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Rust Diseases on Switchgrass (*Panicum virgatum*)

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By

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Switchgrass (*Panicum virgatum*) is a perennial C_4 grass native to the central prairies of North America. Recent development of switchgrass as a sustainable biofuel feedstock has raised interest in diseases that could impact switchgrass, especially when grown under monoculture conditions. Among the known diseases of switchgrass, rust diseases are the most widespread and could potentially impact yield and biomass quality of new switchgrass cultivars. Two species of rust fungi, *Uromyces graminicola* and *Puccinia emaculata*, are known to infect switchgrass, with *P. emaculata* being the primary or sole rust pathogen on switchgrass in southern US states. The relative importance of each of the two species in the North Central region, however, was unknown. In this study, the rust fungi on switchgrass samples collected from various locations in Nebraska, Illinois, Indiana, Missouri and Wisconsin were identified on the basis of teliospore morphology and DNA sequence analysis. Both *U. graminicola* and *P. emaculata* were found in samples from Nebraska and Wisconsin, whereas *P. emaculata* only was found in samples from the other states. Among 22 switchgrass strains evaluated in a Nebraska varietal

experiment, lowland types were found to be more resistant to rust than upland types. Most upland switchgrass strains were infected by both rust species, while most lowland switchgrasses were infected by *P. emaculata* only. The results indicate that development of new biofuel switchgrass cultivars, particularly those intended for the northern plains, must include improved resistance against both rust species. As a new tool to facilitate future switchgrass research and resistance breeding efforts, a diagnostic polymerase chain reaction (PCR) system was developed whereby the two rust species can be distinguished in individual infection lesions and in infected leaves. The system utilized three sets of primers, one set (UgF and UgR) designed in this study specifically for *U. graminicola* on the basis of its internal transcribed spacer sequence, and the other two being previously-reported primers diagnostic for *P. emaculata* and for all rust fungi.

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Chapter 1 Literature Review

This study was conducted as part of the CenUSA Bioenergy (http://www.cenusa.iastate.edu/) project, a five-year effort funded by the United States Department of Agriculture National Institute of Food and Agriculture (USDA AFRI) to develop new sustainable biofuel systems for the North Central USA. The research reported here was in support of the objective to develop cultivars and mixtures of perennial grasses optimized for bioenergy production, such as switchgrass (*Panicum virgatum*). The focus of my research was to identify the rust pathogens that have the potential to cause problems on new biofuel switchgrass crops in Nebraska and other North Central states.

Panicum virgatum L., known as switchgrass, is a perennial warm-season C_4 grass native to all regions of the continental United States except California and the Pacific Northwest (Vogel, 2004). Switchgrass is climatically adapted throughout most part of the United States on suitable soil types and different cultivars exhibit adaption to ecoregions from which they or their parental germplasm were generated (Vogel, 2004). The wide geographical range of switchgrass is due to its high level of genetic variability within the species (Casler et al., 2011). Generally, most cultivars, lines, or accessions can be divided into two ecotypes, lowland and upland, according to their ecology, physiology, morphology and cytology distinctions (Vogel, 2004). Lowland ecotypes are found on floodplains such as riverine grasslands while upland ecotypes are found on tall grass upland prairies with rare flooding. Generally, lowland types are taller and larger in stem diameter, as well as being coarser and larger in leave shape, than upland types (Vogel, 2004).

Switchgrass has been studied and planted for use in pastures and for soil conservation feature since the 1940s (Vogel, 2004). Plant breeding efforts of USDA and Land Grant University plant breeders and plant material specialists at USDA Plant Material Centers developed the first cultivars that were used for both forage and conservation purposes (Vogel, 2004). In the 1990s the U.S. Department of Energy's Biofuels Feedstock Development Program funded a program aiming at developing herbaceous biomass crops, with switchgrass selected as the model perennial grass bioenergy species and it has been under development as a bioenergy crop since that time (Vogel, 2004, Sanderson et al., 2006; Mitchell et al., 2008).

Since the beginning of agriculture, plant diseases have caused significant losses on all crops. Although the history of human use of switchgrass is relatively short and intensive monoculture cropping of switchgrass has not yet been put into effect, it is anticipated that when switchgrass becomes widely exploited as a biomass crop, pathogen problems could become an increasing threat to switchgrass production. To address the threat of diseases proactively, the main disease problems that potentially impact switchgrass need to be identified and feasible control methods should be evaluated.

To help the reader better understand the background and rationale for this project better, discussions of rust pathogens and rust diseases in general and a review of the research literature pertaining specifically to switchgrass rusts will be provided in the following literature review.

1.1 Rust Fungi: A Brief Introduction

A rust fungus is any one of the more than 6000 species classified in the order *Pucciniales,* subphylum *Pucciniomycotina*, phylum *Basidiomycota*, kingdom *Fungi* (Aime et al., 2014). All rust fungi are obligately biotrophic parasites of plants, which means they can only draw nutrients from living cells or tissues (Duplessis et al., 2011). Many rust fungi can cause destructive diseases on major crops or economic plants. Some representative examples are stem rust on wheat (*Puccinia graminis* f. sp. *tritici*), leaf rust on corn (*Puccinia sorghi*), coffee rust (*Hemileia vastatrix*), and flax rust (*Melampsora lini*) (Flor, 1954; Nutman & Roberts, 1970; Russell, 1965; Singh at el., 2008). Rust fungi, as a group, have a large host range, from ferns to highly evolved *Orchidaceae* (Hiratsuka & Sato, 1982), although individual species tend to infect narrow host ranges. Most rust fungi have complex life cycles involving two phylogenetically unrelated hosts. A heteroecious rust requires both hosts alternating in its lifecycle, while an autoecious rust completes its lifecycle on one host species. Because of the long period of co-evolution between rust fungi and their plant hosts, most species of rust fungi exhibit specificity at the plant species level (Duplessis et al., 2011). In some rust species, subspecies populations or strains can exhibit differential host specificity. For instance, *P. graminis* can infect wheat, barley, oat, and other cereals and grass, but the strains collected from oat or wheat leaves cannot cross-infect the other hosts. According to their host specificity, the strains on oat and wheat are separated into two different "special forms" or *forma specialis* (f.sp.). Thus, *P. graminis* f. sp. *avenae* infects oat, whereas *P. graminis* f. sp. *tritici* infects wheat (Kolmer et al., 2009). Furthermore, a rust species or a *forma specialis* also can consist

of more than one race, each race being distinguished by virulence on particular genotypes of its host species. There also can be the relatively common situation in which a single host species can be infected by multiple rust species. For instance, wheat can be infected by *P. graminis* f. sp. *tritici* causing stem rust, *P. recondite* causing leaf rust and *P. striiformis* causing stripe rust. In this case, we regard each pathogen and host combination as a distinct disease.

The life cycles of rust fungi are made even more complex by the requirement for multiple spore stages. Each spore stage is designated by the spore type and is assigned a numeral. Using the spore taxonomy from Cummins and Hiratsuka (1983), the stages are: aeciospores (I), urediniospores (II), teliospores (III), basidiospores (IV), and spermatia, also called pycniospores (0) (Figure 1). Aeciospores are dikaryotic spores produced within the dikaryotic aecium (pl. aecia), a cup-like structure on the aecial host (the spring host in the case of a heteroecious rust). Aeciospores serve as the initial inoculum to infect the summer host. Urediniospores are dikaryotic spores, typically red to brown, produced in a uredinium (pl. uredinia), or pustule, on the summer host (i.e. uredinial host). Urediniospores are also referred to as repeating spores because they can infect and be produced on the summer host repeatedly as long as the host and environmental conditions permit. Because of the potential for the uredinial stage to cause rapid and massive epidemics, this is the most economically important stage of most rust diseases. Upon impending senescence of the summer host, the uredinium converts to a telium (pl. telia) in which teliospores are produced. Teliospores are unicellular or multicellular spores (each cell being dikaryotic) that function in overwinter survival of the fungus either in the debris from the summer

host or on soil surface. In the next spring, haploid basidiospores are formed from teliospores after nuclei in teliospores undergo karyogamy and meiosis. They infect the spring host, generating haploid hyphae from which the spermagonium (=pycnium) is formed. The spermagonium typically is a flask-shaped structure containing spermatia and receptive hyphae. Spermatia are non-infectious spores; instead, they serve as male gametes, with spermatia from one spermagonium having to be dispersed to the receptive hyphae of another spermagonium. After plasmogamy between spermatia and receptive hyphae of different mating types, the dikaryotic mycelial state is regenerated and aecia are subsequently produced, thus completing the life cycle (Ulloa & Hanlin, 2000).

A rust species with all five stages is called macrocyclic, and a species lacking the aecial stages is called microcyclic (Hiratsuka & Sato, 1982). The causal agent of wheat stem rust, *P. graminis* f. sp. *tritici*, is a good example of a macrocyclic (Fig 1.1) and heteroecious rust, with wheat and barley being the hosts for infection by aeciospores and urediniospores and barberry being the host for infection by basidiospores and production of pycnia and aecia (Barnes, 1979). *Hemileia vastatrix* Berk. & B, the causal agent of coffee rust is an example of a microcyclic (Figure 1.2) and autoecious rust. Its life cycle requires only the uredinial stage on coffee plants; although teliospores and basidiospores are produced, the host for basidiospore infection is unknown and not required for completion of the rust's life cycle (Kolmer et al., 2009).

