DISEASE MANAGEMENT AND MICROALGAL BIOFERTILIZATION FOR RICE PRODUCTION

A Thesis

by

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ABSTRACT

Sheath blight (ShB) caused by *Rhizoctonia solani* AG1-IA, narrow brown leaf spot (NBLS) caused by *Cercospora janseana* and fertility are among the most important factors limiting rice production in the U.S. Greenhouse experiments were conducted to better understand the effects of temperature and cultivar resistance on the biocontrol efficacy of *Bacillus subtilis* strain MBI 600 for management of ShB. Also, lab and field trials were conducted to evaluate the efficacy of fungicides for control of *C. janseana* and NBLS. In addition, greenhouse and field trials were conducted to explore the use of microalgae-based biofertilizers for rice production. In the first study in the greenhouse, plants of two rice cultivars (moderately resistant and susceptible to ShB) were spray treated with strain MBI 600 and subjected to different temperatures for 24 hours in dew growth chambers. Disease severity was assessed after 8 days of incubation. In the second study under *in vitro* conditions, fungicide sensitivity of *C. janseana* based on the percentage relative germination and the effective concentration that inhibited 50% of conidia germination were assessed. A field trial was also conducted in 2012 and 2014 to evaluate the efficacy of fungicides for control of NBLS and yield improvement. In the third study, greenhouse and field trials were conducted to evaluate the effects of N_2-fixing cyanobacterial biofertilizer, microalgal biomass concentrate, and urea fertilizer on rice plant height and yield.

In the first study, temperature significantly affected the relative biocontrol efficacy of strain MBI 600 in reducing ShB development in either cultivar. Its efficacy linearly increased with the increase of temperature, reaching the maximum at 35 or 40°C.

In the second study, the succinate dehydrogenase inhibitor fungicides fluxapyroxad and flutolanil were most and least effective, respectively, in inhibiting *C. janseana* conidia, indicating that there was no cross-resistance between fluxapyroxad and flutolanil. Fluxapyroxad, propiconazole alone and in combination with azoxystrobin

or trifloxystrobin were highly effective controlling NBLS. However, azoxystrobin was not effective to control NBLS.

In the third study in the greenhouse, microalgal biomass concentrate treatment significantly improved rice plant height. However, no biofertilizer treatments improved rice yield in the field.

DEDICATION

To my God and my whole family.

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TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF FIGURES viii

LIST OF TABLES ix

CHAPTER I INTRODUCTION 1

CHAPTER II EFFECT OF TEMPERATURE ON THE EFFICACY OF *Bacillus subtilis* STRAIN MBI 600 FOR BIOCONTROL OF SHEATH BLIGHT IN RICE 4

- Introduction 4
- Materials and Methods 6
- Results 8
- Discussion 11

CHAPTER III EFFECT OF FUNGICIDES ON CONIDIA GERMINATION OF *Cercospora janseana* AND DISEASE SEVERITY ON RICE 14

- Introduction 14
- Materials and Methods 15
- Results 19
- Discussion 24

Page

CHAPTER IV LIMITED EFFECTS OF MICROALGAL BIOFERTILIZERS FOR RICE PRODUCTION IN TEXAS 32

Introduction 32
Materials and Methods 33
Results 38
Discussion 42

CHAPTER V CONCLUSIONS 47

REFERENCES 49

APPENDIX A 61

APPENDIX B 64

LIST OF FIGURES

FIGURE		Page
1	Effect of temperature on *in vitro* mycelial growth of *R. solani*.	9
2	Effect of rice cultivar and biocontrol treatment on severity of sheath blight (ShB) in rice.	9
3	Effect of temperature on sheath blight (ShB) severity of rice plants treated with the biocontrol or untreated (control)...	10
4	Effect of temperature on the relative efficacy of *B. subtilis* strain MBI 600 for biocontrol of sheath blight in rice.	11
5	Relative germination percentage according to each *C. janseana* isolate.	20
6	Relative germination percentage for *C. janseana* isolates according to each active ingredient..	21
7	Narrow brown leaf spot (NBLS) severity in rice fields.	25
8	Rice yield (kg ha^{-1}) according to each fungicide for the field study on 2012 (A), and 2014 (B)..	26
9	Over time effect of different fertilization treatments on rice plant height (cm) for Exp 1 (A), Exp 2 (B), and Exp 3 (C) under greenhouse conditions.	40
10	Effect of different fertilizers on rice yield for the field study during 2013 (A) and 2014 (B).	42

LIST OF TABLES

TABLE		Page
1	Description of fungicides used for the control of NBLS under field conditions	18
2	Analysis of variance for the effect of experiment, isolate, active ingredient, and the interaction of isolate by active ingredient in the relative germination percentage of *C. janseana* isolates	20
3	EC_{50} values (µg mL^{-1}) of five *C. janseana* isolates for different fungicide active ingredients.	23

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa*) is one of the world's most important food crops (Khush 1997). It feeds more than the half of the world's population. However, rice production is affected by various diseases and low nitrogen use efficiency.

Sheath blight (ShB), caused by *Rhizoctonia solani* Kühn AG1-IA [teleomorph *Thanatephorus cucumeris* (A. B. Frank) Donk], is the most important disease in the southern rice-growing region in the United States (Lee and Rush 1983; Marchetti 1983). Up to 50% in yield losses due to sheath blight have been reported (Lee and Rush 1983). Sheath blight can cause symptoms on leaf sheaths, leaf blades and even panicles, resulting in poorly filled grains (Lee and Rush 1983). High rate of nitrogen fertilizer application, susceptible cultivars, cultivation of semi dwarf as well as high plant density aggravates the spread of the pathogen (Wang et al. 2011; Lee and Rush 1983; Kalpana et al. 2006). Currently, rice producers have limited tools for management of this disease other than synthetic fungicides. No accepted levels of resistance are available in the commercial rice cultivars (Mew et al. 2004; Pinson et al. 2005; Xu et al. 2011). Biocontrol agents constitute an environmentally-friendly alternative for the control of sheath blight. *Bacillus subtilis* MBI 600 has been shown to be effective in controlling *R. solani* under lab, greenhouse and field conditions (Kumar et al. 2011a; Kumar et al. 2011b; Kumar et al. 2012). Understanding the impact of temperature on the efficacy of *B. subtilis* will enhance applicability of *B. subtilis* and help to develop an effective biocontrol method for rice sheath blight management. The objective of this study was to determine the effect of temperature on the efficacy of *B. subtilis* MBI 600 for control of sheath blight in rice.

Narrow brown leaf spot (NBLS) is caused by the fungus *Cercospora janseana* (Biswas 2006). Although NBLS was considered a minor disease three decades ago (Ou 1985), it has become one of the major diseases limiting rice production in the United

States, especially on the late plantings and the ratoon crop in Texas and Louisiana (Zhou 2014). The fungus grows slowly, with periods of 20-30 days of incubation reported (Estrada and Ou 1978). Symptoms of this disease are characterized by short, linear red-brown lesions on leaves, restricted between the veins (Biswas 2006). In some cases, *C. janseana* causes grain discoloration and chalkiness, resulting in a decreased market value of rice (Kurniawati et al. 2009). Control of NBLS has been attempted through breeding for race-resistant cultivars; however, rice cultivars may become susceptible within a few years because of the development of new races of the fungus (Sah and Rush 1988). Chemical control can be an effective option to control NBLS. However, failures to control NBLS using some fungicides have been observed in Texas (Zhou personal communication). Understanding the sensitivity of *C. janseana* populations to new and existing fungicides would be helpful to develop more effective fungicide management program for NBLS. The objectives of this study were to determine the inhibitory effect of different active ingredients of fungicides labeled for the control of *C. janseana* under *in vitro* conditions, and evaluate the effect of different fungicides on NBLS severity and rice yield in the field.

Rice is affected by low nitrogen fertilizer efficiency due to nitrogen losses (De Datta and Buresh 1989). It has been mentioned that nitrogen fertilization mainly increases methane emissions (Banger et al. 2012). On the other hand, numerous studies have supported the importance of using cyanobacteria as a biofertilizer in tropical (Shukla and Gupta 1967; Tirol et al. 1982; Mandal et al. 1999; Jha et al. 2013) and temperate rice-growing regions (Henriksson 1971; Reynaud and Metting 1988). Cyanobacteria possess vegetative cells that are responsible for photosynthesis, and heterocystous cells that fix atmospheric N_2 (Sinha and Häder 1996). After cyanobacterial decomposition, most of the fixed products are made available to rice. Cyanobacteria have additional advantages such as, release of phosphate, improvement of soil structure (Mandal et al. 1999), decrease in Fe toxicity (Das et al. 1991), as well as growth-promoting effects in different crops (Shukla and Gupta 1967; Karthikeyan et al. 2007; Prasanna et al. 2013). The use of economically and environmentally-friendly algae-

based biofertilizers in paddy rice fields present a promising alternative of synthetic fertilizers. The objective of this study was to evaluate the contribution of different microalgal biofertilizers including microalgal biomass concentrate, live N_2-fixing cyanobacteria, as well as conventional fertilizer (urea) to rice plant growth and yield.

CHAPTER II

EFFECT OF TEMPERATURE ON THE EFFICACY OF *Bacillus subtilis* STRAIN MBI 600 FOR BIOCONTROL OF SHEATH BLIGHT IN RICE

INTRODUCTION

Rice is one of the world's most important food crops (Khush 1997) and it is a major staple food among two-thirds of the world's population (Batres-Marquez et al. 2009). However, rice production is threatened by various plant diseases. Sheath blight, caused by the soilborne fungus *R. solani,* is the second major disease in terms of economic importance in major rice-growing countries (Banniza and Holderness 2001). In the United States, sheath blight is the most economically-important rice disease (Lee and Rush 1983; Marchetti 1983). Up to 50% in yield losses due to sheath blight can occur on susceptible cultivars (Lee and Rush 1983).

Sheath blight can cause symptoms on leaf sheaths, leaf blades and even panicles (Lee and Rush 1983). Sclerotia and mycelium in infected plant debris are the main source of inoculum for the development of this disease (Ou 1985; Kobayashi et al. 1997). Initial infection starts when sclerotia float and attach rice plant sheaths at the water line in flooded fields. Kumar et al. (2011d) state that symptoms start with green-grey water soaked lesions that first appear on lower rice sheaths at the late tillering or early internode elongation stage, then lesions expand with bleached appearance surrounding by a brown border. They furthermore describe that under favorable conditions with more than 95% relative humidity and temperature of 28 - 32°C, the disease spreads to leaf blades and other upper parts of plants by runner hyphae. The pathogen is also able to infect panicles, resulting in poorly filled grains, especially in the lower portion of the panicle (Lee and Rush 1983). In addition, the disease also causes lodging, resulting in a significant loss in yield.

Use of susceptible semi-dwarf cultivars and modern cultural practices such as high rates of nitrogen fertilizer and high plant density tend to increase the incidence and

severity of sheath blight worldwide (Lee and Rush 1983; Kalpana et al. 2006; Wang et al. 2011). Currently, rice producers have limited tools to manage this disease. No accepted levels of resistance are available in the commercial rice cultivars (Mew et al. 2004; Pinson et al. 2005; Xu et al. 2011). Farmers heavily depend on the use of synthetic fungicides.

Over the past two decades, as chemical alternatives, there has been an increased interest in the biological control of rice diseases (Kumar et al. 2011a). One example of the biological control agent is plant growth-promoting rhizobacteria (PGPR) (Kumar et al. 2011a). *Bacillus* spp. and fluorescent *Pseudomonas* spp. are among the most widely studied PGPRs for control of sheath blight (Kumar et al. 2011d). *B. subtilis* PGPR strains, including strain MBI 600 have demonstrated antifungal activities against *R. solani* (Kumar et al. 2011a; Kumar et al. 2011b; Kumar et al. 2012). Biocontrol properties of strain MBI 600 were compatible with commonly used synthetic fungicides in rice (Kumar et al. 2012). This strain even improved rice seed germination and seedling growth under both laboratory and greenhouse conditions (Kumar et al. 2011a).

Mechanisms of action associated with the biocontrol activities of *B. subtilis* include antibiosis, competition for infection sites, and induced systemic resistance. Antibiosis in *B. subtilis* includes production of iturin and fengycin antibiotics that suppress fungal pathogens (Moyne et al. 2001; Romero et al. 2007). Inhibition of bacterial quorum sensing (QS) as a new biocontrol mechanism has been reported before (Kumar, A. et al. 2011). Bacterial enzymes produced by *Bacillus* isolates can inhibit the QS of phytopathogenic bacteria. Induced systemic resistance by *B. subtilis* has been reported to contribute to antifungal activities (Choudhary and Johri 2008; García-Gutiérrez et al. 2012; Chowdappa et al. 2013). The enhancement of plant growth and health by *Bacillus* PGPRs is due to the synthesis of plant growth regulators such as auxins and gibberellic acid induced by *Bacillus* species. (Broadbent et al. 1977).

Temperature and leaf wetness duration are among the most important environmental factors affecting the growth and activity of plant pathogens (Huber and Gillespie 1992). The optimum temperatures for the mycelial growth of *R. solani* are in

the range of 25 to 30°C (Goswami et al. 2011; Orozco-Avitia et al. 2013). Prolonged periods of leaf wetness duration are necessary for the mycelial progression of *R. solani* on the rice host plant (Yang et al. 1990).

Environmental factors also affect the survival and growth of biocontrol agents and their antimicrobial activities and biocontrol efficacy (Mathre et al. 1994; Schmidt et al. 2004). Understanding the impact of environmental factors on the biocontrol efficacy of *B. subtilis* will enhance applicability of *B. subtilis* and help to develop an effective biocontrol method for rice sheath blight management. The objective of this study was to determine the effect of temperature on antifungal activity of *B. subtilis* strain MBI 600 for control of sheath blight.

