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Biology and Control of Rice False Smut Caused by Ustilaginoidea virens (Teleomorph Villosiclava virens)

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by

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Missouri Southern State University
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Abstract

Rice false smut (FS), a disease caused by *Ustilaginoidea virens* (Cke.) Takahashi (1896), was first reported in northeastern Arkansas counties in 1997. The first objective of this research was to establish a collection of *U. virens* isolates from geographically diverse regions of Arkansas. Three *U. virens* isolates and chlamydospores from 'Templeton' and 'Clearfield-151' rice cultivars were used to determine the effects of temperature and pH on mycelial growth and germination. A nested-PCR protocol and histological methods were used to determine if *U. virens* infects and colonizes rice seedlings and spikelets on panicles. The sensitivity of three *U. virens* isolates was tested to analyze the inhibition of mycelial growth *in vitro* and to establish inhibitory concentrations to six technical and five analytical grade fungicides. Field and greenhouse tests were conducted to determine if fungicide seed treatments using technical grade fungicides could effectively reduce the incidence of *U. virens* rDNA in seedlings as measured by nested-PCR. Finally, field tests were conducted using fungicide seed treatments to control FS at two locations and disease was assessed by a visual disease assessment.

We have an established collection of 190 isolates obtained from nine cultivars in seven counties of Arkansas, USA. Mycelial growth and germination of chlamydospores occurred between pH levels from 5.5 to 8.0. Mycelial growth and germination of chlamydospores occurred at temperatures from 18° to 34°C and from 18 to 26°C, respectively. Nested-PCR tests indicate the protocol is specific and sensitive for detecting *U. virens* in rice. Ribosomal DNA of *U. virens* was detected using nested-PCR from seedlings within three days after emergence from the soil and in 27.5 to 75% of spikelets in booted panicles before exsertion. Selected isolates of *U. virens* were sensitive to fungicides *in-vitro* but results from using nested-PCR in the

greenhouse and field to screen seedlings for *U. virens* rDNA indicated a significant reduction in the incidence of *U. virens* in some seed treatments compared to controls. Seed treatments did not significantly reduce FS disease compared to controls in the field plots when measured by visual disease assessments.

Acknowledgements

I would like to take this opportunity to thank my committee members Drs. Burt Bluhm, Ken Korth, Fred Spiegel and the head of the Department of Plant Pathology, Rick Bennett for their contributions to initiating and ongoing support of this project. A special thanks to my supportive advisor Dr. David O. TeBeest for his tireless encouragement and careful guidance in preparation of this project and thesis. I also would like to honor my late father, Joseph Francis Jecmen DDS who constantly inspired me to pursue my dreams of studying plant sciences. He also passionately encouraged me to develop and contribute my talents to help others as he devoted his life to a ministry of healing and serving people in need.

This project would not have been able to come to fruition without the relentless work in the persistent summer heat and droughts by my advisor Dr. David O. TeBeest and the field staff at the University of Arkansas Research Stations at Newport and Pine Tree. They all deserve big thanks! Other big thanks are due to the Agriculture Diagnostic Laboratory for Research Soil Analyses, Jeff Velie in the Agricultural Statistics lab for statistical data analysis and Alma Laney for advice in sequence analysis and manipulations. A special thank you is warranted for the ongoing support and intellectual challenge of my peers. I am very thankful to have enjoyed the plentiful learning resources of very helpful staff and outstanding Professors here in the Department of Plant Pathology at the University of Arkansas!

Dedication

I would like to dedicate this thesis to the future generations of researchers, for we all stand on the shoulders of those who precede us. No combination of words may ever fully express what a very wonderful opportunity it is to have the privilege of studying what is known and exploring the unknown. I wish you the best in future endeavors!

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I. Introduction and review of relevant literature on infection of rice by *Ustilaginoidea virens*

Rice and rice production

Rice is a complex of two domesticated grass species (*Oryza sativa* L. and *Oryza glaberrima* Steud.). Rice is grown in the warm temperate and subtropical regions of the world (Sweeney and McCouch, 2007). Rice has a high economic importance and is relied upon as a staple crop by one half of the world's population. Rice production in the United States of America (USA) is about 2% of total global production (Anonymous, 2012) and according to the USA Rice Federation 19 billion pounds of rice are produced in the USA annually on 2,750,000 acres. Arkansas produces nearly 1% of the world's rice, valued at nearly $1 billion annually, on approximately 1.3 million acres each year (Anonymous, 2012). Arkansas is the top rice producer among the six rice producing states in the USA. In 2010, approximately 1,785,000 acres in Arkansas were devoted to its production. Rice producers in Arkansas are continually using measures to control important plant diseases including rice blast, sheath blight, stem rot and smuts.

Ustilaginoidea virens (etiological agent of rice false smut)

Two smut diseases are found on rice panicles (Lee and Gunnell, 1992). Kernel smut is a disease caused by a basidiomycete *Tilletia barclayana* (Bref.) Sacc. & P. Syd. in Sacc. [syn. *Neovossia horrida* (Takahashi)]. The other panicle smut of rice is rice false smut (FS) caused by *Ustilaginoidea virens* (Cooke) Takahashi, an ascomycete in the class Sordariomycetes; order Hypocreales; monophyletic tribe Ustilaginoideae, closely related but distinct from the Claviceptaceae and Hypocreaceae clades (Bischoff et al. 2004). False smut was first described as *Ustilago virens* in 1878 from a specimen originating from Tamil Nadu in India (Cooke, 1878).

Tanaka et al. (2008) proposed the teleomorph *Villosiclava virens* because of taxonomic similarities with other *Villosiclava* spp. The teleomorph name, *Claviceps oryzae-sativae* (Hashioka, 1971) has thus recently been amended because phylogenetic studies identified a close association with *Villosiclava* spp. (Tanaka et al., 2008). False smut was first reported in Arkansas in 1997 and has since become increasingly prevalent in Arkansas rice fields. This disease is also emerging as an economically important problem in global rice production (Tsuda, 2006; Webster and Gunnell, 1992; Zhou et al., 2008).

False smut is now widespread throughout tropical Asia, India, Egypt, Italy, Australia, Central and South America and has been in the United States of America since the late 1890's (Lee and Gunnell, 1992; Atia, 2004). Rice is the most ecologically and economically significant host for *Ustilaginoidea virens* Cooke (Takah.) (Lee and Gunnell, 1992). False smut has become an increasingly important rice crop disease since its discovery in Louisiana in 1908, (Fulton, 1908) and in 17 fields in four counties of northeast Arkansas (Cartwright, 1999). It is now widespread in northeastern and central Arkansas Counties (Cartwright et al., 2002; Cartwright and Lee, 2006).

Interest in the control of FS in Arkansas is increasing because it has spread throughout the state and because the fungus produces two toxins, rhizoxin and ustiloxin. The latter is a phytotoxic and mycotoxic microtubule inhibitor that is toxic to humans and animals feeding on rice grain or silage (Koiso et al., 1992; Koiso et al., 1994; Li et al., 1995; Luduena et al., 1994; Miyazaki et al., 2009; Yamashita, 1956). Spore balls and chlamydospores have received much attention as a source of the toxin (Figure 1.1). Atluri (1988) suggested that inhalation of chlamydospores released into the air at harvest may be hazardous to human health. Thus, there may be an undefined public health risk to people and livestock from exposure to toxins from FS.

Grain quantity and quality are both affected by this disease and there are several reports of reduced yields (Chib et al., 1992; Hegde and Anahosur, 2000; Ikegami, 1959). Recently, the International Rice Research Institute (IRRI) indicated that management of FS is a high priority (Mew et al., 2004). Mew et al. (2004), Lee and Gunnell (1992), Biswas (2001) and Tanaka et al. (2008) concluded that the information on the FS-rice pathosystem is inadequate.

Features of the *Ustilaginoidea virens* and the FS-Rice pathosystem

False smut spore balls or sclerotia (sclerotia = pseudomorph) (Figures 1.1 and 1.2) are found growing in association with *Oryza sativa*, on *Zea mays* L. and a few other tropical Graminae hosts that have no significant economic importance (Abbas, 2002). Spore balls are comprised of a proliferation of branched, radial and compacted hyphae that give rise to spherical to elliptical (3-5 X 4-6 µm), warty olivaceous chlamydospores at the terminus (Ou, 1985). The chlamydospores are smooth, round to elliptical when immature but warty, spiny and yellow to orange pigmented when mature. The chlamydospores become olivaceous, globose to irregularly round, as they mature and the surface is ornamented with prominent spines (Kim and Park, 2007). In culture, the chlamydospores germinate and produce fine germ tubes that give rise to 1-3 small ovoid secondary conidia (Hashioka et al., 1951). *Ustilaginoidea virens* can be cultured on potato dextrose agar (PDA) or potato dextrose sucrose agar (PDSA), but the fungus is slow growing and has two different colony types. The two colony types are either green, hardened sclerotia-like after incubation for two weeks or white, spreading mycelia after incubation for three weeks (Figure 1.3). Further, the fungus can also be grown in liquid broth.

Descriptions of the teleomorphic stage, *Villosiclava virens*, include flat, botuliform, reniform, horseshoe-shaped or differently shaped, 1 to several, usually 2, protruding from sclerotia (or pseudosclerotia) overwintering in the field and producing a stalked stromata (=

ascomata) containing perithecia with about 300 asci in the summer or fall of the following season (Ou, 1985; Lee and Gunnell, 1992; Tanaka et al., 2008). The asci are described as cylindrical, hyaline, filiform, unicellular, 120-180 X 4 µm (130-300 X 4-7 µm) and contain eight ascospores that are hyaline, filiform, septate disarticulating at septa to form four part-spores 120-180 X 0.5-1 µm (140-230 X 1.3-1.8 µm) (Hashioka et al., 1951; Ou, 1985; (Tanaka et al., 2008)). The four part-spores are aseptate and are 30-60 µm according to Tanaka et al. (2008). Although the anamorph stage of *U. virens* is common in Arkansas, the teleomorphic or perfect stage (*Villosiclava virens*) has not yet been observed but it may exist in Arkansas rice fields. If the teleomorphic stage is found, future research on FS will need to be considerate of the recombination of genotypes in the population as described by Zhou et al. (2008), contributing to more genetic variability in the population than an asexual population where there is less genetic variability.

Symptoms on rice infected by *U. virens* are entirely absent until after panicles begin flowering, at which time rice grains are replaced by spore balls that erupt through the glumes. The spore balls, comprised of expanding mycelium and layers of chlamydospores, are at first covered by the pale white expanded testa and aleurone membranes of the seed. With further expansion and development of the chlamydospores, the membranes rupture to expose the chlamydospores which appear orange then becoming yellow-orange to olive green then to dark green or nearly black over time (Figures 1.1 and 1.2).

The disease cycle of this fungus-host interaction has not been completely characterized (Biswas, 2001; Lee and Gunnell, 2002; Mew et al., 2004, Fu et al., 2012), but knowledge of the pathogen's life history and infection process is critical for efforts to control this disease (Tang et al., 2012). Two pathways for infection of plants in the disease cycle have been hypothesized for

FS. In the first proposed model, primary infections occur when chlamydospores produce air-borne conidia and/or sclerotia produce air-borne ascospores that germinate in late summer or early fall and land on inflorescences during the flowering stage of rice (Figure 1.4). Another proposed model is that the fungus survives the winter by means of spore balls and/or sclerotia (Figure 1.5). Chlamydospores released from spore balls on seed and/or in or on soil germinate then the hypha colonize seedlings during germination or shortly thereafter. The fungus then colonizes the apical meristematic tissue and then colonizes flowers in the boot prior to emergence of the panicle.

The role of the sclerotia and/or spore balls has been investigated and they are thought to serve as survival structures for inoculum over the winter (Ashizawa and Kataoka, 2005; Biswas, 2001; Ou, 1985; Rathaiah and Bhattacharya, 1993; Raychaudhuri, 1946; Schroud and TeBeest, 2005; Singh and Dubey, 1984; Sreeramulu and Vittal, 1996; Zhou et al., 2003; Zhou et al., 2008). Hisada (1936) observed fields and suspected that infections occur when rice plants are at the booting stage. Hashioka et al. (1951) observed that 99.6% of the spore balls contained intact anthers, suggesting that most infections take place before flowering. Raychaudhuri (1946) observed disintegration of the ovary, while the style, stigmas and the anther lobes containing pollen grains are entirely surrounded by the growing fungus which gives rise to the spore ball structure. The sources of inoculum have been hypothesized to be airborne conidia or ascospores produced from germinated chlamydospores or sclerotia that make contact and infect flowers after germination or alternatively, infection and transmission may occur from dormant mycelium associated with the embryo at planting (Kulkarni and Moniz, 1975; Raychaudhuri, 1946; Singh and Dubey, 1984; Sreeramulu and Vittal, 1996). Mew et al. (2004) suggested that the initial

inoculum is the sclerotia in the soil or the ascospores, although airborne conidia may also produce infections.

In inoculation studies, Ikegami (1960) successfully inoculated rice plants with chlamydospores and ascospores by injecting a spore suspension into the developing panicle enclosed by the leaf sheath. This suggests that infection takes place during a short period of time just before emergence of the panicle from the boot at heading. Ikegami, (1959, 1962) and Barauh, (1992), suggested that primary infections might be originating from spore balls in soil. Chlamydospores of *U. virens* are easily observed on seed coats (Ikegami, 1959, 1962; Koiso et al., 1994; and Raychaudhuri, 1946) as shown in Figure 1.6. Ikegami (1963) inoculated coleoptiles of germinating seedlings with conidial suspensions and found the fungus in sieve tube elements of rice tissues and in the growing points of the main tillers. These data suggest that *U. virens* infects rice seedlings shortly after germination and may maintain this association through the growing season. Ikegami, (1962) also determined that high incidences of disease was correlated with inoculating coleoptiles that were between 3-10 mm in length with yellow chlamydospores, resulting in numerous spore balls forming on the panicles. Root infection occurring from germinating chlamydospores shortly after germination was demonstrated by Shroud and TeBeest (2005) (Figure 1.7). The importance of chlamydospores in soil or on contaminated rice seed has recently been investigated in Arkansas (Brooks et al., 2009; Ditmore and TeBeest, 2006; Schroud and TeBeest, 2005; TeBeest et al., 2010). Recently, Ditmore and TeBeest (2006) and TeBeest et al. (2010) provided potential new insight into the disease cycle by revealing that increased infection levels of rice may be caused by increasing *U. virens* inoculum levels in the soil (Figure 1.8).

Development of FS spore balls is often associated with periods of high humidity, rainfall and cool weather (Karthikeyan and Jebaraj, 2008; Rathaiah et al., 1993) but this has not been studied in the USA. Tsuji et al. (1997) correlated the incidence of FS to temperatures within the range of 13°C to 15°C during the 10 days before heading and reported that a correlation with rainfall was inconsistent.

Molecular detection methods for *Ustilaginoidea virens*

Progress in studying the epiphytology of the rice-FS pathosystem has been hampered by the lack of an efficient visual diagnostic and disease evaluation protocol or technique. Previous studies have shown that *U. virens* hyphae invade and colonize rice coleoptiles at the seedling germination stage and maintain an asymptomatic association with rice sieve tube elements Ikegami (1963). More recently, a growing number of studies have used nested-PCR to detect *U. virens* and other plant pathogens in similar studies to detect where the pathogen DNA is present in plant tissues (Willits and Sherwood, 1999; Zhou et al., 2003; Ashizawa and Kataoka, 2005; Ditmore and TeBeest, 2006). Ashizawa and Kataoka (2005) and Zhou et al. (2003) independently designed nested-PCR primers specific to *U. virens* rDNA. These protocols were used to detect *U. virens* rDNA in total DNA extracted from panicles prior to emergence from the boots (Ashizawa and Kataoka, 2005) or in DNA from rice spikelet, leaf sheath and stem tissues after artificially inoculating rice as described by Fujita et al. (1989) (Zhou et al., 2003). Ditmore and TeBeest (2006) amplified *U. virens* rDNA from seed infested with *U. virens* chlamydospores (Figure 1.7). These reports indicate that *U. virens* rDNA is present in rice. Thus, nested-PCR primers could be used to detect and quantify *U. virens* rDNA from colonized rice tissues at different stages of rice development and determine the distribution of the fungus within specific tissues of plants including spikelets of panicles prior to emergence from the boot.

Methods for controlling false smut in rice

In the past, the incidence and severity of this disease did not warrant control measures (Lee and Gunnell, 1992). Fungicide applications prior to the booting or heading stage have been investigated to suppress the development of FS on panicles (Cartwright et al., 2002; Cartwright and Lee, 2006; Chen et al., 2013; Hopkins et al., 2002; Tsuda et al., 2006). Field tests were conducted by Tsuda et al. (2006), to determine the effect of fungicide applications to flooded paddy water to control FS. The results show that the application of simeconazole granules to the paddy water two to five weeks prior to the heading stage of rice was highly effective against FS and the fungicide application at three weeks was the most effective. Others (Biswas, 2001; Brooks et al., 2009) reported that water management and fertilization affected FS incidence. Cultural practices that have been investigated to reduce FS include no-till practices, furrow irrigation and reduced nitrogen applications (Brooks et al., 2009, 2010). Mew et al. (2004) and Ou S. H. (1985) pointed out that differences in smut incidence and severity of the disease on cultivars planted in the same site or localities do occur. Reduced levels of infection have been observed in a number of cultivars (Biswas, 2001; Brooks et al., 2009; Brooks et al., 2010; Pan et al., 1999; Singh and Gangopadhyay, 1981; TeBeest and Jecmen, 2011), but high levels of resistance to FS have not been reported in cultivars presently grown in the USA (Cartwright et al., 2000; Cartwright et al., 2002; Cartwright and Lee, 2006; Parsons et al., 2003; Wilson et al., 2003; Wilson et al., 2005). It is evident that the current management strategies for the control of FS may be inadequate around the world.

Goals

Understanding how to manage FS has been difficult because the literature is fragmented, unclear or at times, even contradictory. Many questions relevant to the fully integrated control of FS using cultural practices, fungicide applications and deployed resistance remain unaddressed. If the PCR protocols published in the literature are verified, this offers an opportunity to address these important questions. A few directives outlined by Zhou et al. (2003) suggested that future studies on the epiphytology of FS should include utilization of a nested-PCR protocol. Based on these directives, the proposed uses of nested-PCR in this study are, 1) to facilitate further studies of the FS disease cycle, 2) to provide a tool for predicting the incidence of *U. virens* rDNA in plants prior to the onset of visual symptoms and 3) to facilitate the evaluation of fungicide efficacy and cultivation control strategies to improve integrated management strategies for control of FS in rice. The overall goal of this research project was to provide relevant information that will contribute to more effective management of FS in Arkansas.

Hypothesis

After review of the literature and based on preliminary data obtained from the laboratory and field, *U. virens* chlamydospores may either be soil-borne and/or seed-borne. Colonization of the actively growing point (shoot apical meristem = SAM) of tillers may occur after invasion of the seedling coleoptile or root tissues by *U. virens* hyphae and this association may be maintained through vegetative growth through panicle initiation. Colonization of individual spikelets by hyphae may increase upon panicle maturation in the boot prior to emergence under undefined optimal conditions and the spore balls develop on spikelets after panicle emergence shortly after flowering during an undefined duration of time.

The following hypotheses are proposed to research the above goals. It is hypothesized that *U. virens* infects rice seedlings during the germination stage or within a few days after seedling emergence. It is also hypothesized that since *U. virens* may occur in soil and on seed, fungicide seed treatments may be used or developed to primarily prevent infection of rice seedlings by suppressing germination of chlamydospores and fungal growth.

Scientific objectives

We have developed five objectives to test the two hypotheses.

1. To establish a collection of *U. virens* isolates from geographically diverse regions and different cultivars of Arkansas and to use three isolates from the collection to determine the effects of temperature and pH on germination and growth.
2. To determine if a nested-PCR detection protocol is sufficiently specific and sensitive that it provides for accurately determining the timing of infection and colonization of rice seedlings by *U. virens*.
3. To analyze the efficacy of six technical and five analytical grade fungicides to inhibit mycelial growth of three *U. virens* isolates from our collection and to establish the inhibitory concentrations, IC_{50} (inhibitory fungicide concentration at 50% of maximal growth) that limit mycelial growth *in-vitro*.
4. To analyze the effectiveness of fungicide seed treatments in the greenhouse and field using nested-PCR as a diagnostic probe for detecting the presence of *U. virens* rDNA in rice seedlings.
5. To determine if fungicide seed treatments effectively reduce FS disease development in the field by using a visual disease assessment.

Figure 1.1. Examples of a heavily infected selection of mature panicles with FS spore balls occurring on infected ‘Clearfield-151’ at the University of Arkansas, Pine Tree Research Station, near Colt, AR that are dispersing chlamydospores to adjacent panicles. (A. C. Jecmen)

Figure 1.2. Mature FS spore balls occurring on infected ‘Templeton’ panicles at the University of Arkansas, Division of Agriculture, Newport Research Station, in Newport, Arkansas. The spore balls on the infected panicles are dispersing chlamydospores to the adjacent panicles. (A. C. Jecmen)

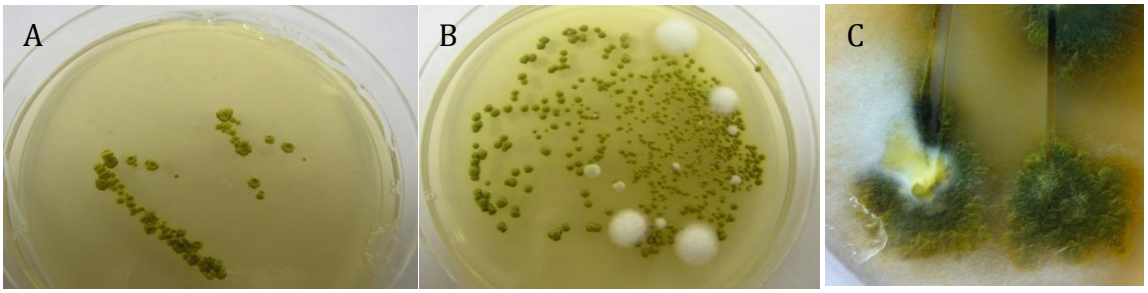

Figure 1.3. Cultures of *U. virens* can be obtained by streaking or spreading chlamydospores from a FS spore ball or culture onto acidified-PDA and incubating at 26 °C.
(A) Colonies after 14 days incubation.
(B) Colonies after 21 days incubation with evidence of conidia and spreading mycelium arising from germinating chlamydospores present.
(C) Colonies after 45 days incubation.
Chlamydospores or conidia from any stage can be transferred to another Petri plate containing PDA to perpetuate the life cycle in culture.

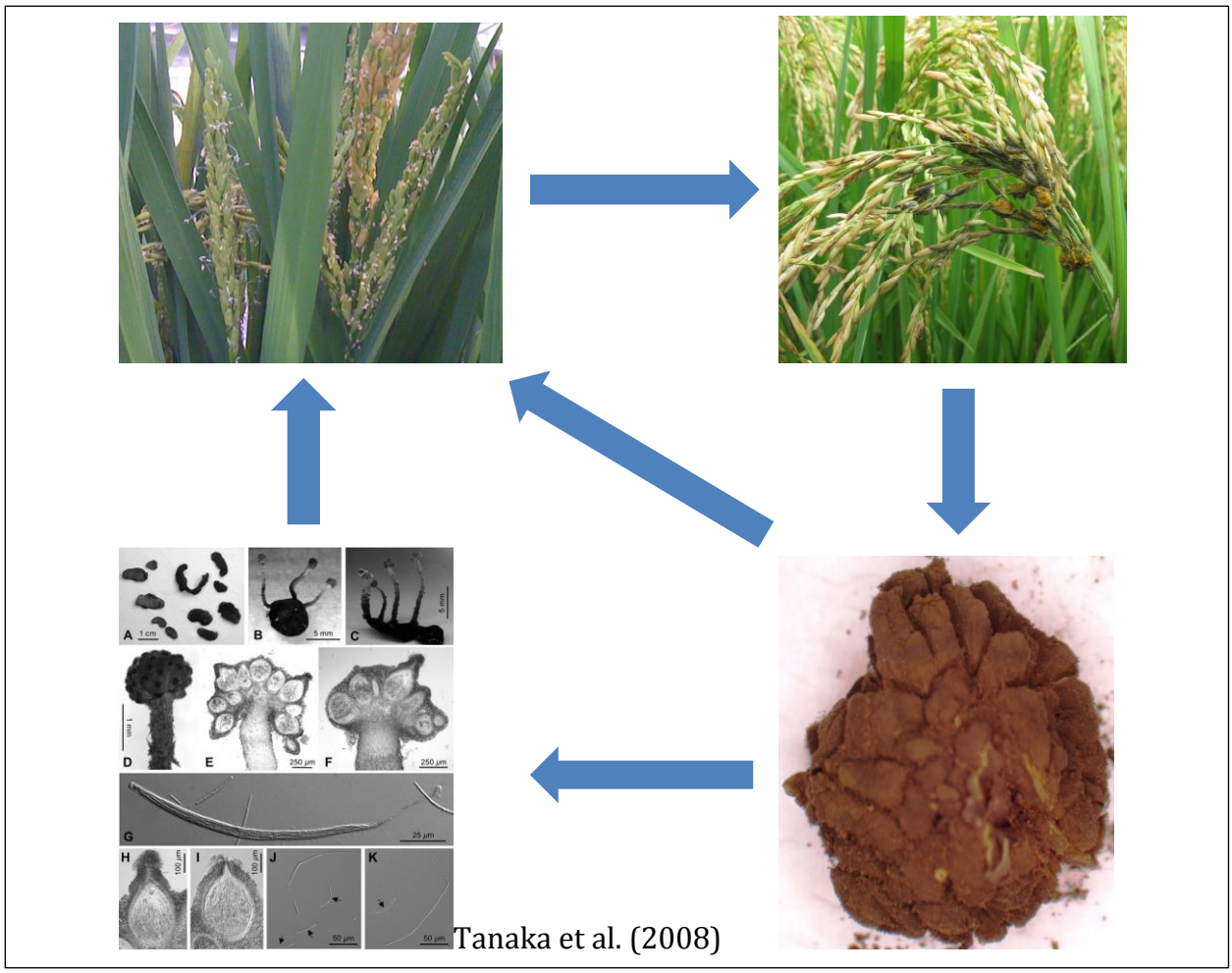

Figure 1.4. A proposed model describing the pathway for infection of plants in the disease cycle of false smut in rice caused by *Villosiclava virens* (anamorph: *Ustilaginoidea virens*). Sclerotia in or on the soil germinate and produce stalked stromata that may release air- or wind-borne ascospores. Alternatively, chlamydospores germinate and produce air- or wind-borne conidia.[A] Primary infection of flowers is thought to occur either by air-borne ascospores as described (Nikata, 1934; Tanaka et al., 2008) produced from sclerotia in the soil (Biswas, 2001; Fu et al., 2012; Ou, 1985; Singh and Dubey, 1984), or by secondary conidia produced from germinated chlamydospores (Kulkarni and Moniz, 1975; Mew et al., 2004; Rathiaiah and Bhattacharya, 1993; Raychaudhuri, 1946; Singh and Dubey, 1984; Sreeramulu and Vittal, 1966). The primary infection site was assumed to be at the stigma, ovary and young grain tissues (Padwick, 1950; Wang, 1992). However, spraying rice panicles after heading does not result in diseased panicles (Deng, 1989; Fujita et al., 1989; Wang et al., 2004), although applying chlamydospores suspensions to fertilized and unfertilized ovaries with a camel hair brush results in successful inoculation (Kulkarni and Moniz (1975). Panicles injected with spores in the boot stage has produced infected panicles (Ashizawa et al., 2011; Fujita et al., 1989; Guo et al., 2012; Ikegami, 1960; Kulkarni and Moniz, 1975; Tanaka et al., 2011; Tang et al., 2012; Wang et al., 1996; Wang et al., 2008; Yoshino and Yamamoto, 1952; Zhang et al., 2004; Zhou et al., 1999). Interestingly enough, a species-specific nested-PCR protocol to amplify the ITS rDNA-of *U. virens* has detected *U. virens* rDNA from whole panicles at the booting stage before rice heading (Ashizawa and Kataoka, 2005), which agrees with previous reports of the fungus invading the rice plant at the booting stage or earlier (Fujita et al., 1989, Hisada, 1936; Ikegami, 1961).

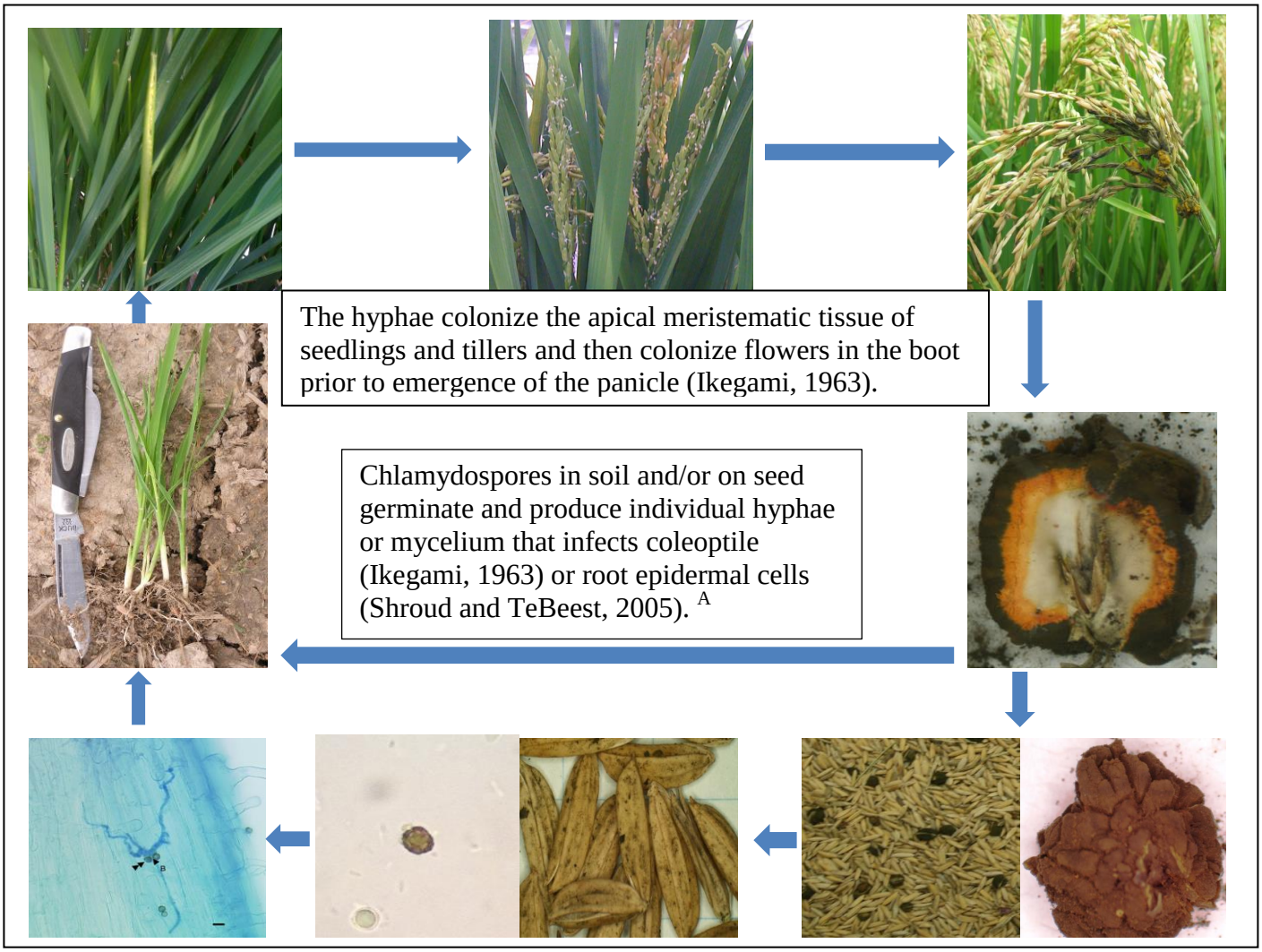

Figure 1.5. Another proposed model describing the pathway for infection of plants in the disease cycle of false smut in rice caused by *Villosiclava virens* (anamorph: *Ustilaginoidea virens*).
[A] Chlamydospores from *Ustilaginoidea virens* may overwinter on seed surfaces (lower middle left) (Brooks et al., 2009; Ditmore and TeBeest, 2006; Koiso et al., 1994; Raychaudhuri, 1946; TeBeest et al., 2010), seed lots sometimes contain numerous false smut spore balls (lower middle) and spore balls may also persist on and/or in soil (lower middle right) (Barauh, 1992; Ditmore and TeBeest, 2006; Ikegami, 1959, 1960; Shroud and TeBeest, 2005; TeBeest et al., 2010). Experiments to determine if false smut is seed transmitted by inoculating seeds with chlamydospores have not been conclusive by Kulkarni and Moniz, (1975) and Biswas (2001). However, colonization of the coleoptile (Ikegami, 1962, 1963; Tang et al., 2012) and roots (Shroud and TeBeest, 2005), by *U. virens* hyphae is described. Interestingly, Wang and Bai (1997) described sowing seeds that were artificially inoculated with chlamydospores of *Ustilaginoidea albicans* resulted in successful infection of rice. Seedlings were previously shown to contain rDNA from *U. virens* after emergence from soil amended with *U. virens* chlamydospores Ditmore and TeBeest (2006). Spore balls contain intact anthers (Hashioka et al., 1951), intact styles, stigmas and anther lobes with pollen while the ovary is destroyed (Raychaudhuri, 1946).

Figure 1.6.

Seeds of rice cultivar ‘Cheniere’ are visibly contaminated with *U. virens* chlamydospores (seen as dark specks) on the surface (right), compared with clean seed (left) (A. C. Jecmen).

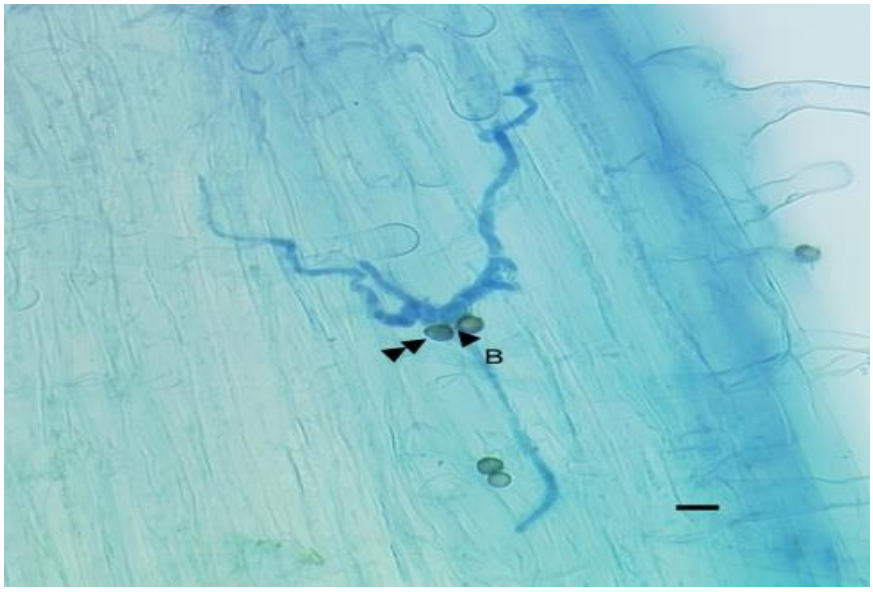

Figure 1.7. *Ustilaginoidea virens* chlamydospores (double arrow) germinating (Single arrow) on a 'Francis' root provides evidence of infection of rice, (Bar = 8 microns) (Shroud and TeBeest, 2005).

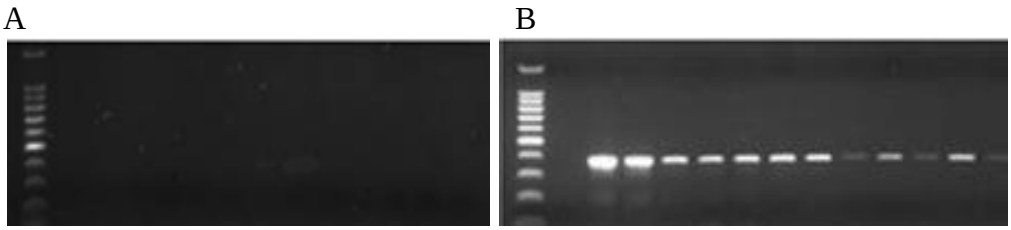

Figure 1.8. Plants grown from infiltrated seed were colonized by *U. virens.*
(A) PCR data showing control plants with no amplification indicating the absence of DNA consistent with *U. virens* rDNA.
(B) rDNA consistent with *U. virens* was detected in plants grown from infiltrated seed as indicated by a positive reaction with specific PCR primers (Ditmore M., J.W. Moore and D.O. TeBeest 2007).

II. Isolation, culture, preservation and the effects of pH and temperature on germination of chlamydospores and growth

Introduction

Rice false smut (FS), caused by *Ustilaginoidea virens* (Cooke) Takahashi, was first reported in Arkansas in 1997 (Cartwright et al., 2000) and is a growing concern for rice producers. The disease causes numerous yellow, rusty or burnt orange spore balls to develop on the panicles of diseased plants, which then turn dark green or black upon maturation of the grain in early fall. False smut is considered an economically destructive disease of rice in humid areas (Abbas et al., 2002; Lee and Gunnell, 1992; Mulder and Holliday, 1971).

Perusal of the Arkansas cryogenic fungal pathogen collections in the Department of Plant Pathology at the University of Arkansas revealed that isolates of *U. virens* were not present in the collections prior to initiation of this study. An isolate collection is a valuable resource to initiate studies on genetic and pathogen diversity within a population. And it is important to studies on the environmental effects of pH and temperature on germination and growth.

Hashioka et al. (1951) reported that germination of chlamydospores and mycelial growth is best at 28°C and that no growth occurred at 36°C. The study also identified mycelial growth as optimum at pH 6.02 to 6.72 and suboptimum at pH 2.77 and 9.05. Tsai et al. (1990) reported that the optimal pH range for conidium germination was between 5.0 and 8.0 and an optimal temperature of 25°C for conidium germination. The effects of either pH or temperature on mycelial growth were not studied by Tsai et al. (1990).

In this section of this study, the first objective was to prepare a collection of *U. virens* isolates from spore balls for use in experiments of this study and for future work to study the genetic diversity of isolates, in fungicide sensitivity assays and in virulence tests. The second

objective was to identify the pH conditions that were optimal or adverse for germination of chlamydospores and mycelial growth of three *U. virens* isolates found in Arkansas. Lastly, the third objective was to identify the temperature conditions that were optimal or adverse for germination of chlamydospores and for mycelial growth of three *U. virens* isolates.

Materials and Methods

Collection of samples to prepare *U. virens* isolates

Spore balls were collected from infected rice panicles of 'Clearfield-151', 'Clearfield-161', 'Cybonnet', 'Francis', 'Rice Tec CL XL 729', 'Taggart' and 'Templeton' cultivars provided by Dr. R. D. Cartwright and Dr. D. O. TeBeest and from my samples collected between 2008-2013. The panicles were obtained from rice fields in Arkansas, Jackson, Poinsett, St. Francis and White counties of Arkansas. The fields from which samples were obtained had recent but ongoing histories of FS epidemics. Samples from the cultivars in fields at each location consisted of 20-30 infected heads. The spore ball samples were stored in the laboratory in paper bags placed in Rubbermaid® tubs at 18°C until processed for isolation of *U. virens*.

Isolation of *U. virens* from spore balls

Single spore balls were removed from infected rice panicles and sectioned with a flamed scalpel. A piece (approximately 2 X 2 mm) of each spore ball was placed into a 1.5 ml microcentrifuge tube containing 1 ml of 0.8% Clorox™ solution, vortexed for one minute to mix the chlamydospores and then incubated for one additional minute. The resulting spore suspensions were diluted with distilled water to a final concentration of approximately 3.0×10^3 chlamydospores per ml^{-1}. Ten µl of a suspension were streaked onto acidified potato dextrose agar (PDA) (Difco™ Potato Dextrose Agar, Becton, Dickinson and Company, Sparks, MD) in 100 x 15 mm polystyrene Petri dishes (VWR, International). The PDA was amended to contain

1.0 µl ml^{-1} of streptomycin/ampicillin stock solution, containing 0.1 g l^{-1} ampicillin and 0.01 g l^{-1} streptomycin. Approximately 0.1 ml l^{-1} glacial acetic acid was added to adjust the pH of the PDA to 6.2 and the dishes were incubated at 26°C for two weeks. After incubation for 14 to 21 days, individual colonies were transferred onto a similarly prepared fresh PDA dish. Three colonies were selected at random from the collection of isolates, that originated from a spore ball from different rice cultivars 'Clearfield-151', 'Francis' and 'Templeton' cultivars (Table 2.1) for other work reported in this study as representative isolates I-6E, I-7E and I-8E respectively.

Cryogenic storage of the isolate collection

A total of 190 *U. virens* isolates were obtained by the isolation procedures described above after manipulating chlamydospores from spore balls (Table 2.1). Isolates were sub-cultured and grown on acidified PDA for two weeks prior to cryopreservation. Approximately 200 mg of tissue from a colony of each isolate was placed into a 2.0 ml polypropylene cryogenic preservation vial (#985746 Cryule® vial Wheaton Manufacturers Millville, N.J.) containing 1.0 ml of 30% sterile glycerol (CAS NO: 56-81-5 Mallinckrodt Baker, Inc. Phillipsburg, NJ). The Cryule vials were then labeled appropriately and placed into 96 well cryofreezer boxes prior to deposition in a Harris Manufacturing Co. (Asheville, NC) cryogenic freezer at -80°C.

Identification of single chlamydospores isolates as *U. virens*

Light microscopy was used to examine morphological characteristics of the spined chlamydospores, germinating chlamydospores, hyphae and secondary conidia in order to identify isolates as *U. virens*. Two slides per sample were prepared for observing chlamydospore morphology and germination at 24 hour increments until 72 hours after incubation. Twenty five chlamydospore samples from each spore ball were observed and this was repeated twice.

Identification of isolates with selective nested-PCR primers

A subset of 24 isolates I-1D (Cybonnet), I-2D (Cybonnet), I-3D (CL-161), I-4D (Templeton), I-5D (Rice Tec CL XL 729), I-6E (Clearfield-151), I-7E (Francis), I-8E (Templeton), I-9E (Clearfield-151), I-10E (Taggart), 3C (cultivar unknown), 1D (cultivar unknown), 3D (cultivar unknown), ST#3A (Clearfield-161), ST#8A (Rice Tec CL XL 729), G101A (Cybonnet), 203C (Cybonnet), I-1A (Cybonnet), I-2A (Cybonnet), I-4A (Templeton), I-5A (Rice Tec Cl XL 729), ST#1A (Clearfield-151), 4A (cultivar unknown), 109A (Cybonnet) and G102A (Cybonnet) from the isolate collection was tested for identity using DNA extracted from the isolate tissues by using the DNA QuickExtract™ system from Epicenter® following the manufacturer's protocol reacted with nested-PCR with primers selective for *U. virens* (Zhou et al., 2003). Approximately 0.1 g of mycelium from each isolate was scraped from 14 day old cultures grown on PDA. The harvested mycelium was placed into 2.0 ml tubes containing 100 µl of extraction buffer. The samples were incubated in water baths at 65˚ C for 6 min, then 98˚ C for 2 min and stored at 4° C until used to extract total DNA. Allele-specific nested-PCR primers US1-5/US3-3 and US2-5/US4-3 as designed by Zhou et al. (2003) (Table 2.2) were used to amplify the 380-bp and 232-bp rDNA fragments of the previously mentioned 24 *U. virens* isolates, respectively. Polymerase chain reactions consisted of DNA template, primary or nested-PCR primers, and 2 X Taq-polymerase master-mix from GenScript and PCR grade water. Negative reagent controls consisted of PCR reagents in a reaction with no template and a positive control from isolate I-6E were included in these tests.

