny when tilted or too bare and patchy. The placement of a five millimeter diameter *T. harzianum* and *M. anisopliae* plugs and disk containing commercial BLB stopper at the center of the bacterial lawn were done on separate plates after 48 hours of incubation of the bacterial lawn at 37°C. Three replicates were made. The set-ups were initially observed 48 hours after the placement of the fungal plug.

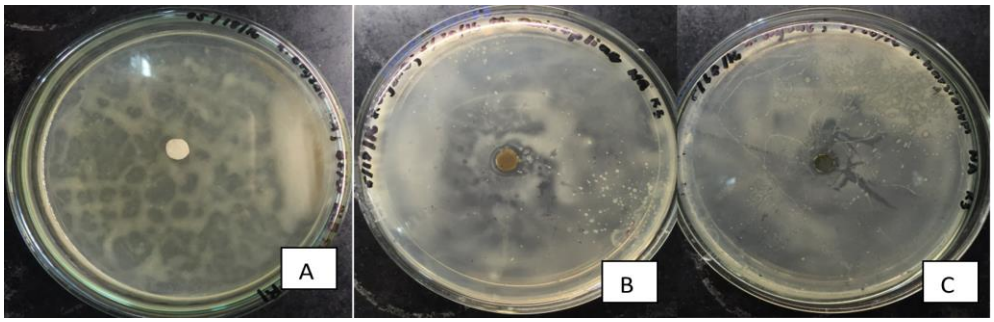

Figure 7. Antagonistic test against *X. oryzae*. A 5 mm diameter of *T. harzianum* (A) and *M. anisopliae* (B)plugs and commercial BLB stopper discs (C) were transferred at the center of two-day old *Xanthomonas oryzae* lawn. The *X. oryzae* (300 µm) was spread all over the Nutrient Agar media in three separate petri plates using Spread Plate technique prior to the transfer of fungal plug.

Trichoderma harzianum

The results for the inhibition of *X. oryzae* against *T. harzianum* was negligible because of the incompatibility of medium. *X. oryzae* and the *T. harzianum* failed to grow together in the same medium, which, in this case, was Nutrient agar. The growth of *X. oryzae* was only observed on Soybean agar, while the growth of *T. harzianum* was only observed in Potato Dextrose Agar. Although Nutrient Agar is a general media, the components and the conditions required for the growth of *X. oryzae* were not supplemented by N.A.

Metarhizium anisopliae

M. anisopliae also showed growth inhibition against *X. oryzae* with the production of clear zones after 48 hours of incubation. On replicates 1 and 2, clear zones of 0.2 cm were observed while on replicate 3, a clear zone of 0.4 cm was observed. However, formations of clear zones are considered inconclusive.

Commercial BLB Stopper (Positive Control)

Experimental results revealed that BLB Stopper was not able to inhibit *X. oryzae* due to the absence of inhibition zone in all replicates. Results are inconclusive.

***Trichoderma harzianum* as potential biocontrol for BLB**

Based on the results, the spraying of the spore suspension of *T. harzianum*at 10 $x10^2$ cfu/ml after the first week and second week of spraying has significantly reduced the DS and DI of the plant leaves infected with BLB by 5.34%. *T. harzianum* and positive control also showed no significant difference for all the three replicates with obtained p-values of 0.315, 0.618, and 0.725, respectively. According to a study conducted by Gangwar (2013), the reduction of the severity of the disease is an indication that the *T. harzianum* as a fungal bioagent is capable of proliferating and establishing on the surface of the rice host.

