Infection of Rice by the False Smut Fungus, *Ustilaginoidea virens*

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**ABSTRACT**

False smut, caused by *Ustilaginoidea virens*, was recently found in Arkansas and it has now been identified in most counties in which rice is grown. The disease is normally identified by the presence of orange and black sporophores that appear on the maturing heads or panicles. The disease cycle for this emerging disease of rice is poorly understood and this lack of understanding has hampered development of effective management strategies for this disease. From 2008 through 2010, field and greenhouse experiments were conducted with the following objectives: 1) to describe the development of the symptoms of disease on two rice cultivars with different pedigrees at two locations, 2) to examine the potential for the disease to be seedborne and/or soilborne, and 3) to begin preliminary investigations on assessing disease severity and its impact on yield on selected cultivars across two locations in Arkansas. The results of these experiments suggest that false smut is both seedborne and soilborne and that spores can persist on seeds and in soil for several years. These results suggest several ways in which cultural, genetic, and chemical strategies might be investigated using molecular-based methods to better understand and manage this emerging disease.

**INTRODUCTION**

False smut of rice is caused by the fungus *Ustilaginoidea virens*. This clavicipitaceous pathogen has been in the United States for many years, but was first reported in Arkansas in 1997 (Cartwright and Lee, 2001; Wilson et al., 2005). It has been previously reported that this disease does not typically affect yield, but quality issues remain important due to production of ustiloxin, a microtubule inhibitor toxic to animals (Koiso
et al., 1994; Miyazaki et al., 2009). More recently, the literature suggests that yields can be significantly reduced (Zhou et al., 2003).

Knowledge concerning the disease cycle and epidemiology of *U. virens* is minimal and incomplete or even contradictory (Lee and Gunnell, 1992). Spore balls are believed to germinate late in the growing season and infect rice flowers (Brooks et al., 2010; Lee and Gunnell, 1992; Cartwright and Lee, 2001). Some investigators have successfully inoculated plants by injecting boots prior to flowering or by inoculating flowering panicles (Ikegami, 1963). It has been reported that the number of spore balls found on mature panicles or the degree of blanking (chaffing) may be related to the level of resistance in the cultivar (Cartwright et al., 2003).

Understanding the disease cycle is critical in order to maximize benefits of control strategies. In 2005, Schroud and TeBeest reported that spores of the fungus germinated on rice roots and infected plants within a few hours after inoculation. In 2007, Ditmore et al. showed that three-week-old seedlings and mature plants grown from infested seeds in pasteurized soils contained DNA sequences consistent with those of *U. virens* as shown by polymerase chain reaction (PCR). These results also suggested that rice plants grown from infested seeds could be infected by the fungus when inoculated this way.

The overall goal of the research reported here was to investigate the disease cycle so that a more complete understanding of how the disease develops can improve management. The specific objectives of the work reported here were as follows: 1) to describe the development of the symptoms of disease on two rice cultivars with different pedigrees at two locations, 2) to examine whether the disease might be seedborne and/or soilborne, and 3) to begin preliminary investigations on the assessment of disease resistance on selected cultivars across two locations in Arkansas.

**PROCEDURES**

Three cultivars, ‘Cheniere’, ‘Francis’, and ‘Cybonnet’, were used in preliminary field and greenhouse tests conducted in 2008 and 2009. Two other cultivars, ‘Clearfield 151’ and ‘Templeton’, were used in the field tests conducted in 2010 as described below. For the preliminary greenhouse tests and the 2008 field test at the Rice Research and Extension Center, seeds of Cheniere and Francis were used from a single seed lot obtained from the foundation seed program at the Rice Research and Extension Center in Stuttgart, Ark., before false smut was found in Arkansas. These seeds had been stored at -4 °F in our laboratory. Seedlings grown from these seeds in pasteurized soils were verified as healthy and not infected by false smut by PCR tests as described below. For the preliminary field test conducted at Hogue Lake in 2009, seeds of Cybonnet rice were obtained from a commercial source. For the field tests conducted in 2010, cleaned and naturally infested seeds of two rice cultivars, Clearfield 151 and Templeton, were obtained by Dr. R. Cartwright, UA Cooperative Extension Service from several Arkansas rice producers in 2010. Seeds of these cultivars were stored at room temperature (24 °C) until used. Infested seed lots were visibly contaminated with sporophores and some seeds were visibly contaminated (blackened) with false smut spores.
Greenhouse Tests