MATERIALS AND METHODS

Effect of temperature on mycelial growth of *R. solani*. An *in vitro* Petri dish assay was conducted to determine the effect of temperature on the mycelial growth of *R. solani* on potato dextrose agar (PDA) medium. A PDA agar plug (0.5 cm in diameter) grown with 2-day-old *R. solani* was placed at the center of a Petri dish containing PDA (10 cm in diameter). Plates were sealed with parafilm, placed in plastic bags, and incubated at 10, 15, 20, 25, 30, 35, or 40°C in dew growth chambers for 24 h. There were three plates per temperature treatment and the diameter of mycelial growth was measured after 24 h of incubation.

Effects of cultivar and temperature on antifungal activity of *B. subtilis*. This study was conducted in the greenhouse at Texas A&M AgriLife Research and Extension Center at Beaumont. Two rice cultivars were used: Jasmine 85 (moderately resistant to sheath blight) and Presidio (susceptible to sheath blight). Rice seeds were surface sterilized in 3% sodium hypochlorite solution for 5 min, washed three times with distilled water, and kept in Petri dishes filling with distilled water at 28°C. After 3 days, germinated seeds were planted in 2.5-Liter-pots containing a soil mix of League type soil (pH 5.5, 3.2% sand, 32.4% silt, 64.4% clay, and 3.8 - 4.8% organic matter) and potting mix (Potting mix, Miracle-Gro) in a rate of 1:1 (v/v). Four seeds of each cultivar

were planted per pot. After 13 days of planting, plants were thinned to three plants per pot.

B. subtilis MBI 600 was harvested by washing off the bacteria in sterile distilled water from 5-days-old cultures grown in King's B agar medium using sterile cotton swabs. The concentration of bacterial suspension was determined by hemocytometer and adjusted to 1×10^8 cells/mL. Tween 20 (Sigma-Aldrich) was added at 0.1% (v/v) to the adjusted suspension. At 16 days after seeding, plants were sprayed with a suspension (1×10^8 cells/mL) of *B. subtilis* MBI 600 until run-off (approximately 10 mL/plant). The treated plants were air dried, and each plant was inoculated with *R. solani* by placing one PDA plug (0.5 cm in diameter) grown with 2-day-old mycelium on the surface of sheath above the soil line. Plants inoculated with *R. solani* without treatment of *B. subtilis* served as the positive controls. Treated plants were placed and kept at 10, 15, 20, 25, 30, 35, or 40°C in dew growth chambers (~100% humidity) for 24 h. Treated plants were then moved out the dew chambers and air dried. Plants were covered with 2-liter transparent soft drink bottles to maintain a high level of humidity inside the bottles and maintained in a greenhouse with temperatures ranging from 22 to 33°C under natural light conditions. After 8 days in the greenhouse, disease severity was measured according to the 0-9 scale, where 0 represented no disease symptoms and 9 represented more than 80% of rice tissue affected (Groth et al. 1990).

Data analyses. The experimental design was a split-split plot design, with the cultivar as whole plots, biocontrol treatment as subplots, and temperature as sub-subplots. All the factors were randomized. Each temperature treatment was conducted in the same dew chamber. The experiment was conducted three times. Relative efficacy of biocontrol at specific temperature was calculated based on the following formula: ((Disease severity of the untreated control - Disease severity of biocontrol treatment) ÷ Disease severity of the untreated control) × 100.

Mycelial growth and disease severity data were subjected to analyses of variance using SAS 9.3 (SAS Institute Inc., Cary, NC). Differences among treatment means were estimated using Fisher's protected least significance difference (LSD) at $P = 0.05$.

RESULTS

Effect of temperature on mycelial growth of *R. solani*. The effect of temperature on mycelial growth of *R. solani* were highly significant ($P < 0.01$). Mycelial diameter of *R. solani* increased with the increase of temperature from 10 to 25°C, but decreased with the increase of temperature from 25 to 40°C (Fig. 1). The maximum growth of *R. solani* mycelium occurred in the range of 20 to 30°C while the minimum growth was in the range of 10 to 15°C and 35 to 40°C.

Effect of cultivar and temperature on antifungal activity of *B. subtilis*. The effects of cultivar and biocontrol treatment on severity of sheath blight were highly significant ($P < 0.01$). Severity of sheath blight was significantly lower on the cultivar Jasmine 85 than Presidio (Fig. 2). The biocontrol treatment significantly decreased sheath blight severity on both cultivars, reducing disease severity by 47% on Presidio and 42% on Jasmine 85.

The effects of temperature and biocontrol treatment on severity of sheath blight were highly significant ($P \leq 0.01$). Sheath blight disease development with or without biocontrol treatment responding to various temperatures showed a similar bell-shaped curve, but significantly less disease were developed with the biocontrol treatment ($P \leq 0.05$) (Fig. 3). Sheath blight severity in the non-biocontrol treatment increased with the increase of temperature from 10 to 25°C, and decreased with the increase of temperature from 25 to 40°C. Sheath blight severity in the biocontrol treatment increased with the increase of temperature from 10 to 30°C, and decreased with the increase of temperature from 30 to 40°C, downing to the lowest at 40°C.

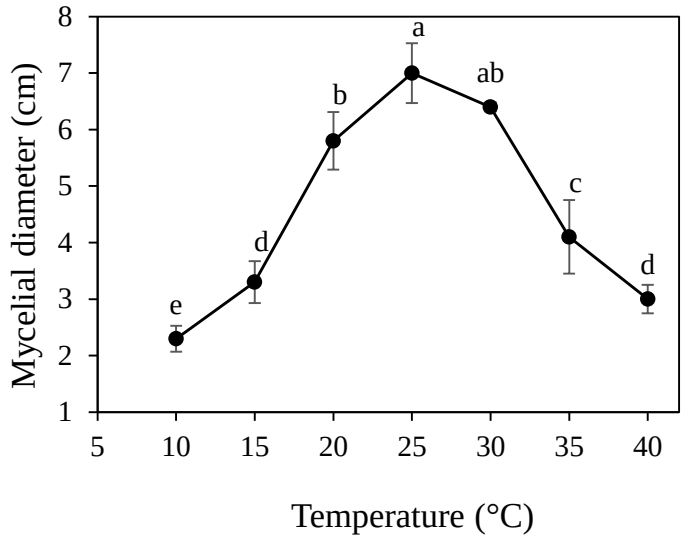

Fig. 1. Effect of temperature on *in vitro* mycelial growth of *R. solani*. The diameter of mycelium growth on PDA was measured at 24 h of incubation. Dots with the same letter are not significantly different according to LSD test ($P = 0.05$). Error bars represent the standard deviation of the mean.

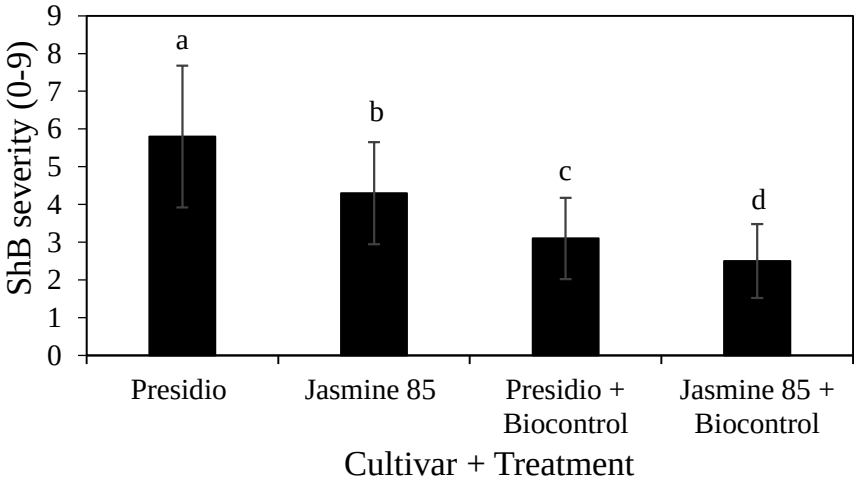

Fig. 2. Effect of rice cultivar and biocontrol treatment on severity of sheath blight (ShB) in rice. Columns with the same letter are not significantly different according to LSD test ($P = 0.05$). Error bars represent the standard deviation of the mean.

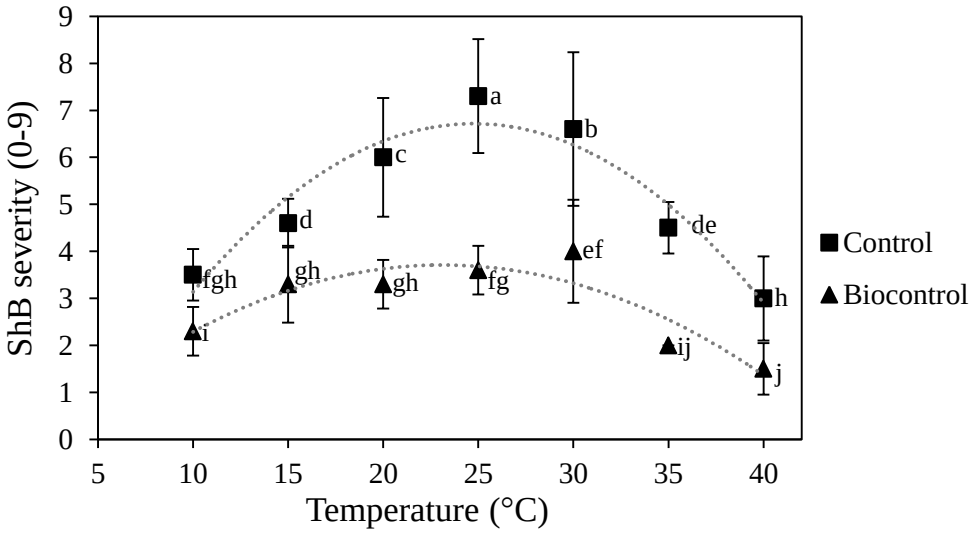

Fig. 3. Effect of temperature on sheath blight (ShB) severity of rice plants treated with the biocontrol or untreated (control). Data are averaged over the two rice cultivars Presidio and Jasmine 85 evaluated. Symbols with the same letter are not significantly different according to LSD test (P = 0.05). Error bars represent the standard deviation of the mean. Multiple regression equations for the untreated control and biocontrol treatments, respectively, were y= -0.0164 x^2 + 0.8136 x - 3.3571 (R^2 = 0.9185) and y = -0.0082 x^2 + 0.3812 x - 0.7 (R^2 = 0.8221) where y = disease severity (0-9) and x = temperature (°C).

The relative efficacy of the biocontrol treatment linearly (R^2 = 0.5737, P < 0.05) increased with the increase of temperature from 10 to 40°C (Fig. 4). The relative efficacy at 10, 15, 20, 25, 30, 35, and 40°C was 34, 28, 45, 51, 39, 56, and 50%, respectively.

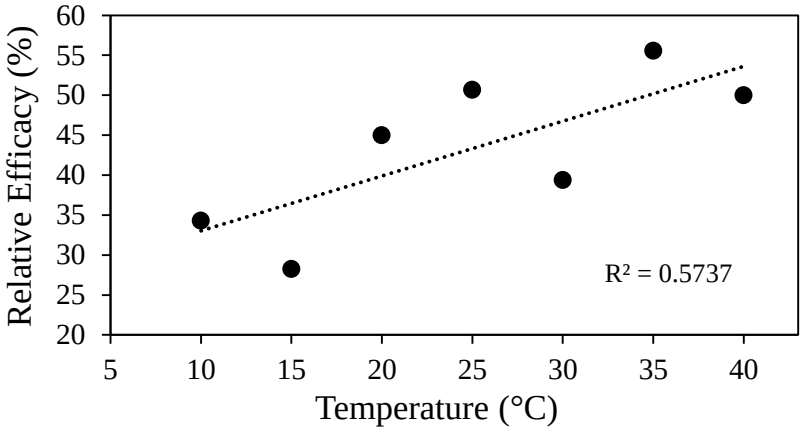

Fig. 4. Effect of temperature on the relative efficacy of *B. subtilis* strain MBI 600 for biocontrol of sheath blight in rice.

DISCUSSION

This study elucidates the impact of micro-environmental conditions on the development of sheath blight and the biological control effectiveness of *B. subtilis* strain MBI 600. The optimum temperature for the sheath blight development in rice was in the range of 25 to 30°C, which is consistent with the range of temperature most favorable for rice sheath blight development under field conditions reported in a previous study (Rush and Lee, 1992). Our greenhouse inoculation study also indicates that the development of sheath blight was slow at lower temperature (10 or 15°C) and suppressed at higher temperature (35 and 40°C). These trend patterns on the effects of temperature on sheath blight development in rice are in a good agreement with the patterns on the effects of temperature on the growth of mycelium of *R. solani* on PDA medium in the current study.

The biocontrol treatment was effective in reducing sheath blight on both Jasmine 85 and Presidio cultivars (moderately resistant and susceptible to sheath blight, respectively). The moderate level of resistance of Jasmine 85 is conferred by a non-allelic dominant major resistance gene (Pan et al. 1999). The biocontrol treatment

reduced sheath blight by 47% in Presidio and 42% in Jasmine 85, demonstrating that the effect of biocontrol is similar along the level of susceptibility or resistance of the cultivar. The best reduction in disease severity occurred when Jasmine 85 was treated with the biocontrol. These results suggest that the combination of the biocontrol treatment and cultivar resistance can be a more effective approach for management of sheath blight of rice than using the biocontrol agent alone.