Description of the white false smut found in this study

In 2011, 2012 and 2013 two non-melanized specimens of a white FS were observed in our field test plots at the Newport and Pine Tree Research Stations. A spore ball was recovered

from ‘Clearfield-151’ in 2011 and was recovered from ‘Francis’ each year in 2012 and 2013 in field plots at the Newport and Pine Tree Research Stations, respectively. Isolation of single chlamydospores colonies from the two spore balls recovered in 2011 and 2013 was conducted by using the isolation procedures described above for *U. virens*. For high magnification light microscope observation, chlamydospores from each of the spore balls were placed onto glass slides with 100 µl of distilled water. Twenty five chlamydospores from each spore ball were observed for biometric measurements and this was repeated twice. Biometrics of chlamydospores obtained from each spore ball and sporulating structures were then described after calibration of the microscope optical metrics using an objective micrometer.

DNA was extracted from five isolates, I-9A, I-9B, I-9C, I-9D and I-9E, that were isolated from the more mature white spore ball recovered from ‘Clearfield-151’ in 2011 at the Newport Station. DNA was extracted from the spore ball collected in 2012 from two samples of chlamydospores taken directly from the surface due to difficulties isolating viable chlamydospores from the less mature spore ball. Amplified DNA fragments, obtained from each DNA sample using Zhou et al. (2003) nested-PCR primer pairs and ITS1/ITS4 (White 1990) yielded 232 bp and ~600 bp fragments, respectively, were purified with a ZR DNA sequencing clean-up kit (Zymo Research, Irvine, CA) and sequenced at the University of Arkansas DNA Resources Laboratory using the Sanger method of DNA sequencing. Basic Local Alignment Search Tool (BLAST®) was utilized to study sequence similarity of isolate I-9E to other *Ustilaginoidea* sequences in the nucleotide sequence database. Sequence analysis and comparison of isolate I-9E to a selection of *U. virens* isolates (I-6E, I-7E and I-8E) from the isolate collection was performed to study sequence comparisons in the CAP3 Sequence

Assembly Program and BioEdit from the forward and reverse sequences (Hall, 1999; Sievers et al., 2011).

The virulence of I-9E was tested based on the methods described by Wang et al. (2008) using 12 plants per cultivar 'Neptune' and 'Roy J' obtained from RB station. Plants were grown in field soil obtained from the Newport Research Station to near maturity in 3.78 l (1 gallon) pots submerged under 10-15 cm (4-6") of water in 18.9 l (5 gallon) pails. Inoculum was prepared with chlamydospores grown in 25 ml of wheat bran broth (WBB) media and potato dextrose broth (PDB) media in 50 ml PYREX® round media bottles for one week on a rotary shaker at 24°C. The cultures were centrifuged at 7500 rpm for 10 min, the supernatant was decanted and pelleted conidia were re-suspended to a final concentration of 5.0 X 10^4 conidia ml^{-1} in water. Three booted panicles per cultivar from 9 plants (54 totals) were inoculated with between 0.5 and 1 ml of inoculum prepared from WBB or PDB cultures. Inoculum was injected with B-D® 1ml latex-free syringes (No. 309628 Becton Dickinson and CO., Rutherford, N.J., USA) fitted with a B-D® $25_G5/8$ (15.9mm) single use Yale hypodermic needles (Becton Dickinson & CO., Rutherford, N.J., USA) between the penultimate and flag leaf collars prior to panicle emergence. The location of the developing panicle was determined by manual dexterity and injections were made at approximately 45 degree angle to the stem at the tip of the panicle. Measurements of the distance between the penultimate and flag leaf collars were recorded as the boots were inoculated. Six panicles on three control plants (18 totals) were inoculated with water only. After inoculation, plants were placed in dew chambers set at 18°C for 48 hours and then plants were enclosed in 113.5 l (30 gal) opaque plastic trash bags for 120 hours at 24°C under a 10 hour day length of indirect fluorescent lighting. After the incubation period, the bags were removed and plants were moved to a greenhouse. Controls were inoculated with water and treated

similarly. Spore balls that developed on panicles after emergence were counted over a four week period. An additional treatment was made to three of the twelve plants during the last week of spore ball development to induce and promote conidium development on the white false smut spore balls of infected panicles. Plants in their respective pails were placed in a locked restricted access area outside to allow exposure to moisture laden air and to permit dew to form on the white false smutted panicles. All plants were observed daily after inoculation. Spore balls that developed on panicles after emergence were counted over a two week period.

Effect of pH on germination of one-, three- and five-month-old *U. virens* chlamydospores in agar adjusted to pH ranging from 5.5 to 8.0

To determine the germinability of chlamydospores after storage, chlamydospores obtained from spore balls on 'Clearfield-151' and 'Templeton' rice cultivars selected from plants in untreated test plots in our field studies at the University of Arkansas, Pine Tree Research Station, near Colt, Arkansas were stored in the lab at 18° C for one, three and five months after harvest. To determine the effects of pH on germination of chlamydospores obtained from each spore ball, buffers adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 were prepared with 0.1 M solutions containing potassium phosphate monobasic (KH_2PO_4) (VWR, West Chester, PA) and anhydrous potassium phosphate dibasic (K_2HPO_4) (Fisher Scientific, Fair Lawn, NJ). Two spore balls originating from 'Clearfield-151' and two spore balls from 'Templeton' cultivars were used to prepare each repetition of this test. A selection of chlamydospores from each spore ball, were suspended after the storage interval in tubes containing 500 µl sterile buffers (pH = 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) and diluted to a final concentration of 3.0×10^3 chlamydospores ml^{-1} as determined with a hemacytometer. Five hundred µl of each buffer solution were aliquoted into 2.0 ml polyvinyl tubes. Tubes of each buffer were then each inoculated with 500 µl

chlamydospores suspensions (3 x 10^6 chlamydospores ml^{-1}) of each chlamydospores sample in each respective pH buffer to create a final dilution of 3 X 10^3 chlamydospores ml^{-1} and 0.05 M buffer. After 96 hours, five µl of the chlamydospores suspensions from each tube were placed on a glass slide with a coverslip and observed with an Olympus CX31 light microscope under 400X magnification. Chlamydospores were considered germinated if a translucent fusiform germ tube was evident or if they were producing secondary conidia on phialides. Fifty chlamydospores mounted on a slide were counted for germination tests twice per tube with a manual Laboratory Counter (Clay Adams BD Co., Parsippany, N. J.).

The experiment was completed by averaging the two sub-sample measurements (50 chlamydospores) from each tube. There were three replicated tubes for each conidium sample in each repetition of the test. The whole experiment was repeated twice for chlamydospores from each cultivar, age group and pH level.

Growth of *U. virens* isolates I-6E, I-7E and I-8E on agar adjusted to pH ranging from 5.5 to 8.0

Agar (USP Grade CAS NO: 9002-19-0 MP Biomedicals, LLC. Aurora, Ohio) was prepared by using 0.1 M KPO_4 buffers (Monobasic KH_2PO_4, CAS NO: 7778-77-0 (VWR, West Chester, PA); and dibasic, anhydrous, K_2HPO_4, CAS NO: 7758-11-4 (Fisher Scientific, Fair Lawn, NJ)) adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Approximately 30.0 ml of each pH buffered agar was poured into 10.0 cm Petri dishes. A 7.0 mm agar plug taken from the actively growing margin of I-6E, I-7E and I-8E isolate cultures growing on PDA was aseptically transferred to the center of nine agar dishes for each pH. The Petri dishes were sealed with parafilm (Parafilm "M" American National Can™ Chicago, IL) and incubated for three weeks at 26°C. The mycelial growth diameter was measured along two axes and averaged after three

weeks of incubation. The adjusted diameter growth (ADG) value was calculated by subtracting the initial 7.0 mm agar plug diameter from the measurements. These experiments were conducted three times.

Germination of *U. virens* chlamydospores at temperatures 18°C, 22°C and 26°C

One-month-old *U. virens* chlamydospores suspended in de-ionized water (3×10^6 chlamydospores ml^{-1}) were prepared to determine the effect of three temperatures on germination of chlamydospores. One-ml conidium suspensions from 'Clearfield-151' and 'Templeton' spore balls were incubated for 96 hours at 18°C, 22°C and 26°C. The experiment was completed by averaging the two sub-sample measurements (50 chlamydospores) from each tube. There were three replicated tubes for each conidium sample in each repetition of the test. The entire experiment was repeated twice for chlamydospores from each cultivar and temperature.

Growth of *U. virens* isolates I-6E, I-7E and I-8E at temperatures 18°C, 22°C, 26°C, 30°C, 34°C, 36°C and 38°C

The objective of this study was to determine the tolerances of *U. virens* isolates as determined by mycelial growth to temperatures in this range and to identify the optimal temperature for growth. Seven mm agar plugs from the actively growing edges from cultures of isolates I-6E, I-7E and I-8E were transferred to three acidified PDA dishes per isolate. The dishes were sealed with parafilm 'M' and incubated for three weeks in separate chambers at 18°C, 22°C, 26°C, 30°C, 34°C, 36°C and 38° C. Accumulated diameter measurements were calculated by measuring the diameter of the individual colonies along two axes from each plate. The adjusted diameter growth (ADG) value was determined from the average of the two diameter measurements minus the initial 7.0 mm plug diameter. There were three replicates prepared with each isolate at each temperature and the experiment was repeated twice.

Data analysis

The analysis of variance (ANOVA) of the means of treatments for temperature and pH interaction effects in relative growth and in germination studies were conducted for each of the isolates or conidium types by using SAS version 9.2. The experimental design for germination experiments followed a randomized complete block design with replications of each temperature or pH treatments for chlamydospores from each cultivar. Growth studies followed a similar randomized complete block design with three replications for each isolate of each test and all tests were repeated twice. Analysis of variance tables were obtained for each test and all significant interactions were submitted for further analysis and compiled for interpretation in graphs and tables. Standard deviations of data values from the means were calculated for the germination and growth studies. Standard error bars were added to the graphs to compare experimental results among tests defined as SE = Standard Deviation SD/$\sqrt{n}$ to estimate variation around the means of each test repetition. Fisher's least significance difference (LSD) means were calculated (a=0.05) to determine each of the significant interaction effects.

Results

Collection of *U. virens* isolates: 2010 and 2011 surveys

As a result of the field surveys conducted, a total of 190 single chlamydospore isolates were obtained and cryogenically preserved in 30% glycerol at -80°C (Table 2.1). In the survey, a total of 5, 146, 5, 10 and 24 isolates were isolated from various cultivars grown in rice growing counties of eastern Arkansas in 2008, 2009, 2010, 2011 and 2013 respectively (Table 2.3). Five isolates (I-9A, I-9B, I-9C, I-9D and I-9E), fourteen isolates (WFS13A1, WFS13A2, WFS13B2, WFS13B1, WFS13C1, WFS13C2, WFS13D1, WFS13D2, WFS13E, WFS13F, WFS13G, WFS13H, WFS13I and WFS13J) and ten isolates (I-90E1, I-90E2, I-90E3, I-90E4, I-90E5, I-

90E6, I-90E7, I-90E8, I-90E9 and I-90E10) were obtained from white spore balls that were collected from the Newport Research Station, Jackson Co., Arkansas, in 2011, 2013 and from greenhouse inoculation studies at Fayetteville, Washington Co., Arkansas in 2013, respectively. The isolates obtained from the white smut spore balls were added to the isolate collection (Tables 2.1).

Identification and verification of *U. virens*

The diameter of FS spore balls found on rice panicles ranged from 6 to 12 mm wet or from 4 to 8 mm when spore balls were measured air dried. Chlamydospores (Figure 2.1 A., B.), taken from the green spore balls (Figure 2.1 D., E., G., H.) of *U. virens* observed with light microscopy were warty, spiny and olivaceous chlamydospores with diameters ranging from 4 to 5 µm. These observations were consistent with formal descriptions of *U. virens* by Tanaka et al. (2008) and the ultrastructural characteristics described by Kim and Park (2007). Representative samples of FS chlamydospores from infected grain have been observed and depicted by Kim and Park (2007) and Groth (2007). Chlamydospores readily germinated and gave rise to promycelia and translucent fusiform germ tubes. The germ tubes and promycelia produced branching, septate hyphae or phialides bearing a few acrogenous hyaline conidia of two sizes, 1.5 µm and 3.1 µm in diameter. The chlamydospore morphology for typical *Ustilaginoidea virens* was consistent with the orange to olivaceous, spined chlamydospores of *Ustilaginoidea virens* (Cke.) Takahashi (Ou 1985) (Figure 2.1 A.).

In our current collection, 161 isolates were accepted as *Ustilaginoidea virens* (Table 2.1) but the 29 isolates obtained from white spore balls were not similar to *Ustilaginoidea virens.* The mature chlamydospores of the white isolates were white in color and spines were not present on the surface as described by Wang and Bai (1997). Amplification of DNA obtained from the

24 *U. virens* isolates with primary and nested-PCR primers designed by Zhou et al. (2003) US1-5/US3-3 and US2-5/US4-3 (Table 2.2) produced the expected 380 base pair (bp) and 232 bp amplicons as visualized in 2.5% agarose stained with ethidium bromide as shown in Figure 2.2 with isolates I-6E, I-7E and I-8E. Amplified products obtained from nested-PCR performed on DNA from these *U. virens* isolates were then sequenced at the DNA Resource Laboratory, University of Arkansas. Sequences were obtained by the Sanger method of DNA sequencing. Basic Local Alignment Search Tool (BLAST®) search results using the non-manipulated sequences obtained from ITS1/4 primers indicated a 99% level of similarity of the *U. virens* isolates I-6E, I-7E and I-8E in the cryogenic collection, with *U. virens, U. albicans* and teleomorph *Villosiclava virens* nucleotide accessions in the database.

Description of the white false smut found in this study

Three independent occurrences of white spore balls were observed in our field tests and the chlamydospores are distinguished from *Ustilaginoidea virens* by several morphological features (Figure 2.1 A., B. and C.). These three occurrences were found in plots of selected rice cultivars 'Clearfield-151', 'Francis' and 'Francis' that were planted in May of 2011, June of 2012 and May of 2013 in field tests conducted at the Pine Tree Research Station near Colt, in St. Francis County Arkansas and at the Newport Research Station in Newport, in Jackson County Arkansas, respectively. A high incidence of FS (= green FS) caused by *U. virens* (Cooke) Takahashi was observed throughout the tests at both locations. Among the mature panicles infected by FS, white spore balls were observed on panicles of 'Clearfield-151', 'Francis' and 'Francis' at the Pine Tree and Newport Research Station locations in September of 2011, August of 2012 and September 2013, respectively. In 2011, erumpent white-to-opaque mass of chlamydospores (spore balls) 7.0 to 8.0 mm long and 4.0 to 6.0 mm wide, were found on a

panicle. White chlamydospores from this spore ball were observed under 600X light magnification. Chlamydospores were non-melanized, smooth and subglobose, 4.96 µm long and 4.59 µm wide (n=50, st. dev. 0.44 µm and 0.38 µm, respectively). In 2012, an immature spore ball of white FS was found on the cultivar 'Francis' at the Pine Tree Research Station in a varietal resistance test (TeBeest and Jecmen, 2012). These chlamydospores were morphologically consistent but slightly smaller than the chlamydospores found in the mature white spore ball observed in 2011 at the Newport Station. Chlamydospores from the spore ball found in 2012, were 3.93 µm long and 3.35 µm wide respectively (n=50, st. dev. 0.267 µm and 0.38 µm, respectively) and did not germinate. Chlamydospores collected in 2011 from 'Clearfield-151' began to germinate in sterile water after 48 hours at 25°C. The hyaline chlamydospores imbibed water, swelled and translucent fusiform germ tubes developed. The germ tubes produced either branching septate hyphae or phialides bearing a few acrogenous hyaline conidia of two sizes, 1.58 µm long and 3.1 µm wide (n=50, st. dev. 0.39µm and 0.45µm, respectively), at the terminus. Chlamydospore morphology from each white spore ball was not comparatively consistent with the melanized, olivaceous, warty and spined of the anamorphic stage of *Ustilaginoidea virens* (Cke.) Takahashi (Kim and Park, 2007; Ou, 1985) (Figure 2.1 A., B.) Five and fourteen isolates from white spore balls that resembled descriptions of *U. albicans* were obtained from a single spore balls collected in 2011 and 2013, respectively, using methods developed for isolating *U. virens*. White erumpent colonies from isolation cultures were selected and placed into 30% glycerol and placed into cryogenic storage at -80°C in the Department of Plant Pathology at the University of Arkansas, Fayetteville, AR.

Total DNA was extracted from two colonies of a representative isolate obtained from white spore balls (I-9E) grown on PDA and two conidium swabs from the surface of the white

spore ball obtained in 2012. The DNA was amplified by nested-PCR using primer sets US1-5/US3-3 and US2-5/US4-3 and by conventional PCR using primer sets ITS-1/ITS-4 (Table 2.2). Amplification with the nested-PCR and conventional PCR of the rDNA ITS region produced 232 bp and ~600 bp fragments of rDNA, respectively. A BLAST® search using the nested-PCR 232 sequences conducted in GenBank (NCBI) revealed the sequences from the white smut were 99% identical with rDNA sequences from *Ustilaginoidea albicans* strain BH accession no. HM439355 (230 of 235 bp matching, 99% identity, E value 2 e-115), *Ustilaginoidea virens* isolate SX0201 accession no. FJ824824.1 (230 of 233 bp matching, 99% identity, E value 6e-115), and *Villosiclava virens* isolate HNHS-1 (= teleomorph of *Ustilaginoidea virens*) accession no. JX427552.1 (230 of 235 bp matching, 99% identity, E value of 2e-115). A BLAST® search using the ~600 bp sequences conducted in GenBank revealed that the sequences obtained from isolates from the white false smut were 99% identical with rDNA sequences of *Ustilaginoidea albicans* strain BH accession no. HM439355.1 (596 of 602 bp matching, 99% identity, E value 3e-75); *Ustilaginoidea virens* isolate ZJ04 accession no. FJ848738.1 (596 of 601 bp matching, 99% identity, E value of 3e-75); and *Villosiclava virens* accession no. JX427552.1 (596 of 601 bp matching, 99% identity, E value of 3e-75). Pairwise alignments in CAP3 Sequence Assembly Program and BioEdit of the 232 bp fragment and ~600 bp fragment sequences obtained from *U. virens* isolates I-6E, I-7E, I-8E and *Ustilaginoidea* isolate I-9E and DNA samples from the white spore ball surface were indistinguishable among the isolates and revealed 100% sequence similarity of the sequences.

Virulence tests of isolate I-9E resulted in 60 white false smut spore balls developing on inoculated rice plant cultivars, ‘Neptune’ and ‘Roy J’, whereas no false smut or white false smut spore balls were observed on any of the 18 panicles of three water inoculated control plants.

Booted panicles that were inoculated when the distance between the flag and penultimate leaf collars was 5 to 13.5 cm resulted in spore balls developing on the panicles. Spore balls began to develop from 16 days after inoculations were made and development continued over a two week period of time. Twenty booted panicles from the virulence test developed 52 white false smut spore balls when inoculated with wheat bran broth inoculum and eight spore balls were produced from potato dextrose broth inoculum of *Ustilaginoidea* isolate I-9E, respectively.

Spore ball production was also successfully induced by placing three of the twelve plants outside during the last week of development during intermittent rainy weather with day/night temperatures ranging from 32° to 14°C (Figure 2.3 A.). Spore ball and chlamydospore morphology was examined under a light microscope and was found to be consistent with spore balls and chlamydospores of the isolate I-9E that was used to inoculate the plants. Spore balls were wholly white from the center to the outer layers and chlamydospores isolated from the spore balls resulting from boot inoculations were white/opaque in appearance and had a smooth surface (Figure 2.3 B., C.). Ten additional isolates were obtained in 2013 from the spore balls that formed on 'Neptune' resulting from inoculations with isolate I-9E in the greenhouse and these were added to the isolate collection on November 12, 2013.

False smut is an emerging disease that occurs in all rice producing counties of Arkansas. White false smut (WFS) was first reported in Japan in 1991 (Honkura et al., 1991) and in China in 1992 (Wang and Bai, 1997) where WFS and FS alike are considered to be a threat to rice production (Wang et al., 2008). To our knowledge, this is the first report of a WFS infecting rice in the USA and outside of China or Japan.

Effect of pH on germination of one-, three- and five-month-old *U. virens* chlamydospores in agar adjusted to pH ranging from 5.5 to 8.0

Experiments were conducted to determine the effect of pH on germination of one-, three- and five-month-old *U. virens* chlamydospores stored at 18°C. Analysis of variance on the germination means from each cultivar conidium group, age and pH level indicated significant interactions among pH levels and among three- and five-month-old chlamydospores from each cultivar (Table 2.4). The data from one-month-old chlamydospores shows that germination of chlamydospores from 'Clearfield-151' at pH 5.5 to 7.0 ranged from 19 to 27.5% and that germination of chlamydospores from 'Templeton' at pH 5.5 to 6.5 ranged from 28 to 45% (Figure 2.4). The data from one-month-old chlamydospores show a 61% reduction above pH 7.0 in mean germination of chlamydospores from 'Clearfield-151' at pH ranging from 7.5 to 8.0 and a 68% reduction from pH 7.5 in mean germination of chlamydospores from 'Templeton' at pH 8.0. Initial germination was highest at pH levels ranging from 5.5 to 7.0 for chlamydospores from both cultivars at all age groups. Germination of chlamydospores for all age groups was highest for 'Templeton' chlamydospores at pH 6.0 and 6.5. A high percentage of one- and three-month-old chlamydospores germinated for both cultivars across the range of pH 5.5 to 7.0 and a significant reduction in the percent of chlamydospores that germinated was observed at all conidium ages at pH 8.0. The percent germination of five-month-old chlamydospores after incubation, was less than one-or three-month-old chlamydospores at pH 5.5 to 7.5. The percent germination of five-month-old chlamydospores was remarkably lower compared to one or three conidium ages at pH levels 7.5 to 8.0 (Table 2.4).

Effect of pH on growth of *U. virens* isolates I-6E, I-7E and I-8E in buffered agar

Experiments were conducted to test the effect of pH on growth of isolates I-6E, I-7E and I-8E by measuring the adjusted diameter growth (ADG). The results indicate there are significant differences in growth under the different pH conditions between isolates I-6E, I-7E and I-8E (Table 2.5). The statistically significant pH levels optimal for growth ranged between pH 5.5 and 6.5 for all the isolates (Figure 2.5). A significant reduction in growth was observed for all isolates at pH 8.0. The reduced growth at high pH conditions (pH > 7.5), may indicate the possibility for compromised growth in the field where high pH conditions in the soil have been determined.

Effect of temperatures 18°C, 22°C and 26°C on germination of *U. virens* chlamydospores

Results from experiments that tested the effect of temperatures ranging from 18°C, 22°C and to 26° C on germination of *U. virens* chlamydospores showed lower germination rates of 'Clearfield-151' and 'Templeton' chlamydospores at 18°C compared to 22°C and 26°C (Figure 2.6). The percentage of chlamydospores from both cultivars germinating increased above 18°C and was highest at 22°C and 26°C after 96 hours of incubation.

Growth of *U. virens* isolates I-6E, I-7E and I-8E at temperatures 18°C, 22°C, 26°C, 30°C, 34°C, 36°C and 38°C

Results from experiments that tested the effect of temperatures including 18°C, 22°C, 26°C, 30°C, 34°C, 36°C and 38°C on growth of isolates I-6E, I-7E and I-8E showed that the optimal temperature for mycelial growth ranged from 22 to 30°C (Figure 2.7). At 18°C and 34°C, there was a significant reduction in growth of isolates I-6E and I-8E but not I-7E. All isolates did not grow at 36°C and 38°C after three weeks incubation. Seven mm diameter agar plugs of each isolate that was incubated at the two higher temperatures were transferred to six

fresh PDA plates and incubated at 26°C for an additional three weeks to test viability. The transfers did not resume growth, indicating mortality of the cultures grown at 36°C and 38°C.

Discussion

We established a working cryogenic culture collection of 190 isolates at the University of Arkansas, in the Department of Plant Pathology from successful isolations of *Ustilaginoidea virens* and *Ustilaginoidea* sp. from panicles. The culturing and preservation of single spore isolates of *U. virens* is important because it enabled us to preserve a diverse population that may provide a reference for future ecological, pathogen diversity, fungicide sensitivity and epiphytological studies. This collection is an essential resource for future work because of the possibility for genetic variability and potential differences in fungicide sensitivity present in the population (Lu et al., 2009; Sun et al., 2013; Zhou et al., 2008). Isolates from other cultivars, locations and outbreaks of FS can be added to the collection to represent a more diverse selection of the Arkansas population.

The first occurrence of white false smut was described in a Shenyang suburb in China in 1987 by Wang and Bai (1997) and in Japan by Honkura et al. (1991). There have since been at least two other reports of white false smut balls on rice in China, one in Xiangshan in Zhejiang province and the other in Mianxian in Shaanxi province (Jin et al., 2012). The causal agent of white smut was described as a new species of *Ustilaginoidea, U. albicans,* by Wang and Bai (1997). Wang et al. (2008) describe sowing seed that was artificially inoculated with the germinating chlamydospores of *U. albicans* resulted in successful infection. In the absence of a type specimen and availability of other *Ustilaginoidea* spp. for examination, *Ustilaginoidea albicans* is accepted taxa for the current study because the description by Wang and Bai in 1997 presents *Ustilaginoidea albicans* S. Wang et J. K. Bai sp. nov., as a new morph of the false smut

fungus in conformity with ICBN Art. 59.6. However, *U. albicans* is currently being re-considered to be "apparently a white mutant of the rice false smut fungus" *U. virens* by Tanaka et al. (2008). It is suggested by Jin et al. (2012) "that these albinotic isolates were the mutants of sporulating degeneration of *Ustilaginoidea virens*". Thus, further work may be needed to compare the white isolates obtained in this study to other named *Ustilaginoidea* spp. specimens to be taxonomically validated as a separate taxon. Although morphological comparison of isolates from white false smut spore balls to formal descriptions of *U. virens* indicates that *U. albicans* may be a separate species from *U. virens*, comparisons of the rDNA ITS1-4 sequences of *U. virens* and *U. albicans* do not indicate any variation among the two species. The sequence analysis in this study indicate 99% sequence identity between rDNA sequences obtained from amplicons acquired by reacting ITS1-4 and US4-3/US2-5 primers with three *U. virens* (I-6E, I-7E and I-8E) and rDNA from white false smut spore balls and isolates (I-9E) from Arkansas to *U. virens* and *V. virens* in the NCBI BLAST® database. A separate analysis of the rDNA-ITS regions of *U. virens* and albinotic isolates by Jin et al. (2012) indicated 99.6% identity to the known wild isolates of *U. virens*. These sequence analyses do not definitively distinguish the rDNA ITS sequences of the white false smut isolates from the green false smut isolates. A mutable gene or genes controlling color and surface ornamentation may exist in the green and white smut isolates, thus we may not identify variation in the rest of the genome. To determine if variation does exist in the rDNA among green and white smut isolates, additional genes such as large subunit (5S, 5.8S and 28S) and/or small subunit (18S) rDNA, inter-genic spacer (IGS), for comparisons could be considered for future study.

Successful inoculations using isolate I-9E were made using 'Neptune' and 'Roy J' rice cultivars with methods similar to those used by Wang et al. (2008). The spore balls developed

on rice panicles under cultivation in the greenhouse but they did not completely develop and sporulate. The surrounding membrane of these spore balls failed to break open as seen in the field. Based on these observations, we hypothesized that the lack of dew was the limiting factor in FS development under greenhouse conditions. Development of white false smut spore balls was rapidly induced by placing three plants from the inoculation experiment outside in a contained, restricted access area during the last week of spore ball development where they were subjected to dew every night. This lack of spore ball development in in the greenhouse has previously hindered studies of false smut and may indicate the actual environmental conditions that favor disease development as previous studies have reported (Ashizawa et al., 2011; Bhagat and Prasad, 1996; Ikegami, 1963; Lee and Gunnell, 1992; Rao, 1964; Singh, 1974; Singh et al., 1987; Wang et al., 2008). The importance of dew and available moisture during false smut and white false smut development on panicles should be in consideration for future greenhouse experiments.

Experiments were conducted to determine the effect of temperature and pH on growth of *U. virens* using isolates I-6E, I-7E and I-8E (Table 2.4). Environmental factors such as pH and temperature are shown in this study to influence the germination of *U. virens* chlamydospores. These are important but often over-looked components of the epiphytological factors that may influence FS development in the field. Hashioka et al. (1951) previously reported that chlamydospores germination and mycelial growth was best at 28°C and that no growth occurred at 36°C. Hashioka et al. (1951) also showed that mycelial growth was optimal between pH 6.02 to 6.72 and suboptimal at pH 2.77 and 9.05. Tsai et al. (1990) indicated that the optimal pH range for germinating chlamydospores was 5.0 to 8.0 and optimal at 25°C for germinating chlamydospores. In the germination tests of this study, the percent germination of

chlamydospores from both cultivars was observed across the range of pH 5.5 to 8.0 and a reduction in percent germination was observed from one-month-old chlamydospores at pH 8.0 and three- and five- month-old chlamydospores at pH levels 7.5 and 8.0. Viability of five-month-old chlamydospores was reduced compared to one- and three-month-old chlamydospores especially at pH 8.0 indicating a suboptimum pH for germination contrary to findings of Hashioka et al. (1951) and Tsai et al. (1990) These tests identified *in-vitro* conditions that might limit optimal growth for *U. virens*, which are present in localized areas of rice production field soil.

The germination and growth tests in this study show that optimal chlamydospore germination occurred at 18°C to 26°C and optimal mycelial growth occurred at 18°C to 34°C. The growth study demonstrated that temperatures above 34°C inhibited mycelial growth and temperatures above 36°C were lethal, confirming the report of Hashioka et al. (1951). If temperature and pH have an influence on mycelial growth and germination *in-vitro*, this study suggests that these environmental conditions may have an influence on the germination and growth of *U. virens in-vivo* and warrants further study to test how these conditions apply to the epiphytology of FS. Temperature and pH may influence the development of FS hyphae in the soil and later the colonization of panicles as they develop in the plant. The pH and temperature at planting and panicle development may affect the germination of chlamydospores, colonization of rice tissues and disease development. The germination and growth data might also suggest that *U. virens* may have unidentified structural proteins or interrelated metabolic pathways that could be altered due to conformational or protonation changes induced by host or environmental pH and could be investigated further.

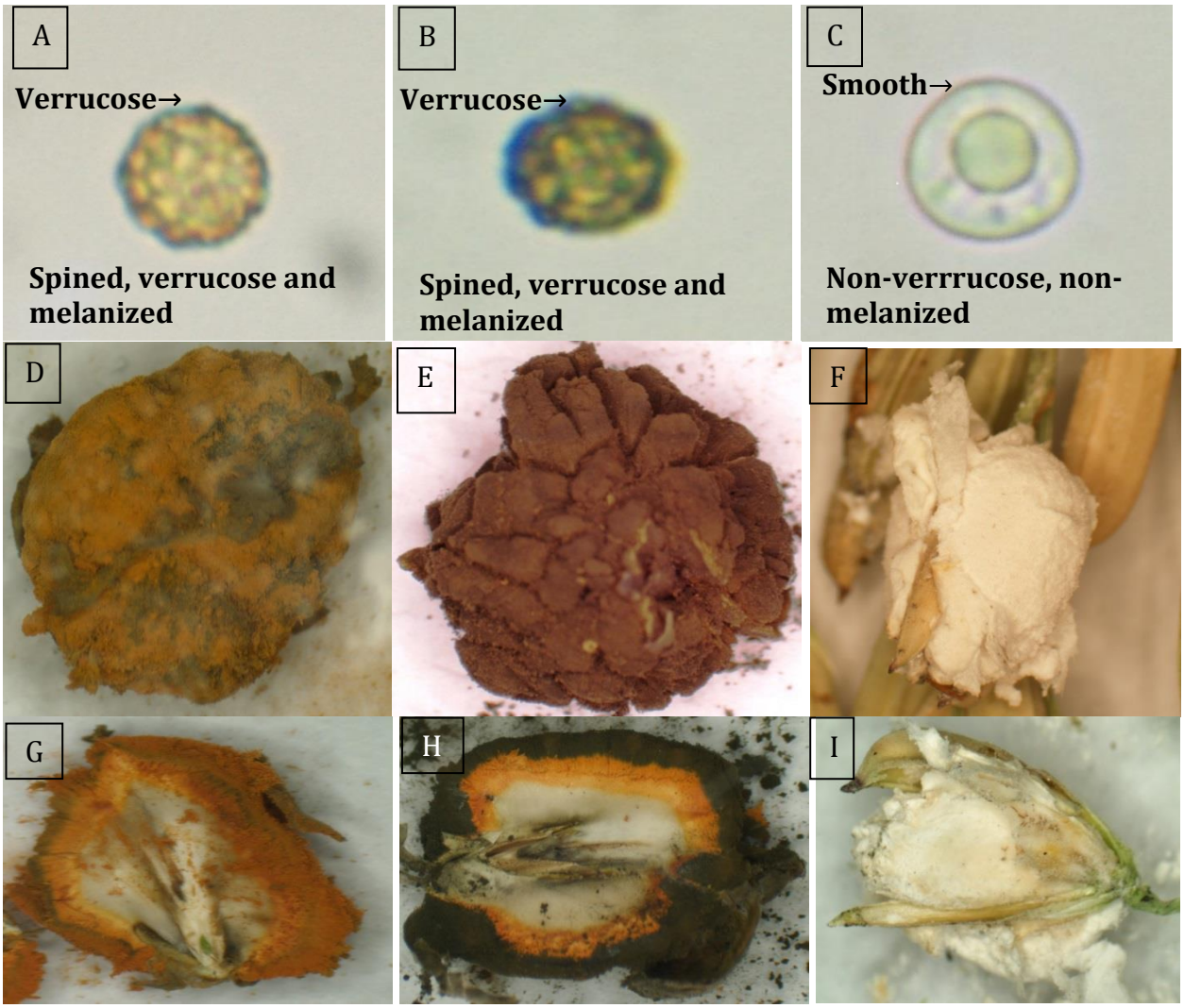

Figure 2.1. Images of false smut chlamydospores and spore ball color morphs obtained during the laboratory study of false smut at the University of Arkansas, Fayetteville, AR.

A.B. Wet slide mounted with *Ustilaginoidea virens* chlamydospores originating from smutted panicles of 'Clearfield-151' from the Pine Tree Research Station, near Colt, Arkansas in 2013. The spiny, verrucose and dark melanized chlamydospores are representative of typical *Ustilaginoidea virens* chlamydospores.

C. The hyaline, smooth, non-verrucose and non-melanized chlamydospores are representative of a *Ustilaginoidea virens* color morph found in our field plot studies on rice cultivar 'Clearfield-151' at the Newport Research Station in 2011 and on 'Francis' and 'Francis' cultivars at the Pine Tree Research Station in 2012, 2013 respectively.

D.E. Typical FS spore balls with melanized rusty-orange to black chlamydospores.

F. A white smut spore ball recognized as white false smut that gave rise to isolates I-9A, I-9B, I-9C, I-9D and I-9E by isolation manipulations.

G. H. False smut spore balls were bisected to reveal layers of conidium colors ranging from white to yellow on the inner layers, to orange to rust-orange in the middle layers, to olive green to dark black on outermost layers from 'Clearfield-151'.

I. This white false smut spore ball was bisected to reveal the non-melanized chlamydospores from inner to outer layers.

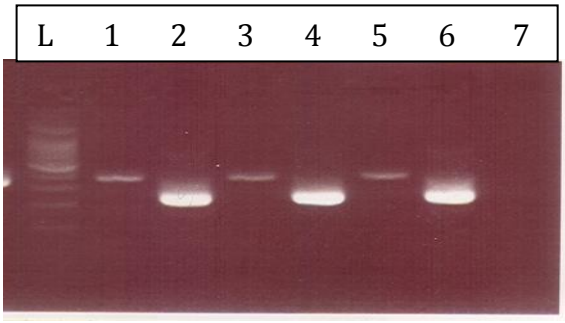

Figure 2.2. Resolved amplicon products in 2.5% agarose stained with ethidium bromide showing amplicons obtained from reacting DNA extracted from *U. virens* isolates I-6E, I-7E and I-8E with primary (1°) and nested (2°) primers US1-5/US3-3 in primary and US2-5/US4-3 nested-PCR reactions.
Lanes: L, 1 kb ladder; lane 1, I-6E US1-5/US3-3; lane 2, I-6E US2-5/US4-3; lane 3, I-7E US1-5/US3-3; lane 4, I-7E US2-5/US4-3; lane 5, I-8E US1-5/US3-3; lane 6, I-8E US2-5/US4-3; lane 7, (-) PCR reagent control.

Figure 2.3. Images of spore balls and white false smut (WFS) chlamydospores obtained from a virulence study of WFS at the University of Arkansas, Fayetteville, AR.
A. Image of white false smut (WFS) spore balls on an infected 'Neptune' rice panicle resulting from inoculating rice plants at the booting stage prior to exertion of the panicle with isolate I-9E.
B. White false smut spore ball bisected to reveal the non-melanized chlamydospores from inner to outer layers.
C. Chlamydospores obtained from the white spore ball

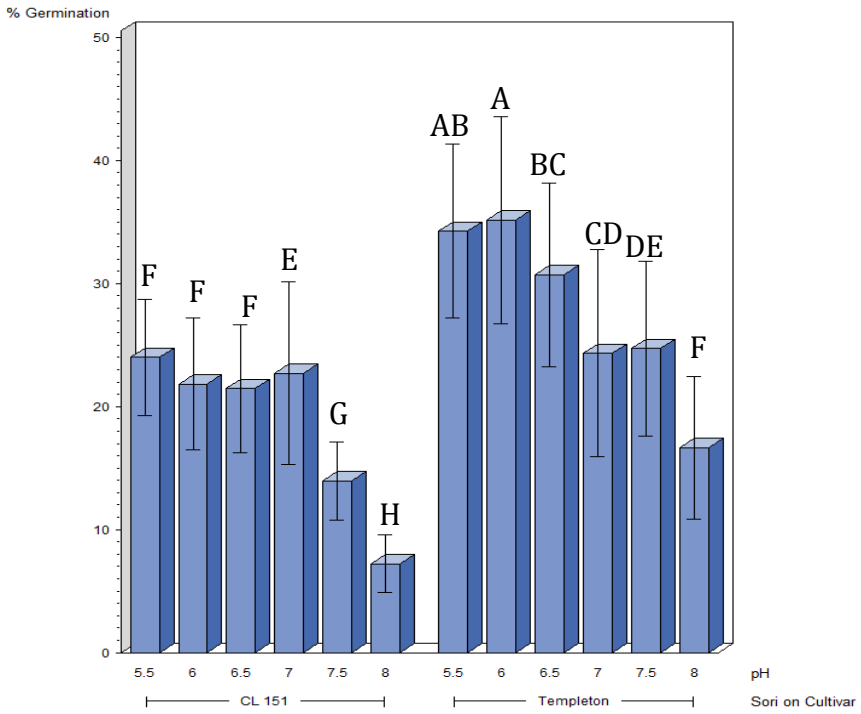

Figure 2.4. Percent germination of one month-old chlamydospores obtained from spore balls occurring on rice cultivars 'Clearfield-151' and 'Templeton' after germination in 0.1M KPO_4 buffers at pH levels ranging from 5.5 to 8.0 after 96 hours incubation. Standard error bars (SE = SD/$\sqrt{}$ *n*) indicate a measure of standard error of the means among repetitions of the test. Bars with a common letter above are not significantly different at *a*=0.05.

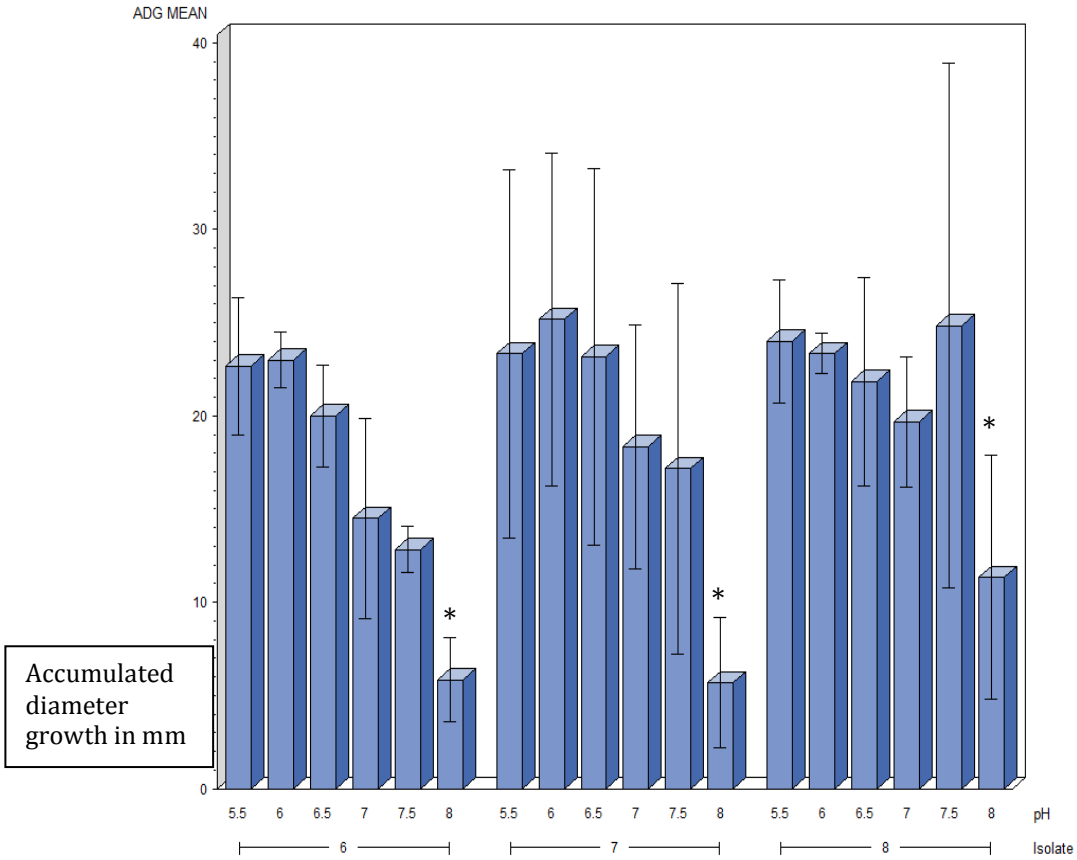

Figure 2.5. Mycelium diameter of *U. virens* colonies calculated as accumulated diameter growth (ADG) of isolates I-6E, I-7E and I-8E grown for three weeks on 0.1M PO_4 pH buffered agar ranging from pH 5.5, 6.0, 6.5, 7.0, 7.5, to 8.0. Bars (SE = SD/ $\sqrt{}$ *n*) indicate a measure of standard error of the means among repetitions of the test. Bars with a star above indicate significant deviation between the observed value and the mean growth of each isolate.