Greenhouse experiments were conducted to determine if Cheniere seedlings grown from healthy seeds were infected by spores in pasteurized soils amended to contain different levels of inoculums. In these experiments, a greenhouse soil mix composed of a Captina silt loam:potting soil:sand (4:4:1) was used. Forty grams of air-dried greenhouse soil mix was placed into 2 cm × 2 cm peat pots. Since viable and proven cultures of *U. virens* known to be virulent to rice were not available, spores of the fungus were collected from pseudomorphs found on panicles of field-grown Francis. Panicles and seeds containing the pseudomorphs had been stored in the laboratory at 24 °C until used. Pseudomorphs were suspended in water and mixed briefly in a Waring blender to loosen spores from the pseudomorph. Final spore concentrations were determined by hemacytometer.

Treatments consisting of 10 mls of spore suspensions were added to each peat pot to achieve final concentrations of 0, 25, 250, 2,500, and 25,000 spores/g air-dried soil. The soil in each peat pot was thoroughly mixed after infestation. Five to six seeds of Francis obtained from the foundation seed program and known to be free of false smut were planted into each peat pot 24 to 48 hr after infestation. After planting, all peat pots were uniformly watered with tap water and placed in a greenhouse at 28 °C with a 15-hour daylength provided by halogen lamps. All treatments were planted in three peat pots (replications) and the experiment was conducted twice. Three weeks after planting, three seedlings were harvested from each peat pot (nine seedlings per treatment). Seedlings were immediately placed in plastic bags and stored at -20 °C until tested for the presence of *U. virens* DNA as described below. Plants were not grown to maturity in these greenhouse tests but previous results showed that the fungus could be found in leaf, stem, and neck tissues after seedling infection was established (Ditmore et al., 2007; Ditmore and TeBeest, 2006).

Preliminary Field Tests Conducted in 2008 and 2009

Preliminary field tests were conducted in 2008 at the Rice Research and Extension Center, Stuttgart, Ark., (Dewitt silt loam) and in a commercial field in 2009 near Hogue Lake, Ark., (Hilleman silt loam) to examine if false smut was soilborne. In 2008, 10 grams of Francis seeds were planted in mid-May in three rows 50-cm long with 15-cm between rows in 120 microplots in a field with a history of the disease. The field had been planted in rice in 2006 and in corn, *Zea maydis*, in 2007 (S. Brooks and M. Anders, pers. comm.). The plots were grown in a single paddy and irrigated and fertilized as recommended (Cartwright et al., 2003). In 2009, field tests were conducted near Hogue Lake, Ark., with cultivar Cybonnet. The field had a history of false smut and had been planted in soybean in 2008 (R. Cartwright, pers. comm.). In 2009, plots were 25-feet long by 5-feet wide and planted with 100 grams of untreated seeds. Seedlings were sampled 3 weeks after emergence and tested for infection by false smut as described below. The total numbers of sporophores in the plots were counted at harvest.
**Field Tests Conducted in 2010**

Field tests were conducted in 2010 at the Pine Tree Research Station, Colt, Ark. (Calloway silt loam) and at the White County Research station near Newport, Ark., (Forrestdale silt loam) with cultivars Clearfield 151 and Templeton. The experiments at Pine Tree were conducted in a field with a history of false smut, while the experiments conducted at Newport were in a field that had never been planted in rice. Treatments consisted of four seed treatments and were planted in a randomized complete block design. Only the results with Clearfield 151 will be reported here.