In the current study, the biocontrol treatment was effective in reducing sheath blight severity at each of the seven temperatures evaluated. However, the relative efficacy of the biocontrol treatment varies with temperature. Its relative efficacy at 10, 15, 20, 25, 30, 35 and 40°C was 34, 28, 45, 51, 39, 56, and 50%, respectively, indicating that *B. subtilis* was more effective at higher temperatures (35 and 40°C) than at lower temperatures. Results from the regression analysis demonstrate a positive linear relationship of the relative biocontrol efficacy with the increase of temperature. This finding explains why some of the biocontrol agents, including strain MBI 600, frequently lack the consistent performance of biocontrol under field conditions. Temperature in the field varies with location and season, and constantly fluctuates with time during a day. The condition of warm temperatures and heavy dew formation at night is likely to promote infection of *R. solani* but may be not the most favorable for *B. subtilis*. The results of our study not only validate the efficacy of *B. subtilis* for suppression of rice sheath blight under the greenhouse conditions reported in previous studies (Kumar et al. 2011a; 2011b; 2012) but also extend the results of the previous studies by improving our understanding of the impact of temperature on the efficacy of biocontrol agents.

An increase in the relative efficacy of *B. subtilis* with the increase of temperature might be associated with the unique biological properties of *B. subtilis*. *B. subtilis* is tolerant against heat, desiccation and other extreme stresses by forming endospores (Waldburger et al. 1993). Production of endospores from vegetative cells of *B. subtilis* is a mechanism to overcome environmental limitations like increased temperature (Driks 2002, Setlow 2006). Setlow (2006) reported that the major factor determining spore wet

heat resistance is the core water content. Spores formed at higher temperatures generally have lower water contents than do spores formed at lower temperatures, with the later spores having lower wet heat resistance than the former (Setlow 2006). Heat shock treatment of *B. subtilis* cells during sporulation increases the heat resistance of spores formed subsequently, as a consequence of the generation of heat shock proteins (Movahedi and Waites 2000). As a result, it is possible that in the present study higher temperatures were likely to induce *B. subtilis* to produce heat-resistant endospores, resulting in an increase in the relative biocontrol efficacy.

To our knowledge, this is the first study to demonstrate the impact of temperature on the efficacy of *B. subtilis* MBI 600, a promising microbial biocontrol agent. Results of this study can help to improve the biocontrol approach of using *B. subtilis* strain MBI 600. Combined use of *Bacillus subtilis* strain BMI 600 with partially-resistant cultivar can maximize the biocontrol effectiveness and provide a more reliable tool for management of sheath blight in rice.

CHAPTER III

EFFECT OF FUNGICIDES ON CONIDIA GERMINATION OF *Cercospora janseana* AND DISEASE SEVERITY ON RICE

INTRODUCTION

Narrow brown leaf spot (NBLS) is caused by *Cercospora janseana* (Biswas 2006). NBLS occurs in almost all rice-growing countries in Asia, Latin America, Africa, USA, Australia, and Papua New Guinea (Misra et al. 1994). NBLS severity varies from year to year and usually becomes more severe as the rice approaches to maturity (Kurniawati et al. 2009). Although NBLS is considered a minor disease in most countries (Dissanayake and Wickramasinghe 1999), certain rice cultivars have shown a high susceptibility in the United States during 1930 and 1940 (Ou 1985). Recent increases in the incidence of NBLS have been reported in the U. S. rice production belt (Mani et al. 2012). Particularly, NBLS is one of the major diseases limiting the yield potential of rice ratoon crop in Texas and Louisiana (Zhou 2014).

Upon infestation, *C. janseana* enters the host tissues through the stomata, becoming established in the parenchyma immediately beneath the stomata and spreads longitudinally in the epidermal cells (Ou 1985). The mycelium is mostly intracellular, and the conidiophores are produced from sub-stomatal hyphal branches (Tullis 1937). An incubation period of 20 days is necessary before the first few lesions appear, regardless of the growth stage at which the plant was inoculated, and 30 days before the maximum number of lesions is reached (Estrada and Ou 1978). Symptoms development at the late growth stages in rice is due to the slow disease progress by *C. janseana* (Estrada and Ou 1978).

Symptoms are characterized by many short, linear red-brown lesions on the leaf blades, restricted between the veins (Biswas 2006). Lesions are 2-10 mm long and 0.5-1 mm wide, and later they coalesce to form long, threadlike brown lesions parallel to the veins on the entire leaves (Biswas 2006). In resistant cultivars, lesions tend to be

narrower, shorter and dark brown, while in susceptible cultivars, lesions are wider and lighter brown with gray necrotic centers (Biswas 2006). Lesions also occur on leaf sheaths, pedicels and glumes (Ou 1985). In some cases, symptoms may include premature death of leaves and leaf sheaths, premature ripening of kernels and lodging of plants (Kurniawati et al. 2009). Market values of grains decrease because *C. janseana* causes grain discoloration and chalkiness, and reduces the milling recovery (Kurniawati et al. 2009).

Breeding for race-resistant cultivars has been attempted (Ryker and Jodon 1940). However, rice cultivars with vertical resistance may become broken down quickly after their release. The development of new physiologic races of *C. janseana* is plausible since the fungus is known to be a highly variable pathogen (Sah and Rush 1988). Another common approach to manage NBLS is to use synthetic fungicides. Singh (1988) reported that NBLS could be managed with mancozeb in India. In the U. S., propiconazole is commonly used for NBLS. However, ineffective chemical control of NBLS in the southern U. S. Rice Belt region has been experienced. The objectives of this study were to determine the inhibitory effect of different active ingredients of fungicides for control of *C. janseana* and NBLS under *in vitro* and field conditions.

MATERIALS AND METHODS

Isolate collections. Five isolates of *C. janseana* were from leaves collected from rice fields in Texas. Isolate BMT-41 was collected from an organic rice field at the Texas A&M AgriLife Research Center at Beaumont; CN-1-16, CN-2-4, and CN-1-4 were collected in a commercial rice field in China, Texas; and EL-110 was collected from a rice research plot at the Texas A&M AgriLife Research Center at Eagle Lake. All these isolates were collected in 2011. All isolates were maintained in sterile desiccated filter paper at 4°C.

Fungicide sensitivity. Conidia from each of *C. janseana* isolates were streaked on potato dextrose agar (PDA) medium amended with 1 μg mL^{-1} streptomycin, and incubated under a growth chamber condition (25°C; 16 h of light and 8 h of darkness;

86.63 μmol m^{-2} s^{-1} light intensity). After 5 days of incubation, conidia of each isolate were harvested with sterilized cotton swaps and distilled water. The conidial suspension was collected into 15 mL plastic tubes and adjusted to 10^6 conidia mL^{-1}. Aliquots of 200 μl conidia suspension were spread on PDA plates amended with fungicides. Each of the fungicide active ingredients, azoxystrobin, pyraclostrobin, trifloxystrobin, propiconazole, triadimefon and myclobutanil (Bayer CropScience), thiophanate methyl (Makhteshim Agan of North America, Inc.), mancozeb (Bonide Products Inc.), fluxapyroxad and flutolanil (Nichino America, Inc.) were dissolved in 100% sterile distilled water. Salicylhydroxamic acid (SHAM; Alfa Aesar) was dissolved in 100% methanol. Active ingredients from each fungicide were added to autoclaved PDA cooled to 55°C to make a final concentration of 0.1, 1, or 10 μg mL^{-1} . Additionally, 100 μg mL^{-1} of SHAM were added to PDA plates amended with the quinone outside inhibitors (QoI) active ingredients such as azoxystrobin, pyraclostrobin, and trifloxystrobin in order to inhibit the alternative oxidase respiratory pathway (Olaya et al. 1998). Plates amended with SHAM alone served as control plates for treatments with the active ingredients belonging to the QoI, while PDA plates without amendment served as control for the remaining active ingredients. Three plates per active ingredient concentration were used for each isolate. Petri plates were arranged in a completely randomized design.

After incubation at 20°C in darkness for 15 h, conidia germination rate (based on 30 randomly selected conidia) from each plate was determined under the optical microscope with the 40 × magnification by scanning the plate surface. Percentages of relative germination for each plate were calculated as (number of germinated conidia from the fungicide-amended plate ÷ number of germinated conidia from the control plate) × 100. All relative germination percentage data were divided by the constant 1000 and then arcsine transformed. The experiment was replicated three times. Arcsine transformed data were subjected to analysis of variance by the statistical software SAS 9.3 (SAS Institute Inc., Cary, NC). Differences among treatment means were estimated using Fisher's protected least significance difference (LSD) at $P = 0.05$. Means were

back-transformed to percentage relative germination and multiplied by the constant 1000. EC_{50} values for each experiment were calculated by applying the value of 50% in the dependent variable obtained from the regression equation of relative germination percentage on log_{10} transformed fungicide concentration using Microsoft Excel 2013.

Field study. Two field experiments, one in 2012 and the other in 2014, were established in rice fields naturally infested with NBLS at the Texas A&M AgriLife Research Wintermann Rice Research Station, Eagle Lake. The rice cultivar used in both studies was Presidio that was drill seeded at a rate of 89.6 kg ha^{-1} into a Crowley fine sandy loam soil (59% sand, 2% silt, 12% clay, 0.7% organic matter, and pH 5.3). Each plot consisted of six 4.88 m rows, spaced 19 cm between rows. Prior planting, plots received 280 kg ha^{-1} of fertilizer (19-19-19, N-P-K), and then at panicle differentiation 320.56 kg ha^{-1} of fertilizer (21-0-0, N-P-K) was applied in both experiments.

In 2012, 1.26 kg a.i. ha^{-1} of Command 3ME (Clomazone; Helena Chemical Company) and 1.61 kg a.i. ha^{-1} of Propanil 4SC (Propanil; Willowood USA) were applied 11 days after planting. At 32 days after planting, 0.07 kg a.i. ha^{-1} of Permit (Halosulfuron-methyl; Gowan Company) and 0.29 kg a.i. ha^{-1} of Facet 75DF (Quinclorac; BASF Corporation) were applied. At 46 days after planting, 1.61 kg a.i. ha^{-1} of Propanil 4SC and 0.07 kg a.i. ha^{-1} of Permit were applied for weed control. At 47 days after planting, plots were fertilized with 195.03 kg ha^{-1} of urea (46-0-0) and then 0.028 kg a.i. ha^{-1} of the insecticide Mustang Max (S-Cyano (3-phenoxyphenyl)methyl (+) cis/trans 3-(2,2-dichloroethenyl)- 2,2 dimethylcyclopropane carboxylate; FMC Corporation) was applied for control of harmful insects.

In 2014, 0.028 kg a.i. ha^{-1} of RiceBeaux (Propanil and Thiobencarb; RiceCo USA), 0.336 kg a.i. ha^{-1} of Command 3ME, and 0.056 kg a.i. ha^{-1} of Permit were applied 15 days after planting for weed control. At 18 days after planting, 427.04 kg ha^{-1} of fertilizer (21-0-0, N-P-K) was applied to plots. For insect control, 0.028 kg a.i. ha^{-1} of Mustang Max was applied 53 days after planting.

In both experiments, treatments were arranged in a randomized complete block design with eight replicates in 2012 and three replicates in 2014. Fungicides (Table 1)

were applied at 68 and 84 days after planting in 2012 and 2014, respectively. Plots were sprayed with fungicides using a CO_2 pressurized sprayer equipped with a boom of three TeeJet 8002 nozzles spaced 40.64 cm apart that delivered 299.33 L ha^{-1} of final spray volume. NBLS severity was visually rated at 51 and 33 days after treatment application for the first and second experiment, respectively. Disease severity was based on the scale of 0 to 9, where 0 represents no symptoms, 1 represents less than 1% of leaf area affected, 3 represents 1-5% of leaf area affected, 5 represents 6-25% of leaf area affected, 7 represents 26-50% of leaf area affected, 9 represents 51 -100% leaf area affected (Chaudhary 1996).

Table 1. Description of fungicides used for the control of NBLS under field conditions.

Fungicide name	**Rate in mL ha^{-1}**	**% Active ingredient(s)**	**Manufacturer**
Quadris	658	22.9% Azoxystrobin	Syngenta Crop Protection, Inc., Greensboro, NC
Quilt	1,561	7% Azoxystrobin + 11.7% Propiconazole	Syngenta Crop Protection, Inc., Greensboro, NC
QuiltXcel	1,162	13.5% Azoxystrobin + 11.7% Propiconazole	Syngenta Crop Protection, Inc., Greensboro, NC
Sercadis	483	26.55% Fluxapyroxad	BASF Corporation, Research Triangle Park, NC
Stratego	1,330	11.4% Propiconazole + 11.4% Trifloxystrobin	Bayer CropScience, Research Triangle Park, NC
Tilt	700	41.8% Propiconazole	Syngenta Crop Protection, Inc., Greensboro, NC

Rice was harvested with a plot combine at 124 and 132 days after planting in 2012 and 2014, respectively. Grain yield and moisture were determined and rice yields

were adjusted to 12% moisture content. Data was subjected to ANOVA using SAS 9.4 (SAS Institute Inc., Cary, NC). Differences among treatment means were estimated using Fisher's protected least significance difference (LSD) at $P = 0.05$.

RESULTS

Fungicide sensitivity. Highly significant differences were detected in the relative germination percentage among experiments, isolates, active ingredients, and the interaction of isolate by active ingredient (Table 2). Isolate CN-2-4 had the highest germination (89%), while germination significantly decreased to 73, 72, and 71% with isolates CN-1-16, EL-110, and BMT-41, respectively. However, isolate CN-1-4 had the lowest germination (57%) (Fig. 5).

