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Real-time PCR analysis of maize seedlings for assessment of seed treatment efficacy and genetic resistance to infection by Sphacelotheca reiliana

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Real-time PCR analysis of maize seedlings for assessment of seed treatment efficacy and genetic resistance to infection by *Sphacelotheca reiliana*

by

Samuel James Anderson

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Plant Pathology

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Iowa State University
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ABSTRACT

Head smut caused by *Sphacelotheca reiliana* (*S. reiliana*) is a devastating disease of maize that has been reported to account for up to 80% yield loss in fields. This disease exists in many major maize growing regions and the disease incidence is rising. It has become a major constraint in seed production areas as the spores it produces adhere to and contaminate seeds. The fungus infects systemically during the seedling stage and usually does not produce symptoms until the plant reaches its reproductive stages when the fungus replaces the inflorescences with large black masses of teliospores known as sori. When the sori rupture spores contaminate soil and seed which will then serve as a source of inoculum and a dispersal unit for the fungus. Disease management options include host resistance and fungicidal seed treatments but both of these strategies need improvement; resistance in most commercial hybrids is not complete and more seed treatments need to be tested for efficacy against this disease. The strategies for evaluating both of these options require considerable time expenditure, as the plant does not show symptoms of infection throughout the growing season. With this in mind a seedling assay for the evaluation of host resistance and seed treatment efficacy would be advantageous.

A seedling assay was developed using two novel inoculation techniques to consistently and efficiently infect maize seedlings with *S. reiliana*. Seeds were planted in a growth chamber under controlled conditions that were conducive to infection. Presence of the fungus within seedling tissue was detected by a sensitive and specific real-time polymerase chain reaction. Seed inoculation provided more consistent results than soil infestation.
Five commercially available fungicidal seed treatments were tested for their efficacy against head smut infection, using the seedling assay. Infection rates of inoculated, mock-treated controls varied between 60 – 80%. Incidence of infection. Tebuconazole, fludioxonil and sedaxane seed treatments reduced incidence of seedling infection by *S. reiliana* (*p* = 0.01) whereas azoxystrobin did not. Three rates, representing the ranges of rates on the product labels of both tebuconazole and sedaxane, were also tested. No differences in seedling infection were detected among the rates (*p* < 0.01). Sedaxane (0.1 mg·seed$^{-1}$) eliminated detectable seedling infection when teliospores of *S. reiliana* were applied at a rate of 10 spores·seed$^{-1}$. The assay was effective for seed treatment efficacy testing and can be a useful tool to screen experimental fungicides for their efficacy against head smut.

The seedling assay was used to evaluate five maize inbred lines for their ability to resist infection by *S. reiliana*. Inbreds included A619, B68, B73, Mo17 and Va26. Mo17 had lower incidence of infection compared to the other four inbreds (*p* < 0.01) but no other significant differences between inbreds were detected.

We also evaluated maize tissue from a susceptible hybrid in field trials (in California and Texas) with inoculated seed and non-inoculated seed planted into *S. reiliana* infested soil. Seedling and ear shoot tests showed that *S. reiliana* DNA was present at rates as high as 54% of seedlings in Texas and 11% in California and 30% of ear shoots in Texas and 8% in California; despite these rates of infection no symptoms were observed by field scouting. This assay may be useful to compare initial infection of among maize genotypes, but the lack of symptom development indicates that seedling and ear shoot infection are not predictive of disease.
incidence. A high percentage of maize plants infected with *S. reilianum* may not exhibit any symptoms and this knowledge is important for designing management strategies. Further work is needed to better understand the implications of this result on the epidemiology of this disease and the mechanisms for resistance.
CHAPTER 1. INTRODUCTION

General Introduction

Maize (Zea mays L.) is a worldwide staple crop and its production is important for use as food, feed, and fuel. In 2012, the United States alone cultivated maize on over 35 million ha of land and produced nearly 274 million metric tons (FAO, 2012). Uses of maize abound; it is utilized to make a litany of food and feed products for human and non-human animal consumption including high fructose corn syrup, animal feed and maize starches are used in many food applications; it is used widely in industrial processes including the production of ethanol for fuel and beverage, production of solvents and synthesis of plastics; maize is also intensively studied for advances in genetics, agronomy, and biochemistry.

Technological advances in both breeding and agrochemicals have increased maize yield on a yearly basis. Success in breeding for maize lines resistant to disease has been bolstered by molecular breeding techniques such as marker assisted selection whereby a gene or set of genes (QTL) is identified that confers some level of resistance to a particular disease and is selectively bred into the genome of maize lines. Agrochemicals, especially fungicides, have become a crucial component by which disease pressure is relieved. Fungicides are used in several ways: foliar sprays, in-furrow application, or as seed treatment. The use of seed treatments is extremely high; nearly all commercial maize grown in the U.S. and other developed countries is planted with a seed treatment. Prevention measures notwithstanding, maize production is still threatened by disease. In 2012 yield loss due to disease in the United States was estimated at 8.8% which represents just over 1.07 billion bushels or 27 million metric tons (Mueller and Wise, 2013).
Maize head smut is an important disease in many major maize growing regions around the world. In 2012 maize head smut caused the loss of 38,000 tons of maize in the U.S. and Canada (Mueller and Wise, 2013). The disease is easily spread by its durable teliospores that are deposited in the soil of affected fields, dispersed by wind and by adhering to the seeds of uninfected plants. Head smut disease incidence has been rising and measures to prevent its spread need to be strengthened in order to maintain production of grain and healthy maize seed.

Organization of Thesis
This thesis follows a journal paper format. Chapter 1 contains a general introduction to the thesis and a review of literature that is pertinent to the thesis including background information and previous work in fungicides and host resistance within the head smut pathosystem. Chapter 2 is a paper regarding seed treatment efficacy that will be submitted as a journal article (Plant Disease). Chapter 3 is a paper regarding the use of PCR to compare S. reiliana infection among maize genotypes to be submitted to the journal Crop Science.

Pathogen Background and Epidemiology
Head smut, caused by the fungus Sphacelotheca reiliana (Kühn) Clint. (syn. Sporisorium reilianum (Kühn) Langdon and Fullerton), is an important disease of maize and other crop plants in the family Poaceae; it is found almost everywhere that maize or sorghum is grown extensively but is endemic to China and North America; in the United States it occurs regularly in drier maize production areas such as California, Colorado, Kansas, Nebraska, Oklahoma, and Texas. Although it still remains that a majority of the cases of head smut are restricted to these states, the disease has been reported in Minnesota and other Midwestern states (Potter, 1914;
Halisky, 1963; Martinez, 2002). Head smut is an important factor in the production of maize; it has been reported to account for up to 80% yield loss in fields (Frederiksen, 1977; Stromberg, 1984). *S. reiliana* infects maize, sorghum and sudan grass, causing the replacement of female flowering organs with black galls known as sori, which are filled with teliospores. These galls replace inflorescences of infected plants and as such lead to sterility within the affected plant. Given that the fungus replaces the ear of the maize plant; disease incidence is directly related to yield loss (Martinez, 2002). Disease spread and incidence is increasing and as such tools to improve manageability of the disease are being sought.

*Sphacelotheca reiliana* is a basidiomycetous fungus in the family *Microbotryaceae*; more importantly it is in a subdivision known as the Teliomycetes (Clinton, 1902). Teliomycetes (smuts and rusts) produce robust teliospores; these spores can withstand variation in temperature, moisture and sunlight and still maintain viability for years, which is an important factor in the disease cycle. Teliospores of *S. reiliana* can adhere to surfaces such as the seed coat and are subsequently transported to new areas with the possibility of spreading epidemics (Halisky, 1963).

Changes in nomenclature have resulted in the use of several different binomial designations for *S. reiliana*. The fungus has been referred to as: *Ustilago reiliana*, *Sporisorium reilianum*, *Sorosporium reilianum*, *Sporosorium holci-sorghii*, *Sphacelotheca reiliana* f. sp. *zeae* (a variant specific to maize), and *Sphacelotheca reiliana* var. *reiliana* (a variant specific to sorghum). *Sphacelotheca reiliana* is one of the most commonly used binomials for isolates
infecting maize, sorghum and sudan grass; therefore *S. reiliana* will be used hereafter when discussing this fungus (Species Fungorum, CABI, 2013).