**Polymerase Chain Reaction**

**Detection of *U. virens* in Rice Seedlings**

In the greenhouse tests described above, plants were tested for the presence of DNA consistent with *U. virens* in order to determine if plants were infected or colonized in the absence of symptoms. DNA extraction was performed using the DNeasy extraction kit (Qiagen, Germantown, Md.) after grinding in liquid nitrogen. Polymerase chain reaction primers specific for *U. virens* were selected by the comparison of sequence alignments from the ITS region of isolates collected from diverse geographical origins and rice varieties (Ditmore et al. 2007; Zhou et al. 2003). Nested PCR was performed using puReTaq Ready-To-Go PCR Beads (GE Healthcare Bio-Sciences Corp., Piscataway, N.J.) in a PTC-200 Gradient Thermal cycler (MJ Research, Quebec, Canada). In 2010, infection of seedlings was determined following PCR amplification of samples by procedures established as described above and by protocols described by Ashizawa et al. (2010) and Zhou et al. (2003). A seedling (or tissue sample) was considered to be infected or colonized by *U. virens* if bands consistent with infected controls were found in either procedure in each sample in either the primary or nested amplification. Negative controls consisted of seedlings grown from seed collected prior to the occurrence of false smut in Arkansas.

**Symptom Development and Disease Severity**

In order to determine when signs and/or symptoms of false smut appeared in the tests, all plots at Pine Tree and Newport were examined on a 5- to 7-day interval beginning at the cracking stage and ending when the panicles were in the soft dough stage, approximately 2- to 3-wk before harvest. The data recorded described when the first sporophores began to emerge in each plot, growth stage, and treatment. The total number of infected panicles per square meter (an average of two separate counts per plot) was determined for each plot three weeks before harvest.

Data was statistically analyzed using Proc GLM and Analysis of Variance according to SAS (SAS Institute, Cary, N.C.).
RESULTS AND DISCUSSION

Since little is known about the disease cycle, preliminary experiments were conducted to better understand how rice plants were initially infected by examining seedlings and plants that showed no symptoms of infection developing and using histological and molecular techniques. Schrout and TeBeest (2005) had previously shown that rice roots were infected by spores. Zhou et al. (2003) and Ashizawa et al. (2010) have established that rice was infected by *U. virens* before heading using molecular techniques similar to those described by Willits and Sherwood (1999) who showed that *Ustilago hordei* infected barley plants from seeds. Previously, Ditmore et al. (2007) showed that seedlings grown from infested seeds were infected by *U. virens* in greenhouse tests.

**Greenhouse Tests**

This is the first report which shows that *U. virens* could be detected in 3-wk old rice seedlings grown from healthy seeds in infested soils in greenhouse tests (Table 1). The data show that as few as 25 spores/g pasteurized soil resulted in the infection of approximately 44% of the emerging seedlings. Concentrations of 250 or more spores/g soil resulted in 100% infection of the plants. These results led to the preliminary field tests conducted in 2008 and 2009 at Stuttgart and Hogue Lake.

**Preliminary Field Tests Conducted in 2008 and 2009**

The results of the preliminary field tests conducted at the Rice Research and Extension Center at Stuttgart that were planted in mid-May of 2008 showed that a significant proportion of the rice seedlings of Francis, grown from healthy seeds planted in microplots in a field with a history of disease were infected by false smut at harvest (data not shown). The PCR tests indicated that plants were infected within 3 weeks of emergence. In 2009 at Lake Hogue in larger plots established in a field with a history of false smut, we tested seedlings by PCR as described above and we found that 75% of the seedlings were infected by false smut soon after emergence in all plots. And, as in 2008, the sporophores of false smut began to appear near heading. We collected all sporophores from the plots and found that the number of sporophores ranged between 24 and 108/plot (data not shown) in the plots that were not treated with fungicides as recommended. The results of the two preliminary tests conducted in two different locations over two years showed that simply planting rice in a field with a history of disease can result in infection of rice by *U. virens*. In both years, in both locations and with two separate cultivars, significant amounts of infection were found in plots that were not inoculated with spore suspensions at flowering. It is also important to note that seedlings were infected as measured by PCR testing in both tests within weeks after emergence and that neither test was located near other rice fields. These conditions suggest that the fungus is soilborne and can invade seedlings even if infections also occur at the flowering stage.
Field Tests Conducted in 2010