C. janseana isolates presented similar trends in the relative germination percentages along the different active ingredients; therefore, the effect of isolate is combined and depicted in Fig. 6. Any Demethylation inhibitor (DMI) active ingredient (myclobutanil, propiconazole, or triadimefon) resulted in the highest germination percentages in the range of 97 to 135%. Germination decreased to 85% with the dithiocarbamate (DTC) mancozeb; however, this percentage did not significantly differ from the germination obtained with triadimefon. Sensitivity to fungicides was more notorious with the Quinone outside inhibitors (QoI) pyraclostrobin, trifloxystrobin and azoxystrobin, since germinations significantly decreased to 69, 64, and 61%, respectively. A further decrease in germination was obtained with the methyl benzimidazole carbamate (MBC) thiophanate methyl that resulted in 55%. However, the lowest germination resulted with the succinate dehydrogenase inhibitor (SDHI) fluxapyroxad that was 10%. This low percentage germination contrasted significantly with the SDHI flutolanil that resulted in 98% in germination.

Table 2. Analysis of variance for the effect of experiment, isolate, active ingredient, and the interaction of isolate by active ingredient in the relative germination percentage of *C. janseana* isolates**.**

Source	Degrees of Freedom	Mean Square [a]	F Value [a]
Experiment	2	0.070	11.44**
Isolate (I)	4	0.172	28.12**
Active Ingredient (AI)	9	0.963	157.32**
I × AI	36	0.085	14.00**

** = Highly significant ($p<0.01$).
[a] = Values resulted from the analysis of variance of arcsine transformed percentage relative germination.

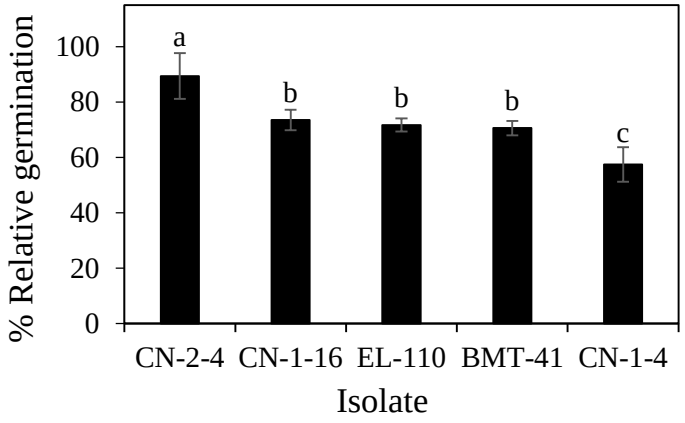

Fig. 5. Relative germination percentage according to each *C. janseana* isolate. Columns with the same letter are not significantly different ($P = 0.05$). Error bars represent the standard error of the mean.

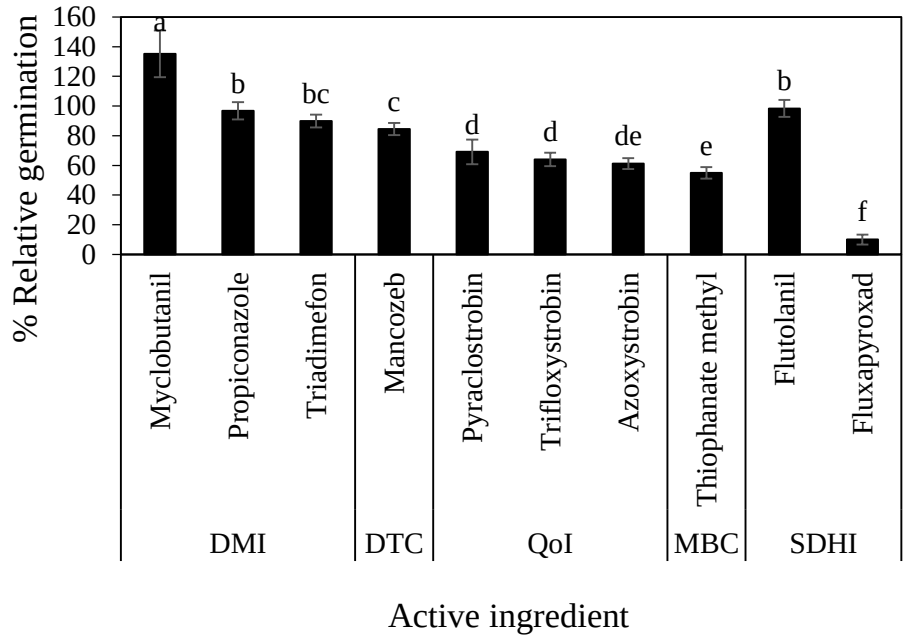

Fig. 6. Relative germination percentage for *C. janseana* isolates according to each active ingredient. Columns with the same letter are not significantly different ($P = 0.05$). Error bars represent the standard error of the mean. DMI, demethylation inhibitors; DTC, dithiocarbamate; QoI, quinone outside inhibitors; MBC, methyl benzimidazole carbamate; and SDHI, succinate dehydrogenase inhibitors.

Because of variance among the EC_{50} values in each experiment for the QoI active ingredients, thiophanate methyl, and mancozeb, EC_{50} values for each experiment are depicted in Table 3. In general, EC_{50} values demonstrated that isolate CN-2-4 was less sensitive to fungicides in contrast to the other isolates. This isolate resulted mainly in EC_{50} values higher than 10 µg mL^{-1} for all QoI (azoxystrobin, pyraclostrobin, and trifloxystrobin), as well as to mancozeb and flutolanil; however, this isolate was sensitive to thiophanate methyl with EC_{50} values in the range of 0.04 to 3.41 µg mL^{-1}, as well as to fluxapyroxad with EC_{50} values of 0.04 to 0.06 µg mL^{-1}. In general, sensitivity to azoxystrobin, pyraclostrobin, and trifloxystrobin, as well as to thiophanate methyl, and mancozeb was isolate-dependent, since each isolate resulted in contrasting

sensitivities along these active ingredients. However, mancozeb resulted in low efficacy to inhibit fungal conidia, since all isolates but CN-1-16 mainly resulted in EC_{50} values higher than 10 µg mL^{-1}. On the other hand, sensitivity to the SDHI fluxapyroxad and flutolanil was active ingredient-dependent, since all isolates were sensitive to fluxapyroxad with EC_{50} values lower than 0.22 µg mL^{-1}. While all isolates were less sensitive to flutolanil, considering that EC_{50} values were higher than 10 µg mL^{-1} along the different experiments (Table 3).

Field study. Significant ($P < 0.05$) and highly significant ($P < 0.01$) differences for the 2012 and 2014 experiments, respectively, were detected in the NBLS severity among the different fungicides. In the 2012 experiment (Fig. 7A), NBLS severity was highest in the untreated control plots (3.4), while disease severity decreased significantly to 2.4, 2.1, 2.0, 1.9, 1.7, and 1.5 with Quadris, Sercadis, Tilt, QuiltXcel, Stratego, and Quilt, respectively. Application of Quilt resulted in least level of NBLS severity, making it become most effective fungicide (56% disease reduction) in 2012. In 2014 (Fig. 7B), plots treated with Quadris had NBLS severity as high as the untreated control. Disease severity significantly decreased to 3.2, 2.7, 2.0, 1.8, and 1.2 with QuiltXcel, Stratego, Quilt, Tilt, and Sercadis, respectively. Application of Sercadis resulted in least level of disease severity, making it become most effective fungicide (70% disease reduction) in 2014. These results demonstrated that disease severity decreased significantly when plots were treated with any fungicide contrasting with the untreated control, with the exception of Quadris that had a poor performance in 2014.

Table 3. EC_{50} values (µg mL^{-1}) of five *C. janseana* isolates for different fungicide active ingredients.

Group name	Active ingredient[a]	BMT-41			CN-1-16			CN-1-4			CN-2-4			EL-110		
		Experiment			Experiment			Experiment			Experiment			Experiment		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
QoI[b]	Azoxystrobin	9.48	5.33	6.05	0.08	5.33	0.03	>10	9.97	0.07	>10	4.47	>10	0.53	>10	4.35
	Pyraclostrobin	0.36	0.36	0.83	3.99	3.89	>10	0.10	0.54	0.01	>10	>10	>10	0.16	0.33	0.34
	Trifloxystrobin	5.56	0.36	>10	>10	>10	>10	0.13	5.16	0.06	>10	0.09	>10	5.79	>10	8.87
MBC	Thiophanate methyl	1.34	5.37	5.98	>10	>10	>10	0.06	3.18	0.03	0.44	3.41	0.04	>10	>10	>10
DTC	Mancozeb	>10	>10	>10	0.40	>10	0.48	>10	>10	0.05	0.36	>10	>10	2.23	>10	>10
SDHI	Fluxapyroxad	0.03	0.04	0.04	0.03	0.21	0.04	0.03	0.04	0.03	0.06	0.04	0.04	0.03	0.04	0.04
	Flutolanil	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10

[a] Active ingredients belonging to the Demethylation inhibitor (DMI) group are not depicted since they had little or no suppression in conidia germination.

[b] Abbreviations: QoI = quinone outside inhibitors; MBC = methyl benzimidazole carbamate; DTC = dithiocarbamate; SDHI = succinate dehydrogenase inhibitors

Significant ($P < 0.05$) differences were detected in rice yield among the different fungicides in both 2012 and 2014. In 2012 (Fig. 8A), application of Sercadis resulted in 11,082 kg ha^{-1} of yield that did not significantly differ from the yield of QuiltXcel, Quilt, Tilt, and Stratego that resulted in 10,960; 10,778; 10,766, and 10,765 kg ha^{-1}, respectively. However, Quadris resulted in 10,614 kg ha^{-1} that was significantly lower than the yield of Sercadis. Nevertheless, untreated control resulted in 10,175 kg ha^{-1} that was lowest in yield. In 2014 (Fig. 8B), applications of Stratego, Quilt, Quadris, QuiltXcel, Sercadis, and Tilt resulted in 8,049; 7,965; 7,807; 7,787; 7,753, and 7,694 kg ha^{-1} of rice yield, respectively, which did not differ significantly among each other. These results demonstrated that plants treated with any fungicide resulted in significantly increased yields compared to the untreated control.

DISCUSSION

The results of conidia germination assays demonstrate a wide range of sensitivity of *C. janseana* from very sensitive to highly resistant levels against major fungicides used for rice disease management. The wide variability of fungicide sensitivity prospects the existence of diverse field populations of *C. janseana* in the Texas rice production areas. This explains why inconsistent or ineffective control of NBLS was observed in the field over years.

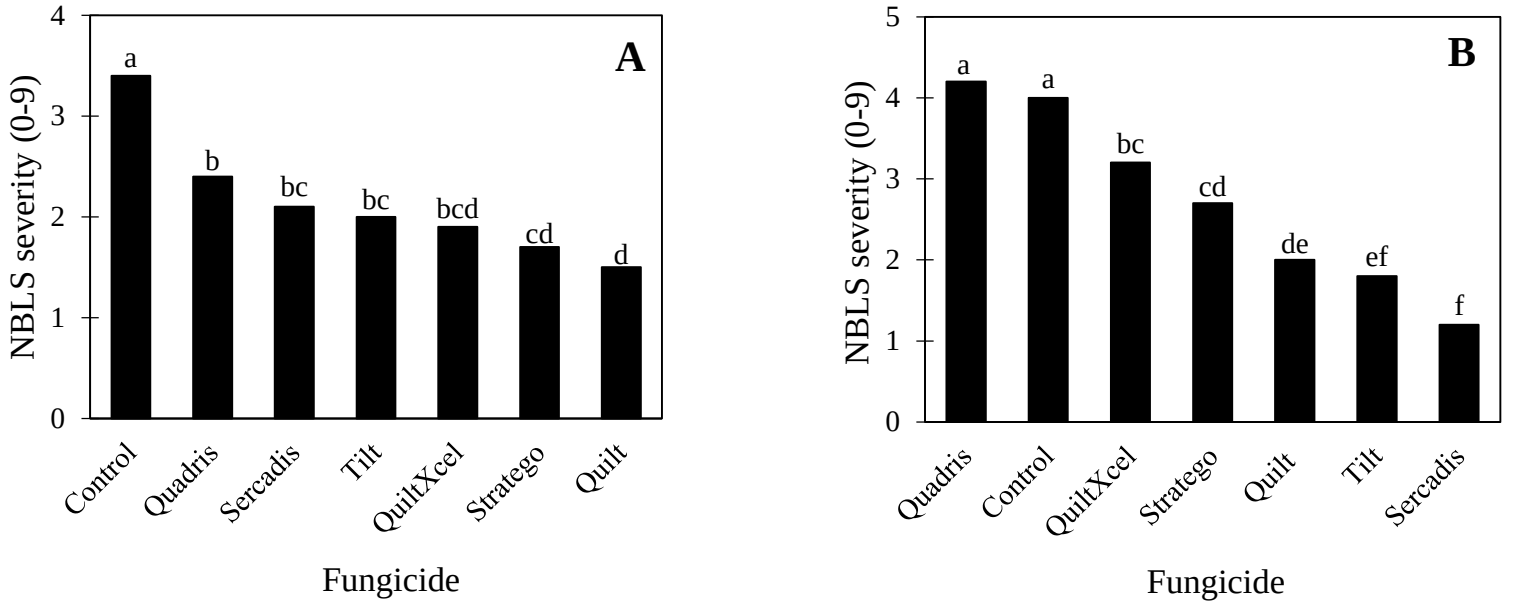

Fig. 7. Narrow brown leaf spot (NBLS) severity in rice fields. A, 2012, B, 2014. Columns with the same letter are not significantly different ($P = 0.05$). Quadris, 22.9% azoxystrobin; Sercadis, 26.6% fluxapyroxad; Tilt, 41.8% propiconazole; QuiltXcel, 13.5% azoxystrobin + 11.7% propiconazole; Stratego, 11.4% propiconazole + 11.4% trifloxystrobin; and Quilt, 11.7% propiconazole + 7% azoxystrobin.