**Disease Cycle**

In maize, the black echinulate teliospores of *S. reiliana* are dispersed during harvest and subsequently spread by the wind or by adhering to the seed coat. They overwinter in the soil or potentially on contaminated seeds. Temperatures of 23-30° C have been shown to be optimum for germination, when the spores form a basidium containing four cells, also known as a promycelium. This structure produces yeast-like haploid basidiospores which reproduce via budding. Switching from the yeast-like stage to a mycelial growth stage has been shown to occur in response to a soil water potential decrease. When hyphae from two compatible strains (differing at mating loci a & b) meet, hyphal conjugation tubes are developed and fuse to form a diploid infection hypha (Halisky, 1963; Schirawski, 2005).

Successful infection takes place when maize is in the seedling stage; *S. reiliana* is thought to infect only during the first 6 weeks of maize growth (Matyac, 1985). In the initial stages of the infection cycle, hyphae form a fungal sheath around the roots and enter the root epidermis via enzymatic degradation of the epidermal cell walls. Subsequent to infection, *S. reiliana* colonizes the plant systemically as a biotroph and hyphae of the fungus exist mostly intercellularly but do not effect a change in plant cells. The fungus grows systemically along with the vegetative apex of the maize plant until vegetative growth stages are complete and the plant reproductive stages begin (Martinez, 2002).
After transition of the plant from the vegetative growth stages to the initial reproductive stage, *S. reiliana* begins necrotrophic pathogenesis wherein fungal hyphae invade host cells and destroy them leading to the last phase of fungal development: sporogenesis. During this stage, the fungus destroys host cells within the plant inflorescences; cells that would have given rise to the tassel and ear are replaced with sporogenous *S. reiliana* cells, usually resulting in sterility (Martinez, 2002; Matyac 1985). What would have been an ear or tassel is now a mass of black spores (a sorus, pl. sori) held together by a thin membrane called a peridium (Figs. 1 & 2). Refer to Figure 3 for a pictographic overview of the disease cycle.

During harvest, the soral peridia of infected plants are ruptured and the teliospores are disseminated via wind or mechanical propulsion. The robust teliospores blend into the soil and will overwinter until conditions conducive to germination and disease production again are present. Teliospores remain viable in the soil for at least three years and are the main source of inoculum, but contaminated seed can also serve as an inoculum reservoir and under seed storage conditions teliospores may remain viable for a much longer period of time (Allan, 1979). Seeds contaminated with teliospores become a unit of dispersal for the fungus and due to the worldwide seed trade, can introduce the fungus to new locations causing new epidemics resulting in crop loss and soil infestation.

**Disease Management**

Common factors among the management of diseases include prevention and therapy. Prevention is accomplished through many routes; those common to maize include host resistance, seed treatments, and pesticide applications. In some pathosystems therapeutic treatments are effective; symptoms are recognized, a pesticide is applied and the disease is
successfully quelled. This becomes difficult or impossible in pathosystems such as maize head smut, in which plants are systemically infected long before any visible symptoms appear, when the damage has already been done and it is too late to employ any management practice.

Management of head smut is currently achieved through two main routes; seed treatment and host resistance. Varying degrees of resistance exist in maize germplasm. Since the fungus invades tissues at the seedling stage it is advantageous in areas of high disease pressure to use seed treatments to reduce infection rates.

Although the disease can be controlled by seed treatment fungicides, few efficacious seed treatments are available against head smut. Tebuconazole is registered for use against head smut in the U.S. and can be effective, but this fungicide is used at high rates and can cause phytotoxicity symptoms on maize (Yang et al, 2014, Zeng et al, 2010). In studies conducted by Stienstra et al (1985) and Wright et al (2006), propiconazole was shown to be effective at reducing disease incidence, but, despite being registered for other diseases of maize, this product is not available as a seed treatment. There is a need for new effective fungicides that will reduce \textit{S. reiliana} infection, particularly at lower rates. Development of seed treatments is an active area in the crop protection industry.

Early research conducted by Stromberg et al. (1984), Whythe and Gevers (1987), Ali and Baggett (1990) and later work by Wang et al. (2008) sought to identify maize lines exhibiting resistance to head smut. In all cases, field trials were conducted and artificial inoculation was used to ensure uniform disease pressure. All studies revealed a continuum of resistance across the inbred lines that were tested ranging from >90\% incidence down to 0\%. Both Stromberg
and Wang tested ‘B73’ and found it to be resistant and Stromberg, Wang and Whythe all found ‘Mo17’ to exhibit resistance. A general conclusion reached by these studies is that the expression of resistance to head smut is additive and quantitative.

Several studies have sought to characterize QTL conferring resistance to head smut. Lübberstedt et al. (1999) identified 3 and 8 QTL that explained 13% and 44% of resistance, respectively. Data from this study led the authors to conclude that resistance is minor or additive but left open the possibility of major resistance genes due to the fact that a few inbred lines displayed extreme resistance over the several years and locations in the study. Li et al. (2007) conducted a comparable study and identified 5 major QTL conferring head smut resistance, three of which were also identified in the study by Lübberstedt. Chen et al. (2008) and Weng et al. (2012) identified a major QTL, \(qHSR1\), on chromosome 2 of maize that seemed to confer a high degree of resistance. These studies accomplished a fine-mapping of this QTL which offered the ability to select for this region using marker-assisted selection (MAS). Zhao et al. (2012) sought to show the effect of this QTL (\(qHSR1\)) by introgression via MAS into ten maize inbred lines. The results showed that for many of the lines tested \(qHSR1\) was able to confer a high degree of resistance, but in some of the extremely susceptible lines introgression of \(qHSR1\) alone was insufficient to achieve a high degree of resistance and the authors concluded that more QTL would be necessary to confer high resistance in these lines.

The majority of the studies seeking to identify maize genes conferring high degrees of resistance have concluded that resistance is quantitative, mostly additive, and highly complex; leading to the study of QTL for resistance. Although this seems to be the consensus, two recent
studies have shown some putative major R-genes conferring resistance to head smut. Wang et al. (2012) conducted a genome-wide association study with the goal of locating genes and QTL conferring head smut resistance. This study revealed 18 candidate genes that were then split into 3 categories based on their putative functions. One category contains R-genes, specifically the gene \textit{GRMZM2G047152} which encodes a protein with an NBS domain, known to be important in disease resistance. The authors of this study concluded that their work could serve as a basis for further characterization of R-genes and QTL for resistance to head smut. In another study, Li et al. (2012) characterized and mapped a resistance gene from the highly resistant maize line ‘R24’. This gene was designated \textit{RsrR} and was hybridized through MAS into two head-smut susceptible lines. The gene was shown to segregate in a 3:1 (resistant to susceptible offspring) fashion and was concluded to be a single dominant gene conferring resistance to head smut.

Head smut infection goes unnoticed until the reproductive stages of maize when symptoms appear. Due to the lack of visible symptoms during disease development, management strategies must be evaluated in field trials over a season-long process, from seed to seed production, which is time consuming and highly variable. Implementation of a seedling assay would be useful for expediting the process of screening both germplasm for resistance and seed treatments for control efficacy.

**Rationale for the Current Work**

Work has been done to identify genes important to native resistance to head smut, such as that tightly linked to the QTL \textit{qHSR1}, and to identify effective fungicidal seed treatments. Fungicides displaying efficacious reduction of head smut serve as a first line of defense against
head smut infection. *S. reiliana* is thought to only infect during the seedling stage (<6 weeks) and therefore if infection can be avoided during this stage (e.g. through the use of fungicidal seed treatments) then losses and proliferation of inoculum can be avoided. Identifying native resistance and introgressing these genes into a breeding program is important in areas at risk for head smut infection in order to preserve yield and prevent the spread of increasing amounts of inoculum. Testing seed treatment fungicides or screening maize genotypes for resistance is challenging because infection is not visible until the final growth stages of the plant; because of this, field trials have been the most commonly used approach. Current methods employ encouragement of natural fungal infestation in the soil by planting susceptible maize crops year after year to increase the inoculum load. Once infestation levels are acceptable, candidate hybrids or treated seeds are planted. This approach involves sowing seed and waiting until maturity to observe the presentation of symptoms, which is inefficient and costly. The success of this approach is also constrained by geographical distribution of the fungus, phytosanitary concerns, within-field variability in soil inoculum and local weather. These factors contribute to wide variability in results and inoculum build-up can cause field testing sites to become unsuitable for subsequent commercial production. Obtaining results using this methodology can take several months and is very labor intensive. Given these constraints; a rapid and precise screening method for *S. reiliana* infection is desirable.