According to Ou (1985), *Xanthomonas oryxzae pv oryzae* enters through the hydathodes at the leaf tip and leaf margin of the rice leaf. Curtis (1943) adds that the cells on the surface of the leaf may be suspended on the guttation fluid as it exudes at night. The cells can then enter the plant either by swimming or through the fluid that is withdrawn into the leaf in the morning (Curtis, 1943). After bacterial multiplication in the intercellular spaces of the underlying epitheme, they can then enter and spread out through the xylem (Noda and Kaku, 1999). Aside from this, Ou (1985) says that Xoo can also enter the xylem through the wounds or openings that resulted from the roots

emerging at the base of leaf sheath. Upon entering the xylem, the bacteria can already interact with the xylem parenchyma cells, moving vertically through the leaf's primary veins, and laterally through the commissural veins (Hilaire *et al.,* 2001). Just after a few days, formation of beads or strands of exudate can already be observed. According to Mew and company (1993), it is a characteristic of Bacterial Leaf Blight that resulted from the bacterial cells that filled the xylem vessels and oozed out from the hydathodes, making it a possible source of secondary inoculum.

There are different mechanisms by which *T. harzianum* can serve as a biocontrol agent against foliar pathogens. Among the most studied mechanisms include mycoparasitism, competition, and antibiosis (Elad, 2000). In antibiosis, Trichoderma species are known to be capable of releasing antibiotics and various chemicals that can harm the pathogens and inhibit their growth (Leelavathi, *et. al.,* 2014).

The fungi's ability to colonize and penetrate root tissues, and to induce series of changes in the plant's morphology and biochemistry, result to an Induced Systemic Resistance in the entire plant (ISR). Induced Systemic Resistance is an important mechanism for biocontrol in vegetative tissues that is caused by different microorganisms including *T. harzianum* to protect the plant from soil or foliar pathogens (Paulitz and Matta, 2000). Aside from this, *T. harzianum* is also known because of its ability of producing protease as one of its biocontrol mechanisms (Elad and Kapat, 1999). Observations from a study conducted by Elad and Kapat (1999) indicate that *T. harzianum* secretes proteolytic enzyme on the leaves infected with a pathogen (*B. cinerea*), which then leads to the reduction of the germination and disease development caused by the pathogen. It was also observed that these proteases secreted by *T. harzianum* were able to deactivate the hydrolytic enzymes produced by the pathogen.

However, results for antagonistic tests of *T. harzianum* against *X. oryzae* in vitro are inconclusive because of the incompatibility of the medium used for the growth of *X. oryzae* and *T. harzianum. X. oryzae* grew best on Soybean agar, while *T. harzianum* grew best on Potato Dextrose Agar (PDA). Although Nutrient Agar (N.A.) is a general media, specific components and conditions required for the growth of *X. oryzae* were not supplemented by N.A.

Separate studies by Leelavathi *et al.* (2014) and Parthasarathy (2014) revealed positive results wherein *Trichoderma* was observed to be highly effective in inhibiting the growth of *X. oryzae* in vitro using Muller Hinton medium. However, when the same methodology was used, isolates of *X. oryzae* still failed to grow on Muller Hinton medium. In addition, other media like Saboraud Dextrose Agar (SDA), Muller Hinton Agar (M.H.A.), and Wakimoto

Agar were also used in an attempt to look for a medium where bacteria and the fungi would grow together for an unbiased antagonistic test, but all of the trials were unsuccessful. Sivasithamparam and Ghisalberti (1998), strains of Trichoderma can also produce 40 metabolites that can contribute to their antibiotic activity, aiding to their effectivity against species of Gram positive and Gram negative bacteria (Leelavathi, *et. al.,* 2014)

According to Bhatttacharjee and company (2014), Trichoderma species are known to produce antibiotics and antifungal toxic metabolites. Because of their ability to secrete enzymes like glucanase, chitinase, cellulose and protease, they are capable of degrading the cell wall of pathogens, which in turn inhibits their growth (Bhatttacharjee *et al.,* 2014). These antibiotics, specifically trichodermin and harzianolide that the Trichoderma strains produce, in combination with hydrolytic enzymes, lead to a higher level of antagonism (Howel, 1998).