Field tests were conducted in 2010 at Pine Tree and at Newport, Arkansas with Clearfield 151 and Templeton to determine if the fungus was seedborne and to determine if seed treatments might be effective in reducing infection. We are only reporting the data from the Clearfield 151 tests in Table 2. According to our nested PCR analysis, approximately 50% of the seedlings grown from infested seeds at Pine Tree were already colonized by *U. virens* within three weeks after emergence. Similarly, 15 of the 30 (50%) seedlings collected at Newport at three weeks after emergence were colonized by *U. virens*. These levels of colonization of seedlings corresponded closely with levels measured in greenhouse tests in which seeds were planted in pasteurized soils. Treatment of seeds with selected fungicides did not appear to have an impact although the number of samples was very low.

The PCR evidence of colonization of seedlings three weeks after emergence was confirmed by counting the number of infected heads at harvest. The occurrence of false smut at Newport in a test that was planted into a field which had never been planted into rice is a strong indication that false smut is seedborne, even if seeds are cleaned. At Pine Tree, we counted the number of infected heads per square meter in the plots and found that the number of infected heads ranged from 13 (infested seeds treated with Maxim/Apron) to 26.5/m² (infested seeds treated with Rancona/Maxim/Nipsit). In contrast, the incidence of disease at Newport was unexpectedly high since it occurred in a field with no history of disease. The incidence of disease at Newport was visually estimated by determining the percentage of panicles infected in the plots. At Newport, the incidence of disease ranged from 43.8% to 66.9% of the heads infected.

Yield data were obtained by harvesting center rows within all plots and each data point represents an average of 4 replications of each treatment at each location. At Pine Tree, adjusted yields ranged between 6181 lb/acre to 6721 lb/acre. There were no significant differences between treatments at Newport although the yields were much lower than yields at Pine Tree even though all plots were planted with the same treatments and seed lots. At Newport, yields ranged between 3395 lb/acre and 5975 lb/acre. The precise effects of false smut on yields could not be fully determined in these tests because all plots were similarly infected at heading.

**SIGNIFICANCE OF FINDINGS**

False smut is an emerging and increasingly significant pathogen of rice in Arkansas. The disease was first reported in a single field in White County, Ark., in 1997. It is now considered to be widespread within the state. Our results show that spores from the disease germinate on and invade roots of rice seedlings (Schroud and TeBeest, 2005) in the field and that the disease can be both soilborne as spores and seedborne as spores. There is little information in the literature on the significance or role of soilborne and seedborne spores because it has long been assumed that rice became infected by spores.
infecting flowers (Cartwright et al., 2001; Lee and Gunnell, 1992). Taken together, the results of our greenhouse, laboratory, and field tests suggest several ways in which cultural, genetic, and chemical strategies might be investigated to better understand and more effectively manage this emerging disease.

ACKNOWLEDGMENTS

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LITERATURE CITED


Fig. 1. The image shows the level of infection of rice panicles by *U. virens* of cultivar Clearfield 151 at Pine Tree in 2010. The plants were grown from cleaned or visibly infested seeds and the level of infection shown is consistent with the severity of the disease observed at harvest in all 40 plots planted with cleaned and infested seeds.
Fig. 2. This image shows the level of infection of Clearfield 151 by *U. virens* observed on plants grown from cleaned or visibly infested seeds at Newport, Ark., in 2010. In contrast to the levels of infection observed at Pine Tree shown in Fig. 1, at Newport approximately half of the panicles were infected by *U. virens* and there were a larger number of sporophores on each of the infected panicles in all of the 40 plots grown from both seed types.
Table 1. The number of Cheniere seedlings infected by *U. virens* after planting healthy seeds into pasteurized potting soils.

<table>
<thead>
<tr>
<th>Spores (no./gm soil)</th>
<th>Plants infected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (a)</td>
</tr>
<tr>
<td>25</td>
<td>44 (b)</td>
</tr>
<tr>
<td>250</td>
<td>100 (c)</td>
</tr>
<tr>
<td>2500</td>
<td>100 (c)</td>
</tr>
<tr>
<td>2,500</td>
<td>100 (c)</td>
</tr>
</tbody>
</table>

* The number of spores per gram soil was established by infesting spores obtained from sporophores collected from Francis with 10 mls of water suspensions of spores.