Methods to obtain quicker results from field trials have been evaluated. In one study conducted by Matyac and Kommedahl (1985), chlorotic flecking along the midrib of maize leaves was investigated as a primary symptom of *S. reiliana* infection, but this method has not proven to be consistent. The polymerase chain reaction (PCR) can be used to detect infection of
plant tissues by pathogens without the need to wait for disease symptoms to appear. A study by Xu et al. (1999) showed that a species specific PCR assay could be used to detect the presence of *S. reiliana* in maize tissues from the node, pith and shank of mature plants that were known to be infected.

In order to facilitate the development of management tactics, our objective was to develop a seedling assay based on real-time polymerase chain reaction (RT-PCR) that allows for early detection of *S. reiliana* infection. An effective assay should allow for detection at the seedling stage (4 weeks after sowing) using growth chamber conditions that are optimal for infection, and seed or soil inoculating procedures that result in consistent exposure of seedling roots to inoculum. This will theoretically allow for precise comparisons among treatments with about one fourth the time expenditure and will allow for the containment of *S. reiliana* propagules, thus reducing the amount of inoculum spread. Assays involving petri dish fungal inhibition are useful but these assays do not well represent the conditions in which maize naturally succumbs to the disease *in planta*. Both fungicide screening and native resistance evaluation could be expedited through a seedling assay which potentially mimics the ability of these two management strategies to control disease in the field.

**REFERENCES**


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Figures

Figure 1. *Sphacelotheca reiliana* infected maize plant showing head smut symptoms on the ear. A mass of fungal teliospores called a sorus replaces the ear.
Figure 2. *Sphacelotheca reiliana* infected maize plant showing symptoms on the tassel. Masses of fungal teliospores (sori) replace the tassel.
Figure 3. Overview of the disease cycle of *Sphacelotheca reiliana*.
CHAPTER 2. A REAL-TIME PCR ASSAY TO DETECT SPHACELOTHECA REILIANA INFECTION IN MAIZE (ZEA MAYS L.) SEEDLINGS AND ITS APPLICATION FOR SEED TREATMENT EFFICACY TESTING

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Abstract
The pathogen responsible for head smut of maize (Sphacelotheca reiliana) was specifically detected in maize seedlings via real-time PCR using oligonucleotides specifically corresponding to a 74 base pair amplicon in the 18s ribosomal region of the S. reiliana genome. Infection rates of 60-80% were detected in inoculated controls onto which no fungicidal seed treatment was applied. Five commercially available fungicidal seed treatments (Raxil, Maxim 4FS, Maxim Quattro, Dynasty, Vibrance) were compared for efficacy toward the reduction of seedling infection. Raxil, Maxim 4FS, Maxim Quattro and Vibrance were all found to reduce the incidence of infection (p<0.05) compared to the control. No difference (p<0.05) was found between the Dynasty treatment and the control. The assay proved valuable for detecting fungal infection at the seedling stage and for differentiating between the efficacies of different seed treatments for reducing the infection incidence.

Introduction
The phytopathogenic fungus Sphacelotheca reiliana (Kühn) Clint. (syn. Sporisorium reilianum (Kühn) Langdon and Fullerton) is the causal agent of maize head smut. The pathogen is prevalent in Asia, Australia, Europe, North America and South America and disease incidence is rising. Devastating epidemics can occur when disease-conducive environmental conditions exist; head smut has been reported to account for up to 80% yield loss in a single field (Frederiksen, 1977). Yield loss occurs when the fungus replaces the ear of a plant with a smut
sorus, generally resulting in 100% yield loss for infected plants. Control of this disease has become increasingly important in areas of both commercial grain production and maize seed production, as spores can contaminate both soil and seed.

In maize, the black echinulate teliospores of *S. reiliana* are dispersed during harvest and subsequently spread by the wind or by adhering to the seed coat. They overwinter in the soil or on contaminated seeds. When conducive conditions are present (optimal soil moisture and temperature), the spores germinate to form a basidium (or promycelium) containing four cells. This structure produces yeast-like haploid basidiospores which reproduce via budding (Martinez et al, 2002). When two compatible strains (differing at mating loci a & b) meet, conjugation tubes are formed and hyphae fuse to form a diploid infection hypha (Schirawski et al, 2005). The infection hypha are able to penetrate the root tissue of maize seedlings up to six weeks after seed germination and the fungus then grows along with the meristematic tissue, effecting minimal or no change in plant cells and generally causing no symptoms during the vegetative stages of maize growth. After the transition of the plant from vegetative growth stages to the reproductive growth stages, fungal hyphae invade host cells and can destroy them leading to the last and most important phase of the fungal life cycle: sporogenesis. In this step the now mostly intracellular fungal cells invade and destroy cells within the plant inflorescences; cells that would have given rise to the tassel and ear are replaced with sporogenous *S. reiliana* cells, usually resulting in sterility of these maize tissues. In place of ears or tassels a mass of black spores (a sorus, pl. sori) is formed enveloped by a thin membrane called the peridium (Martinez et al, 2002; Schirawski et al, 2005).
During harvest, the sori of infected plants are ruptured and the teliospores are disseminated via wind or mechanical propulsion. The robust teliospores blend into the soil and will overwinter until conditions conducive to germination and disease production are again present. Teliospores remain viable in the soil for at least three years and serve as a source of inoculum. Teliospores also stick to seed coats and contaminated seed can serve as an inoculum reservoir and under seed storage conditions teliospores may remain viable for a much longer period of time (Allan, 1979). These contaminated seeds can act as a mechanism of distribution for the fungus transporting it to new areas where it can cause infection and contaminate soils. Prevention of seedborne disease spread is an important goal for the maize seed industry and a major concern for phytosanitary regulatory agencies.

Current management strategies include host resistance and seed- or soil-applied chemical treatment; both of these remain inadequate for head smut control. Currently most commercially available hybrids lack a high degree of resistance to this pathogen and chemical control is a common strategy in high-risk areas. Successful infection takes place when maize is in the seedling stage; *S. reiliana* is thought to infect only during the first 6 weeks of maize growth (Matyac, 1985). Because initial infection occurs during the seedling stage, the disease can be managed by seed treatment fungicides; however, data on the effectiveness of specific active ingredients are very limited. Tebuconazole is registered for head smut and can be effective, but this fungicide must be used at high rates and can cause phytotoxicity on maize (Yang et al, 2014, Wright et al, 2006, Zeng et al, 2010, Stienstra et al, 1985). There is a need to explore new active ingredients that will effectively reduce *S. reiliana* infection at lower rates. The fact that teliospores can adhere to the seed coat and be transported with the seed to
locations previously unaffected by head smut necessitates seed treatments that can eliminate the infection potential of spores on the seed.

Current strategies for screening the efficacy of seed treatments are based on field trials and observations of disease symptoms. Field trials are costly and time-consuming, and they usually rely on natural soil infestation, which leads to highly variable results. Field trials are subject to the effects of weather which can dramatically affect the results. Disease assessments are very laborious and field conditions are commonly unhospitable. In fields heavily infested with head smut, teliospores can contaminate clothing and as such will be transported to new locations. An alternative assay, using an assessment of seedling infection, could greatly facilitate seed treatment testing by providing more rapid and consistent results without the risk of unintentional pathogen spread in the field. We specifically sought to develop and validate an assay that would allow (1) consistent \( S. \) reiliana infection of maize seedlings through a natural infection process, (2) sensitive molecular detection of this infection, and (3) use of this infection/detection procedure to screen seed treatments with different active ingredients for their ability to reduce infection rate. Fulfilling these goals will help expedite the testing of future seed treatment products for their efficacy against head smut thereby reducing time expenditure and cost and allowing new active ingredients to enter the market faster.