The biology of rust fungi, particularly those that infect cereals and grasses, has been extensively studied (Roelfs et al., 1992), and generalities regarding the infection

process can be made. The wind-borne urediniospore is the major inoculum for infection in the field due to the repeating nature of urediniospore production and the resulting abundance of spores. A urediniospore germinates on a plant surface to produce a germ tube after 4-6 hours given free moisture. The germ tube locates the stoma of its host plant and forms an appressorium over the stoma. A penetrate peg is formed from the appressorium and invades the intercellular space of the host plant. Infection hyphae grow intercellularly and the tip forms a haustorial mother cell. From the haustorial mother cell, penetration peg penetrates the cell wall of an adjacent host cell and forms a haustorium between the cell wall and the plasma membrane. Nutrient uptake occurs between the membranes of host cell and the haustorial cell (Kolmer et al., 2009). After successful colonization of host tissue by infection hyphae, uredinia form under the epidermal layer of host plants, and then visible lesions appear. Lesions are usually round to elongate in shape and yellowish to brown in color. In rusts of cereals and other grasses, lesions develop on stems and leaves. Rust fungi, by absorbing nutrients from the host plant, can severely weaken the host (Schumann, 2011). The development of hyphae and uredinia destroys the transpiration and photosynthesis function of host plants (Livne, 1964; McGrath & Pennypacker, 1990). Ultimately, crop yield can be significantly reduced (Schumann, 2011).

For centuries, agriculturalists world-wide have paid attention to the control of rust disease on cereals. Among the available control strategies, resistant cultivars and chemical control are most commonly considered. Since Biffen's study demonstrated the Mendelian inheritance of wheat rust resistance, much progress has been made in generating resistant plants which have race specific rust resistance genes (Biffen,

1907; Mundt & Browning, 1985). However, resistance genes have not offered a sustainable solution. The main disadvantage of genetic resistance is the extensively application of pure resistant lines, which applied too much selection pressure on rust populations. Virulent rust strains were selected very fast as the result (Johnson, 1961; Browning & Frey, 1969).

However, genetic resistance to rust fungi has still been extensively studied and exploited on economic crops like wheat, maize, flax, and soybean. Resistance breeding is also considered as one of the most effective method to control switchgrass rust. There are mainly two kinds of genetic resistance in the pathogen-host interaction. One is vertical resistance and the other is horizontal resistance. These concepts were first introduced by Van der Plank in 1966 to describe polygenic and oligogenic pathogen-host interactions, respectively (Van der Plank et al., 1966).

Vertical resistance corresponds to the gene-for-gene theory, in which single gene controls the resistance response. If a plant has a resistance gene, this R gene can detect a specific virulence gene from a pathogen. Then the plant will exhibit a resistant response to this specific pathogen. The specificity of the response can be at the pathogen species level, and for rust fungi, it is usually at the pathogen race level. For example, a series of Lr proteins (leaf rust resistance protein) and Sr proteins (Stem rust resistance protein) have been isolated from wheat (Kolmer, 1996; Roelfs, 1989). Wheat breeding has made big success in wheat rust control by taking advantage of these genes. But, this strategy also has problems because if only one gene is controlling the resistance response of the host plant, long term exposure to a certain resistance gene can give rust fungi more chance to overcome the resistance,

especially when some resistant cultivars are repeatedly grown in one area without rotation. Also, after the rust pathogen overcomes the resistance, it will rapidly cause losses because of the prevalence of the non-resistant genotype. The outbreak of the variety Ug99 is a good example. After a productive genotype Sr31 was used world-wide for about 30 years, Ug99 overcame Sr31 and immediately became a global threat to wheat production (Wanyera et al., 2006; Singh et al., 2006). So there is a potential risk with the oligogenic resistance strategy. Since there are few studies on the resistance genes in switchgrass, the future task of switchgrass breeding should focus on more durable resistance strategies.

Another aspect of genetic resistance is horizontal resistance. While vertical resistance is controlled by single major genes of host plants, horizontal resistance is controlled by many minor genes (Nelson, 1978). It is also called quantitative resistance or polygenic resistance. In wheat, quantitative trait loci (QTLs) have been mapped in the genome which can significantly reduce rust severity on wheat (Melichar et al., 2008; Santra et al., 2008; Suenaga et al., 2003). This kind of resistance is considered hard to overcome by the pathogens because it is controlled by minor effects of more than one or two genes and the resistance usually involves in multiple rust races. But the limitation of horizontal resistance is the difficulty of identification and transit from one genome to another (Castro et al., 2003).

Chemical control of cereal rust has been initiated, but no fungicide is both technically and economically suitable for cereal rust (Rowell, 1968; Samborski, 1985). A successful chemical application also relies on a precise epidemiological forecast (Rowell, 1968). Plus, the application and storage of chemicals dramatically increases

the expenses of disease control. Known and unknown environmental hazards may be caused by fungicide run-off. Considering the widespread distribution of switchgrass, the environmental risk of fungicide control may be high. So the chemical control of switchgrass rust also would haver limitations.

Destroying the aecial host of cereal rust was also considered to block the emergence of rust by reducing the primary inoculum and genetic diversity (Peterson, 2003). But such procedures only decreases the local inoculum. Studies on wheat stripe rust demonstrated that wind can act as a factor for spreading rust spores over long distance. Central states like Nebraska and Kansas and the Northern Great Plains can receive windborne overwinter urediniospores of *P. striiformis* from Texas and Louisiana and other southern states (Chen, 2005). Also urediniospores from the north can move back to the south during the autumn. This pattern was named as the "*Puccinia* pathway" (Eversmeyer & Kramer, 2000).

1.2 Rust Pathogens of Switchgrass

Two species of fungi *Uromyces graminicola* Burrill and *Puccinia emaculata* Schwein are confirmed in multiple studies to be the rust pathogens of switchgrass (Cummins, 1971; Gustafson et al., 2003). Since the first descriptions of these two species in the 19th century, a number of other fungal species were reported to cause rust in switchgrass, but they were later found to be synonymous with *P. emaculata* or *U. graminicola* (Table 1.1). A third species, *P. graminis,* was reported to be a pathogen of switchgrass (Anonymous, 1960), but the validity of this report has not been confirmed. Cummins (1971) concluded that there was insufficient data to support *P. graminis* as a causal agent of switchgrass rust. *Physopella cameliae*, *Puccinia orientalis*, *Puccinia levis* and *Uromyces setariae*-italicae were all reported as rust pathogens on *Panicum* spp. (Cummins, 1971), but are unlikely to be involved as pathogens of switchgrass in the United States because the distributions of these rust species are in South America or in warm regions in other parts of the world.

Puccinia and *Uromyces* are the two genera in the family of *Pucciniaceae,* containing the largest number of species (Van Der Merwe et al., 2007). They are differentiated morphologically from each other primarily by the number of teliospore cells. Ordinarily, teliospores of *Uromyces* are one-celled, while teliospores of *Puccinia* are two-celled. The morphology of spermogonia, aecia, and uredinia, and the respective spore types produced within these structures is similar between the two genera (Maier et al., 2007; Cummins, 1971). The morphological similarities between *P. emaculata* and *U. graminicola* are particularly apparent (Table 1.2).

In 1904, Fisher brought up the theory that certain species of *Puccinia* and *Uromyces* that infect the same host are more closely related to each other than species within the same genus from different hosts. In another study (Orton, 1912) *Puccinia pammellii* (now considered a synonym of *P. emaculata*) and *U. graminicola* were chosen as examples to support the idea. Orton noted that it was nearly impossible to distinguish the two species in the uredinial stage due to overlapping urediniospore size and similar germ-pore character (Orton, 1912). Based on the observation that *Puccinia* species seem to be more vigorous in its pathogenicity and more environmentally adapted in comparison to *Uromyces* species from the same hosts, Orton proposed the theory that some species of *Uromyces* might simply be the less vigorous form of *Puccinia*.

Results from recent studies on the phylogenetic relationship between *Puccinia* and *Uromyces* tend to support Fisher's theory. In terms of relatedness, host specificity seemed to be more important than the taxonomic genus. Two independent studies (Maier et al., 2007; Van Der Merwe et al., 2007) have shown that the two major genera within *Pucciniaceae*, *Puccinia* and *Uromyces*, are polyphyletic. But within the amalgamated *Puccinia*/*Uromyces* lineage, phylogenetic clades reflected a degree of association with specific hosts. These studies also found that the *Puccinia*/*Uromyces* lineage underwent at least two major diversifications (Van Der Merwe et al., 2008). A more recent study of rust on switchgrass using phylogenetic analyses based on ITS sequencing revealed two monophyletic clades (Kenaley & Bergstrom, 2014). Nucleotide identity and genetic distances between isolates in Clade I (identified morphologically as *P. emaculata*) and Clade II (identified morphologically as *U.*

graminicola) were significantly different, thus providing genetic verification that *P. emaculata* and *U. graminicola* are distinct species.

According to previous experience with cereal rusts (Mundt & Browning, 1985), molecular tools for identifying rust species and for characterizing populations within species will be essential in future efforts to improve genetic resistance to rust diseases in switchgrass and to study the epidemiology of switchgrass rust diseases. Progress has been made in developing such tools for *P. emaculata*. Uppalapati et al. (2013) designed specific PCR primers for *P. emaculata* identification base on the fungal ITS region. Wadl et al. (2011) developed a set of 10 microsatellites for characterizing genetic diversity among 20 isolates of *P. emaculata.* Orquera et al. (2013) described haplotype diversity among *P. emaculata* isolates collected from Iowa, Mississippi, Oklahoma, South Dakota, and Virginia, using three "DNA barcodes": ITS, TEF1a, and βtub. From the same rust collection, they identified 18 microsatellite loci for examining genetic diversity within *P. emaculata* (Orquera et al., 2014). DNA-based tools have not been developed, however, for *U. graminicola*.