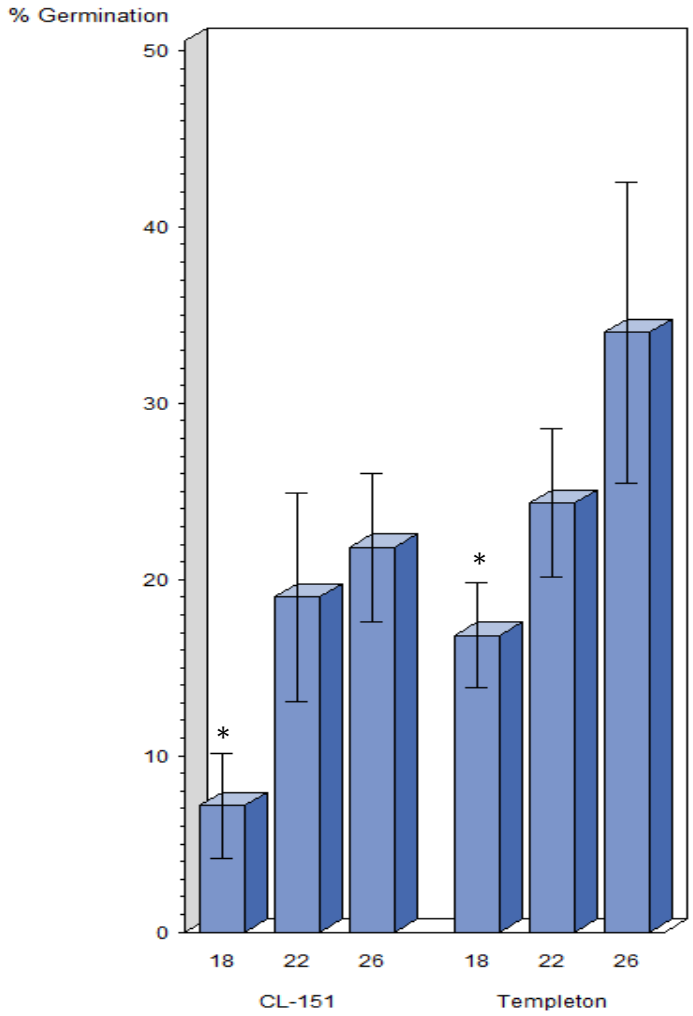

Figure 2.6. Percent germination of chlamydospores obtained from spore balls occurring on rice cultivars 'Clearfield-151' and 'Templeton' after 96 hours of incubation, at temperatures of 18°C, 22°C to 26°C. Standard error bars (SE = SD/ $\sqrt{}$ *n*) indicate a measure of standard error of the means among repetitions of the test. Bar with a star above indicates significant deviation between the observed value and the % germination of each conidium type.

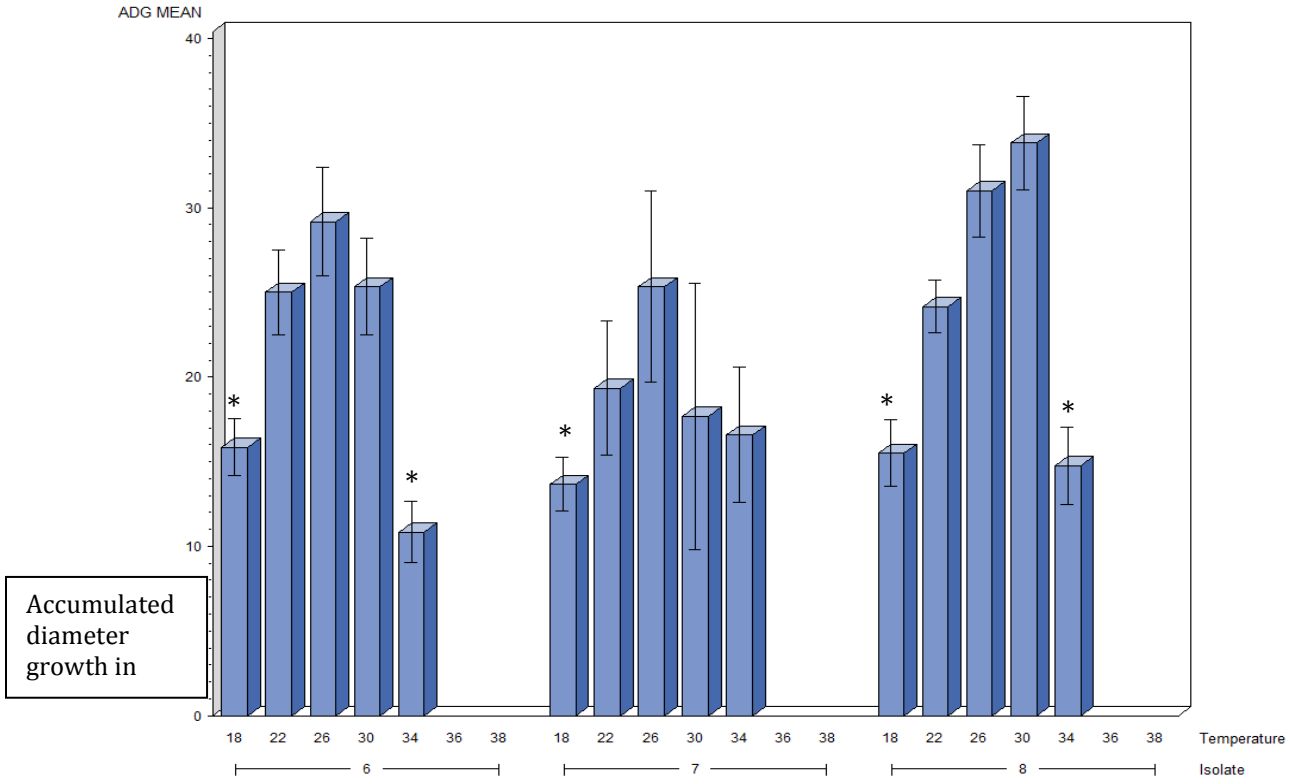

Figure 2.7. Mycelium diameter of *U. virens* colonies calculated as accumulated diameter growth (ADG) of Isolates 6 (I-6E), 7 (I-7E) and 8 (I-8E) grown for three weeks of incubation at temperatures ranging from 18°C, 22°C, 26°C, 30°C, 34°C, 36°C to 38°C. Standard error bars (SE = SD/ $\sqrt{}$ *n*) indicate a measure of standard error of the means among repetitions of the test. Bars with a star above indicate significant deviation between the observed value and the mean growth of each isolate.

Table 2.1. Isolates of *Ustilaginoidea* obtained from various rice cultivars grown in Arkansas. The isolates were placed in 30% glycerol and preserved in the collection for storage at -80°C in the Department of Plant Pathology, University of Arkansas in Fayetteville, AR.

Location [A]	County [B]	Cultivar [C]	Isolate # [D]	Tube # [E]	Deposition Date [F]
Pine Tree	St. Francis	Unknown	1A	U-1-1	8/13/10
Pine Tree	St. Francis	Unknown	1B	U-1-2	8/13/10
Pine Tree	St. Francis	Unknown	1C	U-1-3	8/13/10
Pine Tree	St. Francis	Unknown	1D	U-1-4	8/13/10
Pine Tree	St. Francis	Unknown	2A	U-1-5	8/13/10
Pine Tree	St. Francis	Unknown	2B	U-1-6	8/13/10
Pine Tree	St. Francis	Unknown	2C	U-1-7	8/13/10
Pine Tree	St. Francis	Unknown	2D	U-1-8	8/13/10
Pine Tree	St. Francis	Unknown	3A	U-1-9	8/13/10
Pine Tree	St. Francis	Unknown	3B	U-1-10	8/13/10
Pine Tree	St. Francis	Unknown	3C	U-1-11	8/13/10
Pine Tree	St. Francis	Unknown	3D	U-1-12	8/13/10
Pine Tree	St. Francis	Unknown	5A	U-1-13	8/13/10
Pine Tree	St. Francis	Unknown	5B	U-1-14	8/13/10
Pine Tree	St. Francis	Unknown	5C	U-1-15	8/13/10
Pine Tree	St. Francis	Unknown	5D	U-1-16	8/13/10
Pine Tree	St. Francis	Unknown	6A	U-1-17	8/13/10
Pine Tree	St. Francis	Unknown	6B	U-1-18	8/13/10
Pine Tree	St. Francis	Unknown	6C	U-1-19	8/13/10
Pine Tree	St. Francis	Unknown	6D	U-1-20	8/13/10
Pine Tree	St. Francis	Unknown	7A	U-1-21	8/13/10
Pine Tree	St. Francis	Unknown	7B	U-1-22	8/13/10
Pine Tree	St. Francis	Unknown	7C	U-1-23	8/13/10
Pine Tree	St. Francis	Unknown	7D	U-1-24	8/13/10
Statewide	Poinsett	Clearfield-161	ST#3A	U-1-25	8/13/10
Statewide	Poinsett	Clearfield-161	ST#3B	U-1-26	8/13/10
Statewide	Poinsett	Clearfield-161	ST#3C	U-1-27	8/13/10
Statewide	Poinsett	Clearfield-161	ST#3D	U-1-28	8/13/10
Statewide RREC	Arkansas	Templeton	ST#4A	U-1-29	8/13/10
Statewide RREC	Arkansas	Templeton	ST#4B	U-1-30	8/13/10
Statewide RREC	Arkansas	Templeton	ST#4C	U-1-31	8/13/10
Statewide RREC	Arkansas	Templeton	ST#4D	U-1-32	8/13/10
Statewide RRVT	White	Rice Tec CL XL 729	ST#8A	U-1-33	8/13/10

Statewide RRVT	White	Rice Tec CL XL 729	ST#8B	U-1-34	8/13/10
Statewide RRVT	White	Rice Tec CL XL 729	ST#8C	U-1-35	8/13/10
Statewide RRVT	White	Rice Tec CL XL 729	ST#8D	U-1-36	8/13/10
G & H Farms	Arkansas	Cybonnet	G101A	U-1-37	8/13/10
G & H Farms	Arkansas	Cybonnet	G101B	U-1-38	8/13/10
G & H Farms	Arkansas	Cybonnet	G101C	U-1-39	8/13/10
G & H Farms	Arkansas	Cybonnet	G101D	U-1-40	8/13/10
G & H Farms	Arkansas	Cybonnet	G104A	U-1-41	8/13/10
G & H Farms	Arkansas	Cybonnet	G104B	U-1-42	8/13/10
G & H Farms	Arkansas	Cybonnet	G104C	U-1-43	8/13/10
G & H Farms	Arkansas	Cybonnet	G104D	U-1-44	8/13/10
G & H Farms	Arkansas	Cybonnet	G107A	U-1-45	8/13/10
G & H Farms	Arkansas	Cybonnet	G107B	U-1-46	8/13/10
G & H Farms	Arkansas	Cybonnet	G107C	U-1-47	8/13/10
G & H Farms	Arkansas	Cybonnet	G107D	U-1-48	8/13/10
G & H Farms	Arkansas	Cybonnet	G109A	U-1-49	8/13/10
G & H Farms	Arkansas	Cybonnet	G109B	U-1-50	8/13/10
G & H Farms	Arkansas	Cybonnet	G109C	U-1-51	8/13/10
G & H Farms	Arkansas	Cybonnet	G109D	U-1-52	8/13/10
G & H Farms	Arkansas	Cybonnet	G406A	U-1-53	8/13/10
G & H Farms	Arkansas	Cybonnet	G406B	U-1-54	8/13/10
G & H Farms	Arkansas	Cybonnet	G406C	U-1-55	8/13/10
G & H Farms	Arkansas	Cybonnet	G406D	U-1-56	8/13/10

G & H Farms		Arkansas	Cybonnet	G407A	U-1-57	8/13/10
G & H Farms		Arkansas	Cybonnet	G407B	U-1-58	8/13/10
G & H Farms		Arkansas	Cybonnet	G407C	U-1-59	8/13/10
G & H Farms		Arkansas	Cybonnet	G407D	U-1-60	8/13/10
Hogue Lake		Poinsett	Cybonnet	101A	U-1-61	8/13/10
Hogue Lake		Poinsett	Cybonnet	101B	U-1-62	8/16/10
Hogue Lake		Poinsett	Cybonnet	101C	U-1-63	8/16/10
Hogue Lake		Poinsett	Cybonnet	101D	U-1-64	8/16/10
Hogue Lake		Poinsett	Cybonnet	203A	U-2-65	8/16/10
Hogue Lake		Poinsett	Cybonnet	203B	U-2-66	8/16/10
Hogue Lake		Poinsett	Cybonnet	203C	U-2-67	8/16/10
Hogue Lake		Poinsett	Cybonnet	203D	U-2-68	8/16/10
Hogue Lake		Poinsett	Cybonnet	305A	U-2-69	8/16/10
Hogue Lake		Poinsett	Cybonnet	305B	U-2-70	8/16/10
Hogue Lake		Poinsett	Cybonnet	305C	U-2-71	8/16/10
Hogue Lake		Poinsett	Cybonnet	305D	U-2-72	8/16/10
Hogue Lake		Poinsett	Cybonnet	406A	U-2-73	8/16/10
Hogue Lake		Poinsett	Cybonnet	406B	U-2-74	8/16/10
Hogue Lake		Poinsett	Cybonnet	406C	U-2-75	8/16/10
Hogue Lake		Poinsett	Cybonnet	406D	U-2-76	8/16/10
Hogue Lake		Poinsett	Cybonnet	408A	U-2-77	8/16/10
Hogue Lake		Poinsett	Cybonnet	408B	U-2-78	8/16/10
Hogue Lake		Poinsett	Cybonnet	408C	U-2-79	8/16/10

Hogue Lake	Poinsett	Cybonnet	408D	U-2-80	8/16/10
Hogue Lake	Poinsett	Cybonnet	409A	U-2-81	8/16/10
Hogue Lake	Poinsett	Cybonnet	409B	U-2-82	8/16/10
Hogue Lake	Poinsett	Cybonnet	409C	U-2-83	8/16/10
Hogue Lake	Poinsett	Cybonnet	409D	U-2-84	8/16/10
Hogue Lake	Poinsett	Cybonnet	I-1A	U-2-85	9/24/10
Hogue Lake	Poinsett	Cybonnet	I-1B	U-2-86	9/24/10
Hogue Lake	Poinsett	Cybonnet	I-1C	U-2-87	9/24/10
Hogue Lake	Poinsett	Cybonnet	I-1D	U-2-88	9/24/10
G & H Farms	Arkansas	Cybonnet	I-2A	U-2-89	9/24/10
G & H Farms	Arkansas	Cybonnet	I-2B	U-2-90	9/24/10
G & H Farms	Arkansas	Cybonnet	I-2C	U-2-91	9/24/10
G & H Farms	Arkansas	Cybonnet	I-2D	U-2-92	9/24/10
Statewide	Poinsett	Clearfield-161	I-3A	U-2-93	9/24/10
Statewide	Poinsett	Clearfield-161	I-3B	U-2-94	9/24/10
Statewide	Poinsett	Clearfield-161	I-3C	U-2-95	9/24/10
Statewide	Poinsett	Clearfield-161	I-3D	U-2-96	9/24/10
Statewide RREC	Arkansas	Templeton	I-4A	U-2-97	9/24/10
Statewide RREC	Arkansas	Templeton	I-4B	U-2-98	9/24/10
Statewide RREC	Arkansas	Templeton	I-4C	U-2-99	9/24/10
Statewide RREC	Arkansas	Templeton	I-4D	U-2-100	9/24/10
Statewide RRVT	White	Rice Tec CL XL 729	I-5A	U-2-101	9/24/10
Statewide RRVT	White	Rice Tec CL XL 729	I-5B	U-2-102	9/24/10
Statewide RRVT	White	Rice Tec CL XL 729	I-5C	U-2-103	9/24/10
Statewide RRVT	White	Rice Tec CL XL 729	I-5D	U-2-104	9/24/10

Statewide RREC	Poinsett Co	Clearfield-151	I-6A	U-2-105	9/24/10
Statewide RREC	Poinsett Co	Clearfield-151	I-6B	U-2-106	9/24/10
Statewide RREC	Poinsett Co	Clearfield-151	I-6C	U-2-107	9/24/10
Statewide RREC	Poinsett Co	Clearfield-151	I-6D	U-2-108	9/24/10
Statewide RREC	Arkansas	Francis	I-7A	U-2-109	9/24/10
Statewide RREC	Arkansas	Francis	I-7B	U-2-110	9/24/10
Statewide RREC	Arkansas	Francis	I-7C	U-2-111	9/24/10
Statewide RREC	Arkansas	Francis	I-7D	U-2-112	9/24/10
Hogue Lake	Poinsett	Cybonnet	I-1E	U-2-113	8/16/10
G & H Farms	Arkansas	Cybonnet	I-2E	U-2-114	8/16/10
Statewide	Poinsett	Clearfield-161	I-3E	U-2-115	8/16/10
Statewide RREC	Arkansas	Templeton	I-4E	U-2-116	8/16/10
Statewide RRVT	White	Rice Tec CL XL 729	I-5E	U-2-117	8/16/10
Statewide RREC	Poinsett	Clearfield-151	I-6E	U-2-118	8/23/10
G & H Farms	Arkansas	Francis	I-7E	U-2-119	9/24/10
G & H Farms	Arkansas	Templeton	I-8A	U-2-120	10/15/10
G & H Farms	Arkansas	Templeton	I-8B	U-2-121	10/15/10
G & H Farms	Arkansas	Templeton	I-8C	U-2-122	10/15/10
G & H Farms	Arkansas	Templeton	I-8D	U-2-123	10/15/10
G & H Farms	Arkansas	Templeton	I-8E	U-2-124	10/15/10
Statewide RREC	Poinsett	Clearfield-151	ST#1A	U-2-125	10/15/10
Statewide RREC	Poinsett	Clearfield-151	ST#1B	U-2-126	10/15/10
Statewide RREC	Poinsett	Clearfield-151	ST#1C	U-2-127	10/15/10
Statewide	Poinsett	Clearfield-151	ST#1D	U-2-128	10/15/10

RREC					
Pine Tree	St. Francis	Unknown	4A	U-3-129	10/15/10
Pine Tree	St. Francis	Unknown	4B	U-3-130	10/15/10
Pine Tree	St. Francis	Unknown	4C	U-3-131	10/15/10
Pine Tree	St. Francis	Unknown	4D	U-3-132	10/15/10
Hogue Lake	Poinsett	Cybonnet	109A	U-3-133	10/15/10
Hogue Lake	Poinsett	Cybonnet	109B	U-3-134	10/15/10
Hogue Lake	Poinsett	Cybonnet	109C	U-3-135	10/15/10
Hogue Lake	Poinsett	Cybonnet	109D	U-3-136	10/15/10
Hogue Lake	Poinsett	Cybonnet	207A	U-3-137	10/15/10
Hogue Lake	Poinsett	Cybonnet	207B	U-3-138	10/15/10
Hogue Lake	Poinsett	Cybonnet	207C	U-3-139	10/15/10
Hogue Lake	Poinsett	Cybonnet	207D	U-3-140	10/15/10
Hogue Lake	Poinsett	Cybonnet	308A	U-3-141	10/15/10
Hogue Lake	Poinsett	Cybonnet	308B	U-3-142	10/15/10
Hogue Lake	Poinsett	Cybonnet	308C	U-3-143	10/15/10
Hogue Lake	Poinsett	Cybonnet	308D	U-3-144	10/15/10
Hogue Lake	Poinsett	Cybonnet	410A	U-3-145	10/15/10
Hogue Lake	Poinsett	Cybonnet	410B	U-3-146	10/15/10
Hogue Lake	Poinsett	Cybonnet	410C	U-3-147	10/15/10
Hogue Lake	Poinsett	Cybonnet	410D	U-3-148	10/15/10
Hogue Lake	Poinsett	Unknown	1A	U-3-149	10/15/10
Hogue Lake	Poinsett	Unknown	1B	U-3-150	10/15/10
Hogue Lake	Poinsett	Unknown	1C	U-3-151	10/15/10
Hogue Lake	Poinsett	Unknown	1D	U-3-152	10/15/10
G & H	Arkansas	Cybonnet	G102A	U-3-153	10/15/10

Farms					
G & H Farms	Arkansas	Cybonnet	G102B	U-3-154	10/15/10
G & H Farms	Arkansas	Cybonnet	G102C	U-3-155	10/15/10
G & H Farms	Arkansas	Cybonnet	G102D	U-3-156	10/15/10
Newport	Jackson	Clearfield-151	I-9A	U-3-157	3/1/12 [G]
Newport	Jackson	Clearfield-151	I-9B	U-3-158	3/1/12 [G]
Newport	Jackson	Clearfield-151	I-9C	U-3-150	3/1/12 [G]
Newport	Jackson	Clearfield-151	I-9D	U-3-160	3/1/12 [G]
Newport	Jackson	Clearfield-151	I-9E	U-3-161	3/1/12 [G]
Newport	Jackson	Taggart	I-10A	U-3-162	5/17/12
Newport	Jackson	Taggart	I-10B	U-3-163	5/17/12
Newport	Jackson	Taggart	I-10C	U-3-164	5/17/12
Newport	Jackson	Taggart	I-10D	U-3-165	5/17/12
Newport	Jackson	Taggart	I-10E	U-3-166	5/17/12
Pine Tree	St. Francis	Francis	WFS13A1	U-3-167	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13A2	U-3-168	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13B2	U-3-169	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13B1	U-3-170	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13C1	U-3-171	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13C2	U-3-172	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13D1	U-3-173	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13D2	U-3-174	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13E	U-3-175	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13F	U-3-176	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13G	U-3-177	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13H	U-3-178	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13I	U-3-179	10/29/13 [G]
Pine Tree	St. Francis	Francis	WFS13J	U-3-180	10/29/13 [G]
Fayetteville	Washington	Neptune	I-90E1	U-3-181	11/12/13 [G]
Fayetteville	Washington	Neptune	I-90E2	U-3-182	11/12/13 [G]
Fayetteville	Washington	Neptune	I-90E3	U-3-183	11/12/13 [G]
Fayetteville	Washington	Neptune	I-90E4	U-3-184	11/12/13 [G]
Fayetteville	Washington	Neptune	I-90E5	U-3-185	11/12/13 [G]
Fayetteville	Washington	Neptune	I-90E6	U-3-186	11/12/13 [G]
Fayetteville	Washington	Neptune	I-90E7	U-3-187	11/12/13 [G]
Fayetteville	Washington	Neptune	I-90E8	U-3-188	11/12/13 [G]
Fayetteville	Washington	Neptune	I-90E9	U-3-189	11/12/13 [G]
Fayetteville	Washington	Neptune	I-90E10	U-3-190	11/12/13 [G]

Table 2.1. Isolates of *Ustilaginoidea virens* obtained from various rice cultivars grown in Arkansas.
[A] Location of fields where panicles infected with FS were sampled.
[B] County where the fields or sampling locations were located.
[C] Cultivar from which the infected panicles were obtained.
[D] Isolate number and letter that designates from which respective panicle the sample originated.
[E] Tube number, designating the first letter (U) as *Ustilaginoidea*, *-n-* the middle box number, in which the isolate is contained and *-n* the last number designating the isolate in a series.
[F] Date of deposition in the Harris cryogenic freezer located in Lab 221 Plant Sciences Building, University of Arkansas in Fayetteville, AR and maintained at -80°C.
[G] Isolates obtained from white false smut spore balls consistent with descriptions of *Ustilaginoidea albicans*.

Table 2.2. Nucleotide sequences of polymerase chain reaction (PCR) primers designed by Zhou et al. (2003) used in this study.

Primer [A]	Sequence (5'-3') [B]	Length (nucleotides) [C]	Amplicon length (bp) [D]	
US1-5	CCGGAGGATACAACCAAAAAAACTCT	26	380	[A]
US3-3	GCTCCAAGTGCGAGGATAACTGAAT	25	380	[B]
US2-5	CAATGCATGTCTGAGTGGATTTTTG	25	232	[A]
US4-3	CCAACACCAAGCGCAAGACAGA	22	232	[B]

[A] Identification of the respective primary and nested-PCR primers used in this study as described by Zhou et al. (2003). US1-5 and US2-5 are forward primer sequences; US3-3 and US4-3 are reverse primer sequences.

[B] Sequence of each primer used in this study.

[C] Length or the number of nucleotides contained in each primer sequence.

[D] The length of the amplicon product expected when reacted with *Ustilaginoidea virens* template DNA.

Table 2.3. A summary list containing the number of isolates which are morphologically consistent with formal descriptions of *U. virens* and *U. albicans* as described by Tanaka et al. (2008), Honkura et al. (1991), Wang and Bai (1997) and ultrastructural characteristics described by Kim and Park (2007).

Location [A]	County [B]	Year [C]	Cultivar [D]	Isolates [E]
Fayetteville	Washington	2013	Neptune	10 [F]
G & H Farms	Arkansas	2010	Templeton	5
G & H Farms	Arkansas	2009	Cybonnet	33
Hogue Lake	Poinsett	2009	Cybonnet	45
Hogue Lake	Poinsett	2009	Unknown	4
Newport	Jackson	2011	Clearfield-151	5 [F]
Newport	Jackson	2011	Taggart	5
Pine Tree	St. Francis	2013	Francis	14 [F]
Pine Tree	St. Francis	2009	Unknown	28
Statewide	Poinsett	2009	Clearfield-161	9
Statewide	Poinsett	2009	Clearfield-151	4
Statewide RREC	Poinsett	2009	Clearfield-151	5
Statewide RREC	Arkansas	2009	Templeton	9
Statewide RREC	Arkansas	2008	Francis	5
Statewide RRVT	White	2009	Rice Tec CL XL 72	9
			Total	190

[A] Location of fields or greenhouse where panicles infected with FS were sampled.
[B] County where the fields or sampling locations were located.
[C] Year the samples were collected.
[D] Cultivar from which the infected panicles were obtained.
[E] Number of isolates obtained from each Location, County, Year and Cultivar.
[F] White false smut isolates were consistent with descriptions of *Ustilaginoidea albicans*.

Table 2.4. *U. virens* isolates selected for use in germination and growth studies.

Location [A]	County [B]	Cultivar [C]	Isolate # [D]	Date Isolated [E]	Tube# [F]
Statewide	Poinsett	Clearfield-151	I-6E	August 16, 2010	U-2-118
RREC	Poinsett	Francis	I-7E	September, 2008	U-2-119
G&H Farms	Arkansas	Templeton	I-8E	October 15, 2010	U-2-120

[A] Location of fields where panicles infected with FS were sampled.
[B] County where the fields or sampling locations were located.
[C] Cultivar from which the infected panicles were obtained.
[D] Isolate number and letter that designates from which respective panicle the sample originated.
[E] Date of deposition in the Harris cryogenic freezer located in Lab 221 Plant Sciences Building, University of Arkansas in Fayetteville, AR and maintained at -80°C.
[F] Tube number, designating the first letter (U) as *Ustilaginoidea* species, -*n*- the middle box number, in which the isolate is contained and -*n* the last number designating the isolate in a series.

Table 2.5. Percent germination of chlamydospores taken from three- and five-month-old FS spore balls originating from rice cultivars 'Clearfield-151' and 'Templeton' and incubated in PO_4 buffers at pH levels ranging from 5.5 to 8.0

Treatment pH [A]	Mean growth (mm)			
	3-month		5-month [B]	
	Clearfield-151	Templeton	Clearfield-151	Templeton [C]
5.5	18.4 B	24.8 A	8.0 AB	11.6 A[D]
6.0	19.5 B	28.4 A	10.5 A	10.8 A
6.5	20.0 B	26.2 A	8.6 AB	6.8 B
7.0	24.0 A	26.8 A	5.8 BC	3.5 CD
7.5	10.8 C	20.2 B	3.3 CD	4.5 BC
8.0	6.0 D	11.8 C	1.5 D	1.9 D

[A] PO_4 buffers prepared with pH ranging from 5.5, 6.0, 6.5, 7.0, 7.5 to 8.0, were used in these experiments conducted in an incubator at 26°C average temperature for 96 hours.

[B] Percent germination was determined from three and five month old chlamydospores by counting the number of germinated chlamydospores out of 50 chlamydospores twice from each sample and the counts were averaged. There were three replications of chlamydospores from each cultivar, age and pH level and the tests were repeated twice.

[C] Chlamydospores originating from 'Clearfield-151' and 'Templeton' were obtained from their respective cultivar after storage at 18°C.

[D] Means of 'Clearfield-151' and 'Templeton' conidium germination in each age group in a column followed by a common letter, are not significantly different, LSD at a=0.05.

Table 2.6. The effect of pH on mycelial growth of *Ustilaginoidea virens* isolates grown on agar at pH levels ranging from 5.5 to 8.0. [A]

Treatment pH [B]	Mean growth (mm) [C]
5.5	30.3 A
6.0	30.8 A
6.5	28.7 AB
7.0	24.5 C
7.5	25.3 BC
8.0	14.6 D
P-value <.0001	<.0001

[A] Experiments were conducted in an incubator at 26°C average temperature. Cultures were grown for three weeks' time. The data are expressed as the means of three replications and two experiments.

[B] Agar was adjusted to the pH values shown with 0.1M PO_4 pH buffer ranging from pH 5.5, 6.0, 6.5, 7.0, 7.5 to 8.0.

[C] Means in each column followed by a common letter are not significantly different at a=0.05.

III. Nested-PCR as a tool for detection of *Ustilaginoidea virens* during asymptomatic colonization of rice tissues.

Abstract

Rice false smut (FS) caused by *Ustilaginoidea virens*, is found in much of the rice growing area of Arkansas. Since the sporulating structures (= spore balls, galls) are not visible until after flowering, when the rice grains begin to fill and mature it is challenging to assess the incidence and severity of FS prior to development of the spore balls. Diagnosing asymptomatic colonization of rice by *U. virens* before visualization of the disease has been limited to standard microbiological isolation procedures. Recently, polymerase chain reaction (PCR) primer pairs specific to *U. virens* based on the conserved internal transcribed spacer (ITS) ribosomal DNA (rDNA) region have been used to determine the presence of *U. virens* in rice leaf, sheath and stem or panicle tissues before and after heading. The objective of this study was to test the specificity and sensitivity nested-PCR to amplify *U. virens* rDNA in total DNA extracts from isolates, rice germling and seedling shoot apical meristematic (SAM) tissues and panicle samples. The results from this work show that nested-PCR consistently and reliably amplified the target rDNA sequence from *U. virens* but did not amplify DNA from non-target species. In sensitivity tests, nested-PCR was determined to be 1000X more sensitive than the primary-PCR reaction. The results of experiments to determine when rice is colonized by *U. virens* show that the fungal rDNA is detected in rice seedlings within three to seven days after germination and is also detected from individual spikelets in the unopened boots of maturing rice plants.

Introduction

In recent years, sporadic outbreaks of rice false smut (FS) have occurred in the rice growing regions of Arkansas (Cartwright et al., 2002). The disease is characterized by the

transformation of individual rice grains on infected panicles into green/orange spore balls covered by an abundance of powdery dark-green chlamydospores late in the season upon maturity (Ou, 1972). The factors contributing to its emergence and epidemics within the last 15 years in Arkansas are not understood due to an inadequate understanding of the disease cycle and the sporadic epiphytology of FS. The pathological events leading to the colonization of rice and development of signs of infection on diseased panicles must be understood before we can develop effective management strategies.

Studies have elucidated the process of invasion and colonization of rice coleoptiles by *U. virens* at the seedling stage (Ikegami, 1962). In 1962, Ikegami inoculated coleoptiles of young seedlings (1 to 22 mm in height) with suspensions of chlamydospores to determine the relationship between the percent of rice infected by *U. virens*, the length of the coleoptile at the time of inoculation and chlamydospores of different maturities (yellow, yellowish-green, greenish-yellow and green). He inoculated coleoptiles between one to ten mm and three to six mm in height with greenish-yellow or yellow chlamydospores, respectively at very early stages of growth. The results of this study indicated a higher percent infection was obtained if the inoculations were made when coleoptiles were >12 mm or >8 mm when inoculated with greenish-yellow or yellow chlamydospores, respectively. Ikegami (1963) applied histological studies to elucidate the mode of rice seedling infection by *U. virens* and the development of hyphae inside plants from a few days after germination up through the mid tillering stage at 61 days after inoculation. In this study, he found hyphae emerging from the germ tube of a germinating *U. virens* conidium between one to two days after inoculation. These hyphae penetrated the cuticle and epidermal wall of the rice coleoptile and within three to four days after inoculation the hyphae grew between the layer of epidermal cells and later grew between

epidermal cells and the next inner cell layer. After 48 days, he observed hyphae growing between the cells of sieve tubes of the host tissues and after 54 and 61 days, hyphae were found within the growing points of the main tiller near the panicle primordium (Ikegami, 1963). These histological studies were complimented with experiments by Shroud and TeBeest (2005) who showed hypha from germinating chlamydospores of *U. virens* infecting and invading rice root tissues. These combined histological studies present evidence that *U. virens* chlamydospores germinate and produce hypha that may colonize rice tissues asymptomatically early in seedling development.

False smut disease can occur if coleoptiles or seeds are inoculated with chlamydospores (Ikegami, 1962, 1963; Schroud and TeBeest, 2005; Wang et al., 2008). Spore balls can also develop on mature rice after planting seed infested and infiltrated with chlamydospores (Ditmore and TeBeest, 2006; Ditmore et al., 2007; TeBeest et al., 2010) or after planting seed in soil infested with chlamydospores (Ditmore and TeBeest, 2006; TeBeest et al., 2010). The reports above all suggest that infection of rice may be occurring at the seedling growth stage. Other studies have shown that FS developed after panicles within boots were inoculated with chlamydospores (Ashizawa et al., 2011; Fujita et al., 1989; Guo et al., 2012; Ikegami, 1960; Kulkarni and Moniz, 1975; Tanaka et al., 2011; Tang et al., 2012; Wang et al., 1996; Wang et al., 2008; Yoshino and Yamamoto, 1952; Zhang et al., 2004; Zhou et al., 1999). False smut disease development resulting from inoculating immature panicles within boots suggests that *U. virens* may colonize spikelets during the booting stage. However, all of these reports present fragmentary evidence since all were done with different cultivars and at different times with different methods. Because it is very difficult to isolate the fungus from plants prior to development of spore balls there is no direct cultural evidence of the fungus within plant tissues

before the appearance of spore balls.

Recently, molecular techniques have facilitated the detection of pathogens in asymptomatic plant tissue and the study of etiological events during infection of barley (*Hordeum vulgare* L.) seedlings by *Ustilago hordei* Bref. (Willits and Sherwood, 1999) and infection of rice by *U. virens* (Ashizawa and Kataoka, 2005; Zhou et al., 2003). Polymerase chain reaction (PCR)-based techniques that selectively amplified *U. virens* ribosomal DNA (rDNA) in rice DNA extracts were initially developed by Zhou et al. (2003). In that study, panicles in the leaf sheaths of rice plants were artificially inoculated with secondary conidia at the booting stage and incubated as described by Fujita et al. (1989). Then DNA was extracted from individual spikelets, leaf, sheath and stem tissues before amplification with the US1-5/US3-3 (primary PCR) and US2-5/US4-3 (nested-PCR) primers. Further, Zhou et al. (2003) conducted histological examinations after dissecting plants under a stereo-microscope and staining with aniline blue. They observed conidia and hyphae on the inner surface of the leaf sheaths around the injection sites and on spikelets, some of which resulted in mycelial invasion of the spikelets. The results showed histological examinations were consistent with nested-PCR detection and that PCR "indicated that the specific primer pairs were useful in directly detecting *U. virens* from infected host tissues prior to the onset of visual symptoms" (Zhou et al., 2003). Further, Ashizawa and Kataoka (2005) used nested-PCR as a diagnostic tool for detecting *U. virens* rDNA using independently designed specific primers. Studies were conducted using the nested-PCR protocol and specific primers to elucidate the infection process of developing rice panicles prior to their emergence from the boot. The results show that rDNA with identity to *U. virens* was amplified from the total DNA samples extracted from panicles prior to emergence of the panicle from the boots. The results also show that DNA amplifications from mature panicles

resulted in more positive samples amplified by nested-PCR than from less mature samples (Ashizawa and Kataoka, 2005). Ditmore and TeBeest (2006) showed that *U. virens* rDNA was detected from seeds infested with *U. virens* spores. As a result of the histological and PCR studies, it seems plausible that *U. virens* infects rice at the seedling stages and that a molecular diagnosis by nested-PCR may be a useful tool to study the association of *U. virens* with asymptomatic rice tissues.

The overall goal of this project was to characterize and determine if PCR is a useful strategy to determine the asymptomatic colonization of rice seedlings by *U. virens*. The five basic hypotheses of this study were: 1) that nested-PCR is an efficient, reliable and specific method to detect *U. virens* and *Ustilaginoidea* isolate I-9E within asymptomatic seedlings and panicles prior to the onset of FS disease; 2) that if *U. virens* chlamydospores germinate on seed surfaces and produce hyphae that invade and colonize the shoot apical meristem (SAM) and seedling tissues, then hyphae should be both visualized by histology and test positive in the nested-polymerase chain reaction (nested-PCR); 3) that if rice seed is contaminated with viable and infectious seed-borne inoculum, then the rate of seedling colonization by *U. virens* over time can be determined by using a specific nested-PCR reacted with DNA extracted from shoot apical meristem tissues sampled after seedling emergence; and 4) that seedling colonization inevitably leads to infection of florets within boots prior to exsertion of the panicles.

Based on these hypotheses, the specific objectives of this study were: 1) to determine if the nested-PCR protocols that amplifies rDNA is a specific and sensitive detection probe for DNA extracted from isolates of *U. virens* in DNA extracted from rice tissues; 2) to determine if hyphae can be visualized in shoot apical meristematic tissue and determine if nested-PCR can verify the presence of *U. virens* rDNA from these tissue samples; 3) to determine the incidence

and time of initial colonization of rice seedlings by detecting *U. virens* rDNA at three-, five- and seven-days after germination and emergence from the soil; and 4) to determine if *U. virens* can be detected in spikelets removed from panicles prior to emergence from the boot.

Materials and methods

Source of isolates and culture preparation

A list of mono-chlamydospore isolates of the fungi used in these tests are shown in Tables 3.1 and 3.2. The isolates of FS and the other named pathogens used in this study were obtained from collections maintained by Dr. D.O. TeBeest and Dr. J. Correll in the Department of Plant Pathology, University of Arkansas. The identified plant pathogenic isolates, the 17 unknown fungal isolates obtained from rice germlings, seedlings and panicles (Table 3.1). The 24 isolates identified as *U. virens* and one isolate of the white smut that shares characteristics of a previously described color morph of *Ustilaginoidea* (= I-9E) (table 3.2) were grown and maintained on acidified (pH 6.2) PDA. A single *U. virens* and non-related isolates were included in nested-PCR specificity tests.

Plant material

Eight rice cultivars, 'Cheniere', 'Clearfield-162', 'Francis', 'Roy J', 'Taggart', 'Wells', 'Clearfield-151' and 'Templeton' were selected and used in this study to represent conventional non-hybrid cultivars commonly grown in Arkansas. Seeds were initially obtained from two sources; Dr. R. Cartwright, UA Extension Service and from the foundation seed program University of Arkansas, Stuttgart, Arkansas. In the greenhouse studies, soils were collected from the same fields used for each test from the Newport Research Station and the Pine Tree Research Station.

Isolation of fungi from rice germlings, seedlings and spikelets from booted panicles

Experiments were conducted to determine if *U. virens* or other fungi could be isolated from selected tissues of rice germlings or seedlings. In these tests, seeds of 'Cheniere', 'Clearfield-151', 'Clearfield-162' 'Francis', 'Roy J', 'Taggart' and 'Templeton' cultivars infested with *U. virens* chlamydospores were germinated in Petri dishes with Whatman No. 4 filter paper and incubated under containment conditions in the laboratory at 24°C. The coleoptiles were removed from the main stems of one-week-old germlings and two- to three-week-old of seedlings and the SAM was transversally sectioned into four 2-2.5 mm pieces from the base using a flamed razor blade while immersed in 0.8% Clorox™ for approximately one minute. The stem pieces were then dipped into sterile distilled water then placed in order (from the stem base upward toward the tip) onto PDA amended to contain glacial acetic acid (100 µl ml^{-1}) and streptomycin/ampicillin (200 µl ml^{-1}). Dishes were incubated for two to three weeks at 23-24°C on a 12 hour day length under fluorescent 15 W lights (General Electric, Cleveland, OH).

Experiments were conducted to determine if *U. virens* or other fungi could be isolated from spikelets found in booted rice panicles. In this study, a spikelet is defined as the combined tissues of an individual floret and pedicel. In these experiments, seeds of 'Clearfield-162' were germinated in five cm (2") Jiffy® peat pots then trans-potted to a 3.78 l (1 gallon) plastic pot filled with field soil from the Newport Research Station. The pots were placed in 18.93 l (5 gallon) buckets and filled with water after tillering to a flood depth of 10 to 15 cm above the soil line. The plants were grown to near-maturity and individual panicles were selected at random from the boots when the boot leaf collar was 8-12 cm above the flag leaf or penultimate leaf prior to exsertion (growth stage R2) (Counce et al., 2000). Booted panicles were removed from

the plant below the panicle internode and their outer leaf sheaths were wiped with a tissue paper saturated in 0.8% Clorox™. The boot was cut to open to remove the panicles from boots using a sterile scalpel and forceps in a transfer hood. Panicles were placed into sterile Petri dishes and individual spikelets were removed from the bracts using flamed forceps and scissors. A total of 92 spikelets from eight panicles were removed and placed on PDA amended to contain ampicillin/streptomycin as previously described.

Petri dishes were incubated for two weeks at 23-24°C on a 12 hour day length under fluorescent light. The samples were observed daily for two weeks before the experiment was terminated. Hyphal tips of fungi that grew from spikelets were transferred onto acidified-PDA dishes amended to contain the streptomycin/ampicillin antibiotics as described. Colony growth, hyphae and sporulating structures were then observed under a microscope after staining with lactophenol/cotton blue for morphological comparison to *U. virens* with light microscopy. The fungal isolates were cultured on PDA and DNA was extracted from each of the unknown fungi for nested-PCR specificity studies. A list of these fungi is shown in Table 3.1.

DNA extraction from cultured mycelium and rice tissues

Plant pathogenic fungi were grown in 100 ml Pyrex bottles containing 50 ml of autoclaved Potato Dextrose Broth (PDB) for use in specificity tests (Table 3.1). The cultures were placed on a rotary shaker and incubated at 26°C for two to three weeks prior to harvesting the mycelia. The isolates were removed from the shaker and filter-separated from the culture medium by using a sterile funnel fitted with a 9.0 cm Whatman No. 4 filter under 27 in $^{-1}$ Hg vacuum. The *U. virens* isolates and isolates of 17 fungi not related to *U. virens* were cultured on PDA prior to extracting DNA from the mycelium.