**Materials and Methods**

**Seed Coating and Fungicide Treatment**
Teliospores of \( S. \) reiliana were collected from naturally infected plants in Sacramento Co., CA. Smut galls were broken up and sieved to remove plant material and other debris. Teliospores were then dried in paper sacks on a greenhouse bench, stored in glass vials, and
shipped to Iowa under the conditions of APHIS-PPQ permit P526P-11-02346. Teliospores were stored at 4° C until use. All tools and materials that contacted teliospores were either bleached or autoclaved according to APHIS permit conditions. Teliospores were surface sterilized to reduce the presence of other pathogens according to the protocol of Bruckart and Eskandari (2002), and viability was checked by spreading a spore suspension on dextrose agar amended with antibiotics and incubating plates at 27° C (Osorio, 1998), which was previously shown to be an optimal temperature for germination (Potter, 1914). Germination was observed under a compound microscope and teliospores were counted as germinated if the promycelium or basidium was present. Teliospores consistently germinated at a rate of approximately 60% after three days at 27° C. In preliminary experiments, several inoculation methods were compared, including soil infestation procedures, direct application of spore suspensions to the germinated seedling, and coating seeds with teliospores. Results (data not shown) were most consistent with the seed coating method.

Preliminary trials were conducted to optimize the concentration of inoculum for seed coating to maximize infection rates while maintaining seedling emergence rates. Three concentrations of inoculum were tested: $10^5$, $10^6$ and $10^7$ teliospores·seed$^{-1}$. It was determined that a concentration of $10^6$ teliospores·seed$^{-1}$ was optimal for infection in untreated controls, and this was used in experiments to represent the maximum inoculum load. In order to target $10^6$ teliospores·seed$^{-1}$ as the inoculum load, teliospores (0.25 g) were suspended in 10 ml of a DAT (3% dextrose w/v, 0.1% agar w/v, 3 drops tween 20 per 100 ml total volume) solution. One hundred g of maize seed of hybrid TA-685-02 (TA Seeds, Jersey Shore, PA) were placed in a Ziploc bag, 10 ml of spore suspension was added, and the bag was lightly kneaded to ensure
even seed coating. Under a sterile biosafety hood, the bag was opened to allow the spore suspension to dry and adhere to the seeds. To confirm the inoculum level per seed, ten dried seeds were arbitrarily chosen from the bag and suspended in 10 ml of water in a 50 ml centrifuge tube with one drop of tween added. The tube was vortexed until the spores were released from the seed coat and samples of the suspension were taken for spore counts on a hemocytometer. Further experiments were conducted with lower inoculum loads, ranging from $10^6$ to $10$ spores·seed$^{-1}$. These spore loads were achieved by ten-fold serial dilutions of the original suspension.

After the seed coating procedure, maize seeds were treated with a fungicidal seed treatment. Seed treatment was performed using a small bowl seed treater (Hege 11) and five commercial seed treatments: fludioxonil (Maxim 4FS, Syngenta); fludioxonil + mefenoxam + azoxystrobin + thiabendazole (Maxim Quattro, Syngenta); azoxystrobin (Dynasty, Syngenta); sedaxane (Vibrance, Syngenta); tebuconazole (Raxil 2.6F, Bayer Crop Science) (Specific treatments and rates are listed in Table 1).

**Growth Chamber Experiments**

Three sets of experiments were conducted. In the first set, several fungicidal seed treatments were compared using seed coated with $10^6$ spores·seed$^{-1}$. In the second set, sedaxane was tested at three different rates using seed coated with $10^6$ spores·seed$^{-1}$. In the third set, the efficacy of sedaxane was tested over a range of inoculum loads on the seed.

Spore-coated and treated seeds were planted at a depth of 4 cm in autoclaved field soil in 150 cm$^3$ cone-tainers (Cone-tainer Nursery, Canby, Oregon, USA) and placed in a growth chamber. The conditions inside the growth chamber were as follows: day temperature, 27° C;
night temperature, 24° C; day length, 15 h; and relative humidity, 70%. Plants were given approximately 10 mL of water on a daily basis and were grown for approximately 4 weeks until growth stage V3, when they were uprooted and processed.

In the first set of experiments, there were seven treatments arranged in a completely randomized design in the growth chamber. Fifteen replicate seeds per treatment were sown, and the experiment was conducted three times. Seed of hybrid TA-685-02 were coated at a rate of $10^6$ teliospores·seed$^{-1}$ and dried. Spore-coated seeds were then treated with fungicides according to the rates shown in Table 1, planted in autoclaved soil and grown in a growth chamber for four weeks as described.

In the second set of experiments, only sedaxane was used, and three rates were compared to a non-treated control. Seed of hybrid TA-685-02 coated at a rate of $10^6$ spores·seed$^{-1}$ were then treated with sedaxane according to the rates shown in Table 1, planted in autoclaved soil and grown in a growth chamber for four weeks. Fifteen kernels per treatment were sown, and the experiment was conducted three times.

In the third set of experiments, sedaxane (0.1 mg·seed$^{-1}$) was tested across inoculum treatment rates of $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, and 10 spores·seed$^{-1}$. Inoculated seeds were planted in autoclaved soil and grown in the growth chamber. Fifteen kernels per treatment were sown, and the experiment was conducted three times.

After plants had grown for four weeks, roots and crown tissue of seedlings were cleansed of visible soil under running tap water and then surface disinfected by submersion in 10% NaOCl solution with agitation for 2 minutes. Plants were rinsed twice in sterile water for 30
seconds each time. Under a sterile biosafety hood, tissue samples were excised from the crown, mesocotyl, seminal root and nodal root tissues. Crown tissue was sampled by removing a cross section of the stem approximately 0.5 cm in length just above the protrusion of the nodal roots. Mesocotyl samples were taken by removing a cross section about 0.5 cm in length halfway between the seed and the nodal root protrusion. Both the seminal and nodal root tissues were sampled by twisting all the roots of one type together and then removing a 0.5 cm cross section near the root base.

Tissue samples were placed in individual 1.2 ml wells in a 96-well grinding plate (Fisher Scientific) and lyophilized at -56° C and 0.002 mBar for 1-2 days. A small metal ball was then placed in each well and the plate was placed in a tissue grinder (Genogrinder). The tissues were ground at 1000 rpm for 1 minute or until tissue was sufficiently powdered.

**DNA Extraction and Real-Time PCR**

In preliminary experiments, previously published primers for conventional PCR (Xu et al. 1999) were not sufficiently sensitive to detect low levels of *S. reiliana* DNA in plant tissue. Therefore, we designed primers for real-Time PCR using Primer Express 3 (Applied Biosystems Int.) to amplify a 74-bp amplicon which is located in the 18s ribosomal ITS region. Primers were synthesized by Integrated DNA Technologies (IDT). Primer design stipulations were as follows: 20 bp in length, minimal secondary structures, and ~50% G-C content. Specificity of the primers was confirmed via a NCBI Blast search that showed the primers had low homology with species that may be found within the same pathosystem including the common smut pathogen *Ustilago maydis*.

Forward Primer Sequence: 5’ – GCT CGC CTT TCG CTC TCT CT – 3’
Reverse Primer Sequence: 5’ – GCC TCC GAA GCC CTG ATA GT – 3’

DNA was extracted directly from teliospores, from axenically grown maize tissue and from maize tissue known to be infected with *S. reiliana*, these samples were used for validation of the primers. Each type of DNA was standardized to a concentration of 10 ng/µl for evaluation of the primers. DNA from teliospores, axenic maize, and infected maize as well as a mixture of teliospore DNA with axenic maize DNA were tested in triplicate. Gradient PCR and varying primer concentrations were used to determine optimal PCR conditions.

Genomic DNA was extracted using the following protocol (Rogers and Bendich, 1985). Six hundred µl of CTAB (cetyltrimethylammoniumbromide) extraction buffer (0.1M Tris-HCl, ph 7.5, 0.7 M NaCl, 10 mM EDTA, 1% CTAB) + 2% 2-mercaptoethanol was added to each well and then tubes were incubated at 65° C for 1 hour and inverted every 10 minutes. The liquid from each sample was drawn off and transferred to a sterile 1.5 ml microfuge tube. Six hundred µl of 24:1 chloroform:isoamyl alcohol was added to each of these tubes and each tube was inverted several times. Each tube was centrifuged for 10 minutes at 13.2·10³ rpm and the upper layer of liquid was drawn off and transferred to a sterile 1.5 ml microfuge tube. Six hundred µl of cold isopropanol was added to each of the tubes which were then centrifuged for 10 minutes at 13.2·10³ RPM. From each tube, the supernatant was poured off and the DNA pellet was retained in the tube. The pellet was then washed with 70% ethanol and dried. The pellet was eluted in 200 µl ultrapure water

All PCR reactions were performed using an Applied Biosystems StepOne Plus real time PCR system and Applied Biosystems 96-well 0.2 ml plates. Twenty µl total reaction volume was
used per sample well; 2 µl of sample DNA extract, 10 µl Applied Biosystems SYBR Green PCR Master Mix, 0.07 µl forward primer, 0.07 µl reverse primer, 7.86 µl ultrapure water. The PCR parameters were as follows: pre-cycle stage, 5 min at 95° C; 40 cycles of denaturing, 1 min at 95° C; and 40 cycles of annealing and extension, 30 s at 60° C.