1.3 Pathogenesis and Epidemiology of Switchgrass Rust

Certain aspects of infection of switchgrass by *P. emaculata* have been studied. Although infection by *U. graminicola* has not been studied, it can be presumed that certain infection phases are similar for both species based on their morphological similarity. Urediniospores of *P. emaculata* adhere to and then germinate on the surface of switchgrass leaves, producing germ tubes from which appressoria are formed over stomata (Uppalapati et al., 2012). Then, chlorosis on leaves was usually found as the first symptom of infection by *P. emaculata* (Li et al., 2009). Necrosis also can result from infection by *P. emaculata* (Gustafson et al., 2003). However, the chlorosis and necrosis on rusted switchgrass leaves is less severe on mildly diseased plants and tends to be less apparent than on rusted wheat leaves (Gustafson et al., 2003). Eventually, uredinia and urediniospores were formed under the epidermal cell layer. Uredinia are yellowish orange to mostly cinnamon in color. During disease development, brown to black spot lesions can be observed on leaf surface (Gilley et al., 2013). Uredinia formed by *P. emaculata* usually formed in lines on the adaxial leaf surface (Bessey, 1890; Gustafson et al., 2003; Gilley et al., 2013). Uppalapati et al. (2013) noticed in their studies involving inoculation of switchgrass with urediniospores of *P. emaculata* that uredinia also formed on stems of switchgrass, but this only happened under laboratory conditions. In late crop season, telia and teliospores can be observed with dark brown to black color. Because the linear arrangement of *P. emaculata* uredinia on switchgrass is similar to that of wheat stripe rust, Gustafson et al. (2003) developed a 10-grade disease rating system for evaluating the severity of switchgrass rust based on the rating scale used for stripe

rust. There was no symptom description for *Uromyces* switchgrass rust. Only Cummins mentioned in his review chapters that the uredinia of *U. graminicola* mostly on adaxial leaf surface having cinnamon to brown colors (Cummins, 1971).

Very little is known in regards to the epidemiology of switchgrass rust. Black (2012) described the chronology of rust epidemics in Tennessee switchgrass fields; the rust pathogen in that study was presumed to be *P. emaculata*, based on that species being reported in Tennessee, but the identity of the pathogen was not confirmed. Uppalapati et al. (2013) reported that sporulation of *P. emaculata* can occur 14 days after inoculation of switchgrass under laboratory conditions, but temperatures, durations of leaf wetness, and other environmental conditions during their experiments were not described. Epidemiological information provided in the fore-mentioned studies pertained to *P. emaculata* and cannot be assumed to apply to *U. graminicola.* Research on cereal rusts provides strong evidence that different rust species of the same host may have very different environmental requirements. In wheat, for instance, leaf rust, stem rust and stripe rust have different optimal temperature ranges, with stripe rust having the lowest (9 to 12 °C) and stem rust having the highest (15 to 30 °C) (Roelfs et al., 1992). Therefore, it is conceivable that the two switchgrass rust species could require different environmental conditions for infection, growth in the host, and sporulation, and, thus, the two diseases could have different epidemiological profiles.

1.4 Host Ranges of the Switchgrass Rust Fungi

The complete life cycle of the two switchgrass rust pathogens has not been fully described. Spore stages that can be easily observed on switchgrass in nature are urediniospores and teliospores (Hirsch et al., 2010; Gilley et al., 2013). The two rust species are also reported to infect and produce these spore stages on other native grass species as well (Table 1.3). The importance of these other native grasses also being uredinial and telial hosts is that the pathogens potentially can reproduce on these grasses, thus generating higher numbers of urediniospores to infect switchgrass and producing teliospores to facilitate local overwintering of these pathogens. The actual contribution of spore production on the other host grass species to rust epidemics in switchgrass, however, is unknown.

The life cycle of macrocyclic heteroecious rusts, such as the wheat rust pathogen *P. graminis f. sp. tritici* , includes basidiospores, which infect the aecial, or spring, host(s), and, aeciospores, which are produced on the aecial host and infect the summer, or uredinial, host. (Agrios, 2005; Schumann & Leonard, 2005). The switchgrass rusts are presumed to be macrocyclic and heteroecious. *Aecidium pammeli* was reported to be the aecial state of *P. panici*, a synonym of *P. emaculata* (Stuart, 1901; Arthur, 1909; Ramachar & Cummins, 1965), and was found on *Euphorbia corollata* L (Stuart, 1901). *Aecidium crotonopsidis* Burr. was reported to be the aecial stage of *U. graminicola* and was found on species of *Croton*, *Sebastiana*, and *Stillingia* which are also members of the Euphorbiaceae (Ramachar & Cummins, 1963). The identity of the fungi producing the aecial stages, however, has not been confirmed using genetic methods. Furthermore, direct evidence of the association of

the aecial fungi with the switchgrass rusts, in the form of successful inoculation of switchgrass with aeciospores, cannot be found in the recent literature.

Yet another possibility as to the life cycle of the switchgrass rusts is that one or both species could be macrocyclic and autoecious, i. e. they produce all spore stages only on grass species. The bean rust pathogen *U. appendiculatus* is an example of a macrocyclic rust in which all spore stages can be found on the same host (McMillan et al., 2003). There is no evidence, however, of aeciospores being produced on switchgrass.

It is important to know the aecial host because it could represent the source of primary inoculum for infection of switch. By knowing the aecial hosts and their distributions, we can ascertain the geographic inoculum source. The distribution of the aecial hosts also can add to our understanding of the epidemiology of the switchgrass rust diseases. For instance, the geographic distribution of the aecial hosts for *P. emaculata* and *U. graminicola* could possibility determine the geographic distribution of the rust species themselves. Furthermore, the identity of the aecial hosts could be important information in the development of management strategies for switchgrass rust diseases, namely eradication of aecial hosts or avoiding areas with aecial hosts.

There also is the possibility that aeciospores might be unimportant as the primary inoculum for infection of switchgrass. Again, using the wheat stem rust pathogen as a model, the primary inoculum could be wind-borne urediniospores dispersed northward from warmer areas where rust pathogens can over-winter as

uredinia on an uredinial host. A macrocyclic rust fungus, thus, can complete its life cycle without the aeciospore stage and could function as a microcyclic rust. If this is the case for the switchgrass rusts, then eradication or avoidance of the aecial host would not be effective as control measures.

1.5 Severity and Distribution of Switchgrass Rust

Reports about switchgrass rust pathogens in the United States were published primarily during two periods of time, the first one starting in the middle of the 19th century, when switchgrass was found entirely in the wild state, and encompassed the 1940s, when the development and use of switchgrass began. The second period started in the 1990s when interest in the development of switchgrass as a new bio-energy crop began to grow.

During the earlier period, most of the reports regarding switchgrass rust in which the pathogen species was identified pertained to *U. graminicola.* In a 1889 report (Webber, 1890), both species were noted in Nebraska and described as being "destructive" but *U. graminicola* was described as "not very common" whereas *P. emaculata* was reported to be "common". In 1941, Cornelius and Johnston regarded *U. graminicola* as the major causal agent on switchgrass. This was the earliest and only documented research about *U. graminicola* on different switchgrass varieties. In their study, 35 varieties collected from Nebraska, Colorado, Kansas, Oklahoma and Texas was planted in Kansas for comparison of forage yield and rust disease severity. They showed switchgrass varieties collected from Oklahoma and Texas to be relatively more resistant to rust than those from Nebraska, Colorado, and Kansas; five cultivars form Nebraska were very susceptible to rust, exhibiting ~50% infection (Cornelius & Johnston, 1941). In 1950, there was another outbreak of rust in Kansas on native grasses grown in nurseries (Cornelius, 1950). Switchgrass cultivars were reportedly heavily infected by both *U. graminicola* and *P. emaculata* in that year. Even the resistant line Blackwell was also attacked. Tiffany and Knaphus conducted a

comprehensive field survey for parasitic fungi from the 1980s to 1990s on Iowa prairies (Tiffany & Knaphus, 1995). *U. graminicola* and *P. emaculata* were identified in mostly mid-north area of Iowa.

In contrast to reports during the earlier period, reports published in the second period almost exclusively pertained to *P. emaculata*, with severe outbreaks of rust caused by this species being reported in South Dakota (Gustafson et al., 2003) and several southern states during the first decade of the 21st century (Frazier et al., 2013; Hirsch et al., 2010; Uppalapati et al., 2013). During this period, there were several studies in which resistance/susceptibility to rust caused by *P. emaculata* was compared among switchgrass strains developed for forage and biofuel use (Gustafson et al., 2003; Zale et al., 2008, Uppalapati et al., 2013); all reported differences among strains, with lowland strains exhibiting greater resistance than upland strains. .

Early and later reports as to the distribution of the two rusts species appear to be contradictory. Orton (1912) in his comparison of *Puccinia* and *Uromyces* described the two switchgrass rust species as having nearly the same geographic distribution, with the distribution of *P. emaculata* extending from Pennsylvania, west to Nebraska and south to the Gulf of Mexico. The distribution of *P. emaculata* on switchgrass report in Farr et al., 1998, however, did not include states in the south, and it was only in the recent 15 years, that several states in the southern U.S. published their first report of *P. emaculata* on switchgrass (Zale et al., 2008; Hirsch et al., 2010; Frazier et al., 2013; Gilley et al., 2013). One possible explanation for the discrepancy is that Orton (1912) may have been refer to the distribution of *P. emaculata* on all grass hosts, whereas the apparent absence of *P. emaculata* from southern states until the

21st century pertained only to switchgrass. It is conceivable that the recent reports of *P. emaculata* on switchgrass involve a new race or f. sp. with greater virulence on switchgrass than previous populations. When reports of *U. graminicola* and *P. emaculata* on all plant hosts are considered (ARS database) it would appear that the two species share a common geographic distribution.

It is also possible that the current distribution of the rust species might be different from what was reported 50 to 100 years ago. The fact that recent reports of rust epidemics made no mention of the presence of *U. graminicola* suggests that the geographic distribution of that species might have diminished. A shrinking of its range could result from loss of or reductions in populations of the aecial hosts and alternate grass host species from conversion of grasslands to agricultural lands. In addition, changes in genetic resistance to rust in switchgrass populations, which might have resulted from widespread planting of forage type switchgrass with greater rust resistance, might also contribute to a shrinking of its distribution.