For the tests using rice seedling tissues, seeds from each cultivar were planted in the

greenhouse and/or field and sampled three weeks after seedling emergence. Seedlings were gently removed from the soil and any remaining soil was washed from seedlings under cold running water for 30 seconds. Roots were removed from the mesocotyl tissues below the SAM and the coleoptiles were removed from the stems to expose the main stems of seedlings or prophylls in germlings. The remaining hypocotyl including the SAM tissue was cut into several pieces (up to 1.0 cm in length) with small surgical scissors that were flamed for 10 seconds between samples. In tests where DNA was extracted from spikelets of booted panicles, each spikelet was sampled after aseptically removing the panicle from the boot. Individual spikelets were placed into an extraction tube with buffer prior to extraction of the DNA as described below.

Extraction of total DNA from rice tissues and mycelium from cultures of all isolates was done according to the Epicentre® QuickExtract™ manufacturer's protocol. One hundred milligrams of either plant tissues from the field or greenhouse samples or mycelium samples from cultures were placed into 1.5 ml polyethylene tubes on ice containing 100 µl of QuickExtract™ Plant DNA extraction buffer. The samples were briefly vortexed and then incubated in water baths in extraction buffer at 65°C for six minutes then incubated at 98°C for two minutes. All DNA samples were stored at 4°C until used as template in PCR.

High-molecular weight DNA was obtained from plant tissues using the Qiagen DNeasy Plant Mini Kit was used for sequence analysis. Seeds of 'Clearfield-151' and 'Templeton' rice cultivars infested with *U. virens* chlamydospores were germinated in 10.0 cm Petri dishes fitted with a Whatman No.4 filter paper and watered with sterile de-ionized water. Sprouted seeds were planted in the greenhouse in five cm Jiffy® peat pots containing field soil from the Newport Research Station. Seedlings were harvested by gently pulling from the soil after the V3 stage

(Counce, et al., 2000), when collar formation was complete. Soil was washed from the plants before the roots were cut and removed below the crown leaving the remaining coleoptile in-tact. Approximately 500 mg of tissue was sampled from the coleoptile and shoot apical meristem of seedlings and frozen under liquid nitrogen then ground into powder in sterile mortar and pestles. The powdered tissue samples were placed into 1.5 ml tubes and processed using DNeasy Plant Mini Kit (50) Cat. No. 69104 (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol.

High-molecular weight DNA was additionally obtained from three-week-old cultures of *U. virens* isolates. Five hundred mg of mycelium were scraped from the surface of cultures of *U. virens* isolates I-6E, I-7E, I-8E, I-9E and I-10E, grown on PDA for three weeks. The mycelium was placed in a sterile mortar and pestle and ground to powder in liquid nitrogen. High-molecular weight DNA was extracted from the ground mycelium using the DNeasy Plant mini kit from Qiagen. The DNA concentration from plant or mycelium samples was determined prior to PCR using a microvolume spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Conditions for detection of *U. virens* by simple and nested-PCR

The conditions for nested-PCR protocols for *U. virens* from plant tissues (Zhou et al., 2003, Ashizawa et al., 2005, 2010) were modified in this study by using Epicentre® Biotechnologies PlantAmp™ PCR System Premix Kit (Cat. No. PA08091K), containing Epicentre® Failsafe™ PCR reaction buffer 2X PreMix C and PCR Enzyme Mix. The Failsafe™ PlantAmp™ protocol was routinely used in this field and greenhouse study to amplify the expected amplicon by reacting extracted plant or fungal DNA with the selective primers in a nested-PCR reaction. Failsafe™ PlantAmp™ was discontinued by the manufacturer, but later modified based on recommendations of a representative from Epicentre to include 1% final

volume polyvinylpyrrolidone (PVP) with Failsafe™ PCR Premix C. After the field and greenhouse experiments were completed in 2011 and 2012, Epicentre PCR Failsafe™ premix products were substituted with 2X Taq Master Mix (GenScript USA Inc. Piscataway, NJ). The 2X Taq Master mix from GenScript was tested and found to be suitable for sequencing. The 2 X Taq Master mix is now used for all nested-PCR diagnostics for detection of *U. virens* rDNA from plant DNA samples.

Ustilaginoidea virens allele-specific primary-PCR primers 5'-ccggaggatacaaccaaaaaaaactct-3' (US1-5), 5'-gctccaagtgcgaggataactgaat-3' (US3-3) and nested-PCR 5'-caatgcatgtctgagtggatttttg-3' (US2-5) 5'-ccaacaccaagcgcaagacaga-3' (US4-3) primer pairs, as designed by Zhou et al. (2003), were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa, USA) and reconstituted to 20 µM (Table 3.3). These primer pairs were used to amplify the 380 and 232-bp conserved rDNA sequences specific to *U. virens,* respectively. Nested-PCR amplification was accomplished in a Bio-Rad PTC-200 Gradient icycler and Bio-Rad C 1000™ thermal cyclers. Each 50 µl PCR reaction was prepared on ice to contain 21 µl of sterile water, 1 µl of each primer, 1.5 µl of DNA template, 0.5 µl FailSafe™ PCR Enzyme Mix and 25 µl of the FailSafe™ Master Mix (alternatively, 25 µl of the FailSafe™ PCR Premix C + 1% Final Volume (1% FV) polyvinylpyrrolidone). The 50 µl reactions prepared for sequencing or routine nested-PCR diagnosis that utilized 2X Taq Master Mix contained 21.5 µl of sterile water, 1 µl of each primer, 1.5 µl of DNA Template, 25 µl 2X Taq Master Mix. A PCR reagent master mix was prepared prior to each PCR reaction and the reagents were aliquoted to 8-tube PCR strips with attached strip caps on ice prior to loading the template DNA. Templates in the primary reactions consisted of 1.5 µl from DNA quick extractions, while the nested reaction utilized 1.5 µl of amplicon from the first reaction product as template. A negative reagent and a

positive (DNA from I-6E) control were included in each set of reactions. Thermocycling conditions for primary and nested-PCR were as follows, initial denaturation was done at 96°C for two minutes to separate DNA into single strands, followed by 30 replication cycles at 95°C for 20 seconds, annealing at 54° C for 30 seconds and finally, extension at 72°C for 30 seconds. After the 30 cycles, final extension was held at 72° C for seven minutes and then held at 4°C until the amplified products were resolved.

After thermo cycling, 10.0 µl of the amplified product + 2.0 µl of loading dye were analyzed by horizontal electrophoresis using 2.5% (wt./vol) LE agarose (Shelton Scientific Inc., Shelton, CT) stained with ethidium bromide (EtBr) in 1X TBE (0.089 M Tris, 0.089 M Borate, 0.002M EDTA, amresco®, Solon, Ohio) buffer for 1-1.5 hour at 92 volts. Amplified DNA products were visualized under ultraviolet light in an Alphaimager Multi image light cabinet atop a transilluminator (Alpha Innotech Corporation, San Leandro, CA, USA). Amplicon band weights were determined by comparison to 100 bp DNA ladder (Promega Corporation Madison WI, USA) with 20% loading dye. Images of the resolved amplicon products were taken using a Toshiba CCD Model KP-M2U camera (Hitachi Denshi Ltd. Japan) with a Close-up +2 lens (Vivitar, Japan) run by MultiCam Studio V 6.7.3.1740 (© Euresys, 1989-2011) software. All photographs were cropped using Microsoft® Office Picture Manager (©Microsoft Corporation, 2006).

The specificity of nested-PCR primers and conditions used for detection of *U. virens* isolates and one *Ustilaginoidea* isolate I-9E

The specificity of the nested-PCR primers and protocols for detection of *U. virens* isolates from rice seedlings using the described primer sets described above was determined in this study to increase user confidence of the PCR protocol in field and greenhouse

epiphytological studies. Four tests were conducted to determine the specificity of primers US1-5/US3-3 and US2-5/US4-3 designed by Zhou et al. (2003) (Table 3.3) by testing the primers to amplify DNA of *U. virens* isolates, pathogens of rice and non-rice plant pathogens and other unrelated and unknown isolates from rice seedlings or spikelets. Specificity tests were concluded by sequencing the amplicon products and comparing the sequences to the published sequences in GenBank using a BLAST® nucleotide search. The results of the tests provided a full evaluation or confirmation that the primers described by Zhou et al. (2003) are specific to Arkansas *U. virens* isolates and to *Ustilaginoidea* isolate I-9E with accessions in the BLAST® database. The following tests evaluated the utility of the protocol by using the designed primers for specificity against several unrelated fungi and for diagnosis of *U. virens* DNA isolated from rice samples obtained from the greenhouse and field.

Part 1: The specificity of nested-PCR to *U. virens* isolates from Arkansas

The objective of this study was to determine the specificity of PCR primers to a selection of 24 isolates morphologically and biometrically consistent with descriptions of *U. virens* and one isolate (I-9E) that is morphologically and biometrically consistent with descriptions of *Ustilaginoidea albicans* (perhaps an albinotic color morph of *Ustilaginoidea* vi*rens,* Jin et al., 2012). DNA extracted from 100 mg of mycelium from each isolate and was reacted with the primer pairs US1-5/US3-3 (1°, primary) and US2-5/US4-3 (2°, nested) in a nested-PCR reaction as described. The amplicon products were resolved as described and the experiments were conducted twice.

Part 2: The specificity of nested-PCR to other rice pathogens

The objective of this experiment was to determine if the nested-PCR protocol and primer sets would amplify products from other rice pathogens. *Magnaporthe oryzae* IC 17,

Magnaporthe oryzae IB49, *Rhizoctonia oryzae* vc 268, *Rhizoctonia oryzae* vc 27, *Rhizoctonia oryzae* vc 77 and *Tilletia barclayana* were grown in PDB and harvested under vacuum in a funnel fitted with a Whatman No. 4 filter paper. DNA was extracted from 100 mg of the fungal hyphae using QuickExtract™ Plant DNA Extraction buffer. The extracted DNA from these isolates was reacted using the nested-PCR protocol described above and these the experiments were repeated twice. The products were then separated and visualized in 2.5% (wt. /vol) agarose stained with ethidium bromide under UV light in the transilluminator.

Part 3: The specificity of nested-PCR to pathogens of other crops

The objective of this experiment was to determine if the described PCR protocol and primer sets would produce amplicons from plant pathogens and *Trichoderma viride* found in rice fields in Arkansas. *Colletotrichum gloeosporioides* f. sp. *aeschynomene* CLA 5A, *Colletotrichum gloeosporioides* f. sp. *aeschynomene* 313, *Colletotrichum sublineolum* CS 571, *Colletotrichum sublineolum* CS 612, *Rhizoctonia solani* vc 11 and *Trichoderma viride* were grown in PDB and harvested under vacuum in a funnel fitted with a Whatman No. 4 filter paper. DNA extracted from 100 mg of mycelium from the isolates using QuickExtract™ Plant DNA Extraction buffer was reacted using the nested-PCR protocol described above and these experiments were conducted twice. The products were visualized in 2.5% agarose stained with ethidium bromide under UV light in a transilluminator.

Part 4: The specificity of nested-PCR to isolates of fungi from crowns of rice germlings, seedlings and spikelets from booted panicles

The objective of this experiment was to determine if potential false positive reactions could result from reactions with DNA of unidentified fungi that were isolated directly from rice germling apical meristem tissue, seedling apical meristem tissue and spikelets. A number of

fungi were isolated from germlings of 'Francis' (14), 'Cheniere' (13), 'Roy J' (2), 'Taggart' (12); from seedlings of 'Roy J' (15), 'Clearfield-151' (20) and 'Templeton' (15); and from 92 spikelets of 'Clearfield-162' panicles. In these experiments, DNA templates from the fifteen isolates of fungi from rice germlings, seedlings and two fungal isolates from spikelets of panicles removed from boots were reacted in a nested-PCR reaction. The products were visualized in 2.5% agarose stained with ethidium bromide under UV light in a transilluminator and the nested-PCR reactions were conducted twice.

Sequence analysis and multiple sequence alignment of the amplified products from nested-PCR

The specificity of primers US1-5/US3-3 and US2-5/US4-3 was first determined by using Basic Local Alignment Search Tool (BLAST®) to examine the database for near matches to the primer sequences. The primers were checked for specificity in the nucleotide BLAST® database for "short and near exact matches" by concatenating the primer sequences after pasting the forward and reverse primer sequences together with a string of ten N's prior to the search.

Nested-PCR amplicons were visualized after separation in 2.5% agarose stained with ethidium bromide under UV light. Amplified DNA products from the nested-PCR reactions of four *U. virens* isolates I-6E, I-7E, I-8E, I-9E and I-10E and ten seedlings from both 'Clearfield-151' and 'Templeton' were sequenced to determine and establish the identity of the amplified products. DNA amplicons were separated from primers, dNTP's and salts with a ZR DNA Sequencing Clean-up Kit ™ (Zymo Research Irvine, CA) then quantified and diluted with the aid of the NanoDrop microvolume spectrophotometer. Sequencing reactions were prepared by diluting each nested primer (US2-5/US4-3) to 3.4 pmol and DNA template was diluted to 3 to 10 ng μl^{-1} in 13 µl total volume reactions. Samples were then submitted to the DNA Resource

Center, Center of Excellence for Poultry Science, University of Arkansas Division of Agriculture, according to guidelines for the sample preparation (http://dnaresourcecenters.uark.edu/SamplePrepGuide_NEW%20DNA.html).

Applied Biosystems BigDye v3.1 chemistry was utilized for the sequencing reactions following the Sanger chain-terminating method of DNA sequencing with fluorescently labeled dd-NTPs. CleanSeq magnetic beads (Beckman Coulter Genomics) were used to remove unincorporated d-NTPs, salts and primers from the reactions. Electrophoresis was performed on an Applied Biosystems 3130 XL Genetic Analyzer. AB1 files were analyzed using the SeqScanner software from ABI Appliedbiosystems.com. Sequences were obtained and a Basic Local Alignment Search Tool (BLAST®) somewhat similar sequence (blastn) search from the nucleotide collection was conducted with each forward and reverse sequence (Altschul et al., 1990 and 1997).

Sequence similarity was compared among *U. virens* isolates and among sequences obtained from seedlings after manipulation to align multiple sequences. Clipped and aligned sequences were produced in the CAP3 Sequence Assembly Program (Sievers et al. 2011) from forward and reverse complementary sequences of each of the *U. virens* isolates I-6E, I-7E, I-8E, I-9E and I-10E and for sequences from ten seedlings each of 'Clearfield-151' and 'Templeton' cultivars. The multiple sequences were aligned using Clustal Omega (EMBL-EBI, Hinxton, Cambridgeshire, UK) and BioEdit version 7.1.3.0 (Hall, 1999) multiple sequence alignment programs to search for similarity among the sequences of amplicons derived from isolates and seedlings.

Sensitivity in *U. virens* detection in the presence of rice DNA

The sensitivity of the described protocol above was tested to simulate amplification of fungal rDNA in DNA samples extracted from plant tissues for primary and nested-PCR. For this experiment, 25 ng DNA μl^{-1} of total DNA from rice was combined with serially diluted DNA from *U. virens* isolate I-6E and reacted with the described primers in PCR reactions. A NanoDrop micro-volume spectrophotometer (Thermo Fisher Scientific Wilmington, DE, USA) was used to determine the first two dilution concentrations and to create a dilution series of the template DNA ranging from 131 ng μl^{-1}, 13.1 ng μl^{-1}, 1,310 pg μl^{-1}, 131 pg μl^{-1}, 13.1 pg μl^{-1}, 1,310 fg μl^{-1}, 131 fg μl^{-1}, 13.1 fg μl^{-1} to 1.31 fg μl^{-1}. The products were then visually resolved in 2.5% agarose stained with ethidium bromide under UV light. This experiment was repeated three times with the same templates freshly diluted each time.

Histology

In preliminary experiments, 'Taggart' seeds infested with *U. virens* were grown in field soil from the Newport Research Station in the greenhouse for two to three weeks. Seedlings were examined under light microscopy for colonization by hyphae of *U. virens* as described by Ikegami (1963). Hypocotyl and SAM tissues from six seedlings were cut, free-hand, into thin longitudinal sections with a scalpel and stained with lactophenol/cotton blue. The tissues were examined after staining with an Olympus CX31 light microscope at 100X and 400X magnifications. Photographs were obtained with an Olympus Evolt E-500 8 Megapixel camera through a microscope adapter lens. Twenty-four 'Taggart' samples including six examined after sectioning from the hypocotyl and SAM region were also tested for *U. virens* using DNA-specific primers in a nested-PCR reaction to confirm the presence of *U. virens*.

Determination of the incidence of *U. virens* DNA in rice seedlings 3, 5 and 7 days after emergence from the soil by nested-PCR amplification

This preliminary experiment was conducted to determine if *U. virens* rDNA could be detected within rice seedling tissues 3, 5 and 7 days after emergence from the soil. Seeds of 'Clearfield-151' and 'Templeton' cultivars that were visibly infested with *U. virens* spores were planted in five cm Jiffy® peat pots containing field soil from the Newport and Pine Tree Research Stations. The seeds were germinated in the greenhouse with an average temperature of 24°C day and 21°C night. Eight seedlings were sampled per replicate at 3, 5 and 7 days after emergence from the soil and tested for *U. virens* rDNA using the described nested-PCR protocol described above. The experiment had four replicates per cultivar in each soil type and the tests were conducted twice. Percent infection was determined for each soil and cultivar combination by dividing the number of samples that tested positive by the total number samples in both repetitions of the test.

Determination of the incidence of *U. virens* DNA in spikelets from rice panicles prior to emergence from the boot by nested-PCR amplification

This test was conducted to determine if *U. virens* rDNA could be detected in samples taken from individual spikelets on panicles prior to emergence from the boot. Seeds infested with *U. virens* chlamydospores were harvested in 2012 from 'Clearfield-162', 'Clearfield-151', 'Francis' and 'Wells' cultivars and planted in five cm square Jiffy® peat pots containing Newport soil inside Tupperware® bins. When the seedlings began tillering, plants from each Jiffy® pot were transplanted into 1 gallon black plastic pots containing Newport soil then submerged under 10 cm of water in large Tupperware® tubs and grown to maturity in the greenhouse. As panicles developed and elongated into the emerging boot, five booted panicles were harvested from each

cultivar by cutting the main stem below the panicle internode. Outer sheath leaves were removed from the boot under aseptic conditions using flamed instruments and the inner sheath leaf was kept intact while in transport to the lab. In the lab, inner sheath leaves were swabbed with a tissue paper saturated in Clorox™ (0.8 %) for one minute and the panicles were then carefully removed using a flamed scalpel and forceps. The panicles were placed into a 10.0 cm Petri dish. Eight spikelets were carefully removed from each panicle using flamed stainless steel forceps and surgical scissors and individually placed into a 1.5 ml polyethylene tube with 100 µl of Epicentre® QuickExtract™ Buffer to extract total DNA. Samples were then placed into water hot baths for DNA extraction as described above. The eight spikelets from five panicles per cultivar were then tested for the presence of *U. virens* rDNA using the specified nested-PCR protocol. A negative reagent and positive (DNA from isolate I-6E) controls were included in each set of reactions.

Statistical analyses

Statistical analyses were performed on the differences among varieties for incidence of *U. virens* rDNA in spikelets obtained from nested-PCR tests in Analysis of Variance (ANOVA) using PROC GLM in SAS version 9.2 (SAS Inc. Cary, NC). The means of treatments were compared following ANOVA using the Fisher's protected least significant difference (FLSD) at (α=0.05).

Results:

Isolates of fungi obtained from seedlings, germlings and spikelets from booted panicles

Colony growth was observed only a few days after plating the transversal sections of seedling, germling and panicle samples on PDA although, none of the fungi observed had the typical morphological or biometric characteristics consistent with *U. virens*. Fungal isolates

were highly prevalent from samples taken from the basal sections of the SAM but no fungi were isolated from the 3rd and 4th samples cut from the base of neither seedlings nor germlings. Only two fungi were isolated from spikelets of 'Clearfield-162' panicles and neither had morphological characteristics consistent with *U. virens*. Of the fungi isolated from seedlings, germlings or panicles, none produced the expected amplicon in a nested-PCR reaction (Tables 3.1, 3.2) with the *U. virens* rDNA-specific primers (Table 3.3). Difficulties associated with isolation attempts from tissue samples were not experienced when isolating *U. virens* directly from chlamydospores of spore balls. Several successful isolations were made from chlamydospores obtained from spore balls originating from infected rice panicles. *Ustilaginoidea virens* may be exceedingly difficult to isolate from plant tissues as none of the fungi isolated from seedlings, germlings or panicles were neither morphologically similar nor did the samples produce the expected nested-PCR products.

Specificity of nested-PCR amplification in detection of *U. virens* isolates from Arkansas

Nested-PCR primers (Table 3.3) were reacted with DNA from 24 *U. virens* isolates in repeated nested-PCR reactions to test for specificity of the protocol to amplify the rDNA of *U. virens* isolates from Arkansas. The resolved nested-PCR products from *U. virens* isolates consistently produced the 232 bp band in 2.5% agarose stained with ethidium bromide (Figures 3.1, 3.2, 3.3, 3.4 and summarized in Table 3.2).

Specificity of nested-PCR to other rice pathogens and pathogens of other crops

Several groups of fungal isolates were used to further determine the specificity of the described nested-PCR protocol using *U. virens*-specific primers. The results show that *Colletotrichum gloeosporioides* f. sp. *aeschynomene CLA 5A, Colletotrichum gloeosporioides* f. sp. *aeschynomene* 313, *Colletotrichum sublineolum* CS 571, *Colletotrichum sublineolum* CS

612, *Magnaporthe oryzae* IC 17, *Magnaporthe oryzae* IB 49, *Rhizoctonia solani* vc 11, *Rhizoctonia oryzae* vc 268, *Rhizoctonia oryzae* vc 27, *Rhizoctonia oryzae* vc 77, *Tilletia barclayana, Trichoderma viride,* produced no positive amplicons in nested-PCR reactions (Figure 3.4 and Table 3.1). Positive amplification products were not obtained from the nested-PCR reactions using DNA template from other unknown unrelated fungal isolates (Table 3.1). Figure 3.4 shows amplicon products from rDNA of 12 *U. virens* isolates used in the specificity tests resolved in 2.5% agarose stained with ethidium bromide.

Specificity of nested-PCR to fungi isolated from crowns of rice germlings, seedlings and spikelets from booted panicles

Nested-PCR tests using the *U. virens* specific primers and template of 17 unknown fungi isolated from germlings, seedlings and spikelets in this test did not produce any amplicon products (Table 3.1). However, germling, seedling and panicle spikelet tissue samples consistently produced the expected amplified 232 bp product, indicating the presence of *U. virens* DNA from the selected tissues (Table 3.10 and 3.11). This data suggests that although DNA with identity to *U. virens* may be present in meristematic tissues and easily identified by PCR, it continues to be exceedingly difficult to isolate *U. virens* hypha from the selected tissues.

Sequence analysis of amplified products obtained from nested-PCR

The specificity of primers US1-5/US3-3 and US2-5/US4-3 was initially determined by a BLAST® search of the concatenated primer sequences using blastn. The results show significant sequence alignment with 59 *Ustilaginoidea virens* and *Villosiclava virens* sequences for both of the forward and reverse primer sets (data not shown) with a Max score of 46.4, E value of 0.016 and Max identity of 100% for 58 out of 59 blastn hits. Two PCR products of 380 bp and 232 bp were consistently observed from primary-PCR and nested-PCR reactions utilizing DNA

extracted from *U. virens* isolates as template in the primary reaction. The amplicon products obtained from nested-PCR amplification of DNA from *U. virens* isolates I-6E, I-7E, I-8E, I-10E, *Ustilaginoidea* isolate I-9E and 10 seedling samples each from 'Clearfield-151' and 'Templeton' cultivars, were sequenced using the Sanger method of DNA sequencing at the University of Arkansas DNA Resources Center. The forward and reverse sequences obtained from the selected five isolates and twenty seedling samples were each subjected to a nucleotide BLAST® search to confirm the sequence specificity. The primer sequences of amplified DNA from isolates and seedlings showed alignment with over 60 *Ustilaginoidea virens and Villosiclava virens* sequences with GenBank Accessions (first 29 shown) (Table 3.4). The BLAST® nucleotide somewhat similar sequences (blastn) query results indicate very strong similarities between isolates I-6E, I-7E, I-8E, I-9E, I-10E and DNA amplified from 'Templeton' and 'Clearfield-151' seedlings tissues, with *Ustilaginoidea virens, Ustilaginoidea* isolate I-9E and *Villosiclava virens* sequences in the database (Tables 3.4, 3.5). Sequences obtained from the nested-PCR product of the rDNA amplified by nested-PCR and blastn search of the DNA sequences obtained from isolates I-6E, I-7E, I-8E, I-9E and I-10E, conferred Maximum identity ranging from 95 to 100%, with sequences of *Ustilaginoidea virens, Ustilaginoidea* isolate I-9E and *Villosiclava virens* in the BLAST® database with E-values ranging from 9e-145 to 4e-86 (Table 3.5). Based on the rDNA amplified by nested-PCR, sequence obtained from sequencing the nested-PCR product and blastn search of the DNA sequences obtained from infected 'Clearfield-151' and 'Templeton' seedlings, conferred Maximum identity ranging from 92 to 100%, with sequences of *Ustilaginoidea virens*, *Ustilaginoidea albicans* and *Villosiclava virens* in the BLAST® database with E-values ranging from 2e-87 to 1e-82 (Table 3.4). This level of sequence similarity of *U. virens* isolates from Arkansas with *U. virens, U. albicans* and *V. virens*

accessions in the BLAST® database is a strong indication that the routinely obtained nested-PCR amplicon of the 232 bp sequence associated with rDNA from *U. virens* is highly similar to that of the isolates *Ustilaginoidea virens*, *Ustilaginoidea albicans*, *Villosiclava virens* and/or plant tissues colonized by *U. virens* in these tests.

The sequences from five chosen *U. virens* isolates I-6E, I-7E, I-8E, I-9E and I-10E (Figure 3.2) and ten amplicons obtained from infected seedling each from 'Templeton' and 'Clearfield-151' (Figure 3.3) were aligned into contiguous base sequences (Tables 3.6 and 3.7) and compared with one another in multiple sequence alignments to determine the identification of the target sequence similarity (Tables 3.8, 3.9) respectively. Alignment of the contiguous complementary forward and reverse sequences obtained from *U. virens* isolates I-6E, I-7E, I-8E, I-9E and I-10E and *Ustilaginoidea* isolate I-9E (Table 3.6) with the Cap3 Sequence Assembly Program and alignment in Clustal Omega and BioEdit revealed no distinguishable variation in the sequences among isolates (Table 3.8). Similarly, a 232 bp nested-PCR product obtained by reacting DNA extracted from ten seedling samples infected with *U. virens* from the field, of each cultivar 'Clearfield-151' and 'Templeton' with primer pair US4-3 and US2-5 was aligned into contiguous sequences (Table 3.7) and were aligned with one another to compare sequence similarity (Table 3.9). The results show that there were only a few base pair differences in the latter sequence portion of the isolate and plant sequences as indicated with the absence of an asterisk (*). Isolates of *U. virens* and isolate I-9E had DNA sequences highly similar to those from the 20 samples of 'Clearfield-151' and 'Templeton' seedlings.

Sensitivity of PCR amplification in *U. virens* detection

The sensitivity of the primary and nested-PCR protocol was determined using serially diluted DNA template from *U. virens* isolate I-6E combined with 25 ng DNA μl^{-1} of template

from rice plant DNA extractions to simulate use for diagnostic purposes. The sensitivity of the primary reaction for detection of *U. virens* DNA was found to amplify 131 ng DNA µl $^{-1}$ however, in the nested-PCR reaction sensitivity ranged from 131 ng DNA µl $^{-1}$ to 13.1 pg DNA µl $^{-1}$ (Figure 3.5). The sensitivity of nested-PCR amplification using Epicentre® PlantAmp™ in the field and greenhouse studies is maintained at the same level by the substituted use of 2X Taq Master Mix from GenScript in the nested-PCR reactions (data not shown).

Histology

In this preliminary experiment, mycelial fragments stained with lactophenol/cotton blue were observed at the base of young leaves, mesial scutellum and in association with the actively growing SAM primordial tissues (axillary bud) in three out of the six 'Taggart' seedlings selected for histological examination (Figure 3.6). Sixteen seedling samples out of 24 from the 'Taggart' seed lot tested positive for *U. virens* DNA in Nested-PCR reactions, including the three of six sectioned and stained seedlings that were observed to contain mycelium in the SAM region. These data suggest hypha from *U. virens* may invade and colonize the actively growing point of the SAM as previously suggested by Ikegami (1963). Colonization of rice by *U. virens* at the germinating seedling stage may be an important step in the disease cycle (Figure 3.7)

Incidence of *U. virens* DNA in rice seedlings 3, 5 and 7 days after emergence from soils in greenhouse experiments

The chlamydospores of *U. virens* have been shown to germinate and develop hyphae that penetrated and invaded the coleoptile and root tissues of seedlings at early stages of development (Ikegami 1962; Ikegami 1963; Schroud and TeBeest 2005). This preliminary experiment was conducted to determine if colonization of rice seedlings by *U. virens* could be detected using nested-PCR at 3, 5 and 7 days after emergence of the seedling from the soil. Total DNA was

extracted from the SAM of seedlings at each stage of development and the described nested-PCR protocol was performed on the samples. The results indicate the presence of DNA associated with *U. virens* in seedlings at 3 days after emergence. The number of seedlings that tested positive for *U. virens* increased between 5 and 7 days when more samples produced the expected amplified product (Table 3.10). The results varied among the different replications for both cultivars, which indicate that either the seedlings were not uniformly colonized or the inoculum source was not evenly distributed within the tissues. The data show an increase in the percentage of seedlings that tested positive for *U. virens* rDNA between 3 and 7 days after emergence. The infection and colonization of seedlings previously described by Ikegami (1963) is an important step in the disease cycle and may now be detectable through nested-PCR (Figure 3.7).

Incidence of *U. virens* DNA in rice panicles prior to emergence from the boot in greenhouse experiments

Panicles from 'Clearfield-151', 'Clearfield-162', 'Francis' and 'Wells' were removed from rice plants prior to emergence of panicles from the boots to determine if *U. virens* rDNA was present in individual spikelets in the absence of external inoculum sources. Eight spikelets, removed from five booted panicles per cultivar, were screened for the presence of *U. virens* rDNA using the described nested-PCR protocol. Results show 75.0%, 57.5%, 40.0% and 27.5% of the 40 spikelets removed from panicles of 'Clearfield-151', 'Clearfield-162', 'Francis' and 'Wells' cultivars tested positive for *U. virens*, respectively (Table 3.11). This data indicates the presence of *U. virens* rDNA within spikelets in the booted panicles of plants grown from infested seed and is an important step in the disease cycle of FS prior to disease development (Figure 3.7).

Discussion

The overall goal of this project was to determine if nested-PCR was a useful tool for examining the colonization of rice seedlings. The specific objectives of this study were: 1) to determine if nested-PCR that amplifies ribosomal DNA (rDNA) is a specific and sensitive detection probe for isolates of *U. virens* and *Ustilaginoidea* isolate I-9E obtained from the field and for fungal DNA extracted from rice tissues, 2) to determine if hyphae can be visualized by sectioning tissues and determine if nested-PCR can be used to verify the presence of *U. virens* rDNA from the tissue samples, 3) to determine the incidence and time of initial colonization of rice seedlings by *U. virens* over time at three, five and seven days after germination and emergence from the soil, using the nested-PCR protocol and 4) to determine if *U. virens* can be detected in spikelets removed from panicles prior to emergence from the boot using the nested-PCR protocol.

Specificity was determined by reaction of the *U. virens* specific primers with 27 unrelated fungal isolates of pathogens of rice, non-pathogens of rice, unknown fungi isolated from rice seedlings, a collection of 24 *U. virens* isolates and one *Ustilaginoidea* isolate I-9E. None of the unrelated fungi produced a positive amplicon, however all 24 of the *U. virens* isolates and *Ustilaginoidea* isolate I-9E isolate produced a positive nested-PCR result. The nested-PCR protocol used for detecting *U. virens* rDNA in this study utilized primers previously designed to be specific to *U. virens* rDNA nucleotide sequences. The results indicate that these primer sets are highly specific for detection of the *Ustilaginoidea virens* isolates and a color morph isolate consistent with descriptions of *Ustilaginoidea albicans* (*Ustilaginoidea* isolate I-9E) present in Arkansas described as a new disease report in chapter two of this thesis. The primer pairs US1-5/US3-3 and US2-5/US4-3 were designed from the ITS1 and ITS2 sequences of *U. virens* (Zhou

et al. 2003) and are shown by multiple sequence alignments in this study to share conserved ITS sequences with *U. virens* isolates obtained from Arkansas rice fields. Sequence data revealed that, in addition to the 24 *U. virens* isolates used in this study, more than 60 other *U. virens, U. albicans and V. virens* isolates also share this conserved ITS sequence in blastn searches. Multiple sequence analysis by Zhou et al. (2003) revealed the nucleotide ITS rDNA sequences of the designed primers were unique to *U. virens* when compared to *Ephelis japonica* and other claviceptaceous fungi. Specificity tests in this study confirm the primary PCR (US1-5/US3-3) and nested-PCR (US2-5/US4-3) primer pairs are specific for *U. virens* rDNA and do not amplify DNA from other non-related pathogens, rice pathogens, unknown and non-pathogenic fungi.

The sensitivity of the primary PCR and nested-PCR protocol was evaluated after preparing a dilution series of the fungal template in the presence of rice template. Sensitivity of PCR in the primary reaction was efficient to 131.0 ng fungal DNA μl^{-1}. However, by utilizing the template from the primary reaction in the nested reaction, the level of sensitivity was enhanced from the primary reaction by 1000X in the nested-PCR reaction to 13.1 pg DNA μl^{-1} of fungal template DNA. Although the sensitivity of the nested-PCR protocol was considered adequate for reliable detection in this test, it was not as sensitive to levels described by Zhou et al. (2003) where nested-PCR was determined to be sensitive down to 23 fg of template DNA.

The methods in this study utilized a rapid DNA extraction method, whereas previous studies utilized phenol extraction methods or DNA centrifuge-technology elution kits that require a great deal of attention to details not suitable for high-through-put applications and which are laborious to use (Willits and Sherwood 1999, Zhou et al. 2003, Ashizawa and Kataoka, 2005). These kits may produce higher quality DNA that may increase the sensitivity of nested-PCR. This study demonstrates that simple extraction methods for DNA extraction from

rice seedlings such as the QuickExtract™ buffer from Epicentre® seem to be adequate for routine diagnosis of fungal DNA from within plant DNA extracts. Based on recommendations of an Epicentre® representative, the addition of polyvinylpyrrolidone to the Failsafe™ PCR Premix C PCR reagents by was done to substitute for the PlantAmp™ protocol. The reliability of the PCR protocol to amplify fungal DNA in QuickExtract™ extractions from plants may depend on the addition of 1% polyvinylpyrrolidone to the final volume in PCR reactions as described with RNA manipulations by Koonjul et al. (1999) and DNA manipulations by Xin et al. (2003).

Histological examinations of six seedlings in this study revealed the presence of observable hypha within the actively growing points of the SAM and associated tissues of the mesial scutellum of three seedlings. These three seedlings and 13 others from the same seed lot (16 totals) of 24 samples also tested positive for *U. virens* rDNA using the specific nested-PCR protocol. This data supports descriptions based on histology by Ikegami (1963). Recently, Ashizawa et al. (2012) described how the fungus invades through a small gap at the apex of a rice spikelet during the booting stage prior to heading. A substantial knowledge gap exists in the lifecycle of FS and the infection process in nature is critical for disease control (Tang et al., 2013). Critical events are undescribed between initial colonization of seedling shoot apical meristematic tissues and colonization of a rice spikelet or inflorescence in the panicles prior to emergence from the boots. This intermediate stage of the life cycle during rice development could be investigated with histolopathological techniques as described by Tang et al. (2013) in a different approach to observe the distribution of the fungus in histopathogenesis studies involving individual germinating seedlings, or plant tillers of colonized plants under *in-vivo* conditions. Based on data obtained in this and other studies, infection of seedlings, germlings and colonization of plants could be examined in future studies. For example, rice seeds or

germlings could be inoculated with a green fluorescent protein (GFP-) labeled strain of *U. virens* (Ashizawa et al., 2012; Zhang et al., 2006; Tanaka et al., 2011) and tissues could be examined at different stages of development with fluorescence microscopy to elucidate where precisely *U. virens* hyphae may reside in rice tissues as they develop over time.

The nested-PCR detection protocol described here was used to determine the incidence of *U. virens* rDNA in association with rice seedlings grown from infested seed by 3, 5 and 7 days after emergence. The results from this study correspond to studies conducted by Ikegami (Ikegami, 1962, 1963) and show when and where the infection of rice seedlings by *U. virens* may take place. The results from the tests by Ikegami indicate that in the 1 to 6 days after inoculating coleoptiles of seedlings with *U. virens* chlamydospores, the cuticle and epidermal wall were invaded and sieve tubes were colonized by infectious hyphae of *U. virens* (Ikegami, 1963). Thus for future study, highly detailed ultrastructural examination of rice seedlings after inoculation of the seed coat or coleoptiles with fresh *U. virens* chlamydospores to elucidate the infection process should also be considered.

The nested-PCR protocol was used to determine the distribution of *U. virens* rDNA among the individual spikelets of panicles from individual plants of four cultivars. Differences in the incidence of *U. virens* rDNA in individual spikelets of individual plants were not statistically different among the cultivars as determined by nested-PCR prior to exsertion of the panicles from the boots (a=0.05). This may have been due to the variance of colonization of spikelets within each panicle among the cultivars. A future study could focus on detecting differences in incidence of colonized spikelets on different portions of the panicles from the main tiller or individual tillers on each plant. Distribution and colonization of the individual spikelets of tillers on individual plants of selected cultivars by *U. virens* may complement differences in

cultivar susceptibility as determined in visual disease assessments of previous field studies (TeBeest and Jecmen, 2011). This work was preceded by Ashizawa and Kataoka (2005) who utilized nested-PCR primers to amplify *U. virens* rDNA that was extracted from whole panicles prior to and after exsertion from the boot. The application of PCR specific tools could be useful in studies to determine the extent of intercellular colonization in resistant or susceptible varieties as done by Willits and Sherwood (1999) or in a future study combined with histology to distinguish the extent of colonization of panicles by *U. virens* in cultivars with differences in susceptibility. Future applications of nested-PCR with real-time quantitative PCR to determine the extent of colonization could be used in conjunction with visual disease assessments in epiphytological studies to help establish the susceptibility of differential rice cultivars to specific isolates. Nested-PCR or real-time-PCR may also be useful as diagnostic tools to determine the extent of rice spikelet and panicle colonization by *U. virens* in efforts to determine the precise role environmental factors may have in relation to pathogenesis during the latency period and disease development.

The histological and molecular data in this study supports previous histological and molecular data in other studies to suggest striking disease cycle similarities of FS (Figure 3.7) to stinking smut in wheat and we are beginning to elucidate the disease cycle of FS based on these similarities. For example, stinking smut or common bunt of wheat (*Triticum aestivum* L.) caused by *Tilletia tritici*, (or *Tilletia laevis*), has been described as a seed-borne pathogen Agrios (2005) and Lipps (2012). To illustrate these similarities, this study and previous studies by Ditmore and TeBeest (2006) indicate that FS is seed-borne and may also be a seed-transmitted disease. The seeds of both rice and wheat may become contaminated when a smutted field is harvested and the grain is processed during handling. This can be seen as a darkened cloud commonly

observed behind a combine while harvesting as the combine wheel disrupts the spore balls or bunt balls (sori) that then release the chlamydospores or teliospores into the entire seed lot and/or field soils. The seeds are then planted the following season, at which time spores of either *U. virens* or *Tilletia* spp. germinate and invade the coleoptile of the developing seedlings prior to emergence from the soil. Histological studies indicate that after conidium germination, infectious hyphae arising from either type of spore invade and colonize the actively growing points of the tiller and may eventually colonize the inflorescences as they develop (Hansen, 1958; and Ikegami, 1963). The fungal hyphae eventually replace the grain on panicles as the individual spikelets are colonized and the fungal tissues then expand and initiate sporulation after flowering upon maturation of the grain (Cashion and Luttrell, 1988; Ashizawa et al., 2011; Ashizawa et al., 2012; and Tang et al., 2012). When the infected grain is harvested by combine, the chlamydospores or teliospores are released from spore balls or sori, respectively, to contaminate the harvested grain to repeat the monocyclic disease cycle (comparison to Lipps, Ohio State University Extension, 2012).

Heald (1921) has shown a direct relationship between the amount of inoculum per 100 grams of seed and the incidence of stinking smut developing in the mature wheat crop. This data suggests that disease levels in other smuts with similar disease cycles may be related to inoculum density on the seed or in the soil. Therefore given the data from these combined studies, future studies on the integrated management of FS could use the nested-PCR protocol to investigate the relationships between the combined effects of varying inoculum densities, resistance of cultivars and/or how environmental factors relate to pathogenesis when assessing the effectiveness of various strategies to mitigate ongoing FS epidemics.

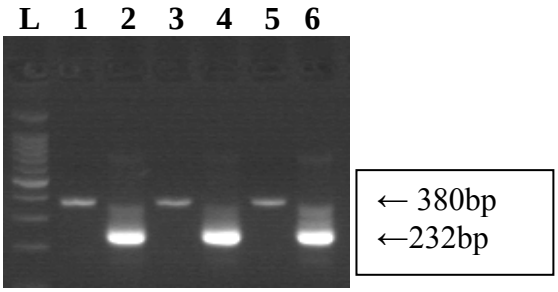

Fig. 3.1. Conventional and nested-PCR amplicon products of rDNA were resolved from *U. virens* isolates I-6E, I-7E and I-8E.

Conventional and Nested PCR amplification of *U. virens* isolates I-6E, I-7E and I-8E was performed using primary (1°), US1-5/US3-3 and nested (2°), US2-5/US3-4 primer sets as designed by Zhou et al. (2003) prior to sequencing. Lanes, L 100 kb ladder; 1, (1°) I-6E; 2, (2°) I-6E; 3, (1°) I-7E; 4 (2°) I-7E; 5, (1°) I-8E; 6, (2°) I-8E.

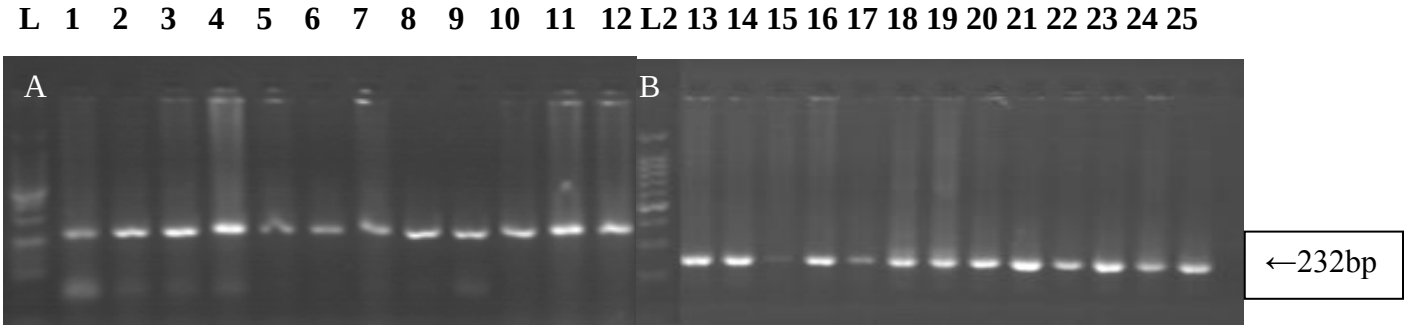

Fig. 3.2. Nested-PCR 232 bp amplicon products were resolved from the rDNA of twenty five *U. virens* isolates.