Plant tissue samples (seminal root, nodal root, mesocotyl, and crown) were analyzed separately by real-time PCR. Individual plants were considered infected if threshold amplification occurred for any one or more of the four types of excised plant tissue. Analysis of variance was performed on infection incidence data using the SAS® software package by generalized linear model with Bonferroni corrections to detect differences between treatments. Data were combined among the three repetitions of each experiment.

Results

*S. reiliana* DNA was detected from all DNA types except for axenic maize tissue and melt curve analysis showed a single peak corresponding to the primer amplicon. A standard curve was developed from a serial dilution of DNA from direct teliospore extraction (Fig. 1); the accompanying amplification curve is shown in Figure 2. This RT-PCR assay reliably detected *S. reiliana* DNA at concentrations as low as 1 pg/µl.

In the first set of experiments, tebuconazole, fludioxonil, and Maxim Quattro (fludioxonil+ azoxystrobin+ thiabendazole+ mefenoxam) reduced *S. reiliana* infection compared to the control (p=0.01), whereas azoxystrobin alone did not. There were no differences in incidence of infection among the three rates of tebuconazole tested (p=0.01), and also no
differences among the tebuconazole, fludioxonil, and combination (Maxim Quattro) treatments (Fig. 3).

Sedaxane reduced infection at all three treatment rates compared to the control (p=0.01) and a pattern of reduced infection was seen with increasing rate of sedaxane, although incidence of infection did not differ among the three rates (Fig. 4). Sedaxane (0.1 mg·seed^{-1}) eliminated detectable infection in seeds coated at a rate of 10 spores·seed^{-1} (Fig. 5).

**Discussion**

Our results indicate that tebuconazole, fludioxonil and sedaxane are effective at reducing infection by *S. reiliana*. The three rates of tebuconazole tested showed no statistical differences, suggesting that the lower range of recommended rates is adequate even with high levels of inoculum. This observation has positive implications in terms of cost efficiency and avoidance of potential phytotoxic effects. The comparison of fludioxonil to azoxystrobin to fludioxonil+ azoxystrobin+ thiabendazole+ mefenoxam showed that fludioxonil was likely the effective active ingredient. Mefenoxam and thiabendazole have not been shown to be effective against head smut; fludioxonil alone reduced infection as effectively as the combination of fludioxonil with other active ingredients (Maxim Quattro) and azoxystrobin alone was not effective. Although the fludioxonil rate in the combination treatment was half that of the fludioxonil alone, the failure of azoxystrobin to significantly reduce infection compared to the control, indicates that fludioxonil was the effective ingredient in the Maxim Quattro combination. In contrast, Wright et al. (2006) found that azoxystrobin significantly reduces the percentage of symptomatic plants. Stienstra et al. (1985) reported variability of fungicidal efficacy for head smut, which may explain some of the discrepancies between our study with
Wright et al. (2006). We found that sedaxane was effective to reduce infection rates and although decreased infection was observed with increased application rates, the rates did not differ among each other. The lower range of recommended rates for sedaxane appears to be adequate for protection from *S. reiliana* infection. Other studies (Stienstra et al., 1985; Wright et al., 2006) reported that propiconazole and carboxin were also effective to reduce smut incidence, but those ingredients were not tested by our assay.

Sedaxane eliminated infection from seed-borne inoculum, when inoculum was applied at a rate of 10 spores·seed⁻¹. The range of inoculum levels per seed under natural conditions is unknown; it seems unlikely to be as high as 10⁶ spores·seed⁻¹ but may be higher than 10 spores·seed⁻¹. Additional data are needed on naturally occurring inoculum levels in order to assess whether current seed treatment practices are adequate to eliminate the risk associated with seedborne inoculum.

In this study, *S. reiliana* inoculum was applied to seed in order to mimic infection from naturally seedborne inoculum and to maximize consistency of infection in the assay. Applying teliospores to seed ensured the presence of high levels of inoculum in the seedling infection court and provided an accurate basis for comparisons among seed treatments. Absolute levels of infection may differ when the primary source of inoculum is soilborne. However, results of the assay developed in this study should still provide a valid basis for expectations of relative performance among seed treatment active ingredients.

Although it is possible to quantify pathogen DNA using the real-time PCR assay developed in this study, the relationship between DNA quantity and symptom development is
unknown, and we limited our interpretation of the PCR results to infection incidence in seedlings and the reduction of this infection incidence with fungicide seed treatments. Although some infected seedlings do not become symptomatic plants (Anderson, 2014), presumably the absence of seedling infection corresponds to reduced risk of disease, and is an appropriate measure of seed treatment efficacy. The assay we developed has the ability to perform DNA quantification and future work could be directed toward establishing the relationships of pathogen DNA levels in different tissues and at different plant development stages to ultimate disease incidence. These results could be used to elucidate disease development and host resistance mechanisms.

In growth chamber assessments under controlled conditions, other fungi related to *S. reiliana* are unlikely to be present and the risk of false positive results is very low. Additionally, tissues were surface-disinfested and the other common smut pathogen in maize (*Ustilago maydis*) does not infect seedling tissues from seedborne or soilborne inoculum. Therefore we feel that the specificity of the assay was adequately confirmed for this use. However, if these primers were to be used for detection of seed contamination, additional validation might be necessary to confirm specificity to *S. reiliana* by testing with related fungi.

The results presented here demonstrate that seed treatment efficacy can be assessed efficiently and accurately using the real-time PCR-based seedling assay. With our growth chamber assay environmental conditions can be controlled to simulate conditions that result in high infection incidence of *S. reiliana*. It is under these conditions that the true potential of a seed treatment to combat head smut is realized. Furthermore, these results indicate that
currently available seed treatments provide significant reductions in *S. reiliana* infection.

Sedaxane, tebuconazole, and fludioxonil were effective alone or in combination with other active ingredients. Although we did not test the combination of sedaxane and Maxim Quattro, this is also a commercially available combination and our results suggest that it should provide effective protection against head smut. The assay developed here can be a useful tool for further evaluation of new active ingredients and combinations.

**REFERENCES**


**TABLES AND FIGURES**

Table 1. Fungicide active ingredients, trade names, and application rates for products tested in growth chamber experiments for control of seedling infection by *Sphaeclotheta reiliana*.

<table>
<thead>
<tr>
<th>Fungicide Active Ingredient</th>
<th>Trade Name</th>
<th>Treatment Rate(s) (mg/kernel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fludioxonil</td>
<td>Maxim 4FS</td>
<td>0.012</td>
</tr>
<tr>
<td>Fludioxonil(Flu)+Azoxystrobin(Azo)+Thiabendazole(Thi)+Mefenoxam(Mef)</td>
<td>Maxim Quattro</td>
<td>0.0064(Flu)+0.0025(Azo)+0.05(Thi)+0.005(Mef)</td>
</tr>
<tr>
<td>Azoxystrobin</td>
<td>Dynasty</td>
<td>0.0025</td>
</tr>
<tr>
<td>Tebuconazole</td>
<td>Raxil</td>
<td>0.019, 0.028, 0.038</td>
</tr>
<tr>
<td>Sedaxane</td>
<td>Vibrance</td>
<td>0.05, 0.075, 0.1</td>
</tr>
</tbody>
</table>

Raxil provided by Bayer Crop Science (Monheim, Germany) all other fungicides provided by Syngenta Crop Protection (Basel, Switzerland).
Figure 1. Standard curve obtained from 10-fold serial dilution of DNA extracted from teliospores of *S. reiliana*. Each concentration was evaluated in triplicate and each point represents one sample. $R^2 = 99.8\%$. Efficiency $= 100.1\%$. 
Figure 2. Amplification plot obtained by 10-fold serial dilution of DNA extracted from spore tissue. Each dilution was tested in triplicate. The leftmost curve represents a starting concentration of 10 ng/µl.
Figure 3. Incidence of infection detected via RT-PCR in seedlings of maize hybrid TA-685-02 treated with fungicidal seed treatments. Treatment names are described in Table 1 and were compared to a control that was inoculated and mock treated with colorant and polymer only. Numbers in parenthesis represent active ingredient rate in mg·seed⁻¹. Each bar represents the average of three trials. Error bars represent standard error.