1.6 Potential Disease Control Strategies of Switchgrass Rust

Rust is gradually considered to be one of the most severe disease threats due to yield in biofuel switchgrasses losses it has led to the production of switchgrass (Sanderson & Adler, 2008; Wadl et al., 2011). Furthermore, with expanding acreage of switchgrass monoculture across the United States, the risk of potential rust outbreaks also could increase. It is likely that the risk of economic damage to switchgrass from rust will mimic what occurred in the cereal rusts. To identify strategies to reduce the impact of rust diseases on switchgrass, we can draw on the experience of rust control on other crops such as wheat and barley. Common cultural practices used in managing other types of diseases such as crop rotation, would not be very practical for switchgrass, because switchgrass grown as a biofuel crop must be maintained for decades. In addition, urediniospores can be blown long distances, so planting switchgrass in a field after crop rotation will not prevent the arrival of inoculum into that field. In the northern US, winter conditions may be too harsh for urediniospores to overwinter. If aeciospores are determined as the early inoculum source in a growth season, removing aecial hosts of switchgrass rust could be a practical method to reduce disease severity or delay the epidemics for weeks. Also eliminating the sexual cycle will reduce the genetic variation in the fungal population (Schumann & Leonard, 2000).

However, eradication of the aecial host is often not economically feasible. Furthermore, that strategy could fail, as demonstrated with wheat stem rust (Agrios, 2005), if urediniospores can be blown stepwise northward from the south to serve as the primary inoculum.

Chemical control is a viable option as shown with the cereal rusts; fungicides have been successfully used in Europe where high yields are desired and prices for wheat are supported (Singh & Saari, 1992). On ornamental switchgrass, Eagle 40W and Heritage 50WDG were proven to provide good protection from rust. Pustule formation can be suppressed on lower leaves and leaf sheaths when treated. Compared with 3336 4.5F, Banner MAXX, Daconil Ultrex, Concert II, Palladium, Medallion 50W, and non-treated control, Eagle 40W and Heritage 50WDG treated switchgrass often gave lower disease rating, providing higher biomass yields in the meantime. Dry biomass yield for the non-treated control was 37% and 40% lower when compared with Heritage 50WDG and Eagle 40W, respectively (Bowen et al., 2012). Chemical application can be very effective when needed but it will greatly increase input costs, which goes against the idea of growing switchgrass as a low input biofuel crop.

Compared with the control strategies above, genetic resistance is the most practical strategy for rust management. Early and more recent studies comparing strains of switchgrass for susceptibility to rusts have shown considerable differences among strains (Gustafson et al., 2003; Zale et al., 2008, Uppalapati et al., 2013), with lowland strains exhibiting greater resistance than upland strains to *P. emaculata*. Thus, sources of resistance to are available within existing switchgrass populations. The genetic and physiological basis for rust resistance in switchgrass, however, is not understood.

Currently, some identified rust resistance characteristics of some lowland cultivars like Alamo can possibly be classified as horizontal resistance (Uppalapati et

al., 2013). Gustafson et al. (2003) have studied susceptibility to the rust associated with *P. emaculata* in four switchgrass cultivars and determined that heritable resistance is available within the species. The heritability of rust resistance offers clear opportunities for breeders to improve yields in rust-prone environments.

1.7 Critical Questions

Many critical questions regarding switchgrass rust disease arise from reviewing the literature. This study attempts to answer these questions: 1) what are the rust species that infect biofuel switchgrass in the North Central states? (Chapter 2); 2) what is the relative contribution of *Puccinia emaculata* and *Uromyces graminicola* on new switchgrass strains? (Chapter 2); 3) can *U. graminicola* and *P. emaculata* be detected and distinguished using gene sequencing based methodology? (Chapter 3).

Tables and Figures

Table 1.1 Synonyms for *Puccinia emaculata* and *Uromyces graminicola*

Puccinia emaculata	*Uromyces graminicola*
*Aecidium pammellii**	*Aecidium crotonopsidis**
Puccinia graminis var. *brevicarpa*	*Caeomurus graminicola*
Puccinia panici	*Caeomurus panici*
Puccinia panici var. *robusta Puccinia pammelii*	*Nigredo graminicola Puccinella graminicola Uromyces panici*
Uredo panici-urvilleani	*Uredo panici*

USDA Fungal Databases

*Names first assigned to fungi on aecial host

Table 1.2 Comparison of morphological spore characteristics of *P. emaculata* and *U. graminicola*
Source: *The Rust Fungi of Cereals, Grasses and Bamboos* (Cummins, 1971).

	Puccinia emaculata	*Uromyces graminicola*
Aecial stage	Occurs on species of *Euphorbia*	Occurs on species of Euphorbiaceae
Aeciospore	Globoid or ellipsoid 20-32*16-23 Wall finely verrucose, hyaline	20-32*16-23 Wall verrucose,colorless
Uredinia	Adaxial leaf surface Cinnamon-brown	mostly on adaxial leaf surface Cinnamon-brown
Urediniospore	(19-)21-27(-30)*(17-)20-24 Ellipsoid or globoid Echinulate Cinnamon-brown Germ spores 3 or 4 Equatorial	(18-)20-25(-28)*(17-)19-23(-25) Ellipsoid or globoid Echinulate Golden or Cinnamon-brown Germ spores 3 or 4 Equatorial
Telia	Adaxial leaf surface Early exposed Pulvinate Blackish brown	Early exposed Pulvinate Blackish brown
Teliospore	Two-celled (27-)33-44(-49)*(15-)17-21(-24) Ellipsoid or narrowly obovoid Smooth Chestnut-brown 3-9 apically	One-celled (20-)23-28(-32)*(12-)17-20(-22) Ellipsoid, oval, obovate, often angular Smooth Deep golden or Chestnut-brown 5-9 apically
Pedicels	Colorless Thin-walled Mostly collapsing	Hyaline to golden Moderately thin-walled Persistent collapsing or not

Table 1.3 Uredinal host list of *Puccinia emaculata* and *Uromyces graminicola.*

Source: USDA ARS fungus-host database (http://nt.ars-grin.gov/fungaldatabases/fungushost/FungusHost.cfm); search date 07/09/2015

	Puccinia emaculata	*Uromyces graminicola*
Host	*Eragrostis ferruginea* *Panicum amarulum* *Panicum amarum* *Panicum capillare* *Panicum commutatum* *Panicum maximum* *Panicum miliaceum* *Panicum philadelphicum* *Panicum* sp. *Panicum virgatum* *Paspalum scrobiculatum* *Paspalum stramineum*	*Aegilops cylindrical* *Panicum altum* *Panicum amarulum* *Panicum amarum* *Panicum anceps* *Panicum antidotale* *Panicum depauperatum* *Panicum hemitomon* *Panicum nephelophilum* *Panicum* sp *Panicum virgatum*

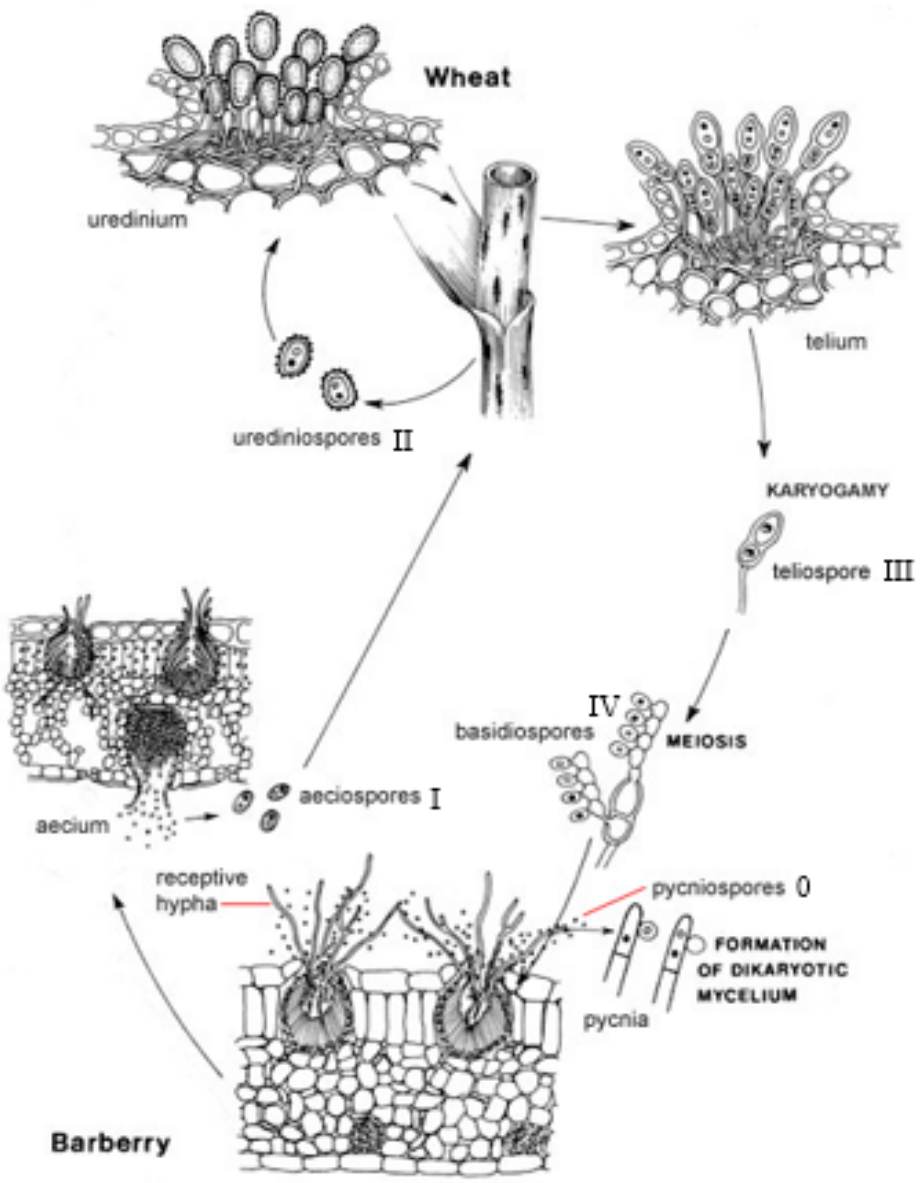

Fig1.1 Life cycle of wheat stem rust (macrocyclic)