A. Nested-PCR amplification of *U. virens* isolates (I-1D through 3D) using template from primary (1°) reaction as template in nested (2° reaction shown) prior to sequencing. Lanes, L= 100 kb ladder; 1 (I-1D); 2 (I-2D); 3(I-3D); 4 (I-4D); 5 (I-5D); 6 (I-6E); 7 (I-7E); 8 (I-8E); 9 (I-9E); 10 (I-10E); 11 (3C); 12(3D).

B. Nested-PCR amplification of *U. virens* Isolates 1D through G102A. Lanes, L2=100 kb ladder; 13 (1D); 14(ST#3A); 15(ST#8A); 16 (G101A); 17 (203C); 18 (I-1A); 19 (I-2A); 20 (I-4A); 21 (I-5A); 22 (ST#1A) 23 (4A); 24 (109A); 25 (G102A).

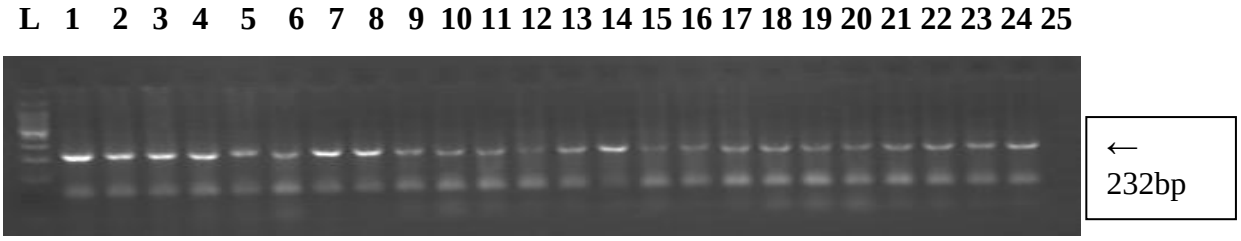

Fig. 3.3. Nested-PCR 232-bp amplified products of were resolved from *U. virens* isolates and rice cultivars 'Templeton' and 'Clearfield-151'.
Lanes, L=100 bp ladder; 1 (I-6E); 2 (I-7E); 3 (I-8E); 4 (I-9E); 5-14 ('Clearfield-151'); 15-24 ('Templeton'); 25 (–) reagent control.

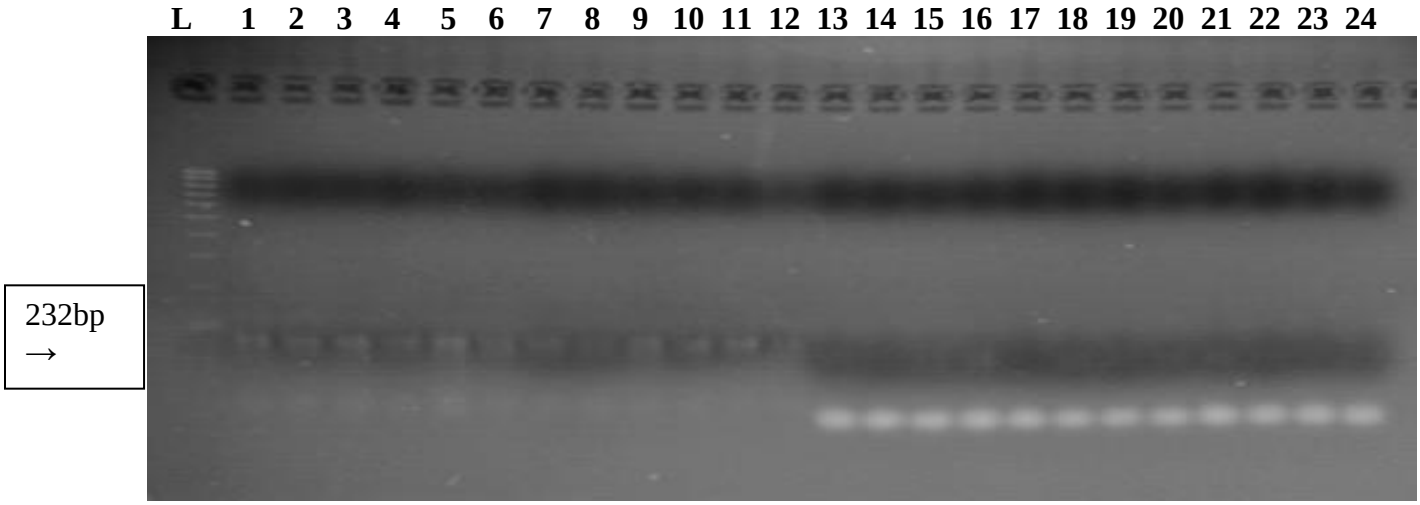

Fig 3.4. Nested-PCR 232-bp amplified products of a specificity test conducted to determine the specific reaction of rDNA from *U. virens* isolates and DNA from other rice pathogens and un-related fungi with the nested-PCR primers.

Resolved nested-PCR amplification products of *U. virens* template from cultures (lanes 1 to 12) and unrelated fungi (lanes 13 to 24) using Zhou et al. (2003) (US1-5/US3-3) primary and (US2-5/US4-3) nested primers reacted with Failsafe™ PlantAmp™ PCR master mix. Lanes: L, 100kb Ladder; 1, I-1D; 2, I-2D; 3, I-3D; 4, I-4D; 5, I-5D; 6, I-6E; 7, I-7E; 8, I-8E; 9, I-9E; 10, I-10E; 11, ST#3A; 12, ST#4A; 13, *Colletotrichum gloeosporioides* f. sp. *aeschynomene* CLA 5A; 14, *Colletotrichum gloeosporioides* f. sp. *aeschynomene* 313; 15, *Colletotrichum sublineolum* CS 571; 16, *Colletotrichum sublineolum* CS 612; 17, M*agnaporthe oryzae* IC 17; 18, *Magnaporthe oryzae* IB 49; 19, *Rhizoctonia solani* vc 11; 20, *Rhizoctonia oryzae* vc 268; 21, *Rhizoctonia oryzae* vc 27; 22, *Rhizoctonia oryzae* vc77; 23, *Tilletia barclayana; 24, Trichoderma viride.*

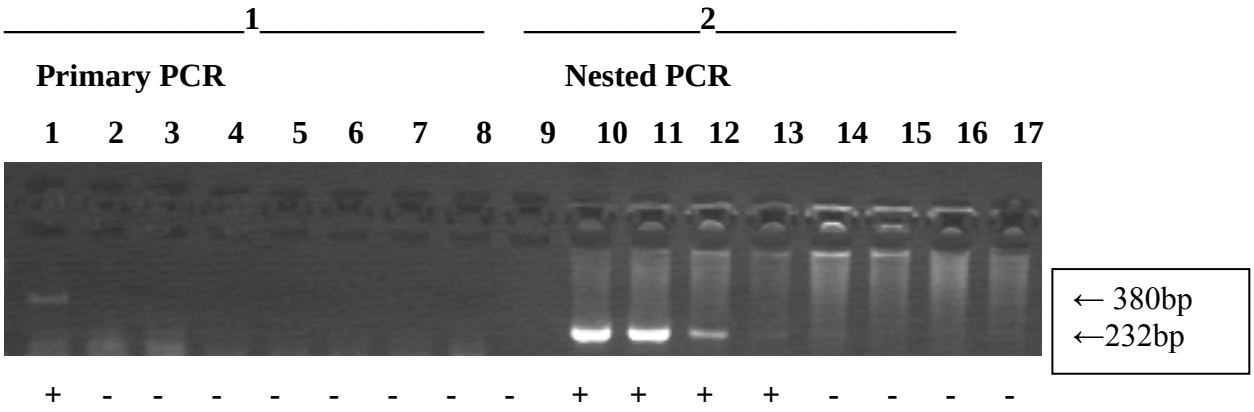

Fig. 3.5. Results of a sensitivity test conducted to identify the limits of the described primary and nested-PCR protocol for detection of *U. virens* rDNA (I-6E from 'Clearfield-151') in the presence of 25 ng rice total DNA.

Nested-PCR amplification of *U. virens* template (I-6E) obtained from serial dilutions was combined with 25 ng/µl rice DNA reacted with Failsafe™ PlantAmp™ PCR master mix from Epicentre® and primers designed by Zhou et al. (2003). The primary round (1°), utilized primers US1-5 and US3-3 and yielded the 380 bp amplicon. The nested-PCR reactions (2°) utilized template from the primary reactions and were reacted using nested-PCR primer pair US2-5 and US4-3 yielded the 232 bp amplicon.

Lanes: L, 100kb Ladder; 1 and 10, 131ng μl^{-1}; lanes 2 and 10, 13.1 ng μl^{-1}; lanes 3 and 11, 1,310 pg μl^{-1}; lanes 4 and 12, 131 pg μl^{-1}; lanes 5 and 13, 13.1 pg μl^{-1}; lanes 6 and 14, 1,310 fg μl^{-1}; lanes 7 and 15, 131 fg μl^{-1}; lanes 8 and 16, 13.1. fg μl^{-1}, 9 and 17 negative control (rice DNA template + PCR reagents).

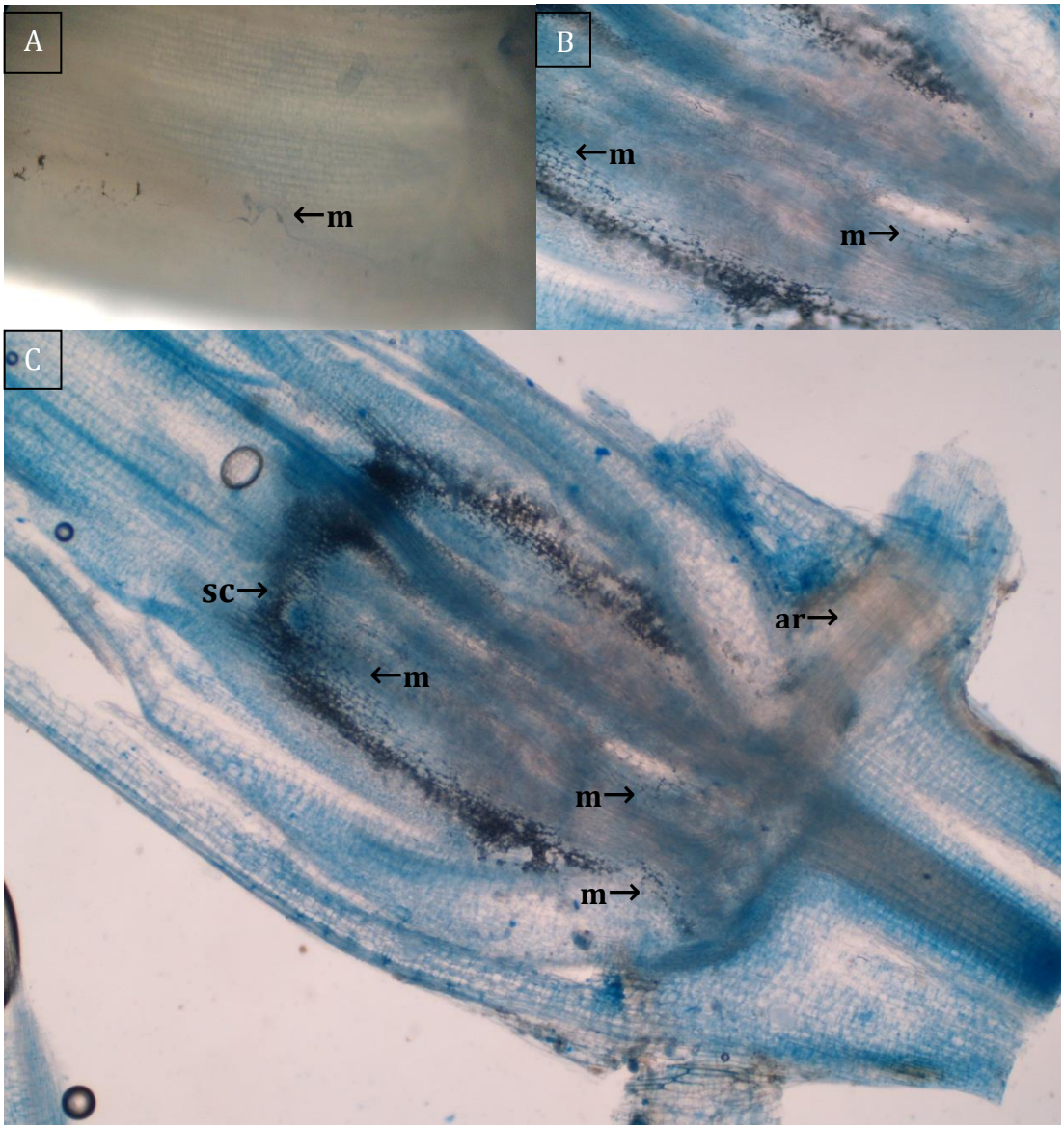

Figure 3.6. Longitudinal shoot apical meristem (SAM) sections obtained from 'Taggart' seedlings 21 days after emergence.
Tissues were sectioned longitudinally, stained dark blue with lactophenol /cotton blue in and observed under 100 X (**C.**) and under 400 X (A. B.**)** magnifications.
A. Mycelium (m) associated with basal parts of young leaves and the axillary bud.
B. Mycelium (m) in association with the scutellum and shoot apical meristem
C. Mycelium (m) in the mesial scutellum (sc) as adventitious roots (ar) develops.

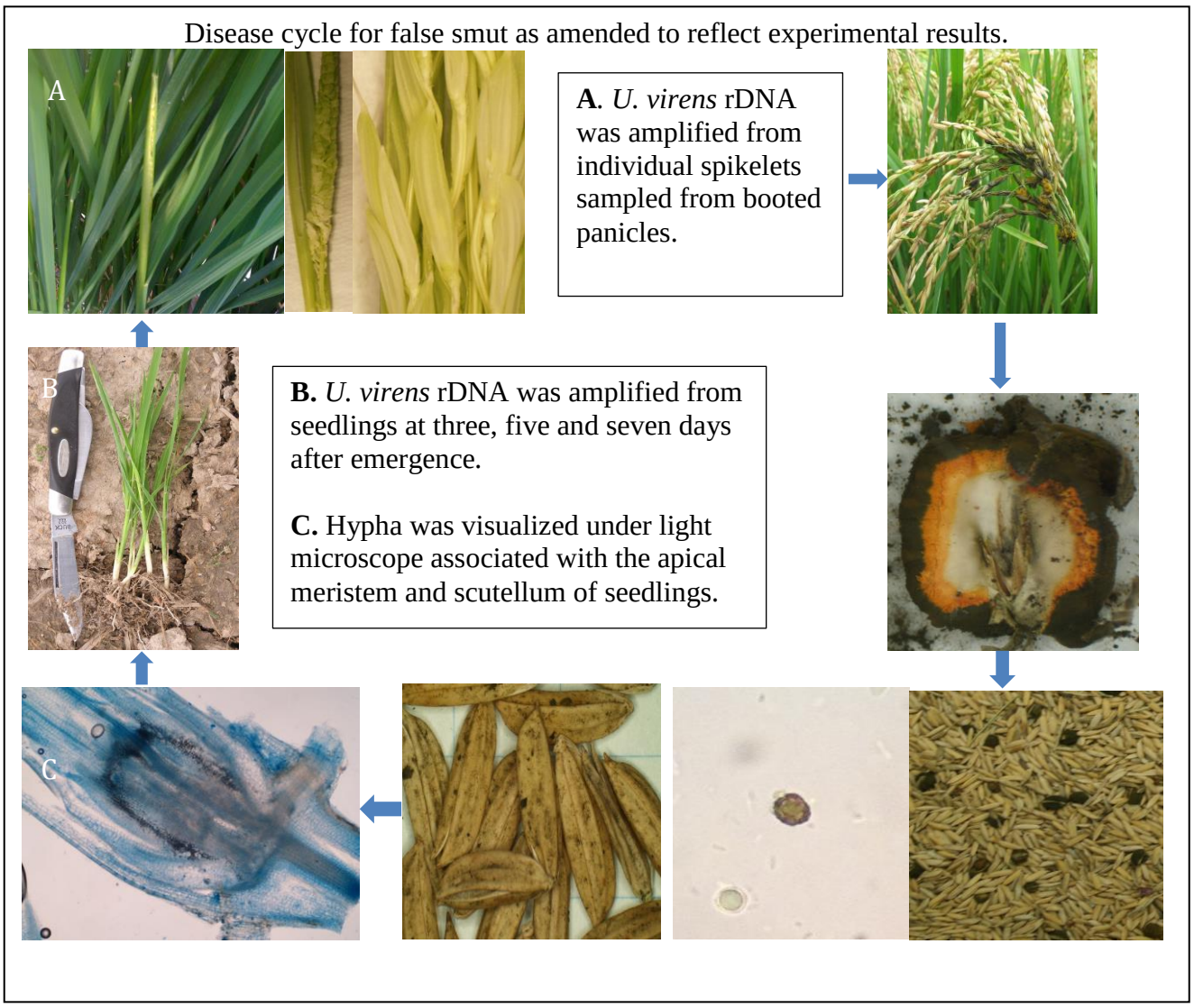

Figure 3.7. Disease cycle for false smut in rice as amended to reflect the experimental results from this study.
A. Seed infested with *U. virens* chlamydospores were grown to near maturity in the greenhouse. Spikelets from booted panicles were removed and screened for *U. virens* rDNA using the nested-PCR protocol (Table 3.11).
B. Seedlings grown from infested seed were sampled at 3, 5 and 7 days after emergence from the soil. Nested-PCR amplification of the samples produced the expected 232 bp rDNA amplicon (Table 3.10).
C. Seedlings grown from seed infested with *U. virens* chlamydospores were sectioned and stained with lactophenol/cotton blue. Hypha was observed in association with tissues of the apical meristematic and mesial scutellum as shown in Figure 3.1.

Table 3.1. Results of specificity tests to determine the reaction of US1-5/US3-3 and US2-5/US4-3 primer sets with pathogens of rice, pathogens of plants other than rice, unknown species of fungi isolated from rice seedlings and *U. virens* isolate I-6E. [A]

Pathogens isolated from rice	**Source**	**PCR result**
Magnaporthe oryzae IC 17	Dr. James Correll	–
Magnaporthe oryzae IB 49	Dr. James Correll	–
Rhizoctonia oryzae vc 268	Dr. James Correll	–
Rhizoctonia oryzae vc 27	Dr. James Correll	–
Rhizoctonia oryzae vc77	Dr. James Correll	–
Tilletia barclayana	'Templeton'	–
Plant pathogens isolated from plants other than rice [B]		
Colletotrichum gloeosporioides f. sp. *aeschynomene* CLA 5A	Dr. D. O. TeBeest	–
Colletotrichum gloeosporioides f. sp. *aeschynomene* 313	Dr. D. O. TeBeest	–
Colletotrichum sublineolum CS 571	Dr. D. O. TeBeest	–
Colletotrichum sublineolum CS 612	Dr. D. O. TeBeest	–
Rhizoctonia solani vc 11	Dr. James Correll	–
Trichoderma viride	Dr. Chuck Kenerley	–
Fungi isolated from crowns of rice seedlings, germlings and booted panicles [C]		
UNF 1 Francis	'Francis'	–
UNF 2 Francis	'Francis'	–
UNF 3 Francis	'Francis'	–
UNF 4 Cheniere	'Cheniere'	–
UNF 5 Cheniere	'Cheniere'	–
UNF 6 Cheniere	'Cheniere'	–
UNF 7 Roy J	'Roy J'	–
UNF 8 Taggart	'Taggart'	–
UNF 9 Taggart	'Taggart'	–
UNF 10 Roy J	'Roy J'	–
UNF 11 Clearfield-151	'Clearfield-151'	–
UNF 12 Clearfiele-151	'Clearfield-151'	–
UNF 13 Clearfield-151	'Clearfield-151'	–
UNF 15 Templeton	'Templeton'	–
UNF 16 Clearfield-162	'Clearfield-162'	–
UNF 17 Clearfield-162	'Clearfield-162'	–
I-6E Clearfield-151	(Positive control)	+ [D]

[A] Isolates were tested for specificity using Zhou et al. (2003) nested-PCR primers selective for *U. virens*. Nested-PCR was performed on the extracted DNA from the isolates and repeated twice for these tests; bands were only observed for the target fungus (I-6E).

[B] *Tilletia barclayana* was collected and isolated from an infected rice panicle of 'Templeton' found at the Pine Tree Research Station, near Colt, Arkansas.

[C] Seedlings of 'Francis', 'Cheniere', 'Roy J', 'Taggart', 'Clearfield-151' and 'Templeton' were grown and fungal isolations were made from the tissues, 2 and 4 mm above the mesocotyl/hypocotyl interface. Ninety two spikelets from eight 'Clearfield-162' panicles were plated on PDA and fungal isolations were made from the resulting mycelial growth.

[D] DNA from Isolate I-6E served as positive control for these tests.

Table 3.2. *Ustilaginoidea virens* isolates used to test the specific reaction of nucleotide primers (US1-5/US3-3, US2-5/US4-3) with rDNA of *U. virens* in nested-PCR reactions. [A]

***U. virens* Isolates** [B]	**Origin** [C]	**PCR result** [D]
I-1D (Cybonnet)	Poinsett	+
I-2D (Cybonnet	Arkansas	+
I-3D (CL-161)	Poinsett	+
I-4D (Templeton)	Arkansas	+
I-5D (Rice Tec CL XL 729)	White Co. RRVT	+
I-6E (Clearfield-151)	Poinsett	+
I-7E (Francis)	Arkansas	+
I-8E (Templeton)	Arkansas	+
I-9E (Clearfield-151)	Arkansas	+
I-10E (Taggart)	Arkansas	+
3C (cultivar unknown)	St. Francis	+
1D (cultivar unknown)	St. Francis	+
3D (cultivar unknown)	Arkansas	+
ST#3A (Clearfield-161)	Poinsett	+
ST#8A (Rice Tec CL XL 729)	White	+
G101A (Cybonnet)	Arkansas	+
203C (Cybonnet)	Poinsett	+
I-1A (Cybonnet)	Poinsett	+
I-2A (Cybonnet)	Arkansas	+
I-4A (Templeton)	Arkansas	+
I-5A (Rice Tec Cl XL 729)	White Co. RRVT	+
ST#1A (Clearfield-151)	Poinsett	+
4A (cultivar unknown)	St. Francis	+
109A (Cybonnet)	Poinsett	+
G102A (Cybonnet)	Arkansas	+

[A] Isolates morphologically consistent with *U. virens* and *U. albicans* descriptions were tested for specificity using Zhou et al. (2003) nested-PCR primers selective for *U. virens*. Two tests were run with the same results, bands were only observed for the target fungus.

[B] Isolates were obtained for this test from the cryogenic collection.

[C] The isolates used in this study were originally obtained in the specified county in Arkansas.

[D] Results from the resolved products of nested-PCR reactions performed on template DNA extracted from each isolate. The extractions and nested-PCR reactions were repeated twice in this experiment.

Table 3.3. Reported and synthesized nucleotide sequences of polymerase chain reaction primary and nested primers designed by Zhou et al. (2003) used in this study.

Primer name	Sequence (5'-3')	Primer length	Amplicon length (bp)
Primary 1° US1-5	CCGGAGGATACAACCAAAAAAACTCT	26	380[A1]
Primary 1° US3-3	GCTCCAAGTGCGAGGATAACTGAAT	25	380 [B1]
Nested 2° US2-5	CAATGCATGTCTGAGTGGATTTTTG	25	232 [A2]
Nested 2° US4-3	CCAACACCAAGCGCAAGACAGA	22	232 [B2]

[A1, A2] Forward primer sequences
[B1, B2] Reverse primer sequences

Table 3.4. The results of the BLAST® search of the forward primer sequence obtained from DNA isolated from 'Templeton' and 'Clearfield-151' rice cultivars reacted with the nested-PCR selective primers US2-5/US4-3. [A]

BLAST® hit [B]	E Value [C]	Max Ident [D]	Accession [E]
Uncultured fungus clone F2-015	1e-82	100%	JX984707.1
Villosiclava virens isolate HNHS-1	1e-82	100%	JX427552.1
Villosiclava virens isolate UV2	1e-82	100%	JQ828996.1
Uncultured eukaryote clone N305T	1e-82	100%	GU941397.1
Ustilaginoidea albicans strain BH	1e-82	100%	HM439355.1
Uncultured fungus clone L042882-122-062-E11	1e-82	100%	GQ999197.1
Ustilaginoidea virens isolate SX0201	1e-82	100%	FJ824824.1
Ustilaginoidea virens isolate ZJ04	1e-82	100%	FJ848738.1
Ustilaginoidea virens isolate ZJ0202	1e-82	100%	FJ848737.1
Ustilaginoidea virens isolate ZJ0201	1e-82	100%	FJ848736.1
Ustilaginoidea virens isolate ZJ01	1e-82	100%	FJ848735.1
Ustilaginoidea virens isolate YN02	1e-82	100%	FJ848734.1
Ustilaginoidea virens isolate YN01	1e-82	100%	FJ848733.1
Ustilaginoidea virens isolate SY06	1e-82	100%	FJ848732.1
Ustilaginoidea virens isolate SY05	1e-82	100%	FJ848731.1
Ustilaginoidea virens isolate SY04	1e-82	100%	FJ848730.1
Ustilaginoidea virens isolate SY03	1e-82	100%	FJ848729.1
Ustilaginoidea virens isolate SY02	1e-82	100%	FJ848728.1
Ustilaginoidea virens isolate SY01	1e-82	100%	FJ848727.1
Ustilaginoidea virens isolate SX0208	1e-82	100%	FJ848726.1
Ustilaginoidea virens isolate SX0207	1e-82	100%	FJ848725.1
Ustilaginoidea virens isolate SX0206	1e-82	100%	FJ848724.1
Ustilaginoidea virens isolate SX0205	1e-82	100%	FJ848723.1
Ustilaginoidea virens isolate SX0204	1e-82	100%	FJ848722.1
Ustilaginoidea virens isolate SX0203	1e-82	100%	FJ848721.1
Ustilaginoidea virens isolate SX0202	1e-82	100%	FJ848720.1
Ustilaginoidea virens isolate SX0104	1e-82	100%	FJ848719.1
Ustilaginoidea virens isolate SX0103	1e-82	100%	FJ848718.1
Ustilaginoidea virens isolate SX0102	1e-82	100%	FJ848717.1

[A] The same 29+ accessions were shown to have homologous sequences to all of the sequences obtained from positive nested-PCR amplicon by amplifying rDNA associated with *U. virens* and *U. albicans* (I-9E) isolates; and 'Clearfield-151' and 'Templeton' seedlings.
[B] Top BLAST® hit from a search in the nucleotide sequence database.
[C] E-value is the number of distinct alignments expected to occur by chance in a database search. The value is the sorting metric and ordinarily gives the same sorting order as the Max score. The E-value of values of e>0.05 indicates the score is significant.
[D] Max Score is the highest alignment (bit score) between the query sequence and the database sequence segment.
[E] Accession number or named identifier of the sequence and isolate name present in the database.

Table 3.5. The most frequently observed BLAST® hits associated with *Ustilaginoidea virens* (teleomorph *Villosiclava virens*) and *Ustilaginoidea* spp. obtained by blastn query search of the sequences obtained from reacting DNA from *U. virens* isolates and DNA from plants in a nested-PCR reaction with specific primers US2-5 and US4-3. [A]

Identifier [B]		Source [C]	BLAST® hit [D]	E value [E]	Max Ident [F]	Accession No.[G]
I-6E	F	CL-151	*V. virens* UV2	6e-90	95%	JQ828996.1
I-6E	R	CL-151	*V. virens*HNHS-1	4e-110	100%	JX427552.1
I-7E	F	Francis	*V. virens* HNHS-1	2e-133	97%	JX427552.1
I-7E	R	Francis	*V. virens* HNHS-1	4e-110	100%	JX427552.1
I-8E	F	Templeton	*V. virens* HNHS-1	9e-145	95%	JX427552.1
I-8E	R	Templeton	*V. virens* HNHS-1	3e-110	95%	JX427552.1
I-9E	F	CL-151	*V. virens* HNHS-1	3e-137	97%	JX427552.1
I-9E	R	CL-151	*V. virens* HNHS-1	1e-108	100%	JX427552.1
I-10E	F	Taggart	*V. virens* HNHS-1	4e-86	98%	JX427552.1
I-10E	R	Taggart	*V. virens* HNHS-1	3e-105	98%	JX427552.1
CL1	F	CL-151	*V. virens* HNHS-1	6e-84	96%	JX427552.1
CL1	R	CL-151	*V. virens* HNHS-1	2e-87	92%	JX427552.1
TM1	F	Templeton	*V. virens* HNHS-1	1e-82	100%	JX427552.1
TM1	R	Templeton	*V. virens* HNHS-1	7e-86	99%	JX427552.1

[A] *U. virens* isolates (I-6E, I-7E, I-8E and I-10E), *Ustilaginoidea* isolate (I-9E) and plant samples (CL1, TM1) indicate high sequence similarities with *Ustilaginoidea virens* (Teleomorph *Villosiclava virens*) in the BLAST® database.

[B] *U. virens* isolates I-6E, I-7E, I-8E, I-10E and *U. albicans* Isolate I-9E, are from the cryogenic collection and plant samples TM1 ('Templeton'), CL1 ('Clearfield-151') were obtained from the fields at the Newport Research Station. F and R indicate the forward (F) and reverse (R) sequences obtained from sequencing using the forward (US2-5) and reverse (US4-3) primers in the reaction (Zhou et al. 2003).

[C] Plant sources from which the isolate or plant samples were originally obtained 'Francis', 'Templeton', 'Taggart' and 'CL-151' ('Clearfield-151').

[D] Top BLAST® hit from a search in the nucleotide sequence database.

[E] E-value is the number of distinct alignments expected to occur by chance in a database search. The value is the sorting metric and ordinarily gives the same sorting order as the Max score. The E-value of values of $e<0.05$, indicates the score is significant.

[F] Max Score is the highest alignment (bit score) between the query sequence and the database sequence segment.

[G] Accession number or named identifier of the sequence and isolate name present in the GenBank database.

Table 3.6. Contiguous sequences obtained from sequencing nested-PCR amplicon obtained from rDNA of *U. virens* isolates. [A]

Isolate name [B]	Contiguous sequences [C]
>I-6E	
	5’- TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG GTGTTGGAAGACGCTCGGCAGGCATGCCCGCCGTAGGGCGCGCAATGTGCGTTAAA GATTCGATGAATCCTGAATTCTGCAGTTCAATTACTTATCGCATTTCGCTGCGTTCTT CATCGATCCGAACCAAGAGTCCGTTTTGAAAGTTTTATTCTTTTGATTTGCAAAATCC CTCAACTGCATTGAA-3’
>I-7E	
	5’- CATTCAGTTATCTTCGAACTTGGAGCAGTCCTGAGGATACAACCAAAGAAAGTCCAT TCAGTTATCCTCGCACTTGGAGCACTCCGGAGGATACAACCAAAAAAACTCTTGTGT TTTCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACA ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGT ATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCT TGGTGTTGGAAATCGGCCCTGCCCGCCAGCCCGGGCGGGCCGCCCCCGAAATGAAT CGGCAGTCTCGTCGCATCCTCCTCTGCTTAATAATTCTGTTATCCTCGCACTTGGAGC ATGCCGAACCAGAATCCGTTTTTGAAAATTTGATTCTTTTGATTGCAAATCACTCTAC AGCATTGAA-3’
>I-8E	
	5’- ATTCAGTTATCCTCGCACTTGGAGCACTTCGGAGGATACAACCAAAAAAACTCTTGT GTTTTCTAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCA ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCC AGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGC GCTTGGTGTTGGAGATCGGCCCTGCCCGCCAGCCCGGGCGGGCCGCCCCCGAAATG AATCGGCGGTCTCGTCGCAGCCTCCTCTGCGTAGTAATTCAGTTATCCTCGCACTTGG AGCACATGCGAAACCACGACTCCGTTGTTTAAAGTTTTGATTCTTTTGATTTGCAAA ATTCCCTCGACTGCATTGAA-3’
>I-9E	
	5’CCATTCAGTTATCCTCGCACTTGGAGCACTCCGGAGGATACAACCAAAAAAACTC TTGTGTTTCTAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTT CAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAG TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCG CCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTT GCCTTGGTGTTGGAAATCGGCCCTGCCCGCCAGCCCGGGCGGGCCGCCCCCGAAAT GAATCGGCGGTCTCGTCGCACCCTCCTCTGCGTAATAATTCAATTATCCTCCCACTTG

GACCACCCGGGAAAATACAGCTCATTAAGAATGTTTGATTCTTTTGATTTGCAAAAC
ACACTCAGACTGCATTGAA-3’

>I-10E

5’-
GAGAAATCTTCTAATCTTATTCAGTTATCCTTGCACTTGGAACACTCCGGAGGATAC
AACCAAAAAAAAATCTTGTGCCTTCAATGCATGTCTGAGTGGATTTTTGCAAATCAAA
ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAG
CGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC
GCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACC
CTCAAGCTCTGTCTTGCCTTTGGTGTTGGAAGACGCTCGAACAGGCATGCCCGCCAT
AGGGGGCCATGTGCGTTCAAGATTCGATGAATCCTGAATTCTGCAGTTACATTACTT
ATCGCATTTCCTGCGTTCTTCATCGATGCCGAACCAAGAGATCCGTTGTTGAAAGTTT
TGATTCATTTTGATTTGCAAAAATCCACTCAACATGCATTGAA-3’

[A] Sequences were obtained from nested-PCR amplicon of each isolate through the University of Arkansas DNA Resources Center using the Sanger method of DNA sequencing with chain terminating fluorescently labeled dd-NTP’s. Template for sequencing was obtained from PCR amplification using nested primers US2-5 and US3-4 and high molecular weight DNA extracted from these isolates as nested-PCR template.

[B] Isolates of *U. virens* selected from the collection for sequencing are representative of ongoing work on their respective cultivars.

[C] Sequences were clipped and aligned in the CAP3 Sequence Assembly Program from forward and reverse complementary sequences of each isolate amplicon product.

Table 3.7. Contiguous sequences obtained from sequencing nested-PCR amplified products obtained from DNA extracted from seedlings grown from 'Templeton' and 'Clearfield-151' seed infested with *U. virens* chlamydospores. [A]

Seedling sample origin [B]	**Contiguous sequences** [C]
>'Clearfield-151' 1 5'- TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCCTTGG TGTTGGA-3'	
>'Clearfield-151' 2 5'- TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCCTTGG TGTTGGA-3'	
>'Clearfield-151' 3 5'- TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCCTTGG TGTTGGA-3'	
>'Clearfield-151' 4 5'- TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG GTGTTGGA-3'	
>'Clearfield-151' 5 5'- TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG GTGTTGGA-3'	
>'Clearfield-151' 6 5'- TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG GTGTTGGA-3'	

>’Clearfield-151’ 7
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGA
>’Clearfield-151’ 8
5’-
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGA-3’
>’Clearfield-151’ 9
5’-
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGA-3’
>’Clearfield-151’ 10
5’-
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGA-3’
>’Templeton’ 1
5’-
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGA-3’
>’Templeton’ 2
5’-
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGA-3’
>’Templeton’ 3
5’-
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT

TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGA-3’
>’Templeton’ 4
5’-
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGA-3’
>’Templeton’ 5
5’-
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGA-3’
>’Templeton’ 6
5’-
TAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAACG
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATT
CTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTGG
TGTTGGA-3’
>’Templeton’ 7
5’-
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGA-3’
>’Templeton’ 8
5’-
CTAATGTTTTTTTTTTAAGACTCACGAAAGCTTTTTTGGTTGTATCCTCCGGAGTCCT
CCAAGTGCGAGGATAACTTTTCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAAT
GAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCG
AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCT
CAAGCTCTGTCTTGCGCTTGGTGTTGGA-3’
>’Templeton’ 9
5’-
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGA-3’
>’Templeton’ 10

5’-
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
GTGTTGGAA-3’

[A] Sequences were obtained from nested-PCR amplicon of each seedling from each cultivar through the University of Arkansas DNA Resources Center using the Sanger method of DNA sequencing with chain terminating fluorescently labeled dd-NTP’s.

[B] Template for sequencing was obtained from PCR amplification using Nested primers US2-5 and US3-4 and high molecular weight DNA extracted from ‘Clearfield-151’ and ‘Templeton’ seedlings grown from seed infested with *U. virens* chlamydospores as nested-PCR template.

[C] Sequences were clipped and aligned in the CAP3 Sequence Assembly Program from forward and reverse complementary sequences of each seedling amplicon product. The multiple sequences were aligned using Clustal Omega, a multiple sequence alignment program to search for similarity among the plant sequences.

Table 3.8. Multiple sequence alignment of sequences from nested PCR amplied products of *Ustilaginoidea virens* isolates I-6E, I-7E, I-8E, I-9E and I-10E. [A]

```
I-6E
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
I-7E
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
I-8E
CTAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
I-9E
CTAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
I-10E
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
 ************************************************************************
I-6E
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
I-7E
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
I-8E
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
I-9E
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
I-10E
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
************************************************************************
I-6E
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
I-7E
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
I-8E
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
I-9E
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
I-10E
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
************************************************************************
I-6E
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
I-7E
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
I-8E
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
I-9E
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCCTTGG
I-10E
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCCTTTG
************************************************************    *  *
```

```
I-6E    GTGTTGGA
I-7E    GTGTTGGA
I-8E    GTGTTGGA
I-9E    TGTTGGA-
I-10E   GTGTTGGA
          *  *   .
```

[A] Multiple sequence analysis are of contiguous sequences obtained from nested-PCR reaction of 2° primers US4-3 and US2-5 with isolates I-6E, I-7E, I-8E, I-9E and I-10E. Sequences indicate a high level of conservation among the samples by the * below each base pairing in a column.

Table 3.9. Multiple sequence alignment of sequences from nested PCR amplified products of 'Clearfield 151' and 'Templeton' cultivars from Newport and Pine Tree Research Stations, AR 2012. [A]

'Clearfield-151'1
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Clearfield-151'2
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Clearfield-151'3
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Clearfield-151'4
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Clearfield-151'5
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Clearfield-151'6
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Clearfield-151'7
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Clearfield-151'8
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Clearfield-151'9
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Clearfield-151'10
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Templeton'1
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Templeton'2
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Templeton'3
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Templeton'4
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Templeton'5
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Templeton'6
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Templeton'7
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Templeton'8
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Templeton'9
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
'Templeton'10
TCAATGCATGTCTGAGTGGATTTTTGCAAATCAAAATGAATCAAAACTTTCAACAAC
****

'Clearfield-151'1
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG

‘Clearfield-151’2
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Clearfield-151’3
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Clearfield-151’4
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Clearfield-151’5
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Clearfield-151’6
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Clearfield-151’7
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Clearfield-151’8
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Clearfield-151’9
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Clearfield-151’10
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Templeton’1
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Templeton’2
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Templeton’3
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Templeton’4
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Templeton’5
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Templeton’6
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Templeton’7
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Templeton’8
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Templeton’9
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
‘Templeton’10
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
****

‘Clearfield-151’1
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Clearfield-151’2
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Clearfield-151’3
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT

‘Clearfield-151’4
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Clearfield-151’5
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Clearfield-151’6
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Clearfield-151’7
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Clearfield-151’8
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Clearfield-151’9
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Clearfield-151’10
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Templeton’1
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Templeton’2
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Templeton’3
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Templeton’4
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Templeton’5
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Templeton’6
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Templeton’7
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Templeton’8
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Templeton’9
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
‘Templeton’10
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
****
‘Clearfield-151’1
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCCTTGG
‘Clearfield-151’2
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCCTTGG
‘Clearfield-151’3
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCCTTGG
‘Clearfield-151’4
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Clearfield-151’5
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG

‘Clearfield-151’6
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Clearfield-151’7
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Clearfield-151’8
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Clearfield-151’9
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Clearfield-151’10
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Templeton’1
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Templeton’2
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Templeton’3
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Templeton’4
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Templeton’5
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Templeton’6
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Templeton’7
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Templeton’8
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Templeton’9
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
‘Templeton’10
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGTCTTGCGCTTG
** * *

[A] Multiple sequence analysis are of contiguous sequences of PCR amplicon of 2° primers US4-3 and US2-5 obtained from 10 ‘Clearfield-151’ (ContigCL1, ContigCL2, ContigCL3, ContigCL4, ContigCL5, ContigCL6, ContigCL7, ContigCL8, ContigCL9, ContigCL10) and from ‘Templeton’ (ContigTM1, ContigTM2, ContigTM3, ContigTM4, ContigTM5, ContigTM6, ContigTM7, ContigTM8, ContigTM9, ContigTM10) seedlings. Sequences indicate a high level of conservation among the samples by the * below each base pairing in a column.

Table 3.10. The percentage of rice seedling tissue samples at 3, 5 and 7 days after emergence (DAE) that reacted with the nested-PCR specific reaction for *U. virens* rDNA. [A]

Soil [B]	Cultivar [C]	DAE [D]		
		3	5	7
Newport	CL 151	47	84	66
	Templeton	28	88	75
Pine Tree	CL 151	31	97	72
	Templeton	43	75	78

[A] All data represents the rounded average percentage of seedlings infected with *U. virens* as detected by nested PCR. The data is the average of four replications of eight seedlings sampled in two repetitions of the experiment.
[B] Soil from each of the Newport and Pine Tree Research Stations was used in this study.
[C] Cultivars 'Clearfield-151' and 'Templeton' were infested with 6.7 and 8.5 spore balls kg^{-1} seed respectively.
[D] Values indicate the number of infected seedlings 3, 5 and 7 days after emergence (DAE) from two experiments with four reps per treatment in each experiment.

Table 3.11. Table of the number of positive nested-PCR reactions of DNA extracted from the spikelets of each panicle with primers specific for *U. virens* rDNA.

Cultivar [A]	Panicle [B]	+ Spikelets [C]	Means [D]	Percent infection [E]
'Clearfield-151'	A	3		
	B	7		
	C	8		
	D	8		
	E	4	6	75 A [F]
'Clearfield-162'	A	0		
	B	8		
	C	7		
	D	8		
	E	0	4.6	57.5 A
'Francis'	A	2		
	B	1		
	C	4		
	D	5		
	E	4	3.2	40 A
'Wells'	A	0		
	B	0		
	C	7		
	D	3		
	E	1	2.2	27.5 A
P value=				0.23

[A] Plants were grown from seeds harvested from infected panicles of four rice cultivars and panicles were removed from plants prior to emergence from the boots from 'Clearfield-151' on April 4, 2013, 'Clearfield-162' on 1-2 2013 and 'Francis' and 'Wells' on March 25, 2013.
[B] A total of five panicles (A through E) was sampled from each cultivar, at three to seven days prior to panicle exsertion from the boot.
[C] Eight spikelets were selected at random from each of the five panicles collected. The number of + Spikelets is the number of spikelets that tested for the presence of *U. virens* rDNA using the described PCR diagnostic protocol.
[D] The mean number represents the number of spikelets out of 40 samples from each cultivar, which tested positive for *U. virens* rDNA.
[E] The percent infection of spikelets out of 40 sampled from each cultivar.
[F] Percent infected values followed by the same letter are not significantly different (a=0.05).