* The percentage of plants infected by *U. virens* was determined by nested PCR analysis of DNA extracted from 18 seedlings (3 seedlings per replication and 9 seedlings for each treatment in two separate experiments) according to protocols published by Zhou et al. (2003). The occurrence of a band equivalent to the expected amplicon in the first or second amplification cycle was considered as evidence of infection. The values in the column showing percent infection followed by the same letter are not significantly different at $P = 0.05$. 
Table 2. Results of a preliminary field test conducted in 2010 at Pine Tree and Newport, Ark., to determine if seed treatments reduced severity of infection by false smut at harvest.

<table>
<thead>
<tr>
<th>Seed type</th>
<th>Treatment</th>
<th>Seedlings infected&lt;sup&gt;x&lt;/sup&gt;</th>
<th>Infected heads&lt;sup&gt;w&lt;/sup&gt;</th>
<th>Yield&lt;sup&gt;v&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Newport (no.)</td>
<td>Pine Tree (no.)</td>
<td>Newport (no./m)</td>
</tr>
<tr>
<td>Cleaned</td>
<td>Untreated controls</td>
<td>ND&lt;sup&gt;u&lt;/sup&gt;</td>
<td>ND</td>
<td>18.8&lt;sup&gt;t&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Rancona</td>
<td>ND</td>
<td>ND</td>
<td>16.9</td>
</tr>
<tr>
<td></td>
<td>Metastar</td>
<td>ND</td>
<td>ND</td>
<td>20.6</td>
</tr>
<tr>
<td></td>
<td>Maxim/Apron</td>
<td>ND</td>
<td>ND</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td>Rancona/Maxim/Nipsit</td>
<td>ND</td>
<td>ND</td>
<td>19.6</td>
</tr>
<tr>
<td>Infested</td>
<td>Untreated controls</td>
<td>2/6</td>
<td>3/6</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>Rancona</td>
<td>2/6</td>
<td>3/6</td>
<td>18.9</td>
</tr>
<tr>
<td></td>
<td>Metastar</td>
<td>3/6</td>
<td>3/6</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>Maxim/Apron</td>
<td>5/6</td>
<td>3/6</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>Rancona/Maxim/Nipsit</td>
<td>3/6</td>
<td>3/6</td>
<td>26.5</td>
</tr>
</tbody>
</table>

<sup>z</sup> Seeds were obtained from a commercial source. Cleaned seed had been sieved to remove sporophores while infested seeds remained visibly contaminated with sporophores of *U. virens*.

<sup>y</sup> Treatments were made in the laboratory and applied at rates recommended by industry using the plastic bag method. One kg of seed was treated with each treatment/seed type combination and 100 gram samples were randomly dispersed into ten 100-gram samples. Four 100-gram samples were used at random to plant the plots at each location.

<sup>x</sup> Seedling infection was determined by PCR analysis of samples collected three weeks after planting. The numerator indicates how many of the six seedlings that were collected from a single plot of each treatment planted with infested seeds were positive for DNA consistent with *U. virens*. DNA was extracted according to protocols established by Epicentre Technologies (Madison, Wis.). Primary and nested PCR was conducted as described by Zhou et al. (2003) and Ashizawa et al. (2010). A seedling was considered infected if an appropriate band (consistent with controls) appeared on agarose gels in either primary or secondary reactions.

<sup>w</sup> The numbers of infected heads were determined from two separate counts per plot and 4 plots per treatment at each location. At Pine Tree, the actual numbers of infected heads per square meter were manually counted while at Newport, the number of infected heads was estimated visually.

<sup>v</sup> Yields were determined after machine harvesting 4 center rows per plot at Pine Tree and 5 center rows per plot at Newport.

<sup>u</sup> ND indicates samples not yet tested.

<sup>t</sup> All data were subjected to Proc GLM and ANOVA and all values within a column are not significantly different at *P* = 0.05.