Source: APS education: stem rust of wheat

http://www.apsnet.org/edcenter/intropp/lessons/fungi/Basidiomycetes/Pages/StemRust.aspx

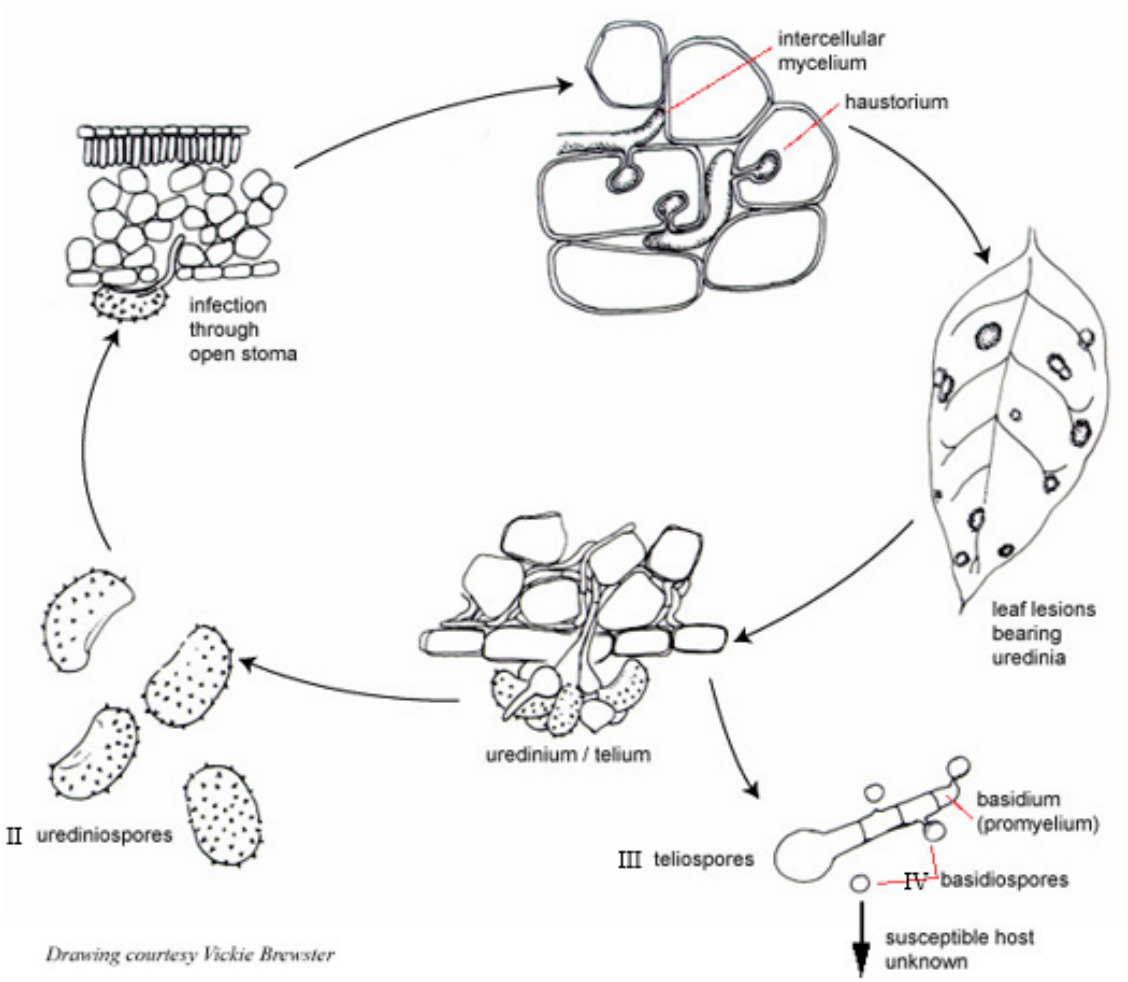

Fig 1.2 Life cycle of coffee rust (microcyclic)

Source: APS education: coffee rust

http://www.apsnet.org/edcenter/intropp/lessons/fungi/Basidiomycetes/Pages/CoffeeRust.aspx

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Chapter 2 Switchgrass rust species in the North Central USA

2.1 INTRODUCTION

One of the earliest reports of rust disease on switchgrass was part of a botanical survey conducted in Nebraska (Webber, 1890). Because fungi were considered to be plants at that time, the rust species *Uromyces graminicola* Burrill and *Puccinia emaculata* Schwein were described as being found on switchgrass (*Panicum virgatum*) and witchgrass (*Panicum capillare*). Two decades later, the two species were thought to have a very wide distribution across North America, as Orton (1912) described the geographic distribution of *U. graminicola* and *P. pammelli* (syn. *P. emaculata*) to be similar, extending from Pennsylvania, west to Nebraska and south to the Gulf of Mexico. Recent reports of rust on switchgrass, however, would seem to challenge the concept that both species are similarly distributed over much of North America. Outbreaks of rust in switchgrass in several southern states (Arkansas, Oklahoma, Mississippi, Tennessee, and Virginia) and South Dakota were attributed to *P. emaculata,* but no mention was made of *U. graminicola* being observed (Frazier et al., 2013; Gilley et al., 2013; Gustafson et al., 2003; Hirsch et al., 2010; Uppalapati et al., 2013; Zale et al., 2008). One explanation for the inconsistency is that each of the two rust species, in addition to infecting switchgrass, has been reported on a broad range of grasses as uredinial hosts and on several species in the Euphorbiaceae as aecial hosts, but there are dissimilarities between the two host ranges, particularly with respect to the aecial hosts. With the disruption of native plant communities from agriculture and urban expansion over the past century, it is possible that populations of the aecial hosts of *U. graminicola* may have been reduced or restricted to a greater

extent than those of *P. emaculata,* causing *U. graminicola* seemingly to disappear from some geographic areas. An alternative explanation is that the geographic distribution of the two rust species may be unaltered, but current switchgrass populations may have differential susceptibility to the rust pathogens. Rust resistance among switchgrass strains was first reported in 1941 by Cornelius and Johnston, at which time *U. graminicola* was the predominant rust species infecting the switchgrass strains they evaluated. If resistance to *U. graminicola* was incorporated into switchgrass cultivars developed and released subsequently, it would make sense that current commercial cultivars or experimental lines would suffer little or no infection by *U. graminicola.* A third explanation is that environmental conditions occurring during the recent switchgrass rust outbreaks may have favored infection by *P. emaculata* over *U. graminicola*. The environmental conditions that control infection, growth, and reproduction by these fungi are unknown and cannot be assumed to be the same between the species.

The rust species that infect current switchgrass populations in Nebraska have not been determined. This information is of vital importance to the development of biofuel switchgrasses because Nebraska is a major center for the development of switchgrass and other perennial grass species for the North Central region. Given the possibilities that there could have been changes in the geographic range of the fungi and the selection for greater resistance to one rust species, it cannot be assumed that both rust species still actively infect switchgrass in Nebraska. Furthermore, findings from the southern US regarding the predominance of *P. emaculata* and the differential susceptibility to rust among new switchgrass populations cannot be translated to

Nebraska and other North Central states because of the considerable climatic differences between the regions. Therefore, the first objective in this study was to identify the causal agent of switchgrass rust disease in Nebraska and other North Central states using classical morphological methods. The second objective was to use available molecular identification methods and phylogenetic analysis to confirm morphological identification. A third objective was to evaluate the relative virulence of *P. emaculata* and *U. graminicola* on new switchgrass strains.

2.2 MATERIALS and METHODS

2.2.1 Sample Collection

Switchgrass leaf samples were collected in late summer 2013 and 2014, when rust was in the telial stage, from locations listed in Table 2.1. Most of the locations were switchgrass varietal experiments and the others were parklands or landscapes. Samples were stored at -20°C until examined for rust and used for further analyses.

2.2.2 Morphological Identification

Rust fungi on infected leaf samples were visually identified to species by teliospore morphology. Transparent tape was used to collect spores from leaf surfaces and then used as a cover slip applied to a glass microscope slide. Slides were examined under 400X magnification and then photographed (Nano). The number and shape of teliospore cells was noted. The length and width of teliospores were measured using reticle calibration. A total number of 150 teliospores of each morphological type from each collection were measured. The results were compared with descriptions of rust species from switchgrass reported in Cummins (1971).

2.2.3 DNA extraction and analysis

To verify the morphological identification, the DNA sequence for internal transcribed spacer (ITS) region was determined for several strains, represented by a single telium, that were identified to species by teliospore morphology. Individual telia were excised from leaves along with a small amount of leaf tissue, and DNA was extracted using NucleoSpin Tissue kit (740952.50). Each single telium sample was

placed in a 1.5 mL microcentrifuge tube containing 80 µL Buffer T1 and 8 µL Proteinase K solution. After vortexing twice for 5 seconds, the sample was incubated at 56°C for 6 hours and vortexed occasionally during incubation. After an additional 80 µL Buffer B3 was added, the sample was vortexed twice for 5 sec and incubated at 70 °C for 5 min. Then add 80 µL ethanol (96–100 %) was added to the lysate and mixed by vortexed twice for 5 secconds. NucleoSpin Tissue XS Column was used to bind DNA. The DNA was washed by adding 50 µL Buffer B5 to NucleoSpin Tissue XS Column and centrifugation at 11,000×g for 1 min twice. At last a total of 20 µL Buffer BE was used to elute DNA.