Flu = Fludioxonil, Azo = Azoxystrobin, Thi = Thiabendazole, Mef = Mefenoxam

Bars marked with an asterisk (*) differed significantly from the control at the p=0.01 level.
Figure 4. Incidence of pathogen infection detected via RT-PCR in seedlings maize hybrid TA-685-02 treated with three rates of sedaxane. Treatments were compared to a control that was inoculated and mock treated with a colorant and polymer only. Numbers in parenthesis represent active ingredient rate in mg·seed$^{-1}$. Each bar represents the average of three trials. Error bars represent standard error. Bars marked with an asterisk (*) differed significantly from the control at the p=0.01 level.
Figure 5. Incidence of infection detected via RT-PCR in seedlings of maize hybrid TA-685-02. Horizontal axis represents inoculum load in spores·seed$^{-1}$. Sedaxane (0.1mg·seed$^{-1}$) was used to treat inoculated seed and was compared to an inoculated control that was mock treated with colorant and polymer. Each data point represents the average of two trials. Error bars represent standard error.
CHAPTER 3. SUSCEPTIBILITY OF MAIZE INBREDS TO SEEDLING INFECTION BY THE HEAD SMUT PATHOGEN, *SPHACELOTHECA REILIANA*

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Abstract

A species specific real-time PCR assay was employed to detect the infection of maize seedlings by the head smut fungus, *Sphacelotheca reiliana*. Comparison of five inbred genotypes representing low, moderate and high susceptibility to the disease showed that the assay could detect differences between an inbred genotype of low susceptibility and the other four, but no other differences were found (p<0.05). The real-time assay was also used to correlate infection detected in field trials at both seedling stage and early reproductive stage with observable symptoms. Field trials conducted in 2012 and 2013 in Texas and in 2013 in California yielded no symptoms (smutted ears or tassels) but infection was detected via real-time PCR at both locations and years as well as all growth stages. The fungus was present in ear shoots without present symptoms. These results indicate that the real-time assay could be a useful tool for screening host resistance. The findings of the field trials indicate that asymptomatic infection is much more common than previously thought; a result that may have interesting implications for the future study of this fungus.

Introduction

Durable genetic resistance to disease could be thought of as the ultimate form of resistance and as such is sought after by the seed industry. Genetic resistance offers many advantages over reliance on chemical treatments and cultural practices. Advantages to the grower include an integrated product with fewer management inputs and less environmental
impact than other practices. The reduction in applications of chemical fungicides is ecologically beneficial as less potentially toxic chemicals are dispersed into the environment. Identification of disease resistance in maize germplasm is one of the major components in successful integrated pest management systems and tools that facilitate this process are vital to plant breeding (McDonald and Linde, 2002).

Early research conducted by Stromberg et al. (1984), Whythe and Gevers (1987), Ali and Baggett (1990) and later work by Wang et al. (2008) sought to identify maize lines exhibiting resistance to head smut. In all cases, field trials were conducted and artificial inoculation, which is common to academic research of head smut but not to industrial testing, was used to ensure uniform disease pressure. All studies revealed a continuum of susceptibility or partial resistance across the inbred lines that were tested, ranging from >90% disease incidence down to 0%. Both Stromberg (1984) and Wang (2008) tested ‘B73’ and found it to be resistant and Stromberg (1984), Wang (2008) and Whythe and Gevers (1987) found ‘Mo17’ to exhibit resistance. A general conclusion reached by these studies is that the expression of resistance to head smut is additive and quantitative.

Several studies have sought to characterize QTL conferring resistance to head smut. Lübberstedt et al. (1999) identified groups of 3 and 8 QTL that explained 13% and 44% of resistance, respectively. Data from this study led the authors to conclude that resistance is minor or additive but left open the possibility of major resistance genes due to the fact that a few inbred lines displayed extreme resistance over the several years and locations in the study. Li et al. (2007) conducted a comparable study and identified 5 major QTL conferring head smut
resistance, three of which were also identified in the study by Lübberstedt (1999). Chen et al. (2008) and Weng et al. (2012) identified a major QTL, qHSR1, on chromosome 2 of maize that seemed to confer a high degree of resistance. These studies accomplished a fine-mapping of this QTL which offered the ability to select for this region using marker-assisted selection (MAS). Zhao et al. (2012) sought to show the effect of this QTL (qHSR1) by introgression via MAS into ten maize inbred lines. The results showed that for many of the lines tested, qHSR1 was able to confer a high degree of resistance, but in some of the extremely susceptible lines introgression of qHSR1 alone was insufficient to achieve a high degree of resistance and the authors concluded that more QTL would be necessary to confer high resistance in these lines.

The majority of the studies seeking to identify maize genes conferring high degrees of resistance have concluded that resistance is quantitative, mostly additive, and highly complex; leading to the study of QTL for resistance. Although this seems to be the consensus, two recent studies have discovered some putative major R-genes conferring resistance to head smut. Wang et al. (2012) conducted a genome-wide association study with the goal of locating genes and QTL conferring head smut resistance. This study revealed 18 candidate genes that were then split into 3 categories based on their putative functions. One category contains R-genes, specifically the gene GRMZM2G047152 which encodes a protein with an NBS domain, known to be important in disease resistance. The authors of this study concluded that their work could serve as a basis for further characterization of R-genes and QTL for resistance to head smut. Another study conducted by Li et al. (2012) characterized and mapped a resistance gene from maize line ‘R24’ which exhibits a high degree of head smut resistant. This gene was designated RsrR and was hybridized through MAS into two head smut susceptible lines. The gene was
shown to segregate in a 3:1 (resistant to susceptible offspring) fashion and so was concluded to be a single dominant gene conferring resistance to head smut.

Head smut infection goes unnoticed until the reproductive stages of maize when symptoms appear. Due to the lack of visible symptoms during disease development, management strategies must be evaluated in field trials over a season-long process, from seed to seed production, which is time consuming and highly variable. Implementation of a seedling assay would be useful for expediting the process of screening both germplasm for resistance and seed treatments for control efficacy. Identification of germplasm with resistance to head smut is currently subject to lengthy field trials with high numbers of replications (Stromberg et al., 1984; Frederiksen, 1977). Fields trials must be performed on a season-long basis due to the inability to observe infection prior to the reproductive stages of maize. Many replications are required if naturally infested fields are used, due to highly heterogeneous spatial distribution of inoculum. In an attempt to reduce variability, artificial inoculation procedures using soil infested with teliospores have been developed which promote more uniform pathogen challenge across treatments. The pitfalls of artificially inoculating soil with teliospores of *S. reiliana* are that extremely large amounts of inoculum are generally needed (Stromberg et al., 1984), and that head smut inoculum has now been introduced into an area that may not have had previous inoculum thereby rendering the newly infested soil undesirable for commercial maize harvest. These methods are costly and time-consuming, and results can be difficult to interpret due to experimental variability. There is a need for testing procedures that both increase precision and decrease time and cost expenditures. An alternative assay, using an assessment of seedling infection, could greatly facilitate selection of resistant genotypes by
providing more rapid and consistent results without the risk of unintentional pathogen spread in the field.

 Accordingly, our objectives were to develop and validate a seedling assay that would allow (1) consistent *S. reiliana* infection of maize seedlings through a natural infection process, (2) sensitive molecular detection of this infection, and (3) use of this infection/detection procedure to evaluate susceptibility/resistance of maize genotypes. We sought to develop a consistent method that would reduce time expenditure, improve homogeneity of inoculum, and allow for control of the environment via a growth chamber assay, therefore providing more precise results than previous methods. Our overall goal was to provide a tool that would facilitate selection of resistant genotypes and quantification of disease resistance for use in breeding programs.