IV. Sensitivity of *Ustilaginoidea virens* isolates from Arkansas to selected triazole and strobilurin fungicides and the efficacy of fungicide seed treatments to suppress rice false smut in the field.

Abstract

False smut is an emerging and economically destructive disease of rice in Arkansas. Foliar fungicides are currently being used to suppress false smut (FS) but are reportedly inadequate to fully suppress this disease. In this work, the sensitivity of three geographically diverse mono-chlamydospore isolates of the FS causal pathogen, *Ustilaginoidea virens*, from Arkansas was tested by determining the inhibitory fungicide concentration at which 50% of maximal radial mycelial growth (IC_{50}) was measured. Fungicide sensitivity laboratory test results showed that four of the five analytical grade fungicides tested were highly fungistatic to at concentrations ranging from 0.11 to 1.1 mg l^{-1}. However, the results from Rancona® 3.8 FS (40.7% ipconazole) tests were highly variable and the fungicide did not appear to inhibit growth at concentrations ranging from 1.1 to 11 mg l^{-1}. The narrow sensitivity range of *U. virens* isolates to the technical and analytical grade triazoles, strobilurins and a combination of triazole/strobilurin fungicides suggests similarities in the inhibitory activity of the fungicides on mycelial growth of *U. virens* isolates.

Field tests were conducted at two locations with two cultivars to determine if seed treatments reduced FS incidence and to determine if the nested-PCR could quantify the effect of fungicide seed treatments on FS incidence by measuring the incidence of *U. virens* rDNA in seedlings. The efficacy of seed treatments was measured by PCR analysis of seedlings and by counting the number of spore balls per m^2 twice per plot. The nested PCR protocol appeared to measure and identifies significant differences in the incidence of *U. virens* rDNA in plants after

treatment with selected fungicides compared to untreated controls. Results from field studies, however, showed that none of the fungicide seed treatments significantly reduced FS incidence in 2011. Further, several treatments appeared to reduce FS disease levels in 2012 on both rice cultivars at both locations, although the results were not statistically significant at $a = 0.05$. This is the first report in the USA of fungicide sensitivity with a selection of *U. virens* isolates that have not been previously exposed to this selection of fungicides. The data from this study may provide information about the effect on growth that these few fungicides may have on *U. virens* isolates. Our findings also may promote further investigation of the use of fungicides as foliar treatments in conjunction with, or alternatively as seed treatments for suppression of FS in the field.

Introduction

Rice FS, caused by *Ustilaginoidea virens* (Cooke) Takahashi, is one of the prevalent and economically destructive fungal diseases that cause localized epidemics in commercial rice fields in Arkansas in recent years. Since consumers demand high quality rice domestically and abroad, control with foliar fungicides is recommended as a management strategy in Arkansas for suppression of FS in fields with a history of disease and fertilized heavily with nitrogen (Cartwright and Lee, 2009). Crop loss is common where fungicide application timing is incorrect or an inadequate concentration of the material is applied (Cartwright, 2000). Applications of fungicides at the booting stage are believed to target two prevention levels as described by Starfield et al. (2008). The first level aims to halt or slow progression of a disease or its sequelae (phytopathological condition resulting from a pre-existing disease) after inception. The second level attempts to reverse, arrest, or delay the progression of disease after the initial onset of signs or symptoms. Further if the disease develops from inoculum in soil or on seed as

shown in Figure 1.5, then information regarding *in-vitro* sensitivity of *U. virens* to these fungicides in the USA or their use as a primary preventative seed treatment would be important, but it has not been reported. Strobilurin fungicides are currently used alone as foliar treatments or in combination with triazole fungicides, for managing FS (Cartwright and Lee, 2011).

The triazole or demethylation-inhibiting (DMI) fungicides inhibit the C-4α-demethylation of lanosterol in sterol biosynthesis of ergosterol in fungi (FRAC group 3) (Brent, 1995). The strobilurin fungicides are the quinone outside-inhibiting fungicides (FRAC group 11) (Bartlett et al., 2002) that target and bind to the quinone outside (Qo) site of the mitochondrial cytochrome bc1 complex III and limits electron transport in cellular respiration and ATP production (Gisi et al., 2002). Resistance or insensitivity to these two classes of fungicides has been reported for several groups of plant pathogens. It is evident from numerous studies that a wide range of fungi and oomycetes are sensitive to DMI and QoI materials, but it is unclear if isolates of *U. virens* from Arkansas are tolerant or sensitive to these fungicides. Based on the seed-borne and transmission hypothesis, fungicides applied to seeds may prevent or reduce colonization of seedlings in the field and be measurable as differences in colonization with nested-PCR or by conducting visual disease assessments among fungicide-treated and non-treated plants.

The objectives of this study are to determine if limited suppression of FS in rice fields is due to a lack of sensitivity by *U. virens* to these two classes of fungicides *in-vitro.* The second objective of this study was to determine if FS can be effectively controlled with fungicides applied as seed treatments. For this research, the activity of DMI and QoI fungicides to inhibit *in-vitro* growth of *U. virens* was measured by determining the inhibitory fungicide concentration by determining the accumulated diameter growth (ADG) of mycelium in PDA Petri plates

amended with varying concentrations of technical and analytical grade fungicides in laboratory tests. The efficacy of the technical grade fungicides as seed treatments was determined in greenhouse and field studies on two cultivars by using a specific nested-PCR protocol to measure the incidence of *U. virens* rDNA in seedlings and by visually quantifying the incidence of FS at maturity.

Materials and Methods

Isolates of *U. virens* used in this study

The three isolates of *U. virens* (I-6E, I-7E and I-8E) used in this study were originally obtained from infected plants growing in fields from eastern Arkansas (Arkansas, White and Poinsett Counties) with no history of fungicide application for FS control (Table 4.1). The isolates were stored in a cryogenic freezer at -80°C in 30% glycerol in the Department of Plant Pathology, University of Arkansas, in Fayetteville, AR. The isolates were grown and maintained on 10.0 cm Petri dishes containing acidified (pH 6.2) PDA at 26°C prior to use in this study.

Mycelial growth inhibition assays

Isolates were tested for sensitivity to the following technical grade fungicides: Stratego® 250 EC (11.8% propiconazole and 11.8 % trifloxystrobin); Tilt® (41.8% propiconazole); Quilt® SE (11.8% propiconazole and 7.0 % azoxystrobin); Raxil® MD, (0.48% tebuconazole and 0.63% metalaxl); Rancona® 3.8 FS (40.7% ipconazole) and copper II carbonate (51.4% Cu) (Table 3.2). Technical grade fungicides Quilt® SE, Raxil® MD, Rancona® 3.8 FS, Stratego® 250 EC and Tilt® were diluted to stock solutions of 1.989 g l^{-1}, 11.60 g l^{-1}, 4.50 g l^{-1}, 2.495 g l^{-1} and 4.318 g l^{-1} respectively in 30% glycerol. Copper II carbonate was suspended in a stock solution of 156.0 g l^{-1} in 30% glycerol. Isolates were additionally tested for sensitivity to the following analytical grade fungicide standards (PESTANAL® Fluka Analytical, Sigma-Aldrich® and St. Louis, MO):

azoxystrobin, trifloxystrobin, propiconazole and tebuconazole. Analytical standards were diluted to stock solutions of 10.0 g l^{-1} in acetone to test final fungicide concentrations in PDA above 0.22 mg l^{-1}; and to 0.1 g l^{-1} in acetone to final fungicide concentrations in PDA below 0.22 mg l^{-1} in order to maximize the solubility of the active ingredients. Final acetone concentrations were at or below 1.1 µl l^{-1} in the fungicide-amended-PDA media. Fungicide and copper II carbonate stock solutions were added in calculated amounts to dilute them to the desired final mg l^{-1} or g l^{-1} concentration to 300 ml of autoclaved PDA. Approximately 30 ml of fungicide-amended-PDA was poured into 10.0 cm Petri dishes when cooled to 55°C in a heated water bath. Technical grade fungicide concentrations in PDA ranged from 0.11, 0.6, 1.1, 2.2, 4.4, 6.6, 8.8 and 11 mg l^{-1} and copper II carbonate was tested from 2.2, 4.4, 6.6, 8.8 and 1.1 g l^{-1} (Table 4.2). Analytical grade fungicide concentrations in PDA ranged from 0.011, 0.055, 0.11, 0.22, 0.44, 0.55, 0.88, to 1.1 mg l^{-1}. Fungicide controls consisted of approximately 30 ml of un-amended-PDA poured into 10.0 cm Petri dishes. These controls were used to determine the maximal mycelial growth of each isolate over the test period. Seven mm diameter plugs were transferred from actively growing cultures of each isolate cultured on un-amended-PDA, to un-amended-PDA control dishes and the fungicide-amended-PDA dishes. Each fungicide sensitivity test replicate consisted of three replicated PDA dishes per isolate for each fungicide and concentration. The fungicide-amended and un-amended PDA dishes with agar plugs of the isolates transferred onto them were incubated in the dark at 26°C for 21 days and the experiments were repeated twice.

The diameter of mycelium growth was measured along two axes from each dish for each replicated fungicide, isolate and fungicide concentration combination at the end of the 21 day incubation period. The accumulated diameter growth was determined for each treatment by averaging the two axial measurements. The adjusted diameter growth (ADG) was then

calculated for each growth measurement by subtracting the initial seven mm diameter of the agar plug from each averaged measurement. Growth on fungicide-amended-PDA was compared to maximal mycelial ADG for each isolate on un-amended-PDA to calculate the IC_{50} values; to determine the inhibitive fungicide concentration at which 50% of maximal growth was observed.

Seed and seed treatments

Two rice cultivars, 'Templeton' (TMPL) and 'Clearfield-151' (Cl-151) were used in greenhouse and in field tests conducted at the Newport and Pine Tree Research Stations, Arkansas in 2011 and 2012 to study the effects of fungicide seed treatments on disease incidence. Plants used to obtain seed for use in these tests had not been previously treated with fungicides and were obtained from variety tests previously conducted at the Pine Tree Research Station. Random seed samples of up to 257 g were obtained from each treated seed lot and the number of FS spore balls per gram was counted then extrapolated to determine the number of spore balls per kg in the seed lot for each variety and treatment. The number of spore balls per kg 'Clearfield-151' and 'Templeton' seed was 48.2 and 36.7 spore balls per kg in 2011 and 5.5 and 7.1 spore balls per kg in 2012, respectively.

Technical grade fungicides used for seed treatments in these tests included; 7.0 % azoxystrobin + 11.7% propiconazole (Quilt®); 40.7% ipconazole (Rancona® 3.8S); 0.48% tebuconazole + 0.66% metalaxyl (Raxil® MD EC); 11.8% propiconazole + 11.8% trifloxystrobin (Stratego®); 41.8% propiconazole (Tilt®); and 51.4% Cu (Copper II carbonate) (Table 4.3). Two fungicide rate treatments low (1X) and high (2X), were used in these experiments, based on the lowest and highest per acre rates the labels recommended for use as foliar applications of the same chemicals on other crops (Table 4.3). All seed treated with fungicides were also treated with Pro-Ized® (a red colorant, 0.283 g kg-1 seed, Bayer Cropscience) to indicate the presence of

fungicides on the seed. Fungicides were applied to the seed with a generic bottle sprayer in a laboratory fume hood by spraying each fungicide reconstituted at the specified concentrations to a final volume of 30 ml per kg of seed. The treated seed was agitated for one minute in 11 x 32 x 24 cm polycarbonate containers and allowed to dry for 48 hours in a fume hood after treatment. After treatment with the fungicides, 105 g of seed for each plot were packaged in #7, 8.9 X 16.5 cm (3.5" X 6.5") manila coin envelopes (OfficeMax Naperville, IL). Seed was transported to the Pine Tree and Newport Research Stations for planting in the appropriated fields at both locations.

Soil characterization

Seventeen and 21 soil samples approximately 0.45 kg each were taken at two m intervals diagonally across the fields at the beginning of the growing season in 2011 and at the beginning and end of the season in 2012 from the Newport and Pine Tree Research Stations, respectively. For comparison with samples at the beginning of the season, samples were obtained from fungicide seed treatment tests at the end of the growing season at Newport in early September, 2012. Soil samples were submitted for analysis by the University of Arkansas, Agriculture Diagnostic Soil Testing Laboratories in Fayetteville, Arkansas. The concentrations of micronutrients (P, K, Ca, Mg, S, Zn, Fe, Mn, Cu and B) in soil were determined using the Mehlich 3 reagent extraction method and analyzed by Spectrol Arcos ICP (Mehlich, 1984). Soil pH was determined using a modified soil water ratio of 1:2 instead of 1:1 as described (Donahue, 1983). Sample supernatants were allowed to equilibrate on a stirring plate for 15 minutes to acquire a reading. Electrical Conductivity (EC) was determined according to the Donahue (1983) method, with modification of equilibration time from 30 minutes to 15 minutes. Soil textural analysis was conducted at the Agricultural Soil Testing Laboratory on nine randomly

sampled soil samples obtained from fields at the Newport Research Station in October, 2012 and nine samples from fields at the Pine Tree Research Station in May, 2013 using the hydrometer method (Bouyoucos, 1962).

Efficacy of fungicide seed treatments as determined by nested-PCR in the greenhouse

Greenhouse experiments were conducted to determine if differences in colonization among rice seedlings after treatment of seed with triazole, strobilurin, acylalanine and general inhibitor activity group fungicides was measured by the nested-PCR protocol. Two inch Jiffy® peat pots were prepared using field soil from the Pine Tree and Newport Research Stations in the Rosen Alternative Pest Control Center, Greenhouse 1.4, at the University of Arkansas in Fayetteville. Seed of 'Clearfield-151' and 'Templeton' cultivars were treated with fungicides as described above. Sixteen rice seeds per treatment were planted in four Jiffy® peat pots per test with four replications of the two cultivars. The Jiffy® pots were arranged in non-perforated solid nursery flats and were sub-irrigated to avoid splashing soil and erosion. The greenhouse conditions in this study averaged 18°C at night, to 24°C in the day and light was on a 12-hour photoperiod.

Eight seedlings were chosen randomly from each replicate (pot) at the four leaf stage, 21 days after emergence (DAE) and placed in 8.9 X 16.5 cm ($3^{1/2}$ X $6^{1/2}$") #7 paper coin envelopes (OfficeMax, Naperville, IL), returned to the lab on ice and stored 4°C until DNA was extracted. The seedlings were washed under tap water to remove soil from the roots and blotted dry. The roots were cut from the seedling and the coleoptile was removed to expose the main stem. One cm sections were cut from the SAM section of each seedling into five pieces. Total DNA was extracted using the Epicentre® QuickExtraction™ protocol and samples were placed at 4°C until *U. virens* rDNA was amplified using the described Epicentre® protocol and primer sets described

in the previous chapter. All manipulations were done in a containment hood and nested-PCR was performed in a dedicated closed-door room apart from the main laboratory. In each set of reactions, negative reagent and positive (DNA from I-6E) controls were included. PCR products were resolved as described and the number of visualized amplified products was tabulated for each replicated treatment by location and cultivar for each year. The percent incidence of infected seedlings was determined from the number of seedlings that produced an amplified PCR product divided by the total number of seedlings (32) that were sampled for each treatment, location and cultivar combination from all replications. The experimental design followed a random complete block design with four replications and was repeated twice with field soils from the Newport and Pine Tree Research Stations in 2011 and 2012.

Efficacy of fungicide seed treatments as determined by nested-PCR in field experiments

Field plots were prepared and established at the Newport and Pine Tree Research Stations, on June 1 and May 17, in 2011 and on May 25 and May 19, in 2012 respectively, to determine if differences in colonization among rice seedlings after treatment of seed with fungicides could be detected using the nested-PCR protocol. Seed from 'Clearfield-151' and 'Templeton' cultivars were treated with fungicides as described above. In each plot, 105 g of seed was planted in nine rows 16.9 cm apart. Individual plots measured 1.52 X 7.62 m with 0.31 m buffer strips between each plot and 1.0 m between replications. Eight plants comprised a sample from each plot and plants were harvested at random from each plot across the four replications after emergence of the seedlings on June 30 and July 1, in 2011 and June 18 and June 19, in 2012. The plant samples were gently removed from the soil with a knife blade, placed in 13 X 7.9 X 27 cm ($5^{1/8}$ X $3^{1/8}$ X $10^{5/8}$") brown paper lunch bags (Best Choice®) then transported in ice chests to the lab where they were stored at 4°C until processing.

Seedlings were washed under cold running water to remove soil then blotted dry on paper towels. DNA was extracted from SAM tissues and the incidence of *U. virens* rDNA in seedlings sampled from all plots was determined as described above for greenhouse studies using the nested-PCR protocol as described. In each set of reactions, a negative reagent and a positive (DNA from I-6E) control were included. The percent incidence of infected seedlings was assessed as the number of seedlings that produced an amplified PCR product divided by the total number of seedlings (32) that were sampled for each treatment, location and cultivar combination from all replications. The experimental design was a randomized complete block design with four replications and the experiments were conducted twice over the course of two years in different fields at the Newport and Pine Tree Research Stations in 2011 and 2012.

Field experiments

Field plots were established with fungicide-treated and non-treated seed at the Newport and Pine Tree Research Stations. Seed was planted on May 17 and June 1, in 2011; on May 19 and May 25, in 2012 at the Pine Tree and Newport Research Stations, respectively. The plots were prepared after conventional disking and using a landplane (Tiger-Mate, DMI Tillage machine) to level the soil. Individual plot sizes in the field measured 1.52 m x 7.62 m with 0.31 m (5' X 25' X 1') buffer strip alleys between plots and one m between replicated plots. A HEGE 180 grain drill was used to plant approximately 105 g of seed per plot in a randomized complete block design (RCBD) with 4 replications of each treatment; each plot consisting of nine rows 16.9 cm (6.65") apart. Alleys were cut with Roundup® (Monsanto Technology LLC.) with a Danville Express No Drift Chemical Applicator 15.14 l (4 gallon) Hand Model DEX 30.

Alleys and plots were treated with several herbicides, with a larger HYPRO roller pump (pto driven) model 6500C herbicide sprayer with a 189.27 l (50 gallon) tank and 17 nozzles

spaced at 53.34 cm (21") intervals on the boom (Tee Jet 8002 VS flat fan spray nozzles). In 2011, 2.84 ml (3 qt.) of A-1 Superwham™, 473.18 ml (2 pt.) of (A-1) Prowl™ and 181.44 g (0.4 lb.) of A-1 Facet™ was applied at planting and 22.18 ml (0.75 oz.) A-1 Permit™ was applied one week after planting per acre. In 2012, 226.8 g (0.5 lb.) of A-1 Facet™ and 59.15 ml (2 oz.) of A-1 Clincher™ were applied at planting and 3.79 l (4 qt) A-1 Stam™ and 14.17 g (0.5 oz.) of A-1 of Permit™ was applied 12 days after seedling emergence per acre. Field plots were fertilized prior to flooding with 200 units of N (197.22 kg (434.8 lbs) A-1 of 46% Urea) at Pine Tree Research Station and 150 units of N (147.92 kg (326.1 lbs) A-1 of 46% Urea) at Newport in 2011 per acre. One hundred fifty units of N were applied in 2012 (147.92 kg (326.1 lbs) A-1 of 46% Urea) at both locations per acre. Each field experiment followed a randomized complete block design with four replications of each treatment and the experiments were repeated twice over the course of two years at the two locations.

False smut incidence was determined in field tests at the growth stage R9 (Counce et al. 2000) when all grains had a brown hull by counting the number of infected rice panicles per m^2 twice per plot. The two measurements were then averaged for each plot before the data was submitted for statistical analysis. Disease assessments were done over the period of a few days at maximal disease development in early October and mid-September in 2011 and in mid-September at both locations in 2012.

Yield of fungicide seed treatment test plots

Two weeks after disease assessments, the experimental plots were harvested with a Kubota RX145 (G1 SkyRoad) combine and harvest weights were determined with an electronic balance (OHAUS I-10). Moisture levels were determined at harvest with the aid of a DICKEY-john GAC® 2100 (Grain Analysis Computer). Weight and moisture content were converted

from lbs. to kg and adjusted to 12% final moisture to normalize the yield data prior to statistical analysis.

Stand count measurements

Stand counts were taken on June 6, 2012 to study the phytotoxic effects of fungicide seed treatments and effects on established plant density from the observed stand reduction in the field tests observed in 2011. Several plots in the 2011 tests had poor stand in some fungicide seed treatment plots resulting from suspected phytotoxicity. The number of seedlings per linear meter in each field plot at Newport and Pine Tree Research Stations in 2012 was counted in two randomly selected rows in each plot 21 days after emergence. The two measurements were averaged to represent the stand counts for each plot. Data are presented as the average of four replications of each fungicide or non-fungicide treatment for each cultivar and location.

Statistical analyses

The experimental design for the *in-vitro* fungicide sensitivity assay followed a modified randomized complete block design with a replicated treatment structure and replicated subplots (isolates) with controls. Each block in each test contained a collection of nine dishes prepared for each concentration of the test. The analysis was performed as described by Reynolds et al. (1997) and May-De Mio et al. (2011) with slight modification. Inhibition of colony diameter growth by fungicides was determined for each isolate by measuring colony growth in two dimensions and adjusted diameter growth (ADG) was calculated from the accumulated growth measurement by subtracting the initial seven mm diameter mycelial plug. The ADG was averaged for each isolate grown on fungicide-amended-PDA and non-amended-PDA. The ADG was regressed using logarithmic trend-lines against the mg l^{-1} fungicide or g l^{-1} copper II carbonate concentrations by utilizing Microsoft Excel to calculate IC_{50} values for each isolate.

The IC_{50} values were calculated as the fungicide concentration that reduced growth by 50% from maximal growth on un-amended-PDA for each isolate using the following equation ½(Y) = ln(X) + (Z). The equations were solved algebraically for X (inhibitory fungicide concentration at 50% maximal growth (IC_{50})). The X represents the fungicide concentrations, Y represents ADG and Z is the constant for the log-fit trend-line. The mean ADG was analyzed using PROC GLM in SAS. The means for each test, fungicide, fungicide concentration and isolate combination were compared using Fisher's least significant difference (FLSD) test at α=0.05.

Field data were analyzed by statistical analyses of the disease incidence (the average number of infected heads per m^2 in four replicates of each treatment) and the yield data in each plot by the appropriate functions of PROC GLM in SAS version 9.2 (SAS Inc. Cary, NC). Data for each location were tested separately. The means of cultivars and treatment combinations were compared using the Fisher's protected least significant difference (FLSD) function at (α=0.05).

Results

Sensitivity of *U. virens* isolates to technical grade fungicides

The sensitivity of the three isolates to each of the technical grade fungicides was determined using logarithmic linear models to fit the ADG for isolates I-6E, I-7E and I-8E regressed against each respective fungicide concentration. The mean IC_{50} calculated values for isolates grown on Copper II carbonate-amended-PDA ranged from 0.47 to 0.62 g l^{-1} (Figure 4.1). Mean IC_{50} calculated values for the technical grade fungicides ranged from; 0.52 to 0.72mg l^{-1} for Quilt® SE; 0.91 to 1.25 mg l^{-1} for Raxil® MD (EC); 0.72 to 0.82 mg l^{-1} for Stratego® 250 EC; and 0.54 to 0.74 mg l^{-1} for Tilt® (Figures 4.2, 4.3, 4.4 and 4.5).

The distribution of isolate ADG regressed against the six technical grade fungicide concentrations showed a log-normal distribution for the fungicides tested except for Rancona®

3.8 FS (Figure 4.6). The isolates did not appear to be sensitive to Rancona® 3.8 FS at the concentrations tested. The ADG inhibition values of isolates grown on Rancona® 3.8 FS-amended-PDA were highly variable although it appears some inhibition was found within the fungicide concentration range for isolate I-8E (Figure 4.6). Isolates I-6E, I-7E and I-8E were not strongly inhibited by the active ingredient, ipconazole, found in Rancona® 3.8 FS, at 0.11 to 11 mg l^{-1} concentrations. As a result, the isolate sensitivity to Rancona® 3.8 FS data was not submitted for further statistical analysis and the IC_{50} was not calculated.

The mean ADG (in mm) of *U. virens* isolates for Quilt® SE, Raxil® MD (EC), Stratego® 250 EC and Tilt® fungicides were significantly different (P=0.0001) from one another, with the higher concentrations significantly reducing growth *in-vitro*. Fungicides, isolates and fungicide concentrations, were significantly different between tests at a=0.05. However, fungicide by isolate combinations were not significantly different (P=0.6047) (Table 4.4). Based on the significant differences identified among fungicides and isolates in the analysis of variance, the IC_{50} values were calculated separately for each fungicide, isolate and fungicide concentration combination based on the replicate means for accumulated diameter growth (Table 4.4).

Sensitivity of *U. virens* isolates to analytical grade fungicides

The mean IC_{50} values were determined from the ADG values for isolates I-6E, I-7E and I-8E to the analytical grade fungicide concentrations using logarithmic linear models to fit isolate ADG (in mm) regressed against the fungicide concentrations. The mean IC_{50} values for the analytical grade fungicides ranged from; 0.13 to 0.19 mg l^{-1} for azoxystrobin; 0.03 to 0.09 mg l^{-1} for propiconazole; 0.09 to 0.16 mg l^{-1} for tebuconazol; and 0.05 to 0.08 mg l^{-1} for trifloxystrobin (Figures 4.7, 4.8, 4.9 and 4.10). The distribution of isolate ADG values regressed against the five concentrations of each of the analytical fungicides followed a log-normal distribution for the

fungicides tested with exception of metalaxyl. The isolates I-6E, I-7E and I-8E did not appear to be sensitive to metalaxyl since inhibition of isolates grown on metalaxyl-amended-PDA was highly variable and no inhibition was found within the fungicide concentration range for any of the isolates (Figure 4.11). Isolates were not strongly inhibited by metalaxyl concentrations ranging from 0.011 to 1.1 mg l^{-1} concentrations and as a result, IC_{50} values were not calculated.

The mean ADG values (in mm) of all three isolates on PDA amended with azoxystrobin, propiconazole, tebuconazol, trifloxystrobin analytical grade fungicides were significantly different (P=0.0001) from one another. Fungicides, isolates and fungicide concentrations, were significantly different between tests at a=0.05 and a three way interaction between fungicide, isolate and fungicide concentrations existed in this test (P<0.0001) (Table 4.4). Based on the significant differences identified among fungicides and isolates in the analysis of variance, the IC_{50} values were calculated separately for each fungicide, isolate and fungicide concentration combination based on the replicate means for accumulated diameter growth (Table 4.4). These results indicated that azoxystrobin, propiconazole, trifloxystrobin, the active ingredients in fungicides currently used to suppress FS in the field, effectively inhibited the growth of *U. virens* isolates *in-vitro*. Mycelial growth inhibition by tebuconazole, a fungicide that is not currently recommended to control FS, indicates that there may be other fungicides within the DMI, QoI, or other activity groups that may effectively inhibit mycelial growth of *U. virens*.

Soil characteristics

Soil was sampled from each field plot location to compare soil properties between locations. Fields at the Newport Research Station in Jackson County, feature a Crowley silt loam that borders on fine sandy loam. Soil at Newport is typically Crowley that is a fine, smectitic, hyperthermic typic Albaqualf, strong to moderately acid soil (pH 5.2 to 6.5) (Johnson

et al., 1999). Textural analysis conducted on eight soil samples from Newport indicated 3.1% sand, 79.2% silt and 17.7% clay (Table 4.5). Soil analysis revealed several significant differences between soils at the Newport and Pine Tree Research Stations for a number of variables.

The fields at the Pine Tree Research Station near Colt, AR in St. Francis County feature a Calloway silt loam. The soil is poorly drained with a clay fragipan about 42.5 cm (16.7") below the surface. The subsoil is mottled gray, yellow and brown silty clay loam that ranges from moderately acid to alkaline (pH 6.2 to 7.9) (Gray and Catlett, 1966). Textural analysis conducted on eight soil samples from the Pine Tree Station indicated 1.6% sand, 75% silt and 23.7% clay. Significant differences were observed for pH, P, Ca, Mg, Na, Mn and B values between locations (a=0.05) (Table 4.6).

Efficacy of fungicide seed treatments in greenhouse experiments as determined by nested-PCR

Fungicide treated seeds were grown in the greenhouse to determine the effects of fungicides on the incidence of *U. virens* rDNA in seedlings by using nested-PCR. Statistical analysis of variance (ANOVA) tests of the incidence of seedlings which gave a positive amplicon in 2012 and 2011 indicated that there were no significant difference between years (a= 0.05), so results were combined from both years as repetitions of the test (Table 4.7).

The incidence of infected seedlings varied significantly by the soil used from each location (a=0.05) in greenhouse tests. Cultivars had significantly different *U. virens* rDNA incidence levels in Newport soils (a=0.05) and Pine Tree (a=0.05) soils. A significantly higher percentage of 'Clearfield-151' seedlings tested positive for *U. virens* rDNA compared to 'Templeton' in soil from Newport and Pine Tree. There was a significantly lower incidence of

U. virens in 'Templeton' seedlings of Quilt SE 2X and Stratego 250 EC 2X treatments in Pine Tree Soil compared to untreated control. None of the fungicide treated 'Clearfield-151' seedlings tested for the incidence of *U. virens* rDNA, had significantly lower incidences than the untreated control in either soil at a=0.05.

Efficacy of fungicide seed treatments in field experiments as determined by nested-PCR

Seeds were treated with fungicides and grown in field tests at the Newport and Pine Tree Research Stations to determine the effects of fungicides on the incidence of *U. virens* colonization in seedlings by using nested-PCR. Results of the field experiments conducted in 2011 indicate that the cultivars had significantly different incidence of seedlings infected by *U. virens* at Newport (a=0.05) and Pine Tree (a=0.05). A significantly higher percentage of 'Clearfield-151' seedlings were infected by *U. virens* than 'Templeton' in Newport and Pine Tree soil suggesting a varietal difference. The incidence of infected seedlings also varied significantly by location (a=0.05). The data shows that the effects of fungicide treatments on both cultivars are significantly different at Newport (a=0.05) and Pine Tree (a=0.05). The percentage of 'Cearfield-151' seedlings that tested positive for *U. virens* in Raxil MD 2X and Tilt 2X treatments at Newport and Stratego 250 EC 2X at Pine Tree, were significantly higher than untreated controls. The percentage of 'Templeton' seedlings that tested positive for *U. virens* in Stratego 250 EC 2X at Newport and $CuCO_3$ 2X, Rancona 3.8 FS 2X, Raxil MD 2X and Stratego 250 EC 2X treatments were found to have *U. virens* incidence significantly higher than the untreated control. One treatment, Quilt SE 2X on 'Templeton' at Pine Tree was found to have *U. virens* incidence significantly lower than untreated control (a=0.05) (Table 4.8).

Results of the field experiments at Newport with 'Clearfield-151' cultivar conducted in 2012 indicated no significant difference in seedling infection levels (a=0.05) (Table 4.9). No

seedlings tested positive for *U. virens* in untreated controls from test plots of 'Clearfield-151' at Pine Tree or of 'Templeton' at either Newport or Pine Tree Research Stations. Results from field tests conducted in 2012 indicated a lower than expected incidence of FS in both fungicide treated and untreated plots, this low level of incidence was measurable at the seedling stage with nested PCR in untreated controls.

Disease incidence in field experiments 2011

Overall, higher disease incidence levels on both cultivars were observed at Newport than at Pine Tree in 2011. High levels of FS occurred in the fungicide treated and control plots of both cultivars in 2011 at Newport (Table 4.10), compared to moderate- to low-disease levels observed at Pine Tree (Table 4.11). The fungicide seed treatments applied at Newport and Pine Tree did not significantly reduce the incidence of disease on rice at the two experimental locations. However, there was a significant interaction between locations (a=0.05) and cultivars (a=0.05) in the fields at both locations in years 2011 and 2012. Therefore, the results from each location and cultivar for each year were analyzed separately and are presented as such.

At Newport in 2011, the incidence of FS in control plots was not significantly higher than the fungicide treated plots of 'Clearfield-151' (Table 4.10). In fact, the control disease incidence level was the lowest significant mean value observed for 'Clearfield-151', but not significant for 'Templeton'. The means for all the treatments on 'Clearfield-151' were tested by Fisher's LSD and no significant differences were observed among the treatments.

Statistical evaluation on disease levels observed at Pine Tree in 2011 indicated that some of the treatments significantly reduced disease incidence (Table 4.11). Two treatments on 'Clearfield-151', (1X Rancona® 3.8 FS and 1X Tilt®) had mean incidence levels lower than the untreated control. The opposite was observed on 'Templeton', where the only two treatments

were higher (1X $CuCO_3$ and 2X Raxil® MD) than the control. Disease incidences for the rest of the treatments were lower on 'Templeton' than the untreated control, although the differences were not significant at Pine Tree for either cultivar (a=0.05).

Disease incidence in field experiments 2012

In 2012, disease levels were moderate to low at Newport and Pine Tree (Table 4.12 and 4.13). This result contrasted with the high levels of FS incidence seen on both cultivars at Newport and the moderate levels seen on both cultivars at Pine Tree in 2011. In the 2012 field test, several of the fungicide seed treatments reduced the incidence of FS at Newport and Pine Tree on 'Clearfield-151' and 'Templeton' with a few exceptions. Fewer treatments at Newport on both cultivars provided complete control, though none of the reductions were significantly different from the untreated control (Table 4.12). In these tests, there was only one completely effective treatment 2X Quilt® SE, at Newport where no FS was found on 'Clearfield-151'. The other treatments reduced disease levels to below the control with exception of 1X Quilt® SE and 1X Copper II carbonate. The treatments that provided complete control on 'Templeton' was 2X Stratego® 250 EC and 2X Quilt® SE. These treatments significantly reduced disease levels compared to the previous year, but none were significantly lower than the untreated control at a=0.05.

In the tests conducted at Pine Tree, the completely effective treatments where no disease was observed in 'Clearfield-151' plots, were 1X Copper II carbonate, 2X Copper II carbonate, 2X Quilt® SE, 1X Rancona® 3.8 FS, 2X Raxil® MD, 1X Tilt® and 2X Tilt® (Table 4.13). However, no other treatments significantly reduced disease levels below the untreated control at a=0.05. Several treatments provided complete control on 'Templeton' at Pine Tree including, 1X Quilt® SE, 2X Quilt® SE, 1X Stratego® 250 EC, 2X Stratego® 250 EC, 1X Tilt® and 2X

Tilt®. However, no disease was observed on the untreated control of 'Templeton' and only one diseased panicle was observed on 'Clearfield-151' in the untreated test plots at Pine Tree.

Yield of fungicide seed treatment test plots

Grain yields from the 2011 tests are presented in Tables 4.14 and 4.15. Fungicide seed treatments with exception to 1X $CuCO_3$, 1X Rancona® 3.8 FS and 1X Raxil® MD at Newport, resulted in significant reductions in yield on 'Templeton' in 2011 but not at Pine Tree. No significant yield reductions were observed among fungicide treatments on 'Clearfield-151' cultivar at plots of either location at a=0.05. Statistical analysis of variance (ANOVA) did however; identify significant differences in yield between the two cultivars and among treatments on 'Templeton' at Newport in 2011.

Statistically significant reductions in grain yields occurred for fungicide seed treatments in 2012 on both cultivars at both locations (a=0.05) (Tables 3.16 and 3.17). At Newport, 2X Tilt® and 2X Quilt® SE on 'Clearfield-151' and 1X Tilt® and 2X Tilt® on 'Templeton' were significantly lower than the untreated control. Similar results were obtained at Pine Tree with exception to 'Clearfield-151', where no significant yield reductions were observed when comparing fungicide treatments to the untreated control. On 'Templeton' cultivar at Pine Tree, significant yield reductions were observed for 1X Quilt® SE, 2X Quilt® SE, 1X Stratego® 250 EC, 2X Stratego® 250 EC, 1X Tilt® and 2X Tilt® treatments.

Stand count

Stand counts were significantly different among the fungicide seed treated and untreated plots (Table 4.18). Seedling emergence and growth in the greenhouse and field were significantly inhibited by Stratego® 250 EC, Quilt® SE and Tilt® seed treatments (Figure 4.12). This stand reduction resulted in reduced seedling density in fungicide seed-treated experimental

field plots for both 2011 and 2012. Stand counts of 'Clearfield-151' and 'Templeton' were reduced in 1X Quilt® SE, 2X Quilt® SE, 1X Stratego® 250 EC, 2X Stratego® 250 EC, 1X Tilt® and 2X Tilt® treatments when compared to untreated control plots at both locations (Table 4.18). Stand reductions in these specific treatments may have significantly contributed to yield reductions as seen in Tables 3.14, 3.16 and 3.17.

Discussion

Three selected reference isolates used in this study; I-6E, I-7E and I-8E were assayed for *in-vitro* sensitivity to fungicides. The technical grade fungicides currently used to manage FS at the booting stage and a few experimental fungicides were applied to rice seed infested with *U. virens* spore balls and chlamydospores to determine if seed treatments would reduce the incidence of FS in the experimental test plots. The described nested-PCR protocol was used for detecting the incidence of *U. virens* rDNA in seedlings to determine the effectiveness of fungicidal seed treatments.

Preliminary experiments were conducted to test the sensitivity of the three isolates to a range of the selected fungicide from 50, 100, 150, 200, 250, to 300 mg l^{-1} where no visible growth appeared as described by Tsuda et al. (2006). This preliminary test showed no growth on PDA amended to contain four fungicide concentrations, including 100 mg l^{-1}, 150 mg l^{-1}, 200 mg l^{-1} and 250 mg l^{-1} (data not shown). The data reported here shows that inhibition of mycelial growth occurred at much lower levels of the technical grade fungicides than the preliminary experiment, ranging from 0.52 to 1.25 mg l^{-1} for four of the five fungicides tested. Copper II carbonate levels reduced growth between 0.47 to 0.62 g l^{-1}. Inhibition of mycelial growth occurred at the lowest levels with analytical grade fungicides in the 0.03 to 0.19 mg l^{-1} range for four of the five fungicides tested.

The impact of variable fungicide resistance among fungal isolates of *U. virens* and the range of sensitivity of isolates infecting rice under field conditions remain unknown in the USA, but the inhibitory or similarly the effective fungicide concentrations have been studied with triazoles, ergosterol biosynthesis (EBI) inhibitors or DMI inhibitors (FRAC group 3) (Tsuda et al., 2006; Chen et al., 2013). Tsuda et al. tested the *in-vitro* sensitivity of *U. virens* to simeconazole and determined that the EC_{50} value was 3 mg l^{-1}. Chen et al. reported that the EC_{50} values for inhibiting mycelial growth of 102 *U. virens* isolates ranged from 0.04 to 0.75 mg l^{-1} for prochloraz, 0.04 to 1.08 mg l^{-1} for difenoconazole, 0.04 to 0.38 mg l^{-1} for propiconazole and 0.03 to 0.57 mg l^{-1} for tebuconazole. The EC_{50} values reported by Chen et al. (2013) are similar to the IC_{50} values determined for the three isolates used in this study.

The mycelial growth based assays have some limitations as recently outlined by Rampersad (2012) even though growth based fungicide sensitivity assays have been the conventional method to measure the sensitivity of isolates to chemicals in many studies. These assays are laborious, require precise factoring with weight to volume ratios and require a significant amount of incubator space to run experiments. These limitations have contributed to their limited use for high-through-put situations where large numbers of isolates or chemicals are being tested (Vega et al., 2012).

In this study, the concentrations of the technical grade fungicides that were tested may not have adequately and/or precisely identified the intermediate sensitivity ranges since commercial formulations were utilized to prepare the active ingredients. More precise effects of the active ingredients found in the Fluka Analytical standards were determined from the IC_{50} values of the active ingredients contained in four of the five technical grade fungicides tested. Given these limitations, future tests using an alternative approach to study fungicide sensitivity

using the tetrazolium salt-based resazurin found in the commercially available alamarBlue® Assay (Invitrogen, Carlsbad, CA, U.S. Patent No. 5,501,959) should be considered. Conidium density and fungicide concentrations could be optimized and applied to study the fungicide sensitivity of *U. virens* as was done to study fungicide sensitivity in the brown rot pathogen *Monilinia fructicola*, by Cox et al. (2009). AlamarBlue® based assays (ABA) use microtiter 96 well plates to test fungicide sensitivity which eliminates the need for bulky Petri dishes and boxes to house the experiment in large incubators. The ABA assay is typically optimized in a liquid broth with conidium suspensions which would expose all of the fungal cells to the chemical active ingredients in an aqueous environment. Once optimized, ABA would easily lend itself to automation and be reproducible for high-through-put applications with multiple test replicates and large numbers of isolates to test fungal sensitivity to numerous chemicals. Six previous studies utilized the alamarBlue® as a redox indicator dye for studying fungicide sensitivity in plant pathogens (Pijls et al., 1994; Pelloux-Prayer et al., 1998; Cox et al., 2009; Rampersad, 2011; Rampersad and Teelucksingh, 2012; Vega et al., 2012). The use of alamarBlue® in conjunction with fungicide-amended-PDA assays may be an additional tool that could, in the near future, vastly improve the way we study fungicide toxicity in plant pathogens.

The sensitivity of *U. virens* isolates to the selected fungicides described here may provide a baseline to test other isolates not previously exposed to fungicides in Arkansas. If all field isolates are determined to be sensitive to the fungicides at the rates tested here, a simple test will enable us to determine effective fungicide treatments and to advise growers which chemicals and rates are needed for effective suppression of growth, development and colonization of rice by *U. virens*. If other fungicide groups are found to be effective for suppression of FS in the long term under various field conditions, the utility of the current selection of fungicides will be diversified

and incorporated in rotation with chemicals that have other modes of action. If more than one fungicide activity group is available and rotated from one cropping season to another, then a reduced risk for the development of fungicide insensitivity should be expected and this should increase the long term utility of fungicide activity. There are numerous reports of phytopathogenic fungal insensitivity to DMI and strobilurin type fungicides conferred by point mutations or alternate metabolic pathways (Zhonghua, 2005). Isolates of *U. virens* and other crop pathogens should be routinely screened and monitored for fungicide insensitivity in fields where fungicide control fails or becomes seemingly inadequate. A timely detection of fungicide insensitivity using mycelial growth assays, alamarBlue® germination assays and PCR-based detection to identify point mutations conferring insensitivity, are specific tools to investigate increased frequency of fungicide insensitive phytopathogenic fungal populations. This knowledge would help growers make alterations in their fungicide management programs to control FS and other crop diseases with more precision.