Aside from this, *Trichoderma* species also have mycoparasitic ability which works against economically important plant pathogens, allowing the development of biocontrol strategies (Harman, *et. al.*, 2004; Motlagh *et al.*, 2013). As aggressive competitors, Trichoderma species do not only grow very fast, but they also rapidly colonize substrates to eliminate pathogens (Papavizas, 1985).

All of these mechanisms can act alone or in combination to increase the efficiency of *T. harzianum* as a biocontrol agent against pathogens that enter through leaves (Leelavathi, *et. al.,* 2014). On separate studies conducted by Gangwar and Sinha (2012) and Kumar and company (2009), the same results were also observed wherein *T. harzianum* was able restrict the severity of *Xanthomonas oryzae* in rice.

Metarhizium anisopliae as potential biocontrol for BLB

The spraying of the spore suspension of *M. anisopliae* at 1.2 x 10^{11} cfu/ml after the first week and second week of spraying was proven to be efficient in suppressing the DS and DI of the plant leaves infected with BLB according to the results. Furthermore, there was an average Disease Severity (D.S.) reduction of 1.06% upon the treatment of *M. anisopliae.*

Biological control with *M. anisopliae* offers a long-lasting control to various insects and plant pathogens (including pathogenic bacteria in rice) without imparting damage to the environment and to non-target organisms (Universal Bio-organic and Multiservices Pvt. Ltd. 2008). Some studies showed that the fungi were internally colonized the plant leaves, petioles and stem (Batta, 2013). However, the mechanism by which *M. anisopliae* enters the plant (for the release of compounds and secondary metabolites) after foliar

spray is still unclear. Additionally, *M. anisopliae* is capable of colonizing plant roots where it concurrently acts as biofertilizer and biopesticide in order to boost plant growth (Gao *et al.,* 2011).

The life cycle of *M. anisopliae*, entomopathogenic fungi, is associated with both the synthesis and secretion of various active metabolites such as extracellular enzymes and low-molecular weight compounds (i.e. toxins). These toxic metabolites or by-products primarily aids the *M. anisopliae* to resist and guard themselves against invading pathogens whether bacteria, fungi or insects. *M. anisopliae*, and entomopathogenic fungi in general, produce a wide variety of secondary metabolites with different activities. Their activities include antibiotic effect, release of cytotoxic substances, insecticidal effect, release of compounds which induce or inhibit growth of pathogen, attractor, repellent, and the like. However, the chemical constitution and metabolic fluxes of the *M. anisopliae* differ depending on ecological conditions. Particularly, *M. anisopliae* secrete a wide array of relatively low molecular weight secondary metabolites wherein some of which have antibiotic properties whereas others are vital pathogenicity determinants. Even though these metabolites are pertained to be toxins, only little is known regarding their properties, production, as well as spatial distribution (Ravindran *et al.,* 2014).

In particular, *M. anisopliae* and other entomopathogenic fungi are considered as unique outstanding microbial entities due to their ingenious ability to synthesize plethora of bioactive compounds and from the dependence of their morphological differentiation. For instance, *M. anisopliae* produces a cyclodepsipeptide called destruxin that inhibits the growth of various pathogenic bacteria (i.e. *Xanthomonas oryzae*) (Male *et al.,* 2009). The secondary metabolites play an essential role in improving the fungal adaptability to endure diverse natural habitats. In addition, these metabolites also act as signaling molecules for the establishment of niche in fungal-plant or fungal-pathogen interactions, and may also serve as stress protectors. Nonetheless, the exact function of many secondary metabolites in *M. anisopliae* is still unknown. Various chemical classes have been identified in *M. anisopliae* and the entire *Metarhizium* genus. These are cytochalasins C and D, myroridins, destruxin A,B and E, viridoxin, swainsonine, helvonic acid, 12-hydroxyovalicin, hydroxy- fungerin, 7-desmethyl analogues of fusarin C and (8Z)-fusarin C, serinocyclins A and B, as well as aurovertins (Male *et al.,* 2009). These metabolites are toxic to various insects and pathogenic microbes (including bacteria like *Xanthomonas oryzae*) (Gao *et al*., 2011; Male *et al.,* 2009). Almost all of these metabolites can be isolated from mycelia or from fermentation extracts of *M. anisopliae*. There is lesser information on the secondary metabolites exclusively present in the conidia of *M. anisopliae*

(Ravindran *et al.*, 2014). These metabolites, especially destruxin, allows morphological and cytoskeletal changes in various insects and pathogenic bacteria. They harmfully affect the pathogen's cellular immune responses namely encapsulation and phagocytosis (Male *et al*., 2009).