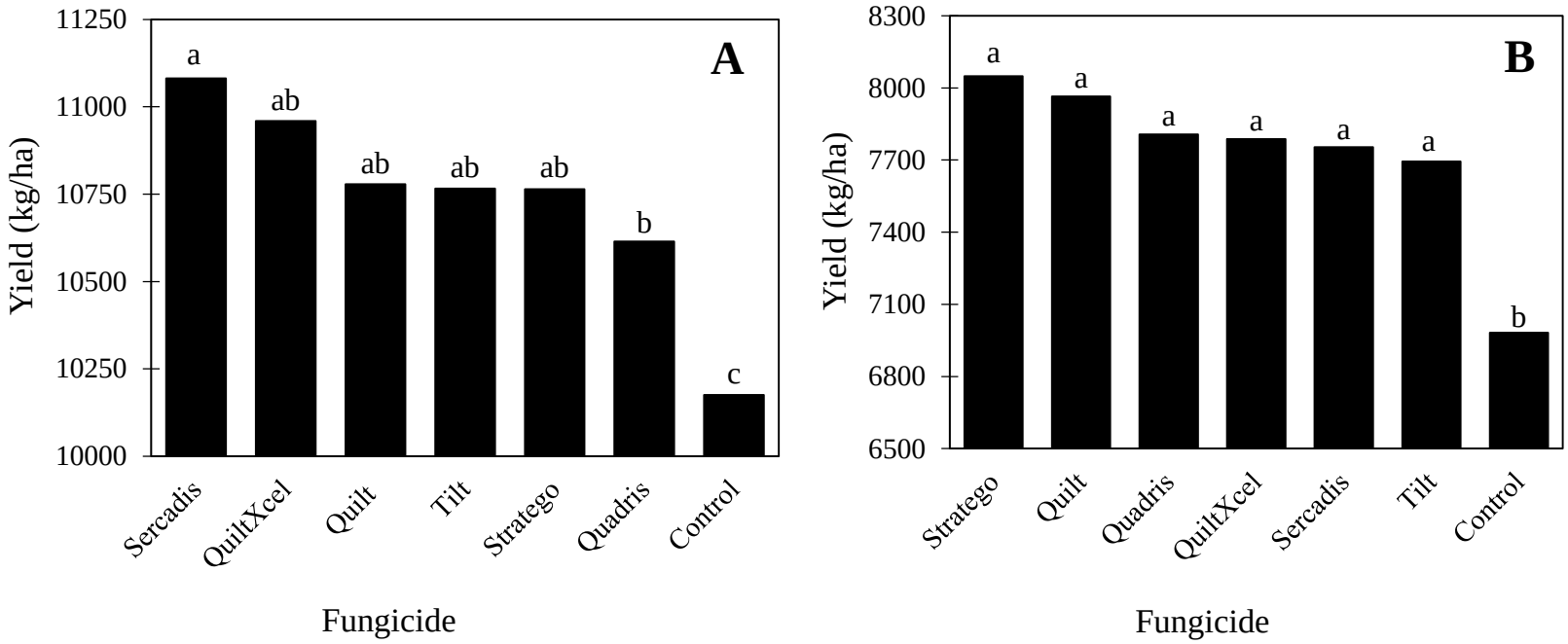

Fig. 8. Rice yield (kg ha^{-1}) according to each fungicide for the field study on 2012 (A), and 2014 (B). Columns with the same letter are not significantly different (P = 0.05). Quadris, 22.9% azoxystrobin; Sercadis, 26.6% fluxapyroxad; Tilt, 41.8% propiconazole; QuiltXcel, 13.5% azoxystrobin + 11.7% propiconazole; Stratego, 11.4% propiconazole + 11.4% trifloxystrobin; and Quilt, 11.7% propiconazole + 7% azoxystrobin.

The pattern of fungicide sensitivities was very different among fungicide groups. DMI active ingredients did not inhibit or even stimulated the germination of *C. janseana*. Similar results have also been reported before. Myclobutanil did not suppress spore germination of the fungal pathogen *Cylindrocladium buxicola* (Henricot et al. 2008). Similarly, myclobutanil and propiconazole did not affect *in vitro* urediniospore germination of *Puccinia hemerocallidis* (Buck and Williams-Woodward 2003). The lack of effect on germination is attributed to the mode of action of DMIs, which inhibits synthesis of ergosterol (Gachomo 2005). Fungal spores are less sensitive to DMI active ingredients because spores already contain enough ergosterol to germinate and produce germ tubes and infection structures. This study focused on the assessment of *C. janseana* conidia mortality and germination inhibition due to the incapability of mycelial growth on artificial fungal growth media. However, it is likely once the fungus has depleted its existing ergosterol after germination, continuing mycelial growth is inhibited because of the inhibition of ergosterol synthesis by DMIs (Gachomo 2005). The results of our field evaluation confirmed the effectiveness of propiconazole in reducing NBLS severity on rice, although propiconazole is unable to affect the germination of *C. janseana*.

Use of the SDHI fluxapyroxad resulted in the highest inhibition of *C. janseana* conidia germination among all the fungicides tested in this study. However, the other SDHI active ingredient, flutolanil, showed no effect on conidia germination. It is a noteworthy observation that there is no cross-resistance of *C. janseana* in SDHI and the conflict sensitivities between these two SDHI active ingredients are consistent on all five isolates evaluated in this study. Resistance to SDHI has been known in the field populations of several fungi, and can lead to a significant loss of fungicide efficacy to control moderately and highly resistant fungal populations (Gudmestad et al. 2013; Ito et al. 2004). Ito et al. (2004) described that resistance to flutolanil is attributed to the result of a single point mutation in the *sdhC* gene, they also stated that this *sdhC* mutation conferred cross-resistance against carboxin that belongs to the SDHI. It has been known that cross-resistance patterns among SDHI fungicides is complex because many mutations in certain fungal pathogens confer full cross resistance while others do not

(Sierotzki and Scalliet 2013). According to our results, *C. janseana* isolates did not present cross-resistance to flutolanil and fluxapyroxad. The loss of efficacy of flutolanil to control *C. janseana* may be explained by high selection pressure to develop resistant populations with the continuous use of flutolanil. Flutolanil has been marketed for rice in the United States since 2000, much earlier than fluxapyroxad that is commercially available after 2012. Fluxapyroxad is likely to be highly effective for management of NBSL as demonstrated in our field evaluations. Considering fast development of resistance to SDHIs, continuous monitoring of fungicide sensitivity in *C. janseana* field populations is needed.

QoI fungicides have been recommended to control fungal spores more effectively than mycelium because spores are more sensitive to these fungicides (Balba 2007). Karadimos et al. (2005) reported complete inhibition of *Cercospora beticola* spore germination with QoI active ingredients: pyraclostrobin and trifloxystrobin at 0.01 and 0.1 µg mL^{-1}, respectively. However, pyraclostrobin, trifloxystrobin, and azoxystrobin tested in this study did not effectively inhibit conidia germination, providing less than 50% of conidia inhibition. Sensitivities of *C. janseana* to QoI active ingredients show a wide range from sensitive ($EC_{50} \leq 1$) to intermediate and to resistant ($EC_{50} > 10$) among isolates. Also there is a wide range of sensitivity values of any given *C. janseana* isolate among different QoI active ingredients, which indicates no cross resistance. The main mechanism conferring high resistance to QoI fungicides is reduced sensitivity to the active ingredients that target an outer, quinone oxidizing site of the cytochrome bc_1 enzyme complex (complex III) (Fernández-Ortuño et al. 2008). Fungi demonstrated variability in sensitivity to different QoI active ingredients. Sensitivities of *Uncinula necator* to azoxystrobin, pyraclostrobin, and trifloxystrobin did not correlate as strongly or uniformly as expected (Wong and Wilcox 2002). Fairchild et al. (2013) reported that 39 isolates of *Alternaria solani* that were resistant to azoxystrobin, were not resistant to pyraclostrobin or famoxadone, demonstrating no cross resistance among these QoI active ingredients. These results demonstrate that although QoI fungicides are generally known to be effective to control fungal spores, conidia of certain *C. janseana*

isolates may not be suppressed by these fungicides, and resistance to one QoI active ingredient in *C. janseana* does not necessarily result in cross resistance to other QoI active ingredients. In the field evaluation, a sole application of QoI did not provide reduction of NBLS as effective as DMI or tank mixture of DMI and QoI. Our *in vitro* fungicide sensitivity assay and field evaluation indicate moderate effectiveness of QoIs for managing NBLS.

Mancozeb is a broad-spectrum fungicide with multi-site modes of action. Mancozeb has provided excellent suppression of spore germination of various fungal genera since it was introduced in 1962 (Gullino et al. 2010). However, fungicide resistance to mancozeb was also reported. The EC_{50} value of the fungal pathogen *Pestalotiopsis theae* reached to 3,940 µg mL^{-1} (Yang and Zhang 2012). Except isolate CN-1-16, mancozeb did not inhibit conidia germination of the *C. janseana* isolates tested in our *in vitro* assay, indicating that mancozeb may present low efficacy to control NBLS.

Previous reports of fungicide evaluations in different fungal pathogens have demonstrated that wild-type or sensitive fungal isolates can be effectively managed by MBC. Conidial germination and growth of *Phoma exigua* was totally inhibited on media containing 50 µg mL^{-1} of the MBC thiophanate methyl (Schmitz et al. 2007). Spore germination of the arbuscular mycorrhizal fungus *Glomus mosseae* was inhibited in the presence of 10 µg mL^{-1} of the MBC benomyl (Chiocchio et al. 2010). However, resistance to MBC is associated with a single gene mutation at beta tubulin, which confers a persistent and complete resistance to this fungicide (Koenraadt et al. 1992). Isolates CN-1-16, and EL-110 showing EC_{50} values higher than 10 µg mL^{-1} of MBC thiophanate methyl indicate development of resistance to this active ingredient, while isolate BMT-41, CN-1-4, and CN-2-4 are sensitive. According to our results, it is likely that some *C. janseana* isolates (CN1-16, and EL-110) must have been exposed and selected by previous uses of MBC. Therefore, MBC may no longer work for these isolates.

Under field conditions, fluxapyroxad (Sercadis) and fungicide containing propiconazole alone (Tilt) or combination of propiconazole with azoxystrobin (Quilt and QuiltXcel,) or trifloxystrobin (Stratego) resulted in best performance on reducing NBLS severity. In contrast, azoxystrobin (Quadris) showed the least effectiveness in NBLS management. This field evaluation is consistent with fungicide sensitivity data, particularly those of EL-110 isolate collected from the site where the field trials were conducted. EL-110 shows consistent inhibition of conidia germination by fluxapyroxad but not azoxystrobin. Regarding rice yield, all fungicide treatments significantly increased yield over the untreated control. Although azoxystrobin by itself did not control NBLS as effective as other fungicide treatments, this active ingredient still increased yield over the untreated control. Similar results were obtained by McCartney et al. (2007), where despite the active ingredient azoxystrobin failed to control septoria tritici blotch caused by *Mycosphaerella graminicola*, this active ingredient increased wheat yield compared with the untreated control. QoI fungicides may have controlled others than primary-target diseases and promote plant heath that contribute to yield increase.

Because of difficulty in growing *C. janseana* on artificial media, the present assessment of conidia germination inhibition shed light on fungicide sensitivities of *C. janseana* to major fungicide active ingredients. With field evaluations, the *in vitro* fungicide sensitivity data will provide comprehensive information to develop fungicide program for NBLS. *C. janseana* isolates show variable sensitivities to different fungicide groups and often present reduced sensitivities that are likely to cause ineffectiveness of fungicides in NBLS management. In the case of SDHI, all isolates tested had high sensitivities to fluxapyroxad that is recently released in the market but low sensitivities to flutolanil. Because the lack of historical information about the fungicide use in the fields where the isolates assessed in this study were collected, we cannot know how development of resistance by *C. janseana* to the fungicide active ingredients occurs. Understanding of the sensitivity *C. janseana* field populations to fungicide can enhance

the development of more effective fungicide management program for NBLS and avoid or delay the development of potential resistance to current effective fungicides.

CHAPTER IV

LIMITED EFFECTS OF MICROALGAL BIOFERTLIZERS FOR RICE PRODUCTION IN TEXAS

INTRODUCTION

Nitrogen (N) is one of the most important limiting factors in rice production (Vaishampayan et al. 2001) since this crop is mainly grown under irrigated conditions where nitrogen fertilizer efficiency is low due to large N losses from flooded soils (De Datta and Buresh 1989). Ammonia (NH_3) volatilization and nitrification-denitrification are the main processes for N losses in flooded rice fields (Irisarri et al. 2007). Additionally, it has been shown that nitrogen fertilizers are the major cause for an increase in methane (CH_4) emissions from rice fields (Banger et al. 2012).