This study examined the utility of using nested-PCR for detecting differences in the rate of colonization of rice seedlings by *U. virens* after treatment of seed with fungicides to control the early infection of seedlings. This study was conducted by extracting DNA from seedlings grown from fungicide treated seed in greenhouse and field experiments and reacting with primers specific for *U. virens* rDNA in nested-PCR reactions. The use of a rapid QuickExtract™ DNA extraction protocol to extract DNA from plant samples and utilization of rapid nested-PCR assays used in this study shortened the time required to study the presence of *U. virens* in the field and greenhouse. No effective control strategy has been proven to eradicate this disease from rice paddies planted from infested seed (Cartwright et al., 2000). Our findings support this notion when comparing disease levels in plots planted with fungicide treated seed to untreated

seed in different years. No seed treatment resulted in lower disease levels that were statistically significant from untreated controls or reliably eradicated FS disease at either field location or cultivar during either year. However, the results show significant differences in the incidence of *U. virens* among fungicide treated and untreated control seedlings from 'Templeton' at Pine Tree as determined by nested-PCR. Results from this study demonstrate the use of nested-PCR to evaluate incidence of *U. virens* from fungicide seed treatments may not be useful but may provide some utility if some adjustments in the number of samples or replications are made to increase the sensitivity and ability of the protocol for distinguishing differences in incidence of *U. virens* in the field samples. The data from 2012 where low *U. virens* incidence levels were detected in some of the treatment plots, suggests that seed treatments may be effective alternatives to foliar fungicide applications to control this disease under the prevailing field conditions that were present in 2012. The perceived disease levels in 2012 among fungicide seed treatments may not be achieved under high inoculum pressure or under the prevailing conducive field conditions as was seen in 2011. Thus the impact of environmental factors and inoculum levels on FS incidence and severity should be taken into consideration in future studies.

The utility of fungicide seed treatments to control FS is a previously uninvestigated component of integrated FS disease management strategies. Seed treatments may be useful in combination with cultural management practices and selected varietal resistance. Our intent was to test the hypothesis devised by Zhou et al. (2003) where it was stated that "molecular diagnostics will help us to determine efficacy of chemical and cultivation control strategies". This study focused on the use of nested-PCR primers in a high through-put application for studying the incidence of seedling infection after treatment of seed with fungicides in the greenhouse and field. In this study, some significant differences in infection levels were found

in ‘Templeton’ at Pine Tree and Newport in 2011 but they did not necessarily correspond to disease assessments in the field. Given this data, the potential may still exist for studying the relationship between fungicide treatments and of *U. virens* incidence in seedlings grown on different cultivars in the field using the molecular techniques described. Data from other studies not addressed in this thesis conducted in the field to describe the reaction of cultivars to FS, suggests that some cultivars may be more or less susceptible than ‘Templeton’ or ‘Clearfield-151’ used in this study (TeBeest and Jecmen, 2011). Variation in the data from the studies on the number of rice spikelets per panicle infected by *U. virens* as determined by nested-PCR indicates that the differences in susceptibility may be measurable using the described techniques and protocols and warrants further study.

In the field experiments using seed treated with several fungicides, the incidences of FS were highly variable in each test. The lack of efficacy may be a result of the rates tested or attributed to unknown epiphytological factors or soil conditions that contributed to variability in the tests. In the fungicide seed treatment field tests, the aggregated distribution of FS contributed to variability within and among experimental plots. This distribution constrained our ability to identify statistically significant differences in disease incidence between some treatments. For example, data from Newport shows that none of the 13 treatments significantly reduced disease levels below the untreated control on ‘Clearfield-151’ and the slight reduction of disease on ‘Templeton’ was not found to be significant in 2011. Only two treatments Tilt® 1X and Rancona® 1X on ‘Clearfield-151’ at Pine Tree, but several treatments on ‘Templeton’ at both locations were found to have lower disease levels than the controls in the field in 2011. However, in 2012 several treatments appeared to have reduced the number of infected plants at both locations on the ‘Templeton’ cultivar but not ‘Clearfield-151’. Previous field tests

conducted by Tsai et al. (1990) with unknown rice varieties treated with eight fungicides showed disease control in plots treated with Daconil WP (clorothalonil) and 58% Ridomil-MZ WP (metalaxyl). Tsuda et al. (2006) applied simeconazole, a triazole fungicide, to floodwater one- to five-weeks before heading to control kernel smut and FS in field trials and they found that an application of simeconazole two- to five-weeks before heading was highly effective for controlling FS although treatment three weeks before heading was the most effective. Chen et al. (2012) tested multiple applications of four triazole fungicides in the field to control FS and found that two applications of three of the four fungicides exhibited greater control than a single application resulting in control of 71.5 to 74.3% disease reduction in years 2010 and 2011 respectively. The data from these studies indicate that the disease may be more adequately controlled if fungicide applications are made at earlier stages of boot development rather than at later stages when the panicle is or has already emerged from boots.

Analysis of all the field studies to control FS with fungicides suggests a need for further review of several factors. First, the treatment and handling of seed during processing and seed treatment can release chlamydospores from spore balls to other seeds in the seed lot. The dislodging of chlamydospores may have resulted in higher infection levels in 2011 due to higher inoculum levels present in the seed than 2012. Initial inoculum levels were different between the years (48.2 and 36.7 spore balls/kg in 2011, 5.5 and 7.1 spore balls/kg in 2012 in 'Clearfield-151' and 'Templeton' seed respectively) and this may significantly contribute to the differences of disease levels as described by Heald (1921). In future tests, seed should be inoculated with uniform spore levels to normalize and minimize the variability associated with varying inoculum levels. Secondly, the variability of disease epidemics may be highly dependent on unknown environmental factors since we observed differences in disease incidence consistently at the two

study locations. More effective disease control at Pine Tree in both years may be partially attributed to differences in soil factors influencing infection of seedlings. A few environmental factors related to pathogenesis that are considered but are not limited to, microbiological communities, pH in the soil and rhizosphere, moisture availability at planting and dew period at flowering. Lastly, that the lower infection levels and differences in yield for some treatments indicate fungicide activity on 'Templeton' but not 'Clearfield-151', may be attributed to differences in cultivar susceptibility. This cultivar difference was found to be statistically significant down to $a<0.05$ in this study and in other work to characterize susceptibility of cultivars to FS not specifically addressed in this thesis (TeBeest and Jecmen, 2011). Thus future tests should take into consideration the susceptibility of the cultivars used in the test and how they react to FS under varying conditions.

The field studies on fungicide seed treatments suggest that the application of a seed treatment may not reduce the amount of functional inoculum interacting with germinating seedlings in plants after germination or in plants prior to the booting stage. This may contribute to the lack of any statistical differences between the control and fungicide treatments. Thus, in future studies, the combined treatment of seed with fungicides taken with steps to minimize variability in disease levels of experimental plots and how other factors related to pathogenesis could affect the results of a field trial should also be investigated.

There was a remarkable reduction of disease, measured by visual assessments in the Quilt SE treated test plots at Pine Tree on 'Templeton' cultivar in 2011. The PCR results from the field plot samples also indicated a significant reduction in *U. virens* incidence from the Quilt SE treated seedlings. This is the only data where the field disease assessments agree with the results from the PCR screening. On the other hand, there were moderate to high disease levels observed

in the field plots of fungicide seed treatments of both cultivars at Newport in 2011. Nested-PCR results from $CuCO_3$, Quilt SE and Tilt treatments on 'Templeton' variety at Newport indicated a significant reduction in *U. virens* incidence that did not compare with visual disease assessments where a high level of FS incidence was observed.

In this study, several triazole and azoxystrobin fungicide treatments reduced seedling emergence, stunted seedlings and reduced overall vigor resulting in a significant reduction in stand development. No studies that I am aware of, have investigated the ability of strobilurin or triazole fungicides to induce phytotoxicity on plant physiology and metabolic processes including cytochrome bc1 in rice. Thus, the phytotoxic cause of the three fungicide active ingredients azoxystrobin, propiconazole and trifloxystrobin should be studied for their effects on physiological and metabolic inhibition of cytochrome bc1 in rice using enzyme kinetic experiments. In other fungicide seed treatment tests not described in this thesis, using 'Templeton' cultivar and combination treatments using Quilt®, Tilt® and Stratego® fungicides combined with Raxil® , an additional seed treatment amendment was used based on the advice of a Valent BioSciences Corporation representative. This product, called Release LC PGR SR# SE 826499 (Valent Biosciences, Libertyville, IL), containing 4% gibberellic acid (GA) was applied to the seed at the recommended rate of 1.8 ml kg^{-1} (1 g a.i. cwt^{-1} or 25 ml cwt^{-1}). In these tests, stand counts were significantly improved over stand counts in the two years of Quilt®, Tilt® and Stratego® seed treatment tests described in this thesis (comparison data not shown). Commercially available gibberellic acid amendments are used on rice by at least one seed company including Horizon Agriculture on commercially available 'Clearfield' rice cultivars. Based on these preliminary results, gibberellic acid may be useful to mitigate the phytotoxic effects of the seed treatments on rice stand and to improve plant density establishment under sub-

optimal conditions. Therefore, further study is warranted on the effects exogenous gibberellic acid may have on host-pathogen interactions and mitigation of phytotoxic stressors (Gangwar et al., 2011).

For future considerations, a more comprehensive understanding of the relationship between, inoculum levels and the environmental factors related to pathogenicity, resistance of differential cultivars to FS and the effects of fungicides (that have demonstrated an inhibitory effect against *U. virens in-vitro*) on reducing ongoing epidemics will enable rice growers worldwide to more effectively and precisely manage FS disease.

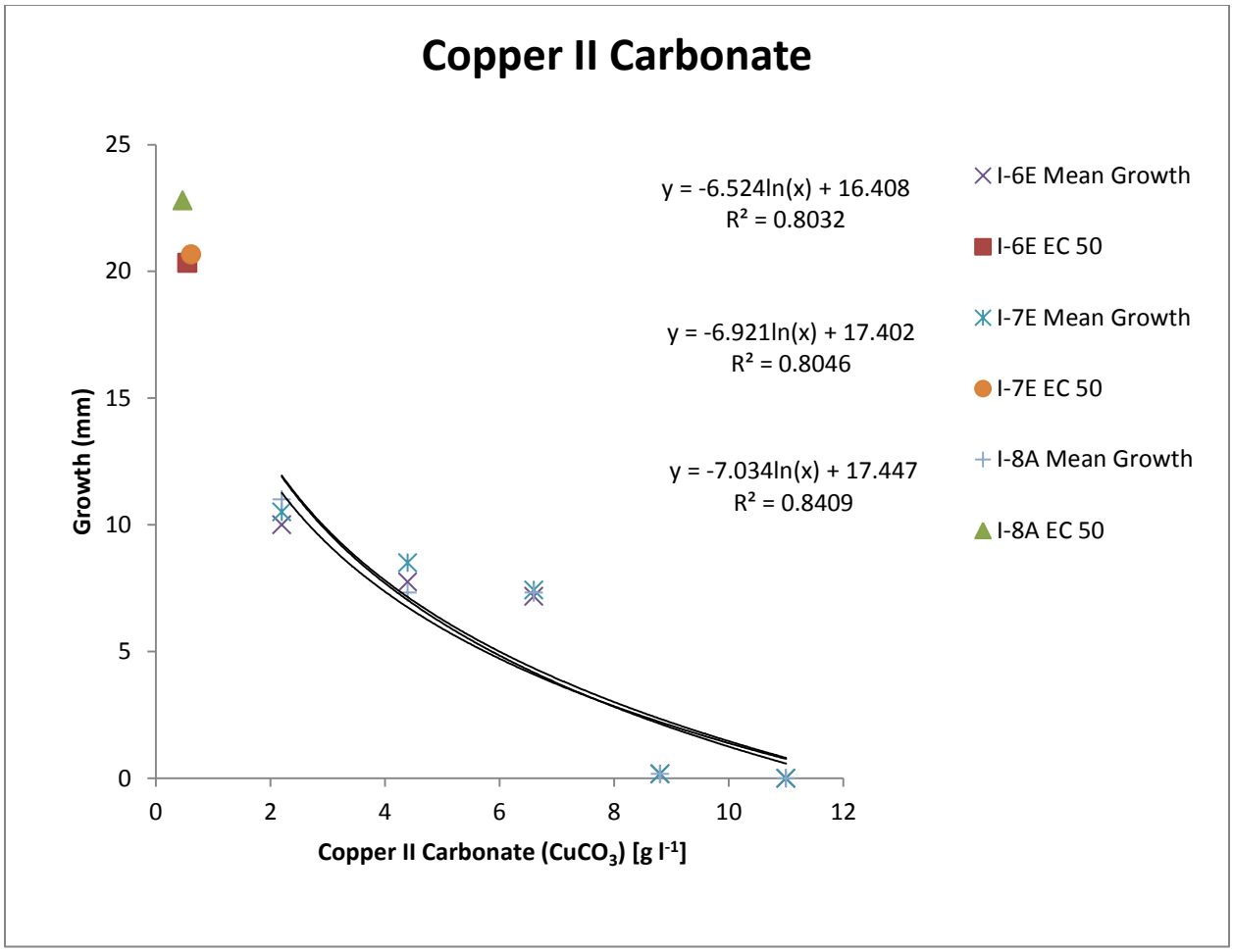

Figure 4.1. The growth of *U. virens* isolates I-6E, I-7E and I-8E expressed as the mean adjusted diameter growth value (ADG) *in-vitro* at 26°C on PDA amended to contain copper II carbonate ($CuCO_3$). Experiments consisted of three replicates of each treatment and two experiments. The EC_{50} (1/2Y = ln (X) + Z) values were calculated from the logarithmic line fit equations obtained from regressing mean growth against fungicide concentrations as the following; I-6E= ■ = 0.55g l^{-1}, I-7E = ● = 0.62 g l^{-1} and I-8E = ▲=0.47 g l^{-1}. Copper II carbonate was omitted from advanced statistical analysis due to its limited commercial interest and use as a modern seed treatment fungicide.

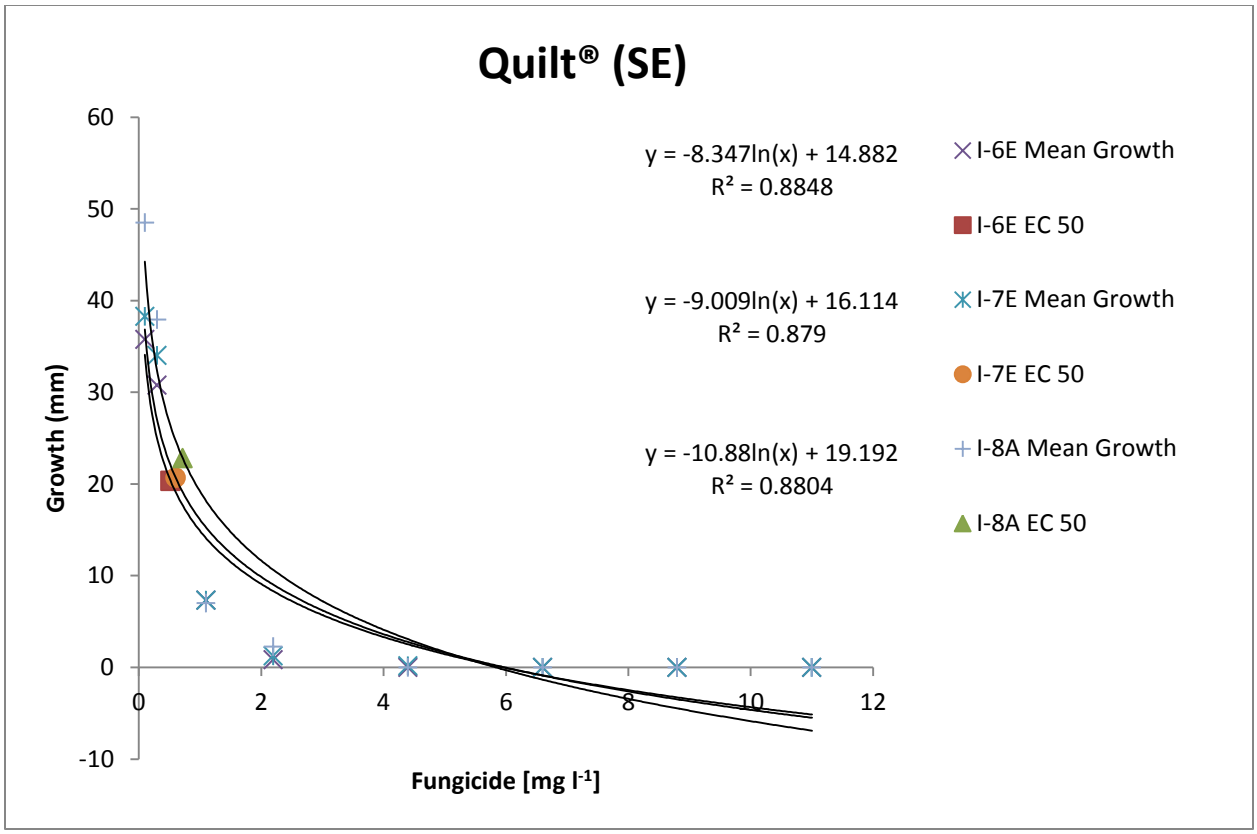

Figure 4.2. The growth of *U. virens* isolates I-6E, I-7E and I-8E expressed as the mean adjusted diameter growth value (ADG) *in-vitro* at 26°C on PDA amended to contain Quilt® (SE) technical grade fungicide. Experiments consisted of three replicates of each treatment and two experiments.
The EC_{50} (1/2Y = ln (X) + Z) values were calculated from the logarithmic line fit equations obtained from regressing mean growth against fungicide concentrations as the following; I-6E = ■ = 0.52 mg l^{-1}, I-7E = ● = 0.60 mg l^{-1} and I-8E =▲=0.72 mg l^{-1}. Fungicide, isolate and fungicide concentrations were significantly different to P=0.0029, P=0.0001 and P=0.0001 at a=0.05 respectively.

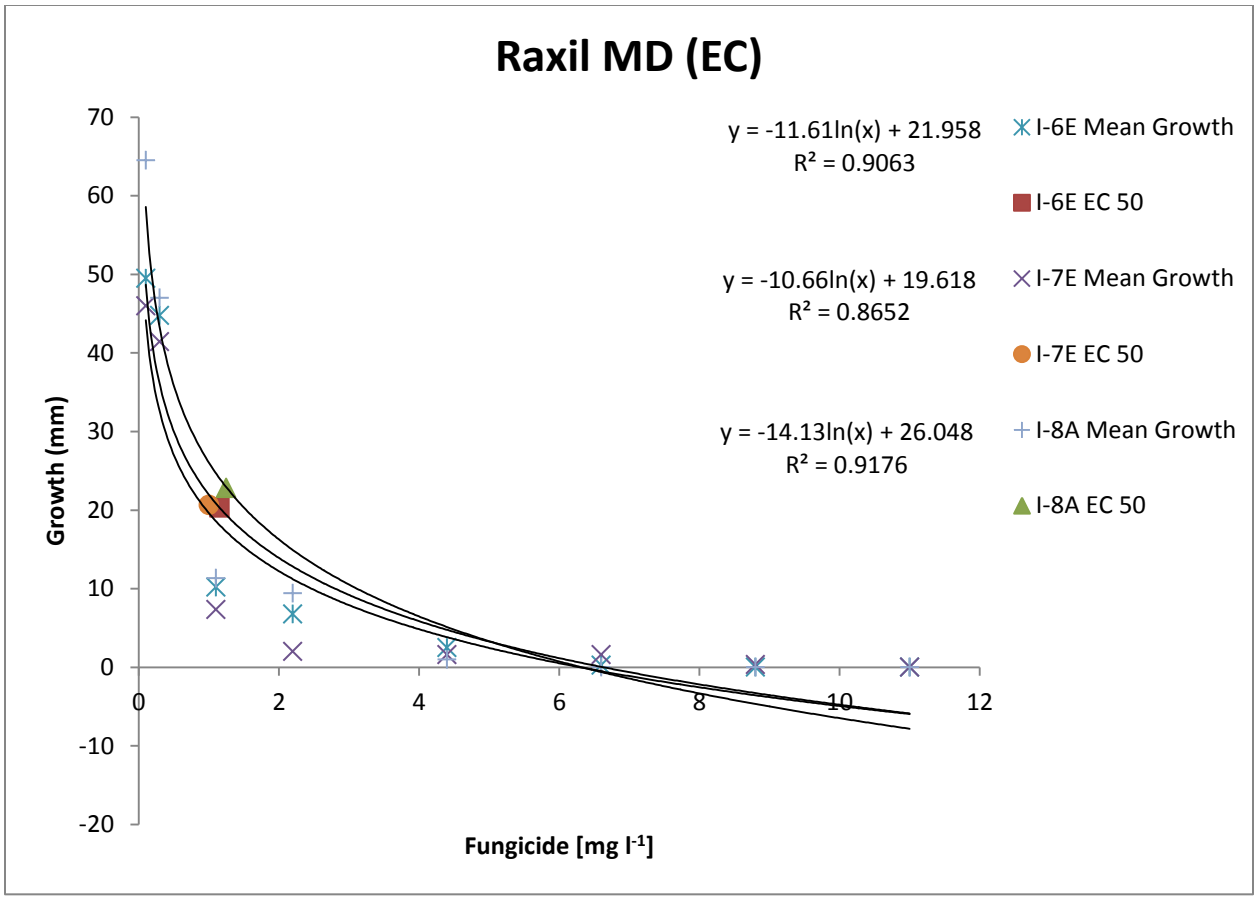

Figure 4.3. The growth of *U. virens* isolates I-6E, I-7E and I-8E expressed as the mean adjusted diameter growth value (ADG) *in-vitro* at 26°C on PDA amended to contain Raxil® MD (EC) technical grade fungicide. Experiments consisted of three replicates of each treatment and two experiments.
The EC_{50} (1/2Y = ln (X) + Z) values were calculated from the logarithmic line fit equations obtained from regressing mean growth against fungicide concentrations as the following; I-6E = ■ = 1.15 mg l^{-1}, I-7E = ● = 0.91 mg l^{-1} and I-8E = ▲ = 1.25 mg l^{-1}. Fungicide, isolate and fungicide concentrations were significantly different to P=0.0029, P=0.0001 and P=0.0001 at a=0.05 respectively.

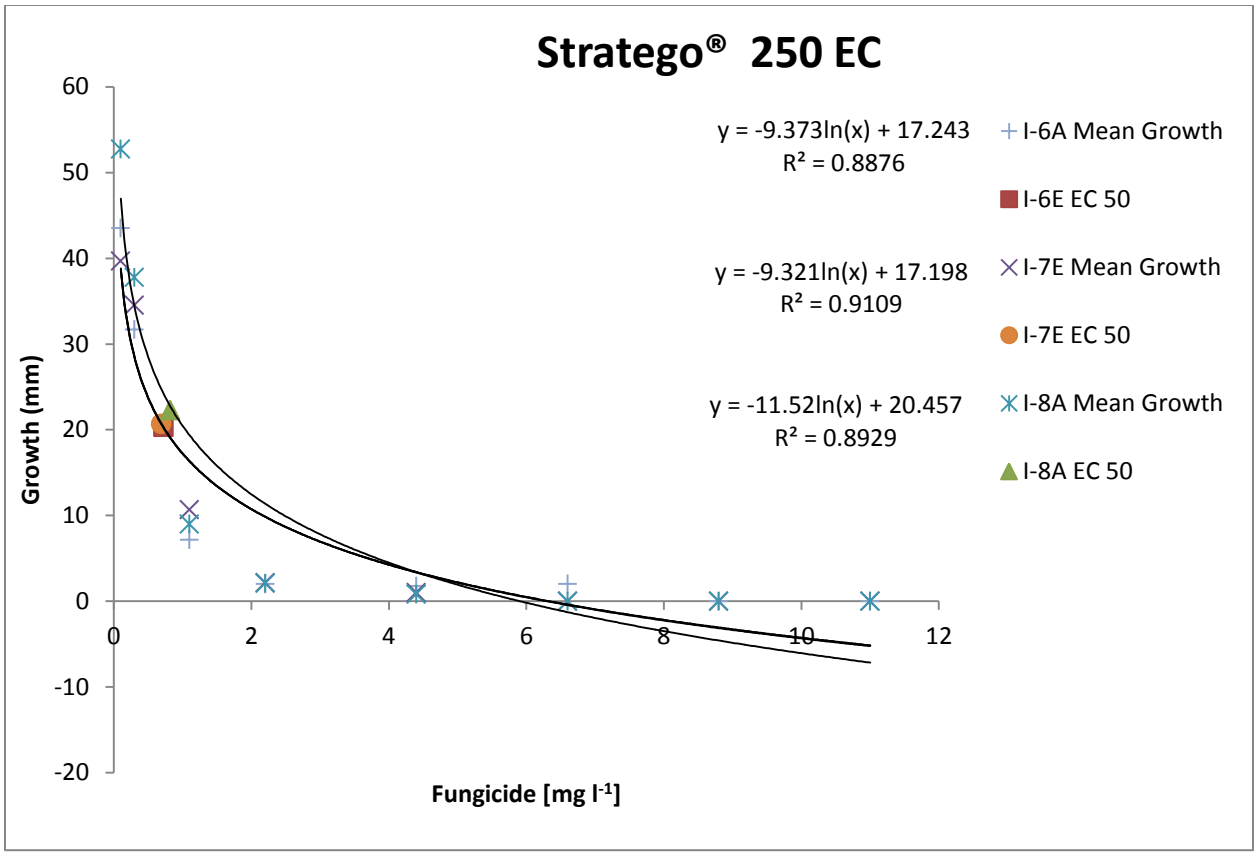

Figure 4.4. The growth of *U. virens* isolates I-6E, I-7E and I-8E expressed as the mean adjusted diameter growth value (ADG) *in-vitro* at 26°C on PDA amended to contain Stratego® (250 EC) technical grade fungicide. Experiments consisted of three replicates of each treatment and two experiments.
The EC_{50} (1/2Y = ln (X) + Z) values were calculated from the logarithmic line fit equations obtained from regressing mean growth against fungicide concentrations as the following; I-6E = ■ = 0.72 mg l^{-1}, I-7E = ● = 0.69 mg l^{-1} and I-8E = ▲= 0.82 mg l^{-1}. Fungicide, isolate and fungicide concentrations were significantly different to P=0.0029, P=0.0001 and P=0.0001 at a=0.05 respectively.

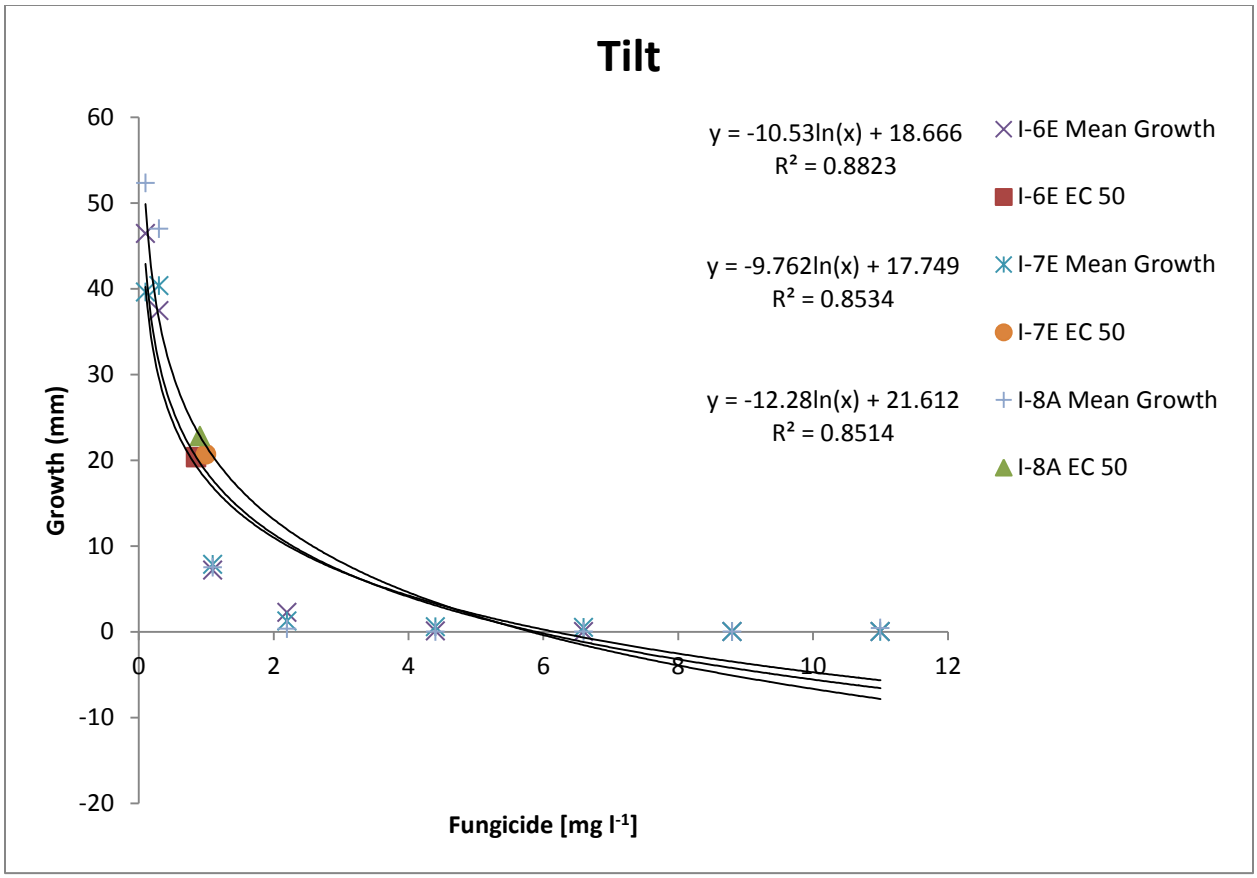

Figure 4.5. The growth of *U. virens* isolates I-6E, I-7E and I-8E expressed as the mean adjusted diameter growth value (ADG) *in-vitro* at 26°C on PDA amended to contain Tilt® technical grade fungicide. Experiments consisted of three replicates of each treatment and two experiments. The EC_{50} (1/2Y = ln (X) + Z) values were calculated from the logarithmic line fit equations obtained from regressing mean growth against fungicide concentrations as the following; I-6E = ■ =0.85 mg l^{-1}, I-7E = ● =0.74 mg l^{-1} and I-8E = ▲=0.91 mg l^{-1}. Fungicide, isolate and fungicide concentrations were significantly different to P=0.0029, P=0.0001 and P=0.0001 at a=0.05 respectively.

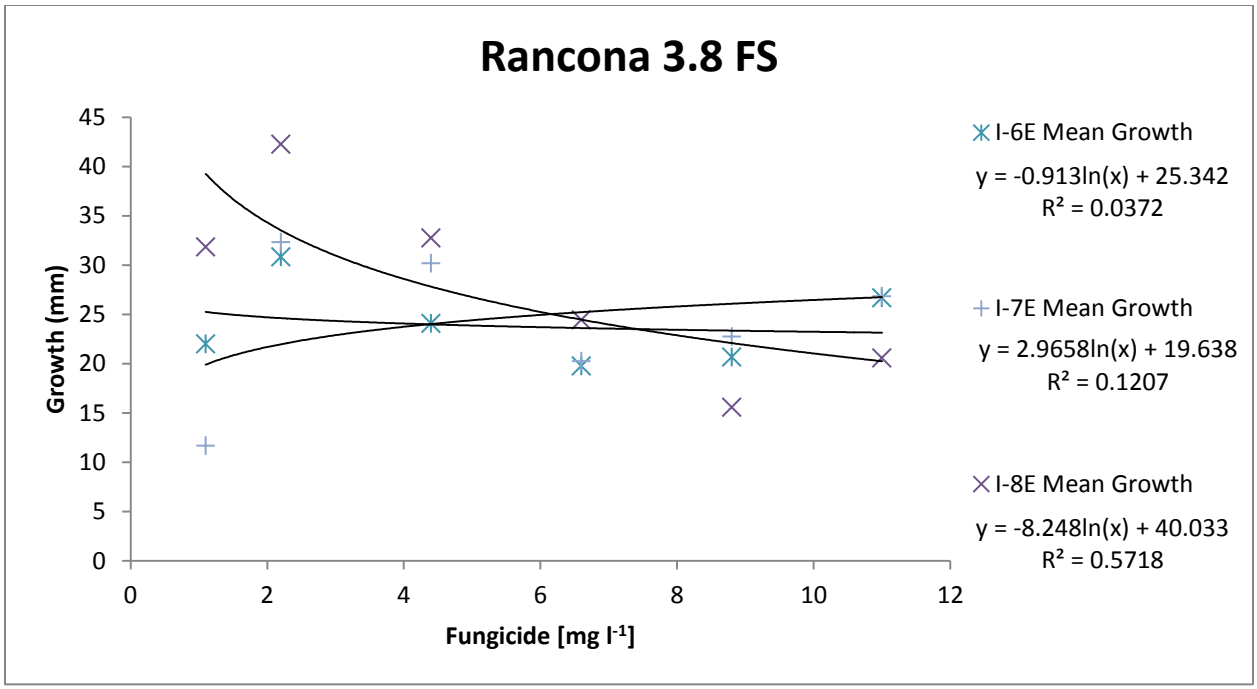

Figure 4.6. The growth of *U. virens* isolates I-6E, I-7E and I-8E expressed as the mean adjusted diameter growth value (ADG) *in-vitro* at 26°C on PDA amended to contain Rancona® 3.8 FS technical grade fungicide. Experiments consisted of three replicates of each treatment and two experiments.
The EC_{50} (1/2Y = ln (X) + Z) values were calculated (but not plotted) from the logarithmic line fit equations obtained from regressing mean growth against fungicide concentrations as the following; I-6E = 239.52 mg l^{-1}, I-7E = 1.42 mg l^{-1} and I-8E = 8.08 mg l^{-1}. Rancona® 3.8 FS was omitted from advanced statistical analysis due to the highly variable calculated growth inhibition values of *U. virens* isolates.

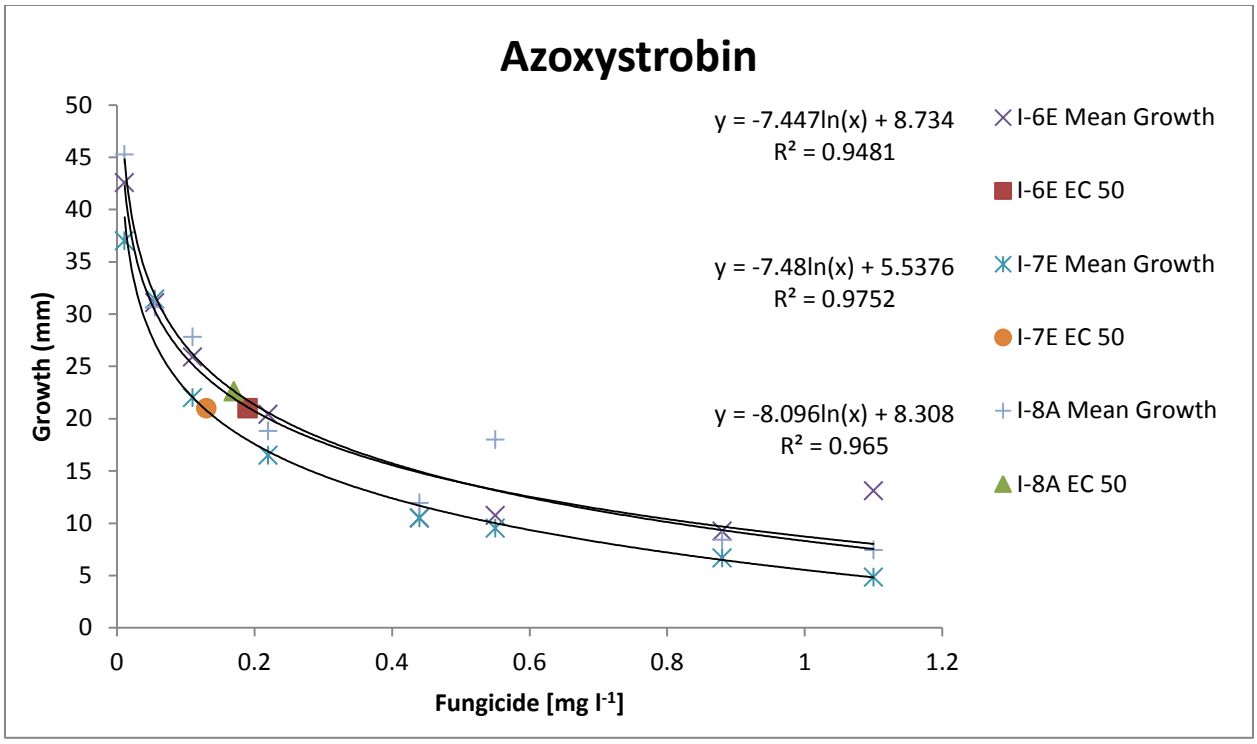

Figure 4.7. The growth of *U. virens* isolates I-6E, I-7E and I-8E expressed as the mean adjusted diameter growth value (ADG) *in-vitro* at 26°C on PDA amended to contain azoxystrobin analytical grade fungicide. Experiments consisted of three replicates of each treatment and two experiments.
The EC_{50} (1/2Y = ln (X) + Z) values were calculated from the logarithmic line fit equations obtained from regressing mean growth against fungicide concentrations as the following; I-6E = ■ = 0.19 mg l^{-1}, I-7E = ● = 0.13 mg l^{-1} and I-8E = ▲ = 0.17 mg l^{-1}. Fungicide, isolate and fungicide concentrations were significantly different to P=0.0001, P=0.0038 and P=0.0001 at a=0.05 respectively.

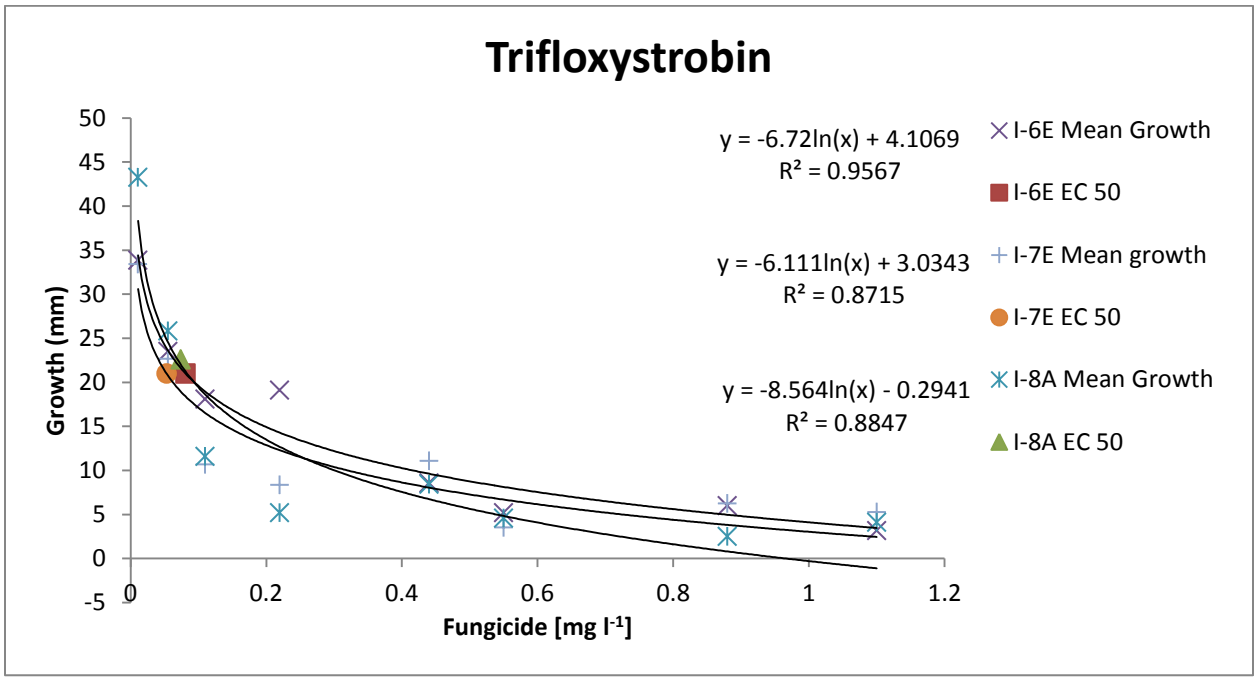

Figure 4.8. The growth of *U. virens* isolates I-6E, I-7E and I-8E expressed as the mean adjusted diameter growth value (ADG) *in-vitro* at 26°C on PDA amended to contain trifloxystrobin analytical grade fungicide. Experiments consisted of three replicates of each treatment and two experiments.
The EC_{50} (1/2Y = ln (X) + Z) values were calculated from the logarithmic line fit equations obtained from regressing mean growth against fungicide concentrations as the following; I-6E = ■ = 0.08 mg l^{-1}, I-7E = ● = 0.05 mg l^{-1} and I-8E =▲= 0.07 mg l^{-1}. Fungicide, isolate and fungicide concentrations were significantly different to P=0.0001, P=0.0038 and P=0.0001 at a=0.05 respectively.

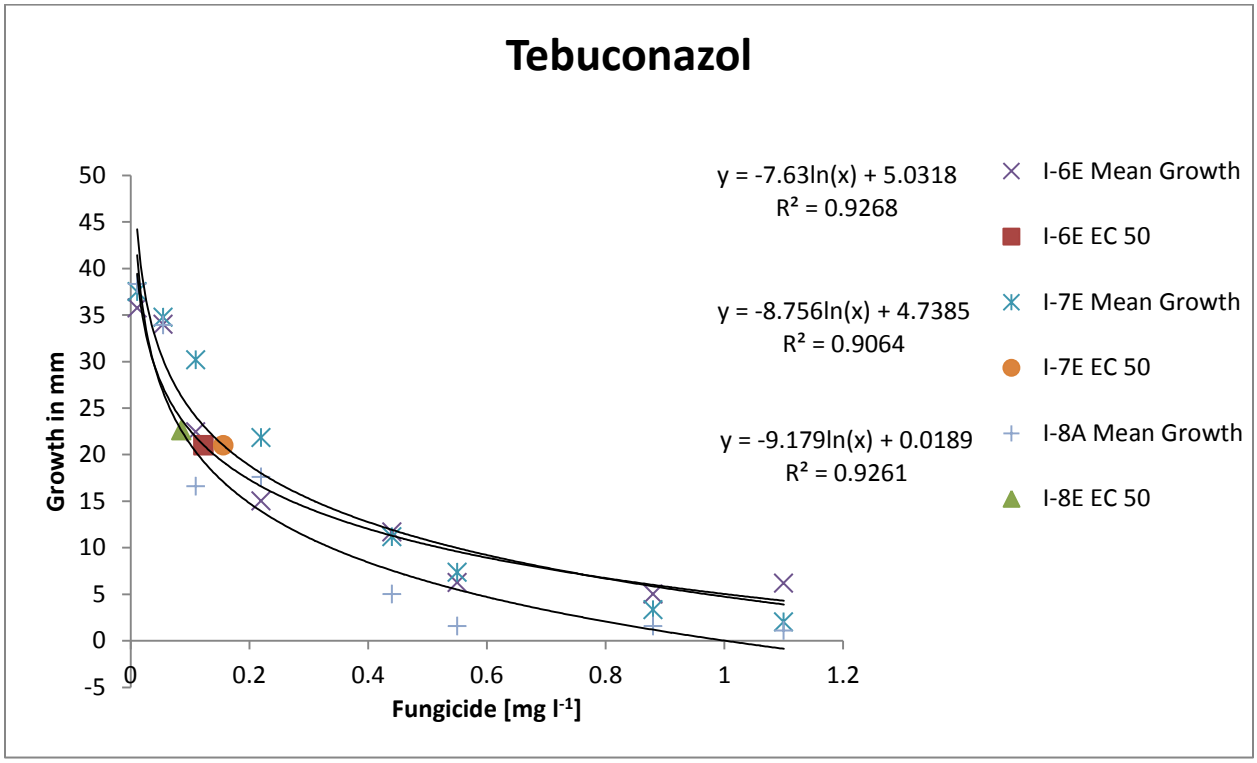

Figure 4.9. The growth of *U. virens* isolates I-6E, I-7E and I-8E expressed as the mean adjusted diameter growth value (ADG) *in-vitro* at 26°C on PDA amended to contain tebuconazole analytical grade fungicide. Experiments consisted of three replicates of each treatment and two experiments.
The EC_{50} (1/2Y = ln (X) + Z) values were calculated from the logarithmic line fit equations obtained from regressing mean growth against fungicide concentrations as the following; I-6E = ■ = 0.123 mg l^{-1}, I-7E = ● = 0.156 mg l^{-1} and I-8E = ▲= 0.085 mg l^{-1}. Fungicide, isolate and fungicide concentrations were significantly different to P=0.0001, P=0.0038 and P=0.0001 at a=0.05 respectively.