The nuclear ribosomal ITS region and the 5'-end of the large subunit were amplified by polymerase chain reaction (PCR) using the primer set ITS1rustF10d and RUST1 (Table 2.2). A DNA fragment of approximately 1,250 bp spanning 3'-end of the 18S rDNA, ITS1, the 5.8S rDNA, ITS2, and the 5'-end of 28S rDNA was amplified. For PCR with individual primer pairs, each reaction mixture contained 2 µl of diluted genomic DNA template, 1× Takara Ex Taq buffer, 250 µM dNTP, 0.5 µM each primer, and 0.5 U Takara Ex Taq Polymerase (Takara, Kyoto, Japan) in a total volume of 50 µl. PCR amplification conditions were 5 min of denaturation at 95 °C, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s and a final extension step of 72 °C for 10 min. Amplicons were excised from 1.0 % agarose gels, purified using the QIAquick Gel Extraction Kit (no.28704) and then sent for sequencing. Sequencing results were used in Basic Local Alignment Search Tool (BLAST) search against the GenBank database for identification.

2.2.4 Phylogenetic Analyses

Sequences generated from single telia samples in this study and 5 additional rust fungi sequences from GenBank Phylogenetic analyses were evaluated (Table 2.3). For all the sequence involved, the partial ITS region was used for phylogenetic analyses. Sequences were aligned using ClustalW (MEGA6). Maximum likelihood (ML) analyses were performed using Tamura 3 - parameter model (bootstrap=1000) and phylogenetic tree visualization were performed by Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA6) software.

2.2.5 Field Trial Design

Switchgrass field experiments were located at the University of Nebraska Agricultural Research and Development Center located near Mead, NE (41.166103^{o} N, 96.482938^{o} W), and in Urbana, IL, West Lafayette, IN, Columbia, MO, and Arlington, WI. These experiments were replications of a switchgrass varietal trial with 22 cultivars seeded in 2012 into randomized blocks. Most cultivars were planted in at least 4 replicate plots. Each plot in the Mead, NE experiment was sampled in late summer 2013 and 2014 by collecting 10 leaves per plot. The 10 leaves were selected at random at a height of roughly 120cm and following an “X” pattern through each plot. The other locations were sampled in 2014 by collecting rust infected leaves from plots selected at random.

2.2.6 Rust Severity Rating

Samples collected from the Mead, NE experiment were rated visually for the severity of rust using a rating system for *P. emaculata* (Gustafson et al., 2003). It utilizes a 0 (no rust) to 9 (most severe) scale (Fig. 2.1). To facilitate statistical analysis,

‘1’ was added to each datum to convert the ratings to a 1 to 10 scale. Results were analyzed using ProcMixed (SAS). Disease rating work was completed by Christy Jochum.

The proportion of telia caused by each rust was determined from leaf samples by selecting three infected leaves at random from each sample. The number of telia corresponding to each species was counted using the tape-mount method. A piece of transparent Scotch® tape (about 2cm * 4cm) was applied onto each leaf at the area with the highest density of telia. The tape piece then was placed onto a glass slide for microscope observation. Telia were recognized as tight clumps of teliospore. Every telium on each slide was identified as either *P. emaculata* telium or *U. graminicola* and the number of telia of each species was counted. The data were collected from 3 replicate leaf samples per switchgrass sample and analyzed in two ways. First, the proportion of telia being comprised of telia of *P. emaculata* or *U. graminicola* was calculated for each sample. This value represented the relative contribution of each species to the disease in that plot. Second, the rust severity rating for each plot was multiplied by the proportion of telia being comprised of telia of *P. emaculata* or *U. graminicola*. This yielded a value that represents the relative susceptibility of the switchgrass strain in that plot to a rust species.

2.3 RESULTS

2.3.1 Field Symptoms and Signs

In June, randomly distributed yellow spots, which are considered to be an early stage symptom of rust infection, were observed on some switchgrass leaves. In late July, yellowish-orange to cinnamon colored uredinia were observed mostly on the adaxial surface of the infected leaves. Uredinia caused yellow to brown lesions as the disease developed. Uredinia usually formed in lines. In late summer, dark brown to black colored telia were observed in on both adaxial and abaxial leaf surfaces. Severity of observed symptoms and signs varied among plants and even among leaves of individual plants (Fig 2.2).

Urediniospores observed in some samples were single-celled, cinnamon-brown in color, ornate, ellipsoidal or globoid with thick cell walls (Fig 2.3A). These characteristics are consistent with descriptions of *P. emaculata* and *U. graminicola*. Two different kinds of teliospores were found. One type was two-celled, narrowly obovoid, and was dark brown in color, 36.69 ± 3.81 µm in length and 19.81 ± 1.73 µm in width (reported as mean ± standard deviation, n = 150). The two-celled teliospores were similar in size and morphological characters to those of *P. emaculata* Schw. reported on *Panicum* spp. (Fig 2.3B, Table 2.1). The other kind of teliospore was one-celled, ellipsoid or oval, obovate, often angular and 24.23 ± 2.76 µm in length and 17.73 ± 1.70 µm in width (n=150). Spore sizes and morphological characters were consistent with those of *U. graminicola* Burr. reported on *Panicum* spp. (Fig 2.3C, Table 2.4).

Both species were found in samples collected from Nebraska locations and Arlington, WI. Only *P. emaculata* was found in samples from Urbana, IL, West Lafayette, IN, and Columbia, MO.

2.3.2 Molecular Confirmation of Rust Species

Phylogenetic analyses using ITS sequence data revealed two groups distinct from other rust species (Group 1 and Group 2; Fig 2.4). The teliospore morphology of Group 1 isolates was consistent with of *U. graminicola*. But the BLAST results of sequences from Group 1 showed unclear taxon affiliation. No sequence in Group 1 could be aligned with any accession in GenBank with >95% nucleotide identity. The reason for lack of sequence identity is that there no sequence for *U. graminicola* in NCBI database. The teliospore morphology of Group 2 isolates was consistent with *P. emaculata* and the BLAST search results of the sequences revealed that the taxa of them were most similar to the rDNA of *P. emaculata* (GenBank Accession No. EU915294.1; >98% identity).

2.3.3 Relative virulence of *P. emaculata* and *U. graminicola* on new switchgrass strains

Mean rust severity scores varied among the switchgrass strains planted in the trial at Mead, NE. Ratings and associated resistance levels (Gustafson et al., 2003) ranged from 0 (highly resistant) to 6 (moderately susceptible), with most entries scoring lower than 3 (moderately resistant). Nearly all lowland types had mean disease severity ratings lower than 3, while nearly all upland types and upland-lowland crosses, had mean disease ratings higher than 3 (Fig 2.5; Table 2.5).

In nearly every switchgrass, *P. emaculata* was the only or predominant rust species. The exception was Sunburst in which over 60% of the telia were caused by *U. graminicola*. Most of the lowland switchgrass strains were infected only by *P. emaculata*, whereas most upland switchgrass strains were infected by both species. When the overall disease severity rating for each switchgrass strain was adjusted to the proportion of rust infection caused by *U. graminicola*, it was very evident that *U. graminicola* caused little if any rust in nearly all switchgrass strains. The exception was Sunburst in which the disease severity rating due to infection by *U. graminicola* exceeded 3.

2.4 DISCUSSION

It was determined in this study that both *U. graminicola* and *P. emaculata* are present in Nebraska and Wisconsin causing rust in switchgrass. The identification of these two species in switchgrass samples from Wisconsin is particularly significant because Wisconsin is another location in which new switchgrass cultivars are being developed and because it represents the first identification of *U. graminicola* and *P. emaculata* in Wisconsin. The two species were clearly identified through teliospore spore morphology (Cummins, 1971). The identification of *P. emaculata* was confirmed by the identity of its ITS sequence to other strains of *P. emaculata*. This method could not be used to confirm the identification of *U. graminicola*, however, because of the absence of *U. graminicola* sequences from the current database. Nevertheless, the results from phylogenetic analysis are consistent with the strains morphological identified as *U. graminicola* belong to one species and being distinct from *P. emaculata*. Sequences generated from *U. graminicola* in this study will be submitted to NCBI and will be important references in future efforts to identifying rust fungi.

In this study *U. graminicola* was not detected in samples collected in Urbana, IL, West Lafayette, IN and Columbia, MO. It is possible that *U. graminicola* does exist in those states but not in the location from which the samples were collected. Nevertheless, the absence of the species from the samples suggests that the species is not uniformly distributed across the North Central region. Further study is needed to determine whether there is an environmental component to the distribution range of this species.

In contrast, *P. emaculata* was found in all samples collected from other states. According to the Fungus-Host Database (http://nt.arsgrin.gov/fungaldatabases/fungushost/FungusHost.cfm; search date 07/09/2015), Minnesota, Wisconsin, Missouri and Indiana are four states in which there were no previous reports of switchgrass rust. These findings, thus, represent the first discovery of *P. emaculata* on switchgrass in these four states. They also support the idea that *P. emaculata* is broadly distributed in switchgrass in both northern and southern regions. Whether this reflects the species being able to overwinter in both regions and produce inoculum locally or whether its spores are dispersed northward from southern regions ala the "*Puccinia* pathway", as the case with wheat stem rust, needs to be investigated.

An important finding from the varietal experiment in this study is that the virulence of *U. graminicola* in comparison to *P. emaculata* can vary depending on the switchgrass population. On most of switchgrass strains planted in the field experiment, *P. emaculata* was the only species detected or caused far higher numbers of infections than *U. graminicola* while the two species appeared to be equally virulent infecting some switchgrass strains, e.g. Kanlow N2 and Sunburst. These results lend support to the explanation that there was a host genetic component to *U. graminicola* not being involved in recent switchgrass rust outbreaks.