Numerous studies have supported the importance of using nitrogen fixing cyanobacteria as biofertilizers in tropical (Shukla and Gupta 1967; Tirol et al. 1982; Mandal et al. 1999; Jha et al. 2013) and temperate rice-growing regions (Henriksson 1971; Reynaud and Metting 1988). The most abundant algal flora in rice fields is composed of *Aulosira fertilissima*, *Anabaena* sp., *Nostoc* sp., and *Scytonema* sp.; moreover, the cyanobacterial strains frequently used for biofertilizer applications include *A. fertilissima*, *Anabaena* sp., *Nostoc* sp., among others (Sinha and Häder 1996).

Many techniques have been developed over time to measure the amount of nitrogen fixed by cyanobacteria including the N dilution method, differentiation in natural 15N abundance (δ15N) method and the most frequently used Acetylene-reducing activity (ARA) measurement method (Roger and Ladha 1992). The percentage of cyanobacteria-derived available nitrogen for rice plants has been documented in the range of 28-40% (Tirol et al. 1982; Mian and Stewart 1985). Heterocystous cyanobacteria contribute on average 20-30 kg N ha^{-1} (Vaishampayan et al. 2001).

Besides N_2-fixation, cyanobacteria can also possess additional advantages, such as phosphate assimilation, and release upon cyanobacterial decomposition (Mandal et al.

1999). An increased level of organic carbon in the soil was reported as an effect of cyanobacterial inoculation (Akhter et al. 2002). Additionally, excretion of extracellular compounds like polysaccharides (Bertocchi et al. 1990) and peptides (Welker and Döhren 2006) results in a rapid regeneration and improvement of soil structure (Mandal et al. 1999), and increased formation of soil organic matter (Das et al. 1991; Mandal et al. 1999).

Growth-promoting effects attributed to cyanobacteria inoculation have been recorded on different crops such as rice (Shukla and Gupta 1967), wheat (Karthikeyan et al. 2007) and tomato (Prasanna et al. 2013). The growth of cyanobacteria in the floodwater causes a decrease in readily available iron, therefore minimizing Fe toxicity in young rice plants in acid soils rich in organic matter, while decomposing algal biomass causes increases in readily available forms of iron and manganese in soils, providing these elements to rice at advanced growth stages where there is an increased demand for these nutrients (Das et al. 1991). Additionally, microalgae have been used for remediation of domestic wastewater and biomass production for biofuels (Rawat et al. 2011).

The use of economically and environmentally-friendly microalgae-based biofertilizers in paddy rice fields presents a promising alternative to synthetic fertilizers. Additional advantages such as incorporation of organic matter, improvement of soil structure, minimization of Fe toxicity, and growth promoting effects make cyanobacterial biofertilization an ideal input for sustainable agriculture. The objective of this study was to evaluate the benefits of microalgae biofertilizers to rice plant growth and yield.

MATERIALS AND METHODS

Microalgae biofertilizers. N_2-fixing cyanobacterial cultures of *Anabaena* sp. UTEX 2576 and *Nostoc muscorum* UTEX 2209s were provided by UTEX The Culture Collection of Algae at the University of Texas at Austin. Previous to treatment application, axenic cultures of *Anabaena* sp. UTEX 2576, *N. muscorum* UTEX 2209s,

and an indigenous strain of *N. punctiforme* bioprospected from the Texas A&M AgriLife Research and Extension Center at Beaumont were scaled up from 0.25 to 1-L glass bottles containing liquid BG-11 minus $NaNO_3$ (BG-11-0) medium (Stanier et al. 1971) maintained at 25°C, with a maximum light intensity of 300 μE m^{-2} s^{-1} from fluorescent lamps with an automated light/dark cycle of 12 h/12h, and constant aeration of normal air infused with + 1.5% CO_2 through 6.35mm diameter glass bubbler tube. Each cyanobacterial culture was then transferred up to 15-L reactor containing liquid BG-11-0 medium and cultivated under greenhouse conditions (25°C +/- 5 degrees, ambient light, and constant aeration + 1.5% CO_2 through a 6.35mm diameter air line with a 100 micron air stone). The microalgal biomass concentrate for all the treatment applications consisted of a polyculture of *Chlorella* sp. and *Scenedesmus* sp. (approx. dry weight = 38 g/L, total N = 442 ppm, P = 670 ppm, K = 500 ppm) that was previously cultivated in a vertical tubular photobioreactor on an artificial wastewater medium (MB3N, based on a protocol from UTEX The Culture Collection of Algae at the University of Texas, which was modified from the protocol of Provasoli and Pintner 1959), centrifuged, and subsequently stored in a -80°C freezer.

During the summer of 2013, indigenous cyanobacterial isolates were collected from paddy rice fields from experimental plots at the Texas A&M AgriLife Research and Extension Center at Beaumont. Rice paddy water was collected into 50 mL conical vials. Vials were centrifuged at approximately 3000 RPM for 5 minutes, decanting the supernatant after centrifugation. Under sterile conditions, pellets were collected and streak inoculated on Petri plates with agar BG-11-0 medium. Plates were incubated at 29°C with a maximum light intensity of 300 μE m^{-2} s^{-1} from fluorescent lamps with an automated light/dark cycle of 12h/12h. Every 24 hours for 2 weeks, green or blue green colonies were observed via light microscopy with the 40 × magnification by inspecting cyanobacterial cells on glass slides. Colonies identified as N_2-fixing cyanobacteria (Bellinger and Sigee 2010) were transferred to new plates containing agar BG-11-0 medium. This process was repeated until axenic cultures were obtained. The axenic indigenous N_2-fixing cyanobacterial isolate (TX-101) was selected for further work.

Isolation and purification of TX-101 was based on a protocol from the UTEX The Culture Collection of Algae at the University of Texas at Austin, which was modified from a previously developed protocol (Hagen and Meeks 1999). Internal transcribed spacer region of this isolate was sequenced following a previously described protocol (Boyer et al. 2001). According to BLAST search isolate TX-101 was identified as *Nostoc punctiforme* (91% identity with GenBank Accession # NC_010628.1). This isolate was transferred to BG-11-0 agar slants for long term storage at ambient room temperature with a maximum light intensity of 300 μE m^{-2} s^{-1} from fluorescent lamps with an automated light/dark cycle of 12h/12h.

Greenhouse studies. Two experiments (Exp 1 and 2) were conducted in the greenhouse of the Department of Plant Pathology and Microbiology at Texas A&M University, College Station, and one experiment (Exp 3) was conducted in a greenhouse at the University of Texas at Austin. Seeds of the rice cultivar Cocodrie were surface sterilized for 5 minutes in 3% sodium hypochlorite solution, and then washed three times with distilled water, kept in Petri plates filled with distilled water inside an incubator at 28°C. After 3 days, 10, 6, and 3 germinated seeds in the Exp 1, Exp 2, and Exp 3 respectively, were seeded in a 2.5-L-pot containing a soil mix of League soil (pH 5.5, 3.2% sand, 32.4% silt, 64.4% clay, and 3.8 - 4.8% organic matter) and potting mix (Sunshine Professional Growing Mix, Sun Gro Horticulture) in a ratio of 1:1 (v/v). Six seeded pots were placed into a polycarbonate tote measuring 58.7 cm length × 42.9 cm width × 31.8 cm height for the Exp 1 and 2. In Exp 3, the polycarbonate tote measured 60 cm length × 30 cm width × 15 cm height. Each polycarbonate tote served as the experimental unit. Three days before treatment application, plants were thinned to 6 and 3 plants per pot for Exp 1 and Exp 2, respectively. In Exp 1 and 2, plants were maintained in the greenhouse under natural light conditions with temperatures ranging from 25 to 35°C. In Exp 3, the temperature in the greenhouse was set to a relatively constant 25°C+/- 5°C with natural light conditions. Treatments were arranged in a randomized block design, with 5 treatments and two replications.

In Exp 1, 21-day-old rice plants in each experimental unit were treated with 2 L of cyanobacterial liquid culture of *Anabaena* sp. UTEX 2576 (Dry weight (DW) = 0.62 g/L) or *N. muscorum* UTEX 2209s (DW = 0.52 g/L); 200 mL of microalgal biomass concentrate; 3g of urea (from 190 kg ha^{-1}); or 7.6 L filtered water that served as the untreated control. Water volume of each experimental unit was maintained at 7.6 L with water weekly refilled. Rice plant heights (the tallest leaf per plant) were measured at 28, 35, 42, 49, 56, 63, 70, and 77 days after planting.

In Exp 2, microalgal biofertilizer treatments were applied at 7 and 28 days after planting. At each application, each experimental unit was treated with 2 L of cyanobacterial liquid culture of *Anabaena* sp. UTEX 2576 or *N. muscorum* UTEX 2209s; 400 mL of microalgal biomass concentrate; or 7.6 L filtered water that served as the untreated control. In the case of urea treatment, 3 g (from 190 kg ha^{-1}) urea was applied only at the first application. Dry weight of UTEX 2576 was 0.14 g/L and 0.1 g/L for the first and second treatment application, respectively, while dry weight for UTEX 2209s was 0.08 and 0.015 g/L for the first and second treatment application, respectively. Water volume of each experimental unit was maintained at 7.6 L with water weekly refilled. Rice plant height was measured at 14, 21, 28, 35, 42, 49, 56, and 63 days after planting.

In Exp 3, 7-day-old rice seedlings in each experimental unit were treated with 15 L of *Anabaena* sp. UTEX 2576 (DW = 0.11 g/L); 15 L of *N. muscorum* UTEX 2209s (DW = 0.12 g/L); 400 mL of microalgal biomass concentrate dissolved in 15 L tap water; 9 g urea (from 190 kg ha^{-1}) dissolved in 15 L tap water; or 15 L of tap water that served as the untreated control. Water volume of each experimental unit was maintained at 7.6 L with water weekly refilled. Rice plant height was measured at 14, 21, 28, 35, 42, 49, 56, 70, and 77 days after planting.

Field evaluation. Field trials were conducted in a rice field at Texas A&M AgriLife Research and Extension Center at Beaumont in 2013 and 2014. Rice seeds were pretreated with Dermacor X-100 (Chlorantraniliprole; DuPont) at a rate of 72.85 g a.i. ha^{-1} and drill-planted at a rate of 89.67 kg ha^{-1} into League soil (pH 5.5, 3.2% sand,

32.4% silt, 64.4% clay, and 3.8 - 4.8% organic matter). Each plot was bordered with metal barriers, forming a rectangle of 5.49 m long per 1.24 m width with 7 internal rows, each one spaced 17.78 cm. In each study, one day after planting, 82.33 kg ha^{-1} of urea was applied to all plots, then plots were temporary flooded for 48 h, then drained. Rice emergence was 14 days after planting. Plots were flushed as needed from emergence to permanent flood

In 2013, rice cultivar Presidio was planted on March 28. Twenty days after planting, 5.23 kg a.i. ha^{-1} of RiceBeaux (Propanil and Thiobencarb; RiceCo USA); 52.54 g a.i. ha^{-1} of Permit (Halosulfuron-methyl; Gowan Company); and 0.42 kg a.i. ha^{-1} of Command 3ME (Clomazone; Helena Chemical Company) were applied for early season weed control. In 2014, rice cultivar Antonio was planted on April 2. At 27 days after planting, 3.48 kg a.i. ha^{-1} of RiceBeaux; 52.54 g a.i. ha^{-1} of Permit; and 0.34 kg a.i. ha^{-1} of Command 3ME were applied for early season weed control. In both years, herbicide applications were done with a 2-person hand-held spray boom (13- 80015 nozzles, 50 mesh screens, 149.66 L ha^{-1} final spray volume). Treatments were arranged in a randomized complete block, with 4 treatments and 4 replications for each study.

Treatments included live N_2-fixing cyanobacteria stock, microalgal biomass concentrate, urea, and untreated control. In 2013, treatments were applied at 36 days after planting. The cyanobacterial stock was a mixture of *Anabaena* sp. UTEX 2576 (DW = 0.14 g/L) and *N. muscorum* UTEX 2209s (DW = 0.27 g/L) in a rate of 1:1 (v/v) applied at 22,026 L ha^{-1}. The microalgal biomass concentrate was applied at 5,580 L ha^{-1}. The urea treatment representing a conventional fertilizer was applied at 331.38 kg ha^{-1}. Rice was harvested on August 5 (130 days after planting). Yield from each plot was converted to kg ha^{-1} at 12% moisture. The average daily minimum and maximum temperature during the rice growing season was 19.0 and 29.5°C, respectively, with an average daily precipitation of 3.24 mm.

In 2014, treatments consisted of live N_2-fixing cyanobacteria, microalgal biomass concentrate, urea and untreated control that were applied twice. The first application at 52 and the second at 81 days after planting. The live N_2-fixing cyanobacterial inoculum

used was an indigenous isolate (TX-101) of *N. punctiforme* applied at a rate of 22,026 L ha $^{-1}$ for each application. Dry weight of TX-101 for the first and second application was 0.13 and 0.11 g/L, respectively. The microalgal biomass concentrate was applied at a rate of 5,580 L ha^{-1} for each application. In the case of urea treatment, 207.11 kg ha^{-1} and 124.26 kg ha^{-1} of urea were applied for the first and second application, respectively. Rice was harvested on August 7 (127 days after planting). Yields were measured as aforementioned. The average daily minimum and maximum temperature during the rice growing season was 19.7 and 29.7°C, respectively, with an average daily precipitation of 3.3 mm.

Statistical analysis. Rice plant height data from the greenhouse experiments and yield data from the field experiments were analyzed by SAS 9.3 (SAS Institute Inc., Cary, NC) to test treatment effects. Differences among treatment means were estimated using Fisher's protected least significance difference (LSD) at $P = 0.05$.