**Materials and Methods**

**Growth Chamber Assay**

Teliospores of *S. reiliana* were collected from naturally infected plants in Sacramento Co., CA. Smut galls were broken up and sieved to remove plant material and other debris. Teliospores were then dried in paper sacks on a greenhouse bench, stored in glass vials, and shipped to Iowa under the conditions of APHIS-PPQ permit P526P-11-02346. Teliospores were stored at 4° C until use. All tools and materials that contacted teliospores were either bleached or autoclaved according to APHIS permit conditions. Teliospores were surface sterilized according to the protocol of Bruckart and Eskandari (2002), and viability was checked by spreading a spore suspension on dextrose agar amended with antibiotics and incubating plates at 27° C (Osorio, 1998), which was previously shown to be an optimal temperature for
germination (Potter, 1914). Germination was observed under a compound microscope and teliospores were counted as germinated if the promycelium or basidium was present. Teliospores consistently germinated at a rate of approximately 60% after three days on agar at 27° C. In preliminary experiments, several inoculation methods were compared, including soil infestation procedures, direct application of spore suspensions, and coating seeds with teliospores. Results (data not shown) were most consistent with the seed coating method.

Maize seeds of five different inbred genotypes (A619, B68, B73, Mo17, and Va26) were obtained from the Germplasm Resources Information Network (GRIN) in Ames, Iowa. These inbreds were chosen because they represent low (A619, Va26), medium (B68), and high (B73, Mo17) levels of resistance to head smut based on their performance in previous resistance trials (Stromberg et al., 1984). Preliminary trials were conducted to optimize the concentration of inoculum for seed coating to maximize infection rates while maintaining emergence rates. Three concentrations of inoculum were tested: $10^5$, $10^6$ and $10^7$ teliospores·seed$^{-1}$. It was determined that a concentration of $10^6$ spores per seed was optimal for infection in untreated controls, and this was used in subsequent experiments. In order to target $10^6$ teliospores·seed$^{-1}$ as the inoculum load, 0.25 g teliospores were suspended in 10 ml of a DAT (3% dextrose w/v, 0.1% agar w/v, 3 drops tween 20 per 100 ml total volume) solution. Surface-sterile seeds of each of the five inbreds were placed into individual Ziploc bags with the spore suspension and the bags were lightly kneaded to ensure even seed coating. Under a sterile biosafety hood, each bag was opened to allow the spore suspension to dry and adhere to the seeds. To quantify the inoculum, ten dried seeds were arbitrarily chosen from the bag and suspended in 10 ml of water in a 50 ml centrifuge tube with one drop of tween added. The tube was vortexed until
the spores were released from the seed coat and samples of the suspension were taken for spore counts on a hemocytometer.

Coated and treated seeds were planted at a depth of 4 cm in autoclaved field soil in 150 cm³ cone-tainers (Cone-tainer Nursery, Canby, Oregon, USA) and placed in the growth chamber. The conditions inside the growth chamber were as follows: day temperature, 27°C; night temperature, 24°C; day length, 15 h; and relative humidity, 70%. Plants were given approximately 10 mL of water on a daily basis and were grown for approximately 4 weeks until growth stage V3, when they were uprooted and processed.

After plants had grown for four weeks, roots and crown tissue of seedlings were cleansed of visible soil under running tap water and then surface disinfested by submersion in 10% NaOCl solution with agitation for 2 minutes. The plants were then rinsed twice in sterile water for 30 seconds each time. Under a sterile biosafety hood, tissue samples were excised from the crown, mesocotyl, seminal root and nodal root tissues. The crown tissue was sampled by removing a cross section of the stem approximately 0.5 cm in length just above the protrusion of the nodal roots. Mesocotyl samples were taken by removing a cross section about 0.5 cm in length halfway between the seed and the nodal root protrusion. Both the seminal and nodal root tissues were sampled by twisting all the roots of one type together and then removing a 0.5 cm cross section near the root base.

Tissue samples were placed in individual 1.2 ml wells in a 96-well grinding plate (Fisher Scientific) and lyophilized at -56°C and .002 mBar for 1-2 days. A small metal ball was then
placed in each well and the plate was placed in a tissue grinder (Genogrinder). The tissues were ground at 1000 rpm for 1 minute or until tissue was sufficiently powdered.

Field Trials

Field trials were conducted in Texas in 2012 and 2013 and in California in 2013. Texas plots were located in Hale Co. and were conducted by the same methods in both years. In California the plots were located in Yolo County near Woodland, CA.

In Texas two treatments were compared using seed of a susceptible hybrid: (1) seeds coated with spore suspension at a rate of $10^6$ spores·seed$^{-1}$ and (2) noninoculated seed planted into soil previously infested with teliospores of *S. reiliana*. Standard fertilization practices were used. Four replicate plots of each treatment were planted with two rows per replicate. Twenty-five plants were randomly sampled from each plot at growth stage V3 and VT for evaluation of infection by real-time PCR. Growth stage V3 samples were taken by uprooting whole plants and sampling the tissues as described for the growth chamber trials. Growth stage VT plants were sampled by removing the ear shoot and excising 0.5 cm of tissue at the tip of the immature cob. After healthy plants reached stage R5, plots were scouted to observe and record the incidence of head smut symptoms.

In California natural infestation was relied upon and seeds of TA-685-02 were planted into soil previously known to be infested with *S. reiliana*. There were 14 replicate plots, 6.1 m long, with 8 rows spaced 76 cm apart. Irrigation and fertilization were provided by subsurface drip tape, and standard practices were used for fertilization and irrigation amounts; weeds were controlled with a pre-emergence application of S-metolachlor (Syngenta Crop Protection) applied according to label recommendations. Sampling time differed from the Texas trials, with
the first samples taken at growth stage V6 and the second sample set at VT. Ten plants were sampled from each of 14 plots for both V6 and VT sampling times. Samples were processed the same way as in the Texas field trials. After healthy plants reached stage R5, plots were scouted to observe and record the incidence of head smut symptoms (Abendroth et al., 2011).

**DNA Extraction and Real-Time PCR**

In preliminary experiments, previously published primers for conventional PCR were not sufficiently sensitive to detect low levels of *S. reiliana* DNA in plant tissue. Therefore, we designed primers for real-Time PCR using Primer Express 3 (Applied Biosystems Int.) to amplify a 74-bp amplicon which is located in the 18s ribosomal ITS region. Primers were synthesized by Integrated DNA Technologies (IDT). Primer design stipulations were as follows: 20 bp in length, minimal secondary structures, and ~50% G-C content. Specificity of the primers was confirmed via a NCBI Blast search and showed that these primers had low homology with species that may be found within the same pathosystem including the common smut pathogen *Ustilago maydis*. Accurate, specific amplification of *S. reiliana* was ensured through the use of specific primers, complete removal of soil, and aseptic sampling of plant tissues.

Forward Primer Sequence: 5’ – GCT CGC CTT TCG CTC TCT CT – 3’

Reverse Primer Sequence: 5’ – GCC TCC GAA GCC CTG ATA GT – 3’

DNA was extracted directly from teliospores, from axenically grown maize tissue and from maize tissue known to be infected with *S. reiliana*, these samples were used for validation of the primers. Each type of DNA was standardized to a concentration of 10 ng/µl for evaluation of the primers. DNA from teliospores, axenic maize, and infected maize were tested in triplicate
as well as a mixture of teliospore DNA with axenic maize DNA. Gradient PCR and varying primer concentrations were used to determine optimal PCR conditions.

Six hundred µl of CTAB (cetyltrimethylammoniumbromide) extraction buffer (0.1M Tris-HCl, pH 7.5, 0.7 M NaCl, 10 mM EDTA, 1% CTAB) + 2% 2-mercaptoethanol was added to each well and then tubes were incubated at 65°C for 1 hour and inverted every 10 minutes. The liquid from each sample was drawn off and transferred to a sterile 1.5 ml microfuge tube. Six hundred µl of 24:1 chloroform:isoamyl alcohol was added to each of these tubes and each tube was inverted several times. Each tube was centrifuged for 10 minutes at 13.2·10³ rpm and the upper layer of liquid was drawn off and transferred to a sterile 1.5 ml microfuge tube. Six hundred µl of cold isopropanol was added to each of the tubes which were then centrifuged for 10 minutes at 13.2·10³ RPM. From each tube, the supernatant was poured off and the DNA pellet was reserved in the tube. The pellet was then washed with 70% ethanol and let dry. Once dry, the pellet was eluted in 200 µl ultrapure water (Rogers and Bendich, 1985).