Vesicular Arbuscular Mycorrhizae (VAM) as potential biocontrol for BLB

Based on the results, the spraying of the spore suspension of VAM at 1.2 x 10^{11} cfu/ml after the first week and second week of spraying has significantly reduced the DS and DI of the plant leaves infected with BLB by 0.293%. This indicates the potential of VAM to be utilized as biocontrol agent against BLB. According to studies, mychorrhiza-induced protection is provided by the improvement of plant nutrition and the consequent compensation of the damages caused by the pathogen (Trotta *et al*. 1996; Fritz 2006; Liu *et al.* 2007)). With the advancement on the understanding of the physiology and regulation of the Arbuscular Mycorrhiza (AM) symbiosis, it was further revealed that symbiosis with AM may involve the activation of plant defense mechanisms and changes in the plant architecture, root exudation, and even in the populations of microbes in the rhizosphere (Azcón-Aguilar and Barea 1996; Whipps 2004). AM symbiosis also confer resistance or tolerance in plants against biotic stresses. The impact of the symbiotic relationship of VAM with plants in terms of resistance and tolerance to biotic stresses depends on the AM fungal isolates and can be modulated by environmental conditions (Pozo and Azcón-Aguilar, 2007).

Many studies have proven that AM symbiosis had greatly influenced the inhibition or the reduction of disease severity and incidence caused by soil-borne pathogens such as fungi, bacteria and oomycetes (Whipps, 2004). Conversely, fewer studies have focused on the effect of AM symbiosis on above-ground diseases caused by biotrophic and necrotrophic pathogens and shoot pathogens.

According to studies, AM symbiosis shows a positive effect on plant resistance against shoot diseases such as diseases caused by the bacterial pathogen *X*. *campetris* in Medicago (Liu *et al*., 2007) and by the necrotrophic fungus *Alternaria solani* in tomato (Fritz *et al.*, 2006; De La Noval *et al*. 2007). Another study also reveals that AM establishment in tomatoes infected with phytoplasma, specialized obligate parasites of phloem tissue, leads to the reduction of the symptoms of the disease (Lingua *et al.*, 2002).

In general, two main mechanisms are considered on how AM symbiosis affects plant pathogens. One is the potential changes in the nutrient levels of the host plant and alterations of the source-sink relation within the plant which may

dictate the susceptibility of the plant to the pathogens. The other possible mechanism is the modulation of the plant defense mechanisms of the plant (Koltai and Kapulnik, 2010).

Further studies revealed that in the general spectrum of protection by mycorrhiza, jasmonates are considered the major regulators in conferring systemic resistance to Mycorrhiza Induced Resistance (MIR) plants. MIR was revealed to be influenced by the priming of JA-dependent defenses of plants (Hause *et al*. 2007 and Hause and Schaarschmidt, 2009). Studies conducted by Pozo *et.al* (2009) have supported that MIR is associated to the priming of JA-dependent defences. In their study, they compared the response of non-mycorrhizal and AM tomato plants to foliar application of the pathogen *Botrytis cinerea.* Results have shown that there was a stronger induction of JA-regulated genes in mycorrhizal plants confirming a primed response. It was also observed that the expansion of the necrotic lesions was strikingly lower in leaves of the mycorrhizal plants. Furthermore, the levels of pathogen in the tissues of the mycorrhizal plants were also lower which confirms an induced systemic resistance in the plant.