RESULTS

Greenhouse studies. Highly significant differences for the Exp 1, 2, and 3 ($P < 0.01$) were detected in plant height among the different fertilizers and different days after planting. In Exp 1 (Fig. 9A), all fertilizers resulted in a progressive increase in plant height until 56 days after planting. During this period, plants treated with the microalgal biomass concentrate or *Anabaena* sp. UTEX 2576 resulted in the two highest plant growth, followed by the treatment with *N. muscorum* UTEX 2209s or urea. While the untreated control resulted in the lowest growth. After 56 days from planting, plants treated with urea continued growing, while plants treated with the microalgal treatments (*Anabaena* sp. UTEX 2576; microalgal biomass concentrate; and *N. muscorum* UTEX 2209s) and untreated control almost ceased vertical growth. At the end of the experiment, height of plants treated with urea surpassed the plant height of the microalgal fertilizer treatments.

In Exp 2 (Fig. 9B), all fertilizers resulted in a progressive increase in plant height until 35 days after planting. During this period, plants treated with microalgal

biomass concentrate resulted in the tallest height, while *Anabaena* sp. UTEX 2576 resulted in the shortest plant height, the rest of treatments (untreated control; *N. muscorum* UTEX 2209s; and urea) resulted in intermediate plant height. After 35 days after planting, plants treated with microalgal biomass concentrate or urea continued growing, while plants treated with *N. muscorum* UTEX 2209s; or *Anabaena* sp. UTEX 2576 almost ceased vertical growth. The microalgal biomass concentrate treatment resulted in the tallest plant height followed by the urea treatment. Height of plants treated with *N. muscorum* UTEX 2209s was not different from the untreated control, while *Anabaena* sp. UTEX 2576 resulted in a lower height than the untreated control.

In Exp 3 (Fig. 9C), all fertilizers resulted in a progressive increase in plant height until 70 days after planting. During this period plants treated with the microalgal biomass concentrate resulted in the tallest height. However, height decreased significantly when plants were treated with urea, *Anabaena* sp. UTEX 2576, or untreated control that resulted in similar heights. Plants treated with *N. muscorum* UTEX 2209s mainly resulted in the shortest height.

Field evaluation. Highly significant differences ($P < 0.01$) for both field trials were detected in rice yield among the different fertilizers. In the two-year field trials, microalgal biofertilizer treatments did not increase yields compared to the untreated control (Fig. 10A and B). The urea treatment produced 4.0 and 2.6 times higher yields than the untreated control in 2013 and 2014, respectively.

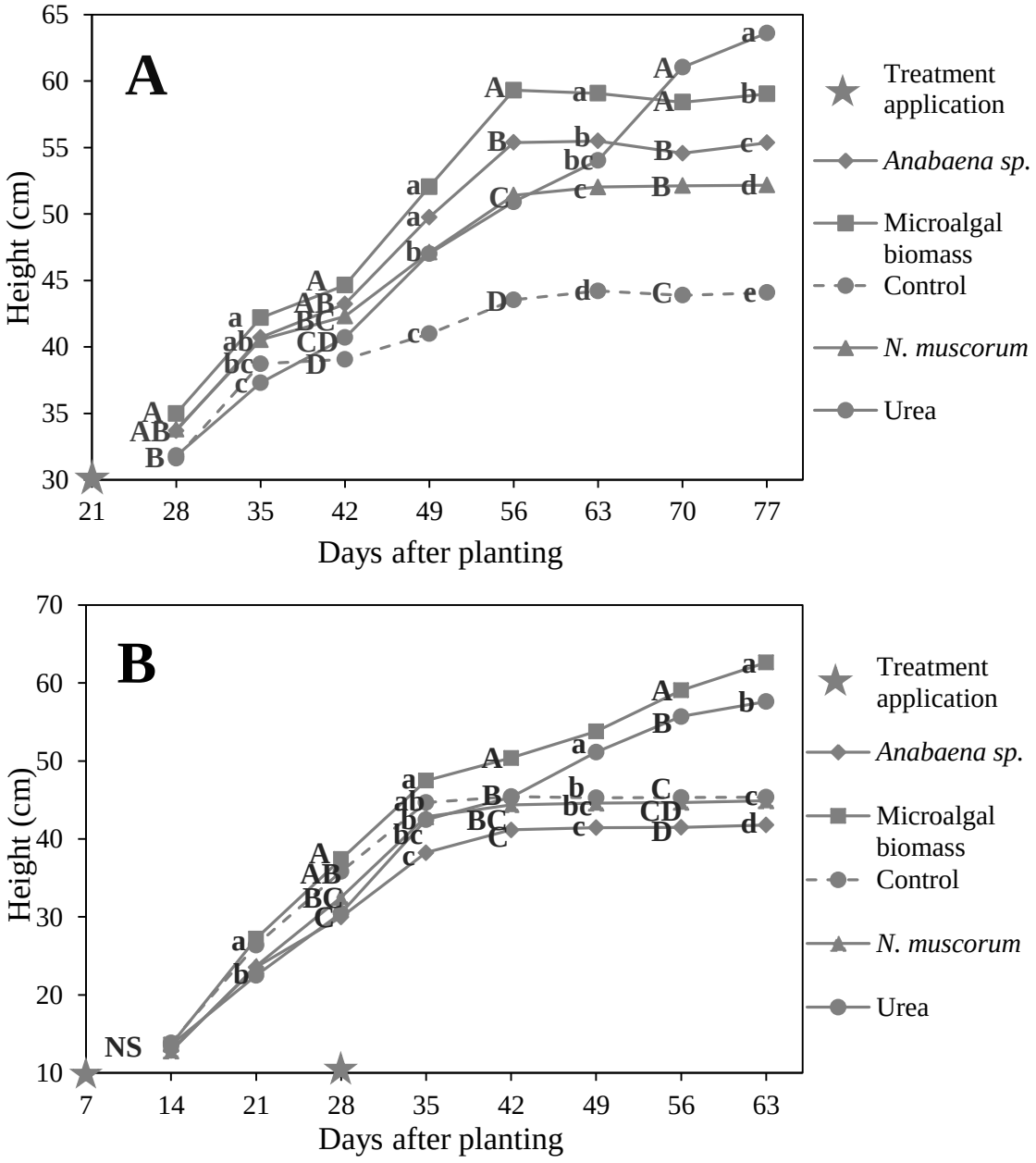

Fig. 9. Over time effect of different fertilization treatments on rice plant height (cm) for Exp 1 (A), Exp 2 (B), and Exp 3 (C) under greenhouse conditions. N_2-fixing cyanobacterial strains applied were *Anabaena* sp. UTEX 2576; *N. muscorum* UTEX 2209s; while microalgal biomass was a concentrate of *Chlorella* sp. and *Scenedesmus* sp. Symbols with the same letter are not significantly different (P = 0.05).

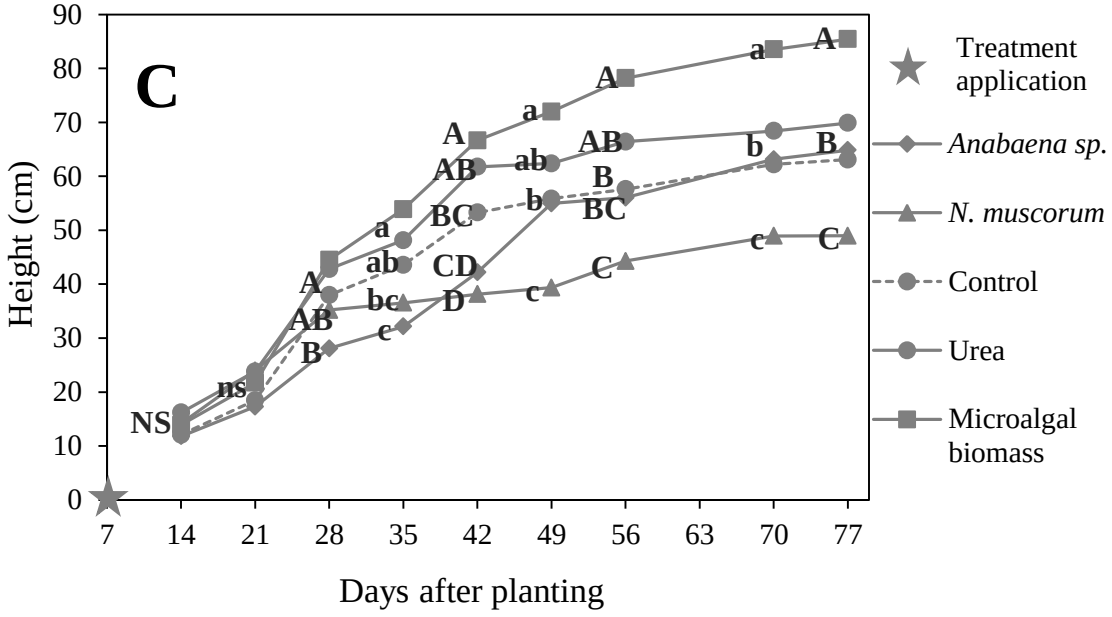

Figure 9 Continued.

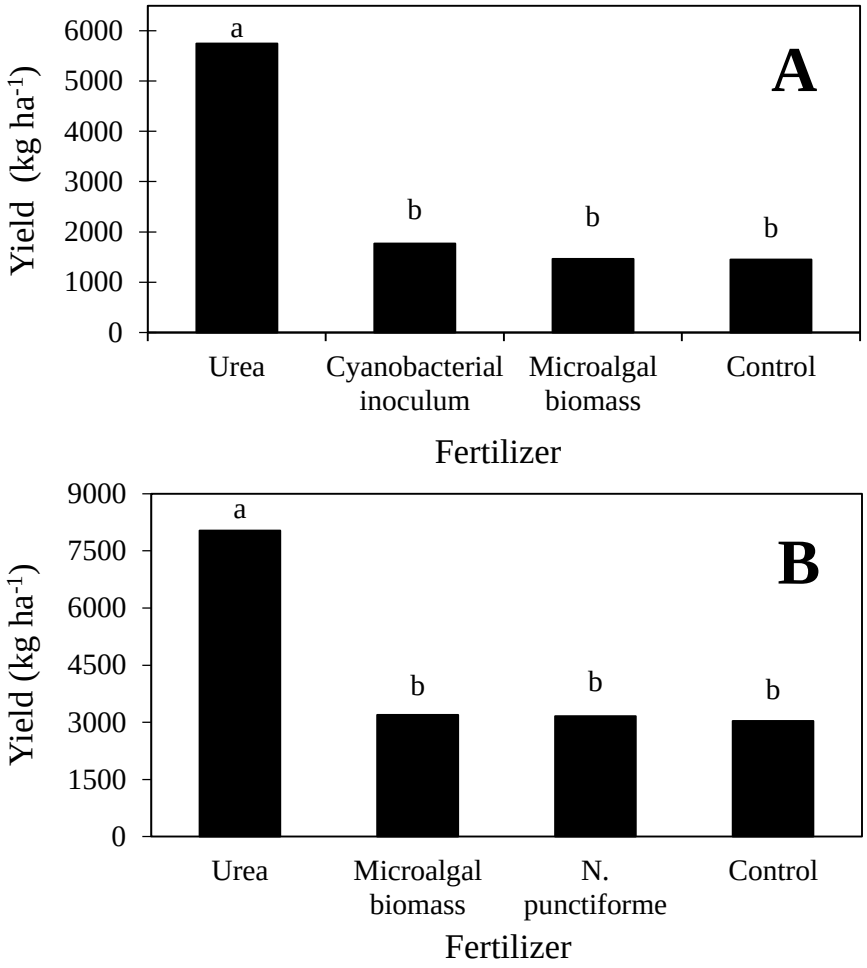

Fig. 10. Effect of different fertilizers on rice yield for the field study during 2013 (A) and 2014 (B). Cyanobacterial inoculum was a polyculture of *Anabaena* sp. UTEX 2576 and *Nostoc muscorum* UTEX 2209s; microalgal biomass was a concentrate of *Chlorella* sp. and *Scenedesmus* sp. Columns with the same letter are not significantly different (P = 0.05).

DISCUSSION

Although in the greenhouse, microalgae-based biofertilizers demonstrated improved results, the potential of these biofertilizers is limited under field conditions due the variable dynamics that have to encounter biological agents in the field that modify

the outcome. This explains why microalgae-based biofertilizer studies are not widely conducted in the United States. However, additional considerations are discussed in order to improve the potential of microalgal biofertilizers.

In the Exp 1 in the greenhouse, the microalgal biomass concentrate resulted in the best plant height among the different biofertilizers. While, in Exp 2 and 3 this treatment yielded the best results at improving plant height in comparison with all other treatments including urea. At the end of either experiment, plants inoculated with microalgal biomass concentrate reached a range of 15 to 22 cm in height over the untreated control. It is likely that the better performance of microalgal biomass concentrate in Exp 2 and 3 was the result of the increased amount used of this biofertilizer in contrast to Exp 1. *N. muscorum* UTEX 2209s or *Anabaena* sp. UTEX 2576 significantly increased plant height over the untreated control in Exp 1. However, in Exp 2 and 3 they did not significantly increase plant height over the untreated control. In Exp 2 and 3, *Anabaena* sp. UTEX 2576 and *N. muscorum* UTEX 2209s, respectively, decreased plant growth in contrast to the untreated control. The inhibition of plant growth (Pedurand and Reynaud 1987) is due the production of cyanotoxins by cyanobacteria (Kós et al. 1995). However, cyanobacterial strains (referred also as *Anabaena*/*Nostoc* sp. strain 7120) used in this study are nontoxic (Rouhiainen et al. 1995). Therefore, decreased plant growth with these cyanobacterial strains in Exp 2 and 3 may be related to environmental factors that limited plant growth.