All PCR reactions were performed using an Applied Biosystems StepOne Plus real time PCR system and Applied Biosystems 96-well 0.2 ml plates. Twenty µl total reaction volume was used per sample well; 2 µl of sample DNA extract, 10 µl Applied Biosystems SYBR Green PCR Master Mix, .07 µl forward primer, .07 µl reverse primer, 7.86 µl ultrapure water. The PCR parameters were as follows: pre-cycle stage, 5 min at 95° C; 40 cycles of denaturing, 1 min at 95° C; and 40 cycles of annealing and extension, 30 s at 60° C.

Plant tissue samples (seminal root, nodal root, mesocotyl, crown, and ear) were analyzed separately by real-time PCR. Individual plants were considered infected if threshold
amplification occurred of any one or more of the four types of excised plant tissue or of the ear shoot tissue. Analysis of variance was performed on infection incidence data using the SAS® software package with general linear models and Bonferroni corrections to detect significant differences between treatment means. Data were combined among replications of the same experiment.

Results

Growth Chamber

*S. reiliana* infection was detected in all five inbred lines at varying incidences (Figure 1) in all three trials. The experiment was conducted three times, though in the final trial the emergence rate for ‘B68’ was so low (possibly due to degradation of seed quality) that in data recorded for trial 3; ‘B68’ was not included. ‘Mo17’ had significantly lower infection incidence than ‘A619’ or ‘Va26’, but did not differ significantly from ‘B68’ or ‘B73’, nor did either of these two differ from ‘A619’ or ‘Va26’. Although ‘B68’ had apparently higher infection incidence than Mo17, this difference was not significant, probably due to the lack of data from the third trial.

Field Trials

Presence of *S. reiliana* was detected in all the types of tissues tested, both in seedlings at stage V3 and V6 and in the ear shoots at growth stage VT (Table 1). Seedling infection ranged from 11% in CA to 54% in the inoculated treatment in TX in 2012, and ear shoot infection ranged from 8% in CA to 31% in the inoculated treatment in TX in 2013. More infection was observed in Texas in general and statistically (p<0.05) higher infection rates were observed among the inoculated treatment compared to the non-inoculated treatment in Texas. No head smut symptoms appeared in any of the field plots in either location.
Discussion

According to results obtained by Stromberg et al. (1984) in field trials, susceptible maize inbreds ‘Va26’ and ‘A619’ showed higher infection rates (56% and 49% incidence, respectively) than did resistant maize inbreds ‘Mo17’ or ‘B73’ (3% and 6% incidence, respectively) and B68 was moderately susceptible, showing 34% disease incidence (p=0.05). Our results showed much higher average infection rates in all five maize varieties, likely due to high inoculum loads and head smut-conducive growing conditions. “Resistant” inbred ‘Mo17’ showed lower (47%, averaged across three trials) infection incidence than did “susceptible” lines ‘Va26’ or ‘A619’ (78% and 76%, respectively, averaged across three trials) (p=0.05), which is consistent with field data from Stromberg. ‘B68’ and ‘B73’ did not differ from either the “resistant” or “susceptible” lines (p=0.05) although ‘B68’ did have a much higher average (79%) incidence than ‘Mo17’. The seedling assay did not discriminate very clearly among the inbreds tested, with infection incidence ranging from 47 to 79% for the most resistant to the most susceptible inbred, compared to a range of 3 to 56% for the same inbreds in the Stromberg et al. (1984) field study. Although a larger sample size may have provided greater power to detect significant differences, the relatively narrower range of mean infection incidences is not ideal for separating genotypes. A lower inoculum dose might improve the separation of genotypes; we tested lower inoculum levels only with a susceptible line, but a range of inoculum levels should be tested against inbreds with partial resistance.

The purpose of the field trials was to establish a relationship between the incidence of S. reiliana infection detected in tissue samples from the field trials and the incidence of disease observed in these trials as smutted tassels or ears. Unfortunately, across both years, both
locations, and both treatments, no symptoms were observed, even when greater than 50% of seedlings and 30% of ear shoots were infected. Based on the 95% confidence intervals of incidence estimates, at least 4.0% of ear shoots were infected even in the lowest-incidence plots (California). Every plant (>4000 plants total) was observed for symptoms, and even if disease incidence were as low as 4.0%, the probability that at least one diseased plant would have been found is greater than 99.9%. Therefore we are confident that the lack of symptom observation was not a sampling error. The occurrence of false positive results for the PCR analysis of tissue samples is unlikely, considering that the amplified sequence does not have high homology with other fungi related to *S. reiliana* (particularly *U. maydis*, which might have been present in the fields), tissues were surface disinfested, and *U. maydis* does not systemically infect maize plants. Our sampling occurred prior to silk emergence, and therefore prior to any *U. maydis* infection that may have occurred (citation).

The discrepancy between infection results and symptom appearance suggests that host or environmental factors suppressed the expression of symptoms. A few recent studies (Ghareeb et al., 2011; Zhang et al., 2013; Zuther et al., 2011) have sought to detail the processes that *S. reiliana* undergoes within corn plants to express smut symptoms, particularly regarding the timing of *S. reiliana* invasion of the inflorescence tissues. Evidence from these studies led the authors to conclude that timing is absolutely critical for the expression of smut symptoms; invasion of inflorescence tissue prior to floral transition will inhibit the ability of the fungus to interfere with the developmental program of maize and will lead to a reduction or elimination of symptom expression (Zhang et al., 2013).
We have developed a sensitive assay with the ability to detect *S. reiliana* presence within maize tissue and differentiate incidences of infection among maize lines. The fact that *S. reiliana* can infect maize without producing smut symptoms in the inflorescences is a limitation for the use of this assay for differentiation of resistance among maize lines. Further research utilizing this assay could focus around quantifying *S. reiliana* DNA in different plant tissues at various growth stages to better understand spatial and temporal activity of resistance mechanisms within the plant. Interestingly, the assumption of many researchers seems to have been that *S. reiliana* infected plants will inevitably show symptoms such as smutted ears or tassels at maturity but we have shown this to most certainly not be the case. For instance, Matyac (1985) reported that colonization of axillary buds or shoot apex when it changes to a reproductive apex results in the replacement of the ear or tassel (respectively) with sori. Contradictory to that, we detected the fungus in the tips of ear shoots without observing symptoms. This discovery has not been reported by other researchers and will have implications in future research on the head smut pathosystem and may be an important factor in the epidemiology of the disease.

**REFERENCES**


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### Table 1. Incidence of infection and observed symptoms of what? in field trials of plants what type of plants from Texas in 2012 and 2013

<table>
<thead>
<tr>
<th>Location</th>
<th>Incidence of Infection (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Observed Symptoms&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Seedlings</td>
<td>Ear Shoots</td>
</tr>
<tr>
<td>Texas 2012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculated</td>
<td>54 (43.7-64.0)</td>
<td>30 (21.2-40.0)</td>
</tr>
<tr>
<td>Natural infestation</td>
<td>30 (21.2-40.0)</td>
<td>-</td>
</tr>
<tr>
<td>Texas 2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculated</td>
<td>47 (36.9-57.2)</td>
<td>31 (22.1-41.0)</td>
</tr>
<tr>
<td>Natural infestation</td>
<td>28 (19.5-37.9)</td>
<td>13 (7.1-21.2)</td>
</tr>
<tr>
<td>California</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-inoculated</td>
<td>11 (6.1-17.0)</td>
<td>8 (4.0-13.6)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Incidence of infection measured by real-time PCR based on 25 plants per repetition and 4 repetitions for trials conducted in Texas and 10 plants per repetition and 14 repetitions for trials conducted in California. Numbers in parentheses are 95% confidence intervals for infection incidence.

<sup>b</sup>Smutted ears and tassels observed by field scouting.

Data not collected for naturally infested ear shoots from 2012.
Figure 1. Infection detected in five different inbred maize lines via RT-PCR. Bars represent averages of three trials; those marked with the same letters did not show significant differences at the p=.05 level using
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