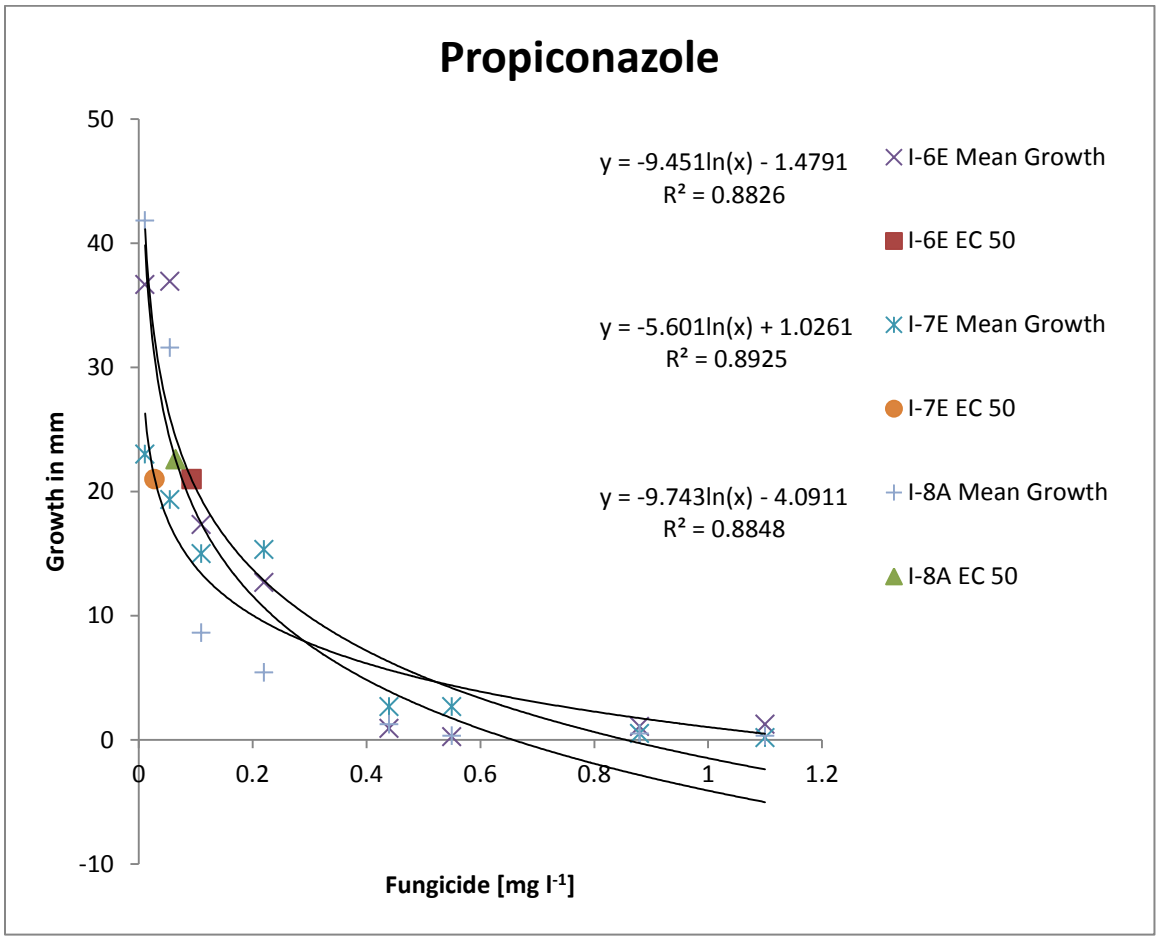

Figure 4.10. The growth of *U. virens* isolates I-6E, I-7E and I-8E expressed as the mean adjusted diameter growth value (ADG) *in-vitro* at 26°C on PDA amended to contain propiconazole analytical grade fungicide. Experiments consisted of three replicates of each treatment and two experiments.
The EC_{50} (1/2Y = ln (X) + Z) values were calculated from the logarithmic line fit equations obtained from regressing mean growth against fungicide concentrations as the following; I-6E = ■ = 0.123 mg l^{-1}, I-7E = ● = 0.156 mg l^{-1} and I-8E = ▲= 0.085 mg l^{-1}. Fungicide, isolate and fungicide concentrations were significantly different to P=0.0001, P=0.0038 and P=0.0001 at a=0.05 respectively.

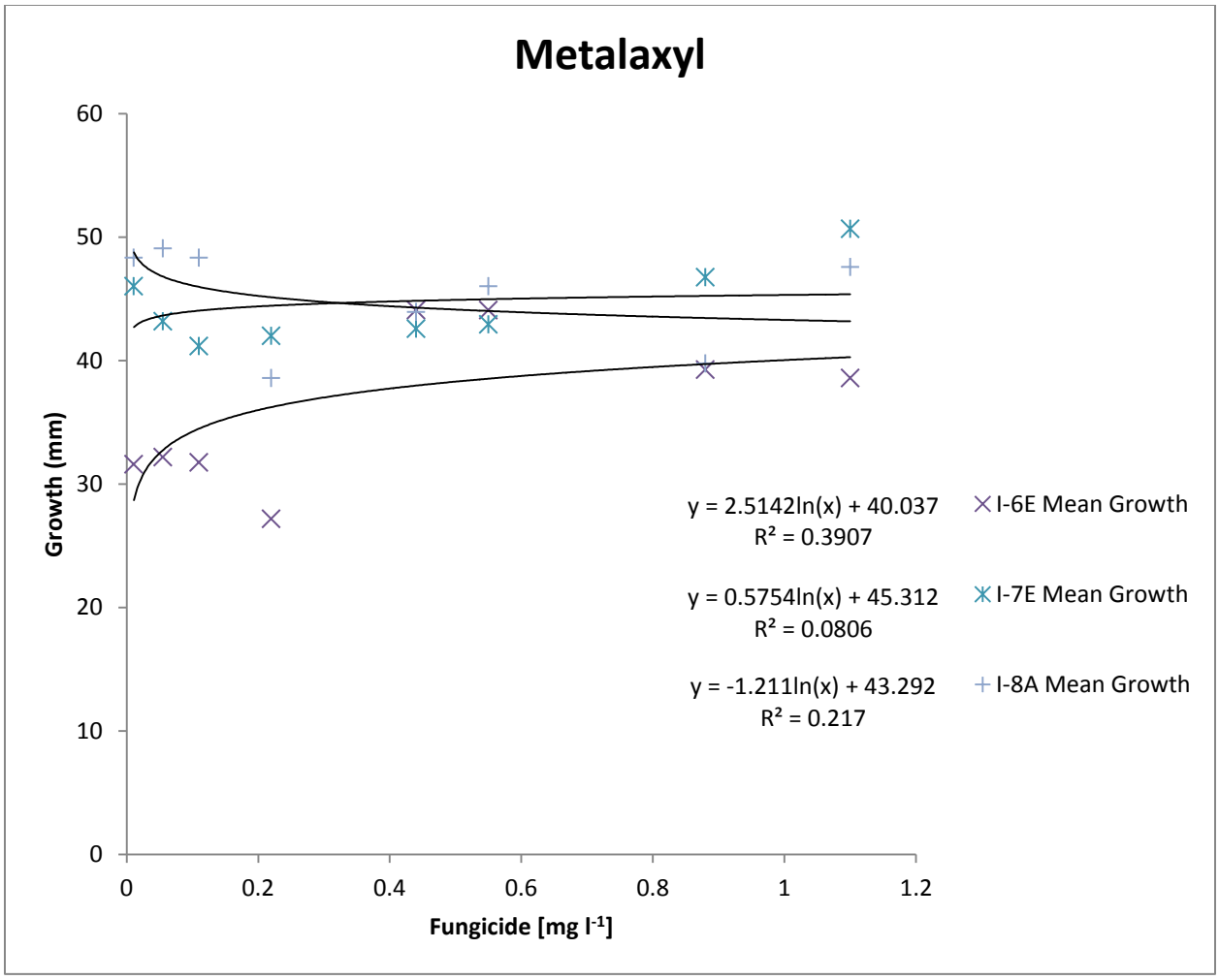

Figure 4.11. The growth of *U. virens* isolates I-6E, I-7E and I-8E expressed as the mean adjusted diameter growth value (ADG) *in-vitro* at 26°C on PDA amended to contain metalaxyl analytical grade fungicide. Experiments consisted of three replicates of each treatment and two experiments.
The EC_{50} (1/2Y = ln (X) + Z) values were not calculated from the logarithmic line fit equations obtained from regressing mean growth against fungicide concentrations. Metalaxyl was omitted from advanced statistical analysis due to the highly variable calculated growth inhibition values of *U. virens* isolates.
Fungicide, isolate and fungicide concentrations were significantly different to P=0.0001, P=0.0038 and P=0.0001 at a=0.05 respectively.

Figure 4.12. Seedlings (2nd, 3rd and 4th from left) exhibiting symptoms of stunting and phytotoxicity from 2X Quilt®, 2X Tilt® and 2X Stratego® fungicide seed treatments grown in the Rosen Alternative Pest Control Center, Greenhouse 1.4 with soil from the Pine Tree (front) and Newport (back) Research Stations.
Treatments from left to right are as follows; Untreated, 2X Stratego® 250 EC, 2X Quilt® SE, 2X Tilt®, 2X Raxil® MD, 2X Rancona® 3.8 FS, 2X copper II carbonate, water control untreated.

Table 4.1. Isolation source of the selected isolates used in this study for testing isolate sensitivity to fungicides.

Location [A]	County [B]	Cultivar [C]	Date collected [D]	Isolate # [E]	Tube# [F]
Statewide	Poinsett	Clearfield-151	8-16-2010	I-6E	U-2-118
RREC	Poinsett	Francis	9-2008	I-7E	U-2-119
G & H Farms	Arkansas	Templeton	10-15-2010	I-8E	U-2-120

[A] Location of fields where panicles infected with FS were obtained.
[B] County where the fields or sampling locations were located.
[C] Cultivars from which the infected panicles were obtained.
[D] Year collected and date of deposition in the Harris cryogenic freezer located in Lab 221 Plant Sciences Building, University of Arkansas in Fayetteville, AR and maintained at -80°C.
[E] Isolate number and letter that designates from which respective panicle the sample originated.
[F] Tube number, designating the first letter (U) as *Ustilaginoidea* species, -*n*- the middle box number in which the isolate is contained, -*n* the last number designating the isolate number in a series.

Table 4.2. List of the fungicides used *in-vitro* to screen the sensitivity of *U. virens* isolates.

Fungicide [A]	Active ingredient (a.i.) [B]	[Total a.i.] (mg l^{-1}) [C]
Copper II carbonate	51.4% Cu	2,200, 4,400, 6,600, 8,800, 11,000
Quilt® SE	7.0 % Azoxystrobin + 11.7% Propiconazole	0.11, 0.6, 1.1, 2.2, 4.4, 6.6, 8.8, 11.0
Rancona® 3.8 FS	40.7% ipconazole	0.11, 0.6, 1.1, 2.2, 4.4, 6.6, 8.8, 11.0
Raxil® MD EC	0.48% Tebuconazol + 0.63 % Metalaxl	0.11, 0.6, 1.1, 2.2, 4.4, 6.6, 8.8, 11.0
Stratego® 250 EC	11.8% Propiconazole + 11.8% Trifloxystrobin	0.11, 0.6, 1.1, 2.2, 4.4, 6.6, 8.8, 11.0
Tilt®	41.8% Propiconazole	0.11, 0.6, 1.1, 2.2, 4.4, 6.6, 8.8, 11.0

[A] Fungicides were selected for use in this study to test the sensitivity of *U. virens* isolates *in-vitro*.

[B] The active ingredient(s) contained within in each fungicide material.

[C] The final concentrations of the active ingredients expressed in mg l^{-1}, were used to evaluate fungicide sensitivity *in-vitro*.

Table 4.3. A list of the fungicide seed treatments used in this study to analyze the effect of treatment of seed with fungicides on the incidence of FS at heading. [A]

No.[B]	Fungicide [C]	Rate [D]	% a.i. [E]
1	Untreated	0.0	Control
2	1X Stratego® 250 EC	4.6 g kg^{-1}	11.8% Propiconazole + 11.8% Trifloxystrobin
3	2X Stratego® 250 EC	9.2 g kg^{-1}	11.8% Propiconazole + 11.8% Trifloxystrobin
4	1XQuilt® SE	4.6 g kg^{-1}	7.0 % Azoxystrobin + 11.7 % Propiconazole
5	2X Quilt® SE	9.2 g kg^{-1}	7.0 % Azoxystrobin + 11.7 % Propiconazole
6	1X Tilt®	2.6 g kg^{-1}	41.8% Propiconazole
7	2X Tilt®	5.2 g kg^{-1}	41.8% Propiconazole
8	1X Raxil® MD EC	3.6 g kg^{-1}	0.48% Tebuconazol + 0.63% Metalaxyl
9	2X Raxil® MD EC	7.2 g kg^{-1}	4.8% Tebuconazol + 6.3% Metalaxyl
10	1X Rancona® 3.8 FS	3.9 g kg^{-1}	40.7% ipconazole
11	2X Rancona® 3.8 FS	7.8 g kg^{-1}	40.7% ipconazole
12	1X $CuC0_3$	1.6 g kg^{-1}	51.4% Cu
13	2X $CuC0_3$	3.2 g kg^{-1}	51.4% Cu

[A] False smut disease incidence analyses were conducted by planting fungicide treated 'Clearfield-151' and 'Templeton' rice seed infested with FS chlamydospores, in a randomized complete block design with four replications at two locations.
[B] Treatment number assigned to the corresponding fungicide seed treatment.
[C] 1X (low rate) and 2X (high rate) fungicides that were selected for use in this study.
[D] Rate of fungicide in grams applied to one kg of seed.
[E] Percentage of active ingredient (a.i.) contained in the commercial fungicide formula.

Table 4.4. Tabulation of calculated inhibitory fungicide concentration values (IC_{50}) of *Ustilaginoidea virens* isolates I-6E, I-7E and I-8E for each technical and analytical grade fungicide.

		IC_{50} values (mg l^{-1})
Fungicide [A]	Isolate [B]	Mean Isolate IC_{50} [C]
$CuCO_3$	I-6E	550 [D]
	I-7E	620
	I-8E	470
Quilt® SE	I-6E	0.52
	I-7E	0.60
	I-8E	0.72
Raxil® MD EC	I-6E	1.15
	I-7E	0.91
	I-8E	1.25
Stratego® 250 EC	I-6E	0.72
	I-7E	0.69
	I-8E	0.82
Tilt®	I-6E	0.57
	I-7E	0.74
	I-8E	0.54
Azoxystrobin	I-6E	0.19 [E]
	I-7E	0.13
	I-8E	0.17
Trifloxystrobin	I-6E	0.08
	I-7E	0.05
	I-8E	0.07
Tebuconazol	I-6E	0.12
	I-7E	0.16
	I-8E	0.09
Propiconazole	I-6E	0.09
	I-7E	0.03
	I-8E	0.07

[A] Fungicides selected for use in this study to test the sensitivity of *U. virens* isolates *in-vitro*.
[B] Isolates assayed for sensitivity to the selection of fungicides.
[C] IC_{50} (fungicide concentration at 1/2 maximal growth) values were calculated from regression curves and the respective means for each fungicide and isolate combination. IC_{50} values were calculated from replicate means across two tests for each isolate fungicide combination.
[D] Mean IC_{50} values of technical grade fungicides were calculated from the means of isolate by fungicide combination.* $CuCO_3$ was omitted from advanced statistical tests.
[E] Mean IC_{50} values of analytical grade fungicides were calculated from the means of isolate by fungicide combination.
Fungicide, isolate and fungicide concentrations were significantly different in these tests at a=0.05.

Table 4.5. Soil for textural analysis was obtained from fungicide seed treatment test plots at the Newport and Pine Tree Research stations in 2011 and 2012.

Location	%Sand	%Silt	%Clay
Newport	3.1	79.2	17.7
Pine Tree	1.6	75	23.7

[A] Micronutrient concentrations were determined by the Mehlich 3 reagent extraction method and analyzed by Spectral Arcos ICP. All data represent the means of nine samples from the Pine Tree Research Station and nine Samples from the Newport Research Station.

Table 4.6. Soil for micronutrient analyses was obtained from fungicide seed treatment test plots at the Newport and Pine Tree Research Stations in 2011 and 2012.

Location	pH	EC	P	K	Ca	Mg
Newport	5.8 a	127.9	79.4 a	99.6	1117.2 a	117.2 a
Pine Tree	7.2 b	189.9	44.3 b	116.9	1776.1 b	279.5 b
LSD	0.25	NS	20.31	NS	153.85	33.52

Location	S	Na	Fe	Mn	Zn	Cu	B [A]
Newport	13.6	29.6 a	363.7	135.9 a	4.07	1.7	0.3 a [B]
Pine Tree	13.9	57.9 b	335.2	235.2 b	4.60	1.4	0.4 b
LSD	NS	19.54	NS	38.69	NS	NS	.09 [C]

[A] Micronutrient concentrations were determined by the Mehlich 3 reagent extraction method and analyzed by Spectral Arcos ICP. All data represent the means of 17 samples from Pine Tree and 21 Samples from Newport.
[B] Variables followed by different letters in a column differ significantly between locations.
[C] NS = not significant (a=0.05).

Table 4.7. The percentage of seedlings colonized by *U. virens*, as determined by nested-PCR in greenhouse studies in years 2011 and 2012. [A]

Treatment [B]	Rate [C]	Greenhouse [D] Newport		Pine Tree [E]	
		CL-151	TMPL	CL-151	TMPL [F]
$CuCO_3$ 2X	3.2g	100 a	21.9 ef	81.3 ab	46.9 c [G]
Quilt® SE 2X	9.2g	78.1 bc	9.4 f	78.1 ab	40.6 d
Rancona® 3.8 FS 2X	7.8g	100 a	15.6 f	81.3 ab	56.3 c
Raxil® MD EC 2X	7.2g	93.8 ab	21.9 ef	100 ab	56.3 c
Stratego® 250 EC 2X	9.2g	81.3 abc	37.5 de	96.9 ab	34.4 d
Tilt® 2X	5.2g	96.9 ab	3.1 f	96.9 ab	56.3 c
Untreated	0.0	62.5 cd	81.3 abc	90.6 ab	90.6 c
P value=		0.0001	0.0001	0.0001	0.0001

[A] Experiments were conducted at the University of Arkansas, Rosen Alternative Pest Control Center, Greenhouse 1.4, fungicide treated seeds for these experiments were planted on June 1, 2011, June 3, 2012. Seedlings were collected three weeks after emergence June 30, 2011 and July 1, 2012.

[B] Seed treatments consisted of copper II carbonate and technical grade fungicides.

[C] The rate in g kg^{-1} (seed) of copper II carbonate or technical grade active ingredient applied to seed.

[D] Experiments were conducted in the Rosen Alternative Pest Control Center in Greenhouse 1.4.

[E] Soils from the Newport and Pine Tree Research Stations were used in this study.

[F] Cl-151 ('Clearfield-151') and TMPL ('Templeton') cultivars were used in this test.

[G] Percent incidence was assessed from eight seedlings collected from each test plot with four replicates and experiments were repeated twice over the course of two years (2011 and 2012) with no statistical significance between years (a=0.05). Analyses were conducted on the average number of seedlings infected for each treatment, location and cultivar combination. There was no significant difference between years at P=0.05, so data from 2011 and 2012 are combined. Means with the same letter are not significantly different (a=0.05).

Table 4.8. The percentage of seedlings colonized by *U. virens* as determined by nested-PCR, collected in field studies in 2011. [A]

Treatment [B]	Rate [C]	Field Location [D]			
		Newport		Pine Tree	
		CL-151	TMPL	CL-151	TMPL [E]
$CuCO_3$	3.2g	28.1 cd	3.1 e	81.3 ab	25 c [F]
Quilt®	9.2g	34.4 c	3.1 e	78.1 ab	3.1 d
Rancona®	7.8g	25 cde	12.5 cde	75 b	25 c
Raxil® MD	7.2g	53.1 b	12.5 cde	78.1 ab	25 c
Stratego®	9.2g	28.1 c	21.9 c	87.5 a	25 c
Tilt®	5.2g	68.8 a	3.1 e	78.1 ab	18.8 b
Untreated	0.0	34.4 c	9.4 de	71.9 b	18.8 b
P value=		0.0086	0.0086	0.0001	0.0001

[A] Fungicide treated seeds for these experiments were planted at the Newport and Pine Tree Research Stations, on June 1 and May 17, 2011 respectively. Seedlings were collected three weeks after planting for nested-PCR analysis.
[B] Treatments consisted of copper II carbonate and technical grade fungicides.
[C] The rate in g kg^{-1} (seed) of copper II carbonate or technical grade active ingredient applied to seed.
[D] Experiments were conducted at the Newport and Pine Tree Research Stations.
[E] Cl-151 ('Clearfield-151') and TMPL ('Templeton') cultivars were used in this test.
[F] Percent incidence was assessed from eight seedlings collected from each test plot with four replicates. Analyses were conducted on the average number of seedlings infected for each treatment, location and cultivar combination.
Means with the same letter are not significantly different (a=0.05).

Table 4.9. The percentage of seedlings colonized by *U. virens* as determined by nested-PCR, collected in field studies in 2012. [A]

Treatments [B]	Rate [C]	Field Location [D]			
		Newport		Pine Tree	
		CL-151	TMPL	CL-151	TMPL [E]
Untreated	0.0	37.5	0	0	0 [F]
Stratego® 2X	9.2g	43.8	-	-	-
Quilt® 2X	9.2g	28.1	-	-	-
Tilt® 2X	5.2g	28.1	-	-	-
Raxil® 2X	7.2g	40.6	-	-	-
Rancona® 2X	7.8g	25.0	-	-	-
Cu_2CO_2 2X	3.2g	62.5	-	-	-
P value=		NS	NS	NS	NS

[A] Fungicide treated seeds for these experiments were planted at the Newport and Pine Tree Research Stations on May 25, 2012 and May 19, 2012 respectively. Seedlings were collected three weeks after planting for nested-PCR analysis.
[B] Treatments consisted of copper II carbonate and technical grade fungicides.
[C] The rate in g kg^{-1} (seed) of copper II carbonate or technical grade active ingredient applied to seed.
[D] Experiments were conducted at the Newport and Pine Tree Research Stations.
[E] Cl-151 ('Clearfield-151') and TMPL ('Templeton') cultivars were used in this test.
[F] Percent incidence was assessed from eight seedlings collected from each test plot with four replicates. Analyses were conducted on the average number of seedlings infected for each treatment, location and cultivar combination.
- = Data not collected in 2012.

Table 4.10. Incidence of rice FS in 'Clearfield-151' and 'Templeton' at the Newport Research Station after treatment of seeds with fungicides, as determined by the number of infected heads per m^2 in 2011. [A]

Treatments [B]	**Rate applied g kg^{-1} seed** [C]		**Clearfield-151 Incidence** [D]	**Templeton Incidence**
$CuCO_3$	1X	1.6	35.6 abc	8.9 z [E]
$CuCO_3$	2X	3.2	38.3 abc	11.9 xyz
Quilt® SE	1X	4.6	27.4 bc	7.5 xyz
Quilt® SE	2X	9.2	38.8 abc	11.6 xyz
Rancona® 3.8 FS	1X	3.9	46.3 ab	7.9 z
Rancona® 3.8 FS	2X	7.8	28.4 bc	10.3 xyz
Raxil® MD EC	1X	3.6	32.6 abc	16.3 xyz
Raxil® MD EC	2X	7.2	35.9 abc	12.1 xyz
Stratego®250 EC	1X	4.6	39.1 abc	11.4 xyz
Stratego® 250 EC	2X	9.2	38.5 abc	10.3 xyz
Tilt®	1X	2.6	52.9 a	9.6 z
Tilt®	2X	5.2	44.4 ab	9.1 z
Untreated		0.0	17.6 c	16.4 xyz
P value=			0.0454	NS

[A] Fungicide treated seeds for these experiments were planted at the Newport Research Station on June 1, 2011 and disease assessments conducted 2 weeks prior to harvest October 2011.
[B] Fungicides that were selected for use in this study.
[C] Rate of fungicide in grams applied to one kg of seed.
[D] Disease incidence was determined from two locations within each test plot at the end of panicle maturation and disease development. Incidence was assessed by averaging two counts of the number of infected panicles in an m^2 of four replicated experimental plots.
[E] Means followed by the same letter in a column are not significantly different (a=0.05).

Table 4.11. Incidence of rice FS in 'Clearfield-151' and 'Templeton' at the Pine Tree Research Station near Colt, AR after treatment of seeds with fungicides, as determined by the number of infected heads per m^2 in 2011. [A]

Treatments [B]	**Rate applied g kg^{-1} seed** [C]		**Clearfield-151 Incidence** [D]	**Templeton Incidence**
$CuCO_3$	1X	1.6	0.6 ab	4.1 xy [E]
$CuCO_3$	2X	3.2	0.9 ab	2.0 xy
Quilt® SE	1X	4.6	1.0 ab	2.0 xy
Quilt® SE	2X	9.2	0.3 ab	1.1 xy
Rancona® 3.8 FS	1X	3.9	0.0 ab	2.5 xy
Rancona® 3.8 FS	2X	7.8	0.9 ab	1.9 xy
Raxil® MD	1X	3.6	0.1 ab	1.3 xy
Raxil® MD	2X	7.2	0.1 ab	3.5 xy
Stratego® 250 EC	1X	4.6	0.4 ab	1.5 xy
Stratego® 250 EC	2X	9.2	0.3 ab	0.4 xy
Tilt®	1X	2.6	0.0 ab	2.6 xy
Tilt®	2X	5.2	0.3 ab	1.1 xy
Untreated		0.0	0.1 ab	3.3 xy
P value=			NS	NS

[A] Fungicide treated seeds for these experiments were planted at the Pine Tree Research Station on May 17, 2011 and disease assessments were conducted 2 weeks prior to harvest, September, 2011.

[B] Fungicides that were selected for use in this study.

[C] Rate of fungicide in grams applied to one kg of seed.

[D] Disease incidence was determined from two locations within each test plot at the end of panicle maturation and disease development. Incidence was assessed by averaging two counts of the number of infected panicles in an m^2 of four replicated experimental plots.

[E] Means followed by the same letter in a column are not significantly different (a=0.05).

Table 4.12. Incidence of rice FS in 'Clearfield-151' and 'Templeton' at the Newport Research Station after treatment of seeds with fungicides, as determined by the number of infected heads per m^2 in 2012. [A]

Treatments [B]	**Rate applied**		**Clearfield-151**	**Templeton**
	g kg^{-1} seed [C]		**Incidence** [D]	**Incidence**
$CuCO_3$	1X	1.6	0.8 bc	0.5 xyz [E]
$CuCO_3$	2X	3.2	2.4 abc	0.3 yz
Quilt® SE	1X	4.6	0.8 bc	0.4 xyz
Quilt® SE	2X	9.2	0.0 c	0.0 z
Rancona® 3.8 FS	1X	3.9	0.4 c	0.6 xyz
Rancona® 3.8 FS	2X	7.8	0.5 c	0.5 xyz
Raxil® MD	1X	3.6	4.3 abc	0.4 xyz
Raxil® MD	2X	7.2	3.5 abc	0.3 yz
Stratego® 250 EC	1X	4.6	0.1 c	1.3 xyz
Stratego® 250 EC	2X	9.2	2.1 abc	0.0 z
Tilt®	1X	2.6	0.3 c	0.5 xyz
Tilt®	2X	5.2	0.3 c	0.3 yz
Untreated		0.0	2.4 abc	0.3 yz
P value=			0.1	0.46

[A] Fungicide treated seeds for these experiments were planted at the Newport Research Station on May 25, 2012 and May 19, 2012 respectfully. Disease assessments were conducted 2 weeks prior to harvest late September, 2012.

[B] Fungicides that were selected for use in this study.

[C] Rate of fungicide in grams applied to one kg of seed.

[D] Disease incidence was determined from two locations within each test plot at the end of panicle maturation and disease development. Incidence was assessed by averaging two counts of the number of infected panicles in an m^2 of four replicated experimental plots.

[E] Means followed by the same letter in a column are not significantly different (a=0.05).

Table 4.13. Incidence of rice FS in 'Clearfield-151' and 'Templeton' at the Pine Tree Research Station near Colt, AR after treatment of seeds with fungicides, as determined by the number of infected heads per m^2 in 2012. [A]

Treatments [B]	**Rate applied g kg^{-1} seed** [C]		**Clearfield-151 Incidence** [D]	**Templeton Incidence**
$CuCO_3$	1X	1.6g	0.0 a	0.3 xy [E]
$CuCO_3$	2X	3.2g	0.0 a	0.3 xy
Quilt® SE	1X	4.6	0.1 a	0.0 xy
Quilt® SE	2X	9.2	0.0 a	0.0 xy
Rancona® 3.8 FS	1X	3.9	0.0 a	0.1 xy
Rancona® 3.8 FS	2X	7.8	0.3 a	0.1 xy
Raxil® MD	1X	3.6	0.1 a	0.3 xy
Raxil® MD	2X	7.2	0.0 a	0.5 xy
Stratego® 250 EC	1X	4.6	0.1 a	0.0 y
Stratego® 250 EC	2X	9.2	0.0 a	0.0 y
Tilt®	1X	2.6	0.0 a	0.0 y
Tilt®	2X	5.2	0.0 a	0.0 y
Untreated		0.0	0.1 a	0.0 y
P value=			0.56	0.32

[A] Fungicide treated seeds for these experiments were planted at the Pine Tree Research Station on May 25, 2012 and May 19, 2012 respectfully. Disease assessments were conducted 2 weeks prior to harvest late September, 2012.

[B] Fungicides that were selected for use in this study.

[C] Rate of fungicide in grams applied to one kg of seed.

[D] Disease incidence was determined from two locations within each test plot at the end of panicle maturation and disease development. Incidence was assessed by averaging two counts of the number of infected panicles in an m^2 of four replicated experimental plots.

[E] Means with the same letter in a column are not significantly different (a=0.05).

Table 4.14. Yield of 'Clearfield-151' and 'Templeton' rice at the Newport Research Station after treatment of seeds with fungicides, as determined by measuring the kg of seed harvested from experimental test plots in 2011. [A]

Treatments [B]	**Rate applied g kg^{-1} seed** [C]		**Clearfield-151 Yield** [D]	**Templeton Yield**
$CuCO_3$	1X	1.6	1745.1 a	2887.4 uv [E]
$CuCO_3$	2X	3.2	1621.4 a	2618.0 x
Quilt® SE	1X	4.6	2206.9 a	2745.2 w
Quilt® SE	2X	9.2	1671.0 a	2397.2 z
Rancona® 3.8 FS	1X	3.9	1821.3 a	2917.1 u
Rancona® 3.8 FS	2X	7.8	1964.7 a	2477.4 y
Raxil® MD	1X	3.6	2358.7 a	2942.2 u
Raxil® MD	2X	7.2	2156.4 a	2810.6 vw
Stratego® 250 EC	1X	4.6	2087.6 a	2607.6 x
Stratego® 250 EC	2X	9.2	1905.4 a	2587.2 x
Tilt®	1X	2.6	2044.3 a	2601.5 x
Tilt®	2X	5.2	1847.5 a	2603.3 x
Untreated		0.0	2242.6 a	2930.7 u
P value=			NS	0.005

[A] Fungicide treated seeds for these experiments were planted at the Newport Research Station on June 1. Yield assessment was completed during harvest in late October 2011.
[B] Fungicides that were selected for use in this study.
[C] Rate of fungicide in grams applied to one kg of seed.
[D] Yield represents adjusted seed weight from four replicated experimental plots for each treatment and cultivar at 12% moisture content.
[E] Means followed by the same letter in a column are not significantly different (a=0.05).

Table 4.15. Yield of 'Clearfield-151' and 'Templeton' rice at the Pine Tree Research Station near Colt, AR after treatment of seeds with fungicides, as determined by measuring the kg of seed harvested from experimental test plots in 2011. [A]

Treatments [B]	**Rate applied g kg^{-1} seed** [C]		**Clearfield-151 Yield**[D]	**Templeton Yield**
$CuCO_3$	1X	1.6	3817.6 ab	3695.4 z [E]
$CuCO_3$	2X	3.2	3787.0 ab	3639.0 z
Quilt® SE	1X	4.6	3695.8 ab	3562.0 z
Quilt® SE	2X	9.2	3662.3 ab	2928.3 z
Rancona® 3.8 FS	1X	3.9	3877.6 ab	3659.7 z
Rancona® 3.8 FS	2X	7.8	3844.1 ab	3444.5 z
Raxil® MD	1X	3.6	3956.8 a	3388.2 z
Raxil® MD	2X	7.2	3834.7 ab	3691.6 z
Stratego® 250 EC	1X	4.6	3879.3 ab	3364.6 z
Stratego® 250 EC	2X	9.2	3674.3 ab	3441.9 z
Tilt®	1X	2.6	3680.6 ab	3426.2 z
Tilt®	1X	5.2	3394.6 ab	3031.9 z
Untreated		0.0	3679.7 ab	3295.8 z
P value=			N.S.	N.S.

[A] Fungicide treated seeds for these experiments were planted the Pine Tree Research Station on May 17, 2011. Yield assessment was completed during harvest in late October 2012.
[B] Fungicides that were selected for use in this study.
[C] Rate of fungicide in grams applied to one kg of seed.
[D] Yield represents adjusted seed weight from four replicated experimental plots for each treatment and cultivar at 12% moisture content.
[E] Means followed by the same letter in a column are not significantly different (a=0.05).

Table 4.16. Yield of 'Clearfield-151' and 'Templeton' rice at the Newport Research Station after treatment of seeds with fungicides, as determined by measuring the kg of seed harvested from experimental test plots in 2012. [A]

Treatments [B]	**Rate applied g kg^{-1} seed** [C]		**Clearfield-151 Yield** [D]	**Templeton Yield**
$CuCO_3$	1X	1.6g	3388.6 a	2782.8 abcdefgh [E]
$CuCO_3$	2X	3.2g	3252.9 abc	2173.3 efghijkl
Quilt® SE	1X	4.6	3023.8 abcde	2230.1 efghijk
Quilt® SE	2X	9.2	1768.7 ijklm	2163.9 fghijkl
Rancona® 3.8 FS	1X	3.9	3267.8 ab	2793.1 abcdefgh
Rancona® 3.8 FS	2X	7.8	2872.5 abcdefg	3185.6 abcd
Raxil® MD EC	1X	3.6	3266.8 ab	2264.5 efghijk
Raxil® MD EC	2X	7.2	3169.6 abcd	2966.5 abcdef
Stratego® 250 EC	1X	4.6	2742.6 abcdefgh	2288.6 efghij
Stratego® 250 EC	2X	9.2	2399.9 abcdefghi	1692.1 jklm
Tilt®	1X	2.6	2745.7 abcdefgh	1420.7 klmn
Tilt®	2X	5.2	2043.8 ghijkl	1337.2 lmn
Untreated		0.0	3191.0 abcd	2341.6 abcdefghij
P value=			0.0001	0.0001

[A] Fungicide treated seeds for these experiments were planted at the Newport Research Station on May 25, 2012. Yield assessment was completed during harvest in late October 2011.
[B] Fungicides that were selected for use in this study.
[C] Rate of fungicide in grams applied to one kg of seed.
[D] Yield represents adjusted seed weight in kg from four replicated experimental plots for each treatment and cultivar at 12% moisture content. Marginally significant differences in cultivars were observed for yield at Newport (P=0.04) and the effects were not treated separately.
[E] Means with the same letter in a column are not significantly different (a=0.05).

Table 4.17. Yield of 'Clearfield-151' and 'Templeton' rice at the Pine Tree Research Station near Colt, AR after treatment of seeds with fungicides, as determined by measuring the kg of seed harvested from experimental test plots in 2012.[A]

Treatments [B]	**Rate applied g kg^{-1} seed** [C]		**Clearfield-151 Yield** [D]	**Templeton Yield**
$CuCO_3$	1X	1.6g	3127.2 abcd	2907.0 abcd [E]
$CuCO_3$	2X	3.2g	3340.4 a	2594.8 abcdefgh
Quilt® SE	1X	4.6	3106.1 abcd	1572.5 h
Quilt® SE	2X	9.2	2307.3 cdefgh	1824.3 gh
Rancona® 3.8 FS	1X	3.9	3304.6 a	3097.8 abcd
Rancona® 3.8 FS	2X	7.8	3214.0 ab	2815.2 abcdef
Raxil® MD EC	1X	3.6	3231.0 ab	2715.4 abcdefg
Raxil® MD EC	2X	7.2	3169.7 abc	2750.3 abcdef
Stratego® 250 EC	1X	4.6	2266.7 defgh	2000.3 efgh
Stratego® 250 EC	2X	9.2	1943.4 fgh	1959.8 fgh
Tilt®	1X	2.6	2342.3 bcdefgh	1200.2 h
Tilt®	2X	5.2	1232.1 h	1344.1 h
Untreated		0.0	2599.7 abcdefgh	2955.0 abcd
P value=			0.0001	0.0001

[A] Fungicide treated seeds for these experiments were planted at the Pine Tree Research Station on May 19, 2012. Yield assessment was completed during harvest in late October 2012.
[B] Fungicides that were selected for use in this study.
[C] Rate of fungicide in grams applied to one kg of seed.
[D] Yield represents adjusted seed weight in kg from four replicated experimental plots for each treatment and cultivar at 12% moisture content. No significant differences between cultivars were observed for yield at Pine Tree for this study (a=0.05).
[E] Means with the same letter in a column are not significantly different (a=0.05).

Table 4.18. Effects of fungicide seed treatments on rice seedling stand count for 'Clearfield-151' (Cl-151) and 'Templeton' at the Newport and Pine Tree Research Stations in 2012. [A]

		Stand count [B]		
		Newport		Pine Tree [C]
Treatment	**Rate**	**Cl-151**	**Templeton**	**Cl-151-Templeton**
1 Untreated	0	19.1 g	31.0 abcd	21.0 a [D]
2 1X Stratego® 250 EC	4.6 g/kg	5.4 hi	10.5 h	12.5 c
3 2X Stratego® 250 EC	9.2 g/kg	4.6 hi	2.8 i	11.3 c
4 1XQuilt® SE	4.6 g/kg	7.8 hi	6.5 hi	15.0 bc
5 2X Quilt® SE	9.2 g/kg	3.0 i	3.9 hi	10.7 c
6 1X Tilt®	2.6 g/kg	4.3 hi	4.4 hi	10.0 c
7 2X Tilt®	5.2 g/kg	1.5 i	2.1 i	11.4 c
8 1X Raxil® MD	3.6 g/kg	26.8 abcdef	29.0 bcd	21.6 a
9 2X Raxil® MD	7.2 g/kg	22.1 efg	31.2 abc	22.3 a
10 1X Rancona® 3.8 FS	3.9 g/kg	24.6 cdefg	37.1 a	24.8 a
11 2X Rancona® 3.8 FS	7.8 g/kg	21.3 fg	31.6 ab	23.7 a
12 1X $CuC0_3$	1.6 g/kg	21.9 fg	24.5 defg	23.9 a
13 2X $CuC0_3$	3.2 g/kg	20.4 fg	28.6 bcde	19.1 ab
P value=		0.0001	0.0001	0.0001

[A] Fungicide treated seed for these experiments was planted at the Newport and Pine Tree Research Stations on May 25, 2012 and May 19, 2012 respectfully. Stand counts were taken 3 weeks after emergence, approximately 4 weeks after planting.

[B] Stand counts were determined by visual counts of the number of seedlings in a one m row twice within each experimental plot.

[C] Stand counts were taken from all fungicide seed treatment experimental plots at the Newport and Pine Tree Research Stations.

[D] Stand count means followed by different letters in a column differ significantly by Fisher's protected LSD (a=0.05). Stand count means were significantly different between cultivars from the Newport Station (P=0.0001) but not from the Pine Tree Station (P=0.13) due to variation around the stand count means. Cultivars from Pine Tree were combined due to this effect (a=0.05).

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Vitae

Oral Presentation

"Investigating effective management strategies for *Ustilaginoidea virens* on *Oryza sativa*" Oral student competition at the 89th Annual SDAPS Meeting, in Birmingham, AL. February 5-6, 2012. Department of Plant Pathology, University of Arkansas, Fayetteville, AR 72701.

Posters

A. C. Jecmen and D. O. TeBeest. 2012. Molecular based tools for diagnosing infection of rice by *Ustilaginoidea virens*. Poster presentation at the Rice Technical Working Group 34th Meeting, in Hot Springs, Arkansas. February 27- March 1, 2012. Department of Plant Pathology, University of Arkansas, Fayetteville, AR.

A. C. Jecmen and D. O. TeBeest. 2014. Discovery of a white false smut infecting rice in Arkansas. Poster presentation at the 91st Annual SDAPS Meeting, in Dallas, TX. February 2-3, 2014. Department of Plant Pathology, University of Arkansas, Fayetteville, AR.

D. O. TeBeest and A. C. Jecmen. 2013. Reaction of selected rice cultivars to infection by *Ustilaginoidea virens*. Poster presentation at the 90th Annual SDAPS Meeting, in Baton Rouge, LA. February 8-10, 2013. Department of Plant Pathology, University of Arkansas, Fayetteville, AR.

D. O. TeBeest and A. C. Jecmen. 2014. Characterization of virulence phenotypes of isolates of Colletotrichum sublineolum from Arkansas on selected grain sorghum hybrids. Poster presentation at the 91st Annual SDAPS Meeting, in Dallas, TX. February 2-3, 2014. Department of Plant Pathology, University of Arkansas, Fayetteville, AR.

Reports

TeBeest, D. O., A. Jecmen and M. Ditmore. 2011. Infection of Rice by the False Smut Fungus, *Ustilaginoidea virens*. In: B.R. Wells Rice Research Studies 2010. R. J. Norman, J.F. Meullenet and K.A.K. Moldenhauer, Eds. Arkansas Agricultural Experiment Station Research Series. 591:70-81.

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Referred Journal Article

Jecmen A. C. and D. O. TeBeest. 2014. Preliminary report on the occurrence of a white smut infecting rice in Arkansas. Short Communication in Journal of Phytopathology. (Accepted for publication March 21, 2014).