Previous studies reported increased plant growth when cyanobacterial biofertilizers were used. Singh et al. (2011) reported increased rice plant height when rice seeds were inoculated with different cyanobacterial strains in contrast to the untreated control. They also concluded that rice growth and development was enhanced by systemic accumulation of phenylpropanoids in rice after cyanobacterial inoculation. In another study, Shariatmadari et al (2011) reported that addition of cyanobacterial extracts can enhance seed germination and plant growth of cucumber, tomato, and squash in contrast to untreated control.

In the field study in 2014, the indigenous N_2-fixing cyanobacterial isolate TX-101 did not result in improved rice yields over the untreated control. Although, indigenous cyanobacterial isolates have more tolerance to the environment where they were collected as previously stated (Manchanda and Kaushik 2000), and their use results in increased yields of different crops (Nisha et al. 2007; Maqubela et al. 2010), our results demonstrated that indigenous cyanobacterial inoculants do not necessarily result in improved yields. Also, the double inoculation of biofertilizers during 2014 in contrast to 2013 did not increase rice yield over the untreated control. These results demonstrate that increased amounts of biofertilizers do not necessarily result in improved rice yields.

Previous reports stated that the relative increase in rice yield with cyanobacterial biofertilizers has been on average 28% in pot experiments, and 15% in field experiments (Roger and Kulasooriya 1980) Similarly, Jha et al. (2013) reported a 21 and 26% rice yield increase during a two-year field study, when plants were treated with cyanobacteria in contrast to untreated control. Our field results during 2013, showed biofertilizer treatments caused a slight increase in rice yield, since there was a 22 and 1% yield increase, compared to the untreated control, when rice plants were inoculated with the N_2-fixing cyanobacterial inoculum and microalgal biomass concentrate, respectively. Similarly, in 2014, a 5 and 4% yield increase, compared to the untreated control, occurred when rice plants were inoculated with the microalgal biomass concentrate and *N. punctiforme*, respectively. However, these yield increases were not statically significant from the untreated control.

These results demonstrate that biofertilizers did not significantly increase rice production in the field. Several factors can affect the potential of cyanobacterial biofertilizers under field conditions. These factors include P deficiency, presence of high concentrations of N in floodwater, low pH and arthropod grazer populations that can limit growth and biological nitrogen fixation activities of cyanobacteria in rice fields (Ladha and Reddy 1995). It has being mentioned that rice canopy plays an important role in the growth of cyanobacteria (Yanni 1992; Choudhary 2009). At later rice vegetative stages, the rice canopy is thicker; therefore, restricting the penetration of light

to the water surface, resulting in the limitation of cyanobacterial growth and N_2-fixation. Additionally, some considerations have been stated in order to ensure the best performance of microalgae biofertilizers under field conditions. It has been recommended applications of 10 - 15 kg ha^{-1} of air-dried cyanobacterial inoculant (Venkataraman 1981) or 100 kg ha^{-1} (90% moisture) of fresh inoculum applied to rice paddy fields 5 days after rice seedling transplantation, being more efficient if applied as a fresh inoculum instead of dry (Yanni and Sehly 1991). Also, it is recommended to apply the cyanobacterial biofertilizer for at least three consecutive cropping seasons in order to ensure the establishment of the inoculated cyanobacteria in rice fields (Venkataraman 1981). These reports demonstrate that success in inoculated N_2-fixing cyanobacterial biofertilizers is contingent on many factors; therefore their potential is limited in the field.

N_2-fixing cyanobacterial treatments have also been used in combination with synthetic fertilizers (Yanni 1992; Yanni and Sehly 1991; Nayak et al. 2004; Singh et al. 1990; Irisarri et al. 2007). Venkataraman (1981) recommended 25 kg ha^{-1} of mineral nitrogen fertilizer when supplemented with algal application. Nevertheless, urea at a rate of 72 kg N ha^{-1} was found to support the best colonization when applied with cyanobacteria (Yanni 1992). Aziz and Hashem (2004) reported that a 20% less of the recommended synthetic fertilizer dose in combination with cyanobacteria resulted in 80.5% increase in rice yield. In another study, plots treated with 30 and 60 kg N ha^{-1} along with the inoculation of N_2-fixing cyanobacteria exhibited the highest percentage increase in terms of algal biomass and ARA (Nayak et al. 2004). Because biofertilizers alone exhibit several limitations in the field, the combination of biological and synthetic fertilizers can ensure higher production in a more environmentally-friendly manner.

Our results demonstrate that under greenhouse conditions microalgal biomass concentrate resulted in a significant improvement in plant height among the different fertilizers. Whereas, in the field, any biofertilizer did not significantly increase rice yield over the untreated control. Also, our results prove that increased amounts of biofertilizers or the use of an indigenous N_2-fixing cyanobacterial isolate does not

necessarily result in better yields. Field conditions represent a challenging environment for the establishment and effectiveness of biological fertilizers. Therefore, according to our results, new approaches should be tested in order to obtain the maximum benefit from biofertilizers. N_2-fixing cyanobacterial genera more adapted to limiting light conditions at later rice vegetative stages should be tested as previously mentioned (De et al. 1998). It is also suggested that the combined use of biological fertilizers and synthetic fertilizers has the potential to achieve a higher productivity in a more sustainable and responsible manner.

CHAPTER V

CONCLUSIONS

The optimum temperature for the sheath blight development in rice was in the range of 25 to 30°C. Development of sheath blight was slow at lower temperatures (10 and 15°C) and suppressed at higher temperatures (35 and 40°C). These trend patterns on the effects of temperature on sheath blight development in rice are in a good agreement with the patterns on the effects of temperature on the growth of mycelium of *Rhizoctonia solani* on PDA medium. The biocontrol treatment reduced sheath blight in 47% in Presidio and 42% in Jasmine 85, demonstrating that the effect of biocontrol is similar on both susceptible and partially-resistant cultivars. The maximum reduction in disease severity occurred when Jasmine 85 was treated with the biocontrol. These results suggest that the combination of the biocontrol treatment and cultivar resistance can be a more effective approach for management of sheath blight of rice than using the biocontrol agent alone. The relative efficacy of the biocontrol treatment varied with temperature. Its relative efficacy at 10, 15, 20, 25, 30, 35 and 40°C was 34, 28, 45, 51, 39, 56, and 50%, respectively, indicating that *Bacillus subtilis* was more effective at higher temperatures (35 and 40°C) than at lower temperatures.

Cercospora janseana isolates showed variable sensitivities to the quinone outside inhibitors (QoI) azoxystrobin, pyraclostrobin, and trifloxystrobin, as well as to thiophanate methyl and mancozeb, with some isolates such as CN-2-4 presenting low sensitivity to most of these active ingredients. On the other hand, all isolates had high sensitivity to the succinate dehydrogenase inhibitors (SDHI) fluxapyroxad that contrasted with the low sensitivity to the SDHI flutolanil, demonstrating no cross-resistance to these active ingredients. Demethylation inhibitor (DMI) active ingredients did not inhibit conidia germination. However, the DMI propiconazole alone or in combination with azoxystrobin or trifloxystrobin reduced narrow brown leaf spot

(NBLS) severity in the field evaluations. Similarly, fluxapyroxad effectively controlled NBLS.

Although in the greenhouse, the microalgal biomass concentrate improved rice plant height, the use of increased amounts of biofertilizers or the use of a N_2-fixing cyanobacterial isolate more adapted to the field conditions did not improve rice yields over the untreated control under field conditions.

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APPENDIX A

SEQUENCING ENDOGENOUS RICE PADDY SAMPLES

COLONY PCR:

Materials:

- Sterile 200µL PCR tubes
- TE Buffer (10mM Tris + 1mM EDTA, pH 8.0) with 1% Triton
- Sterile pipette for transfers
- Chloroform:Isoamyl Alcohol (24:1)

Procedure:

1. Fill a 200µL PCR tube with concentrated sample.
2. Centrifuge at 14,000rpm for 5 minutes.
3. Decant supernatant.
4. Re-suspend pellet in 100 µL TE Buffer with 1% Triton.
5. Vortex the cell suspension for 5-10 seconds.
6. Incubate in a thermocycler at 99°C for 10 min (set the thermocycler with a 10 minute hold at 99°C followed by a hold at 4°C).
7. Remove from thermocycler and add 100µL of Chloroform:Isoamyl Alcohol (24:1).
8. Mix by inversion for 2-3 minutes.
9. Centrifuge at 14,000rpm for 5 minutes.
10. Supernatant (aqueous layer) contains desired PCR product. Remove this layer into a fresh PCR tube without disturbing the phase line.
11. The product can be reserved at 4°C for future use in the short term or -20°C/-80°C if longer storage is required.

ITS AMPLIFICATION:

Add the following components to a PCR tube:

- 40.8µL H2O
- 5 µL 10X Buffer
- 1 µL dNTP (0.2mM each final)
- 1.5 µL MgCl2 (1.5mM)
- 0.25 µL Cyano ITS1-R (Working stock 12µM)
- 0.25 µL Cyano ITS2-F (Working stock 12µM)
- 0.2 µL Invitrogen Platinum TAQ DNA Polymerase
- 1 µL Sample's PCR Product

Incubate in thermocycler under the following program:

- 1 Cycle of: 94°C for 2:00min
- 35 cycles of: 94°C for 1:00/57°C for 1:00/72°C for 4:00
- 1 cycle of: 72°C for 10:00
- 1 hold of: 4°C for ∞

GEL VERIFICATION:

Materials:

- ~30mL 0.6% agarose in 1X TAE
- Gel cast with well template
- 1X TAE
- Tub of ethidium bromide

Procedure:

1. Melt 0.6% agarose in 1X TAE in microwave (~2 min).
2. Allow solution to cool but not solidify.
3. Pour agarose solution into gel cast with well template in place until the bottom of the cast is completely covered. Allow gel to set (15-20 min).
4. Place gel in electrophoresis apparatus. Fill with 1X TAE until the top of gel is completely covered.
5. Load one well with 2µL of DNA ladder.

6. Load other wells with a mix of 5µL amplification product and 1µL 6X loading buffer.
7. Run gel from black to red at 70V, 400amp for ~1.5 hrs.
8. Stain gel in ethidium bromide for ~20min.
9. Visualize gel with UV transilluminator.

- If only one band ~1,600bp – proceed to purification for sequencing.
- If two bands ~1,600bp and ~600bp (or more) – repeat amplification on product with ITS1 and ITS5. Desired product is ~600bp.

PRODUCT PURIFICATION:

1. Follow instructions for GeneJET PCR Purification Kit (#K0702).
2. Verify product integrity and concentration using nanodrop. (Make sure to clean, blank, re-blank, and one measurement with H2O).
3. For samples sent to LIMS for sequencing, rxn must have 2-5ng product for every 100bp.
4. Create two 12µL rxns to sequence, each in 1.5mL tubes:
    - ex: Forward:

        3µL of 13ng/µL product with desired 600bp

        1µL ITS5

        8µL H2O
    - ex: Reverse:

        3µL of 13ng/µL product with desired 600bp

        1µL ITS1

        8µL H2O
5. Enter sequencing request on LIMS (core prep primer-template), 12pmol primer.

APPENDIX B

SAS CODES ACCORDING TO EACH CHAPTER

CHAPTER II

***In vitro* study.**

ANOVA and LSD test

```
proc glm;
class rep temp;
model diameter = temp;
means temp/lsd lines;
run;
```

Greenhouse study.

ANOVA and LSD test

```
 proc glm data= severity;
 class  rep cult treat temp;
 model seve =  rep    cult  rep*cult
 treat   cult*treat rep*cult*treat
 temp       cult*temp treat*temp cult*treat*temp;
 test h=cult   e=rep*cult;
 test h=treat   e=rep*cult*treat;
 test h=cult*treat e=rep*cult*treat;
 means cult/lsd lines e=rep*cult;
 means treat/lsd lines e=rep*cult*treat;
 means temp/lsd lines e=rep*cult*treat*temp;
 lsmeans cult*treat/pdiff lines;
 lsmeans cult*temp/pdiff lines;
 lsmeans treat*temp/pdiff lines;
 lsmeans cult*treat*temp/pdiff lines;
```

run;

CHAPTER III

***In vitro* study.**

Normality of data

```
PROC UNIVARIATE DATA=germination NORMAL PLOT;
VAR germ;
QQPLOT germ /NORMAL(MU=EST SIGMA=EST COLOR=RED L=1);
RUN;
```

ANOVA and LSD test

```
proc glm data=germination;
class rep iso fungic rate;
model germ =rep iso rep*iso fungic iso*fungic rep*iso*fungic rate
iso*rate fungic*rate iso*fungic*rate;
means iso/lsd lines;
means fungic/lsd lines;
means rate/lsd lines;
lsmeans iso*fungic/pdiff lines;
lsmeans iso*rate/pdiff lines;
lsmeans fungic*rate/pdiff lines;
lsmeans iso*fungic*rate/pdiff lines;
run;
```

CHAPTER IV

Greenhouse study.

ANOVA and LSD test including the effect of all days after planting (DAP)

```
proc glm data=height;
class rep fert DAP;
model height =rep fert DAP fert*DAP;
```

means fert/lsd lines;

means DAP/lsd lines;

lsmeans fert*DAP/pdiff lines;

run;

ANOVA and LSD test for each day after planting

proc glm;

class rep fert;

model height = fert;

means fert/lsd lines;

run;

Field study.

ANOVA and LSD test

proc glm;

class rep fert;

model yield =fert;

means fert/lsd lines;

run;