Results from the said studies infer that the inhibition of VAM on the growth of *X. oryzae* is possibly due to mychorrhiza-induced protection and enhanced nutrient levels conferred by AM symbiosis on the rice plants.

Commercial BLB Stopper (Positive Control)

Various chemicals such as bleaching powder and broad spectrum antibiotics have been tested for the chemical control of Bacterial Leaf Blight (BLB) in rice. The use of chemical agents is proven to be effective for controlling diseases by either killing the pathogen or inhibiting the multiplication of the pathogen (Thirumalesh, 2012; Kim, *et al.*, 2015). For this study, BLB Stopper 20sc, a commercial bacteriacide-fungicide agent, was used as the positive control to compare the inhibitory effect of the fungal treatments. For the in-vivo, a Disease Severity (D.S.) reduction of 16% was recorded upon the treatment of positive control.

The active ingredient of the commercial agent is 20% thiadiazole-copper. According Paranjape and company (2014), common bacterial diseases such as blight, leaf spot and blotch are controlled by copper compounds. Copper compounds are known for their antibacterial property and they are commonly used to control diseases in crops like wheat, barley, and rice. Copper ions are toxic to bacteria and they increase the lipophilic character of bacterial cell walls which makes them more susceptible to penetration of substances (Ovadia, 1985). However, due to the phytotoxicity of copper, copper-based bacteriacides

are mixed with copper-chelating fungicides such as as manzoceb or maneb or with Ferric chloride ($FeCl_3.6H_2O$). Some of the major bacteriacides are bronopol, hexachlorophene, acibenzolar-s-meethyl, benzalkonium chloride, kasugamycin, nitrapyrin, oxylinic acid, probenazole, streptomycin and copper-containing bacteriacides such as copper hydroxide and thiadiazole-copper (Paranjapeet *et al.,* 2015). Gleason and company (1993) also verified the efficiency of copper-containing products in the reduction of foliar leaf and fruit spotting.

Several studies have revealed that efficiency of copper-containing bacteriocides, however, this study showed contrasting results in-vitro. This is may be due to the other components of BLB Stopper Sc20 (not specified in the label) which may have reacted with the components of the media used that may have enhanced the growth of the pathogen, rather than suppressing its growth or there is something lacking in the in-vitro for effective reaction to ensue. The inhibitory effect of the commercial agent may not also be evident in-vitro but is more evident when applied in the field due to several factors such as physical conditions and the interaction of the commercial agent with the infected plant and with the pathogen in the plant.

Conclusion

Xanthomonas oryzae is the major and sole cause of Bacterial Leaf Blight (BLB) in rice. Three fungal treatments (*Trichoderma harzianum, Metarhizium anisopliae,* and Vesicular Arbuscular Myccrorhizae) were utilized to determine their probable antagonistic effect to *X. oryzae*. For in-vitro tests, incompatibility of media was the primary reason why the inhibition zones observed in the positive control and in the three (3) fungal treatments are considered inconclusive. For in-vivo tests, statistical analysis of the DS and DI of the plants treated separately with the spore suspensions of *T. harzianum, M. anisopliae,* and VAM were determined to be relatively/ significantly different to the DS and DI obtained for the negative control set-up. This is true for all replicates of plants that were treated with each of the fungal spore suspensions. Results imply that the effectiveness of the three fungal treatments is relatively similar to the effect of the commercial BLB stopper when it comes to the inhibition and antagonism of BLB in rice. Furthermore, calculation of percent reduction of Disease Severities showed that there is a 5.34% reduction of BLB disease in plants treated with *T. harzianum*, 1.06% in plants treated with *M. anisopliae,* and 0.293% in plants treated with VAM. Therefore, the fungi used were confirmed to be effective biocontrol agents against BLB in rice. They are

environment-friendly and cheaper substitutes for chemically-based bactericides against BLB, thus limiting the use of harmful chemicals.

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