Another critical finding from the field experiment is that currently available commercial cultivars and experimental lines of switchgrass are not uniformly resistant to *U. graminicola*, as measured by rust severity ratings adjusted to the relative frequency of infection by *U. graminicola*. The relatively high severity of rust caused by *U. graminicola* on Sunburst indicates that this species does have the

potential to cause significant levels of infection, and points to the need to evaluate all new switchgrass lines for rust in locations where both species are active. On the other hand, the finding that *U. graminicola* caused only very low levels of rust on all the other switchgrass strains is an indication that excellent progress has been made in selecting for and maintaining resistance to *U. graminicola* specifically. Lowland types exhibited resistance to *P. emaculata* as well, suggesting that there are good prospects for using host resistance to manage rust diseases in new biofuel switchgrass. Additionally, these data indicate the field locations near Mead, NE and Arlington, WI are well positioned to conduct breeding and genetics research to address potential rust resistance.

Tables and Figures

Table 2.1 Switchgrass leaf sample collection locations.

Location	Category	Collection Year
Urbana, IL	Varietal trial	2014
West Lafayette, IN	Varietal trial	2014
Columbia, MO	Varietal trial	2014
Arlington, WI	Varietal trial	2014
UNL ARDC Mead, NE	Varietal trial	2013 & 2014
UNL East Campus Lincoln, NE	Restored prairie	2014
Pioneers Park Lincoln, NE	Restored prairie	2014
Homestead National Monument Beatrice, NE	Restored prairie	2014

Table 2.2 PCR primers used in this study.

Primer	Sequence	Target species	Reference
ITS1rustF10d	5′-TGAACCTGCAGAAGGATCATTA-3′	All rust	Uppalapati et al., 2013
RUST1	5′-GCTTACTGCCTTCCTCAATC-3′	All rust	Uppalapati et al., 2013

Table 2.3 Sequences of rust fungi used in phylogenetic analysis

Strain	Host Species	Origin	Accession Number
Puccinia asparagi 89G	Asparagus	MN	AY217137
Puccinia emaculata	Switchgrass	TN	EU915294
Puccinia andropogonis	Not reported	MN	DQ344519
Puccinia graminis f. sp. *tritici*	Wheat (*Triticum* spp.)	MN	DQ417383
Uromyces appendiculatus	Dry Bean	MD	DQ354510.1
WI	Switchgrass	WI	This study
105014	Switchgrass	WI	This study
Pioneers 2	Switchgrass	Lincoln, NE	This study
T1P1	Switchgrass	Mead, NE	This study
T3P1	Switchgrass	Mead, NE	This study
T3P15	Switchgrass	Mead, NE	This study

Table 2.4 *Puccinia emaculata* and *Uromyces graminicola* teliospore size measured in this study and reported by Cummins (1971).

Name	Teliospores in this study		Teliospores in Cummins (1971)	
	NO. Of cells	Size(μm)	NO. Of cells	Size(μm)
Puccinia emaculata	Two	(36.69 ± 3.81) × (19.81 ± 1.73)	Two	(27-)33-44(-49) × (15-)17-21(-24)
Uromyces graminicola	One	(24.23 ± 2.76) × (17.73 ± 1.70)	One	(20-)23-28(-32) × (12-)17-20(-22)

Table 2.5 Rust severity and relative levels of infection by *Uromyces graminicola* (*Ug*) and *Puccinia emaculata* (*Pe*) in switchgrass varietal experiment – Mead, NE, 2014

Switchgrass strain	Ecotype	Mean disease rating	Percentage of infections caused by *Ug*	Percentage of infections caused by *Pe*
NFSG10-11	Lowland	0.9	0	100
Kanlow	Lowland	1.2	0	100
NO94 C2-4	No information	1.3	0	100
NL 94 C2-1	Lowland	1.5	0	100
NL 93-2	Lowland	2.0	0	100
Kanlow N1 Late Mat-High Yield	Lowland	2.0	0	100
Kanlow N1 Early Mat-High Yield	Lowland	2.3	16	84
Blade EG1102	Lowland	2.4	0	100
Kanlow N2	Lowland	2.5	40	60
NSL 2009-1	No information	2.5	0	100
Cave-in-Rock	Upland	2.9	0	100
NFSG10-02	lowland	3.0	1	99
NE 2010 X HYLD-HDMD C1	Upland	3.0	4	96
KxS HP1 NETO2 C2	Lowland-upland cross	3.6	7	93
Shawnee	Upland	3.7	0	100
KxS HP1 NETO2 C1	Lowland-upland cross	3.9	11	89
KxS HP1 High Yield C1	Lowland-upland cross	4.1	2	98
Blade EG2101	Upland	4.2	0	100
Summer	Upland	4.2	7	93
Summer Late Mat. C2	Upland	4.3	10	90
CIR C4	Upland	4.4	12	88
Sunburst	Upland	6.0	62	38

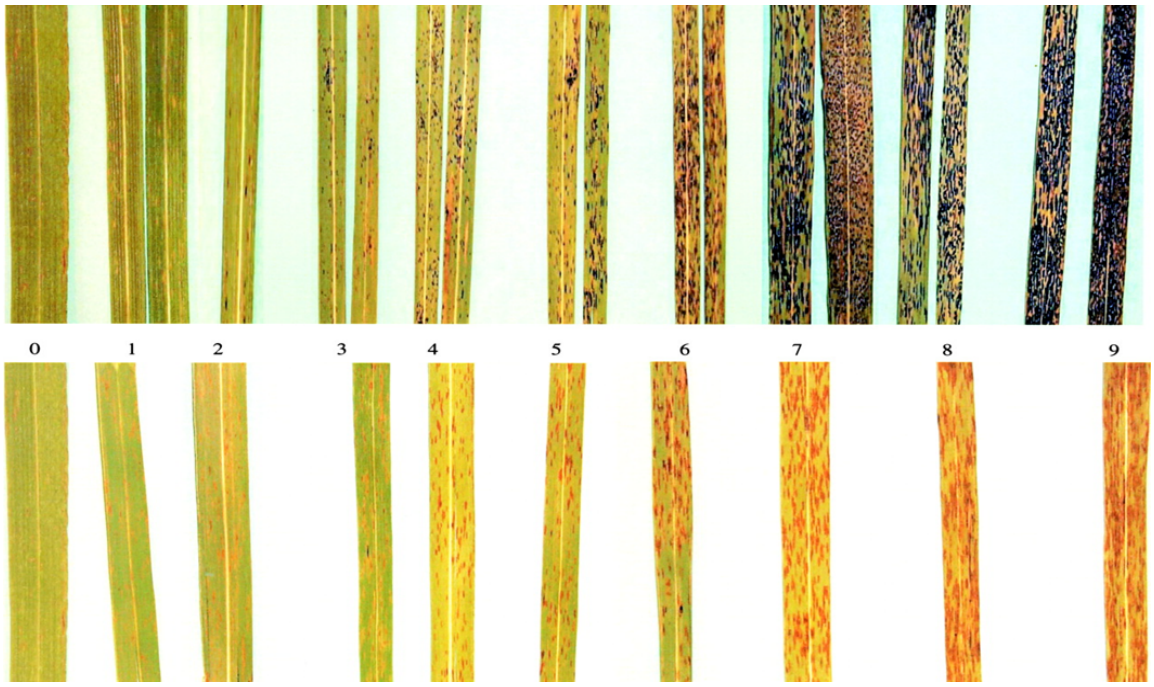

Fig 2.1 Disease rating system for *Puccinia emaculata* telia (top) and uredinia (bottom) infesting switchgrass (Gustafson et al., 2003)

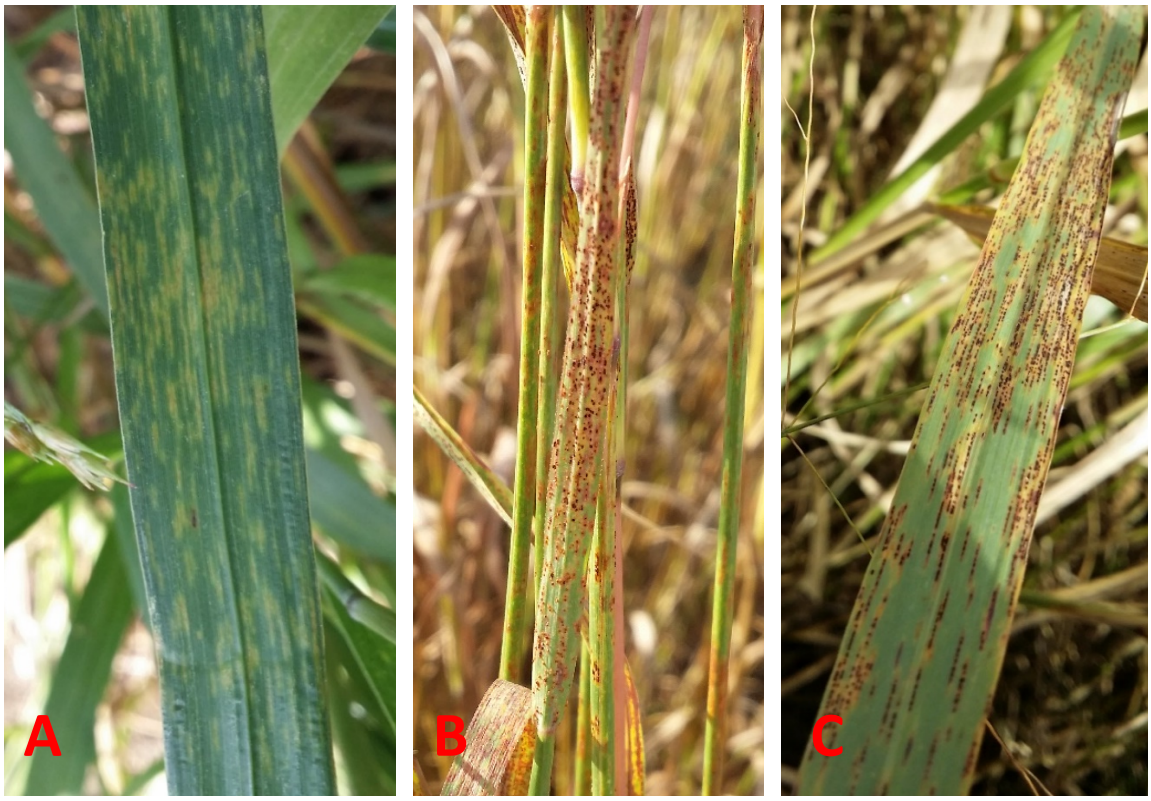

Fig 2.2 (A) Early symptom: yellow spots; (B) Uredinia formed on leaf surface; (C) Telia formed on leaf surface.

Fig 2.3 Morphological characterization of switchgrass rust spores in Nebraska. (A).Urediniospores. (B). Teliospores of *P. emaculata*. (C). Teliospores of *U. graminicola*

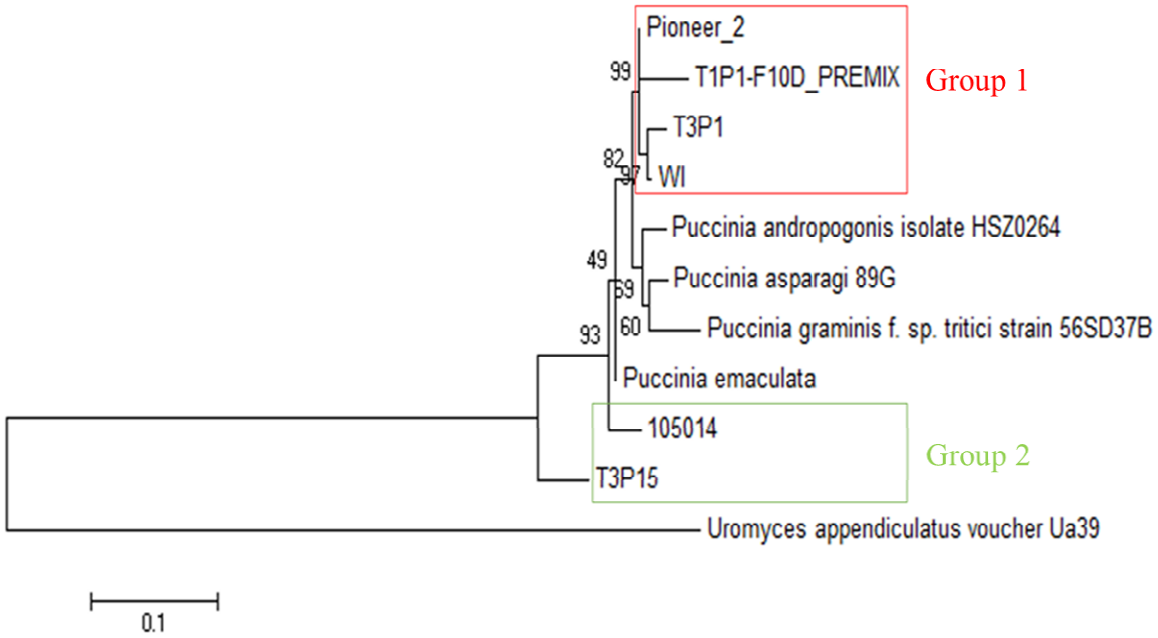

Fig 2.4 Maximum likelihood (ML) phylogenetic tree (Bootstrap=1000). Group 1 and Group 2 contains rust strains identified in this study through teliospore morphology as *Uromyces graminicola* and *Puccinia emaculata.*

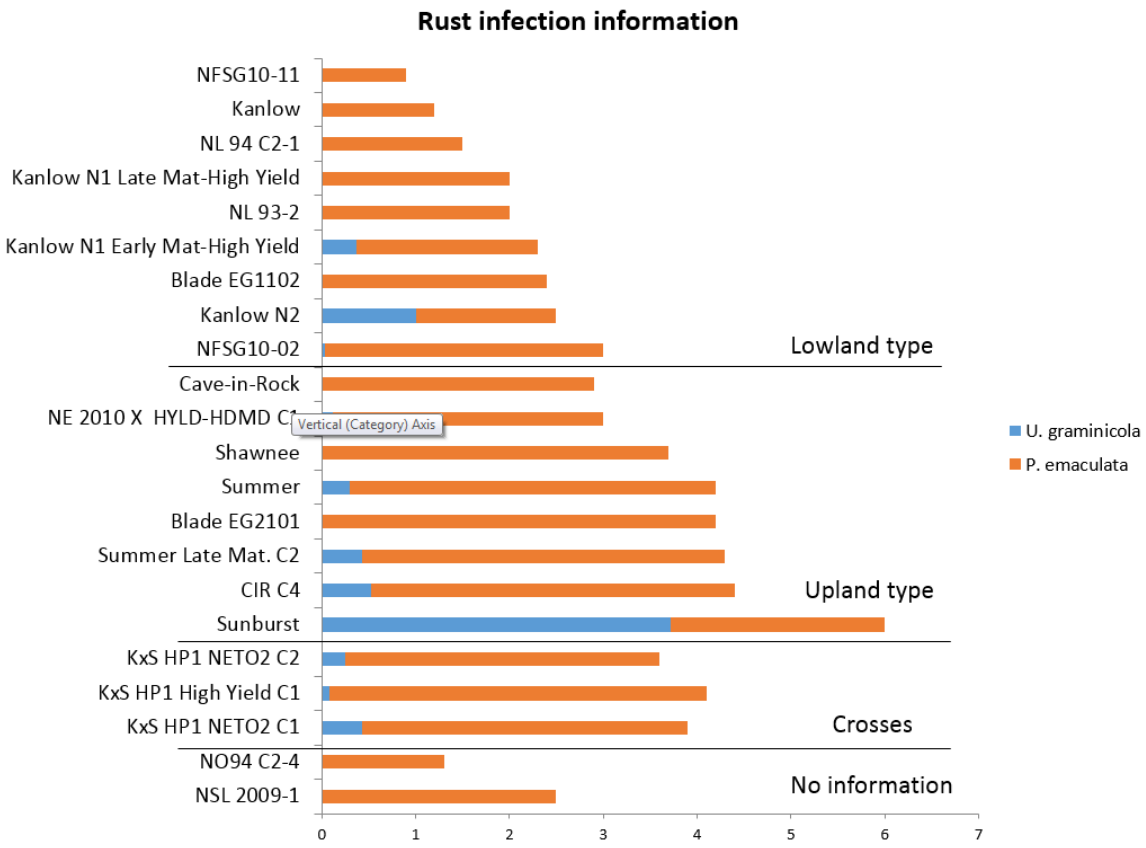

Fig 2.5 Severity of rust caused by *U. graminicola* and *P. emaculata* separately on switchgrass strains in Mead, NE varietal experiment, 2014. Rust severity is denoted by the length of each bar measure on a 0 to 9 scale, with 0 being no rust and 9 being highest severity.

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Chapter 3 Use of polymerase chain reaction (PCR) to identify rust species on switchgrass

3.1 INTRODUCTION

The differentiation of *U. graminicola* (*Ug*) and *P. emaculata* (*Pe*) relies on the morphology of teliospores, which can only be observed microscopically. Teliospores are produced at late stages in the switchgrass growth season and, therefore, the two species cannot be distinguished from each other on the basis of morphology in early stages of rust infection or in other stages of their cycles such as the aecial stage and the uredinial stage. Methods for detecting or identifying the two species that do not rely on morphology are needed for identifying the fungi while in these stages.

Methods that do not rely on morphology also are needed in the breeding and selection of resistance to switchgrass rust. As shown in Chapter 2, both rust species are involved in causing disease in switchgrass in some locations such as Nebraska and Wisconsin. Furthermore each species caused different levels of infection in the field depending upon the switchgrass strains (Chapter 2). The results were obtained by examining teliospores in large numbers of individual pustules. Given the time and labor required, the method would not be practical in analyzing large numbers of samples. This method also would not be effective in identifying a mixed infection if there were a very small number of infections by one species relative to the other. Little is known about the epidemiology of the disease caused by each rust species. Given that both species can cause rust in certain strains, critical epidemiological questions such as the environmental conditions required for infection by each species,

the time when epidemics caused by each species begin to occur, and the progression of epidemics caused by each species can be addressed only if there are methods available to distinguish the two species that are not morphology based.

Along with morphology-based methods, molecular identification methods now play an important role in fungal species identification. Molecular methods have an advantage over morphological methods in not being limited to particular life or disease stages. Methods based on polymerase chain reaction (PCR), such as RFLP, sequencing, and oligonucleotide probing have been widely used (Gardes & Bruns, 1991).

A PCR primer pair specific for *Pe* was designed from the internal transcribed spacer (ITS) regions for *Pe* (Uppalapati et al., 2013). This primer set method made possible the identification of *Pe* in the absence of the telial stage. A comparable set of primers for identifying *Ug*, however, has not been developed. The objectives of this study were, first, to identify a PCR primer set that is specific to *Ug*; and second, develop a PCR system that can be used to detect and distinguish *Ug* and *Pe* on switchgrass leaves using the new *Ug*-specific primer set and the previously described primers for *Pe* (Uppalapati et al., 2013).

3.2 MATERIALS and METHODS

3.2.1 Design of PCR primers for *U. graminicola*

We hypothesized that there are conserved sequences in the *Ug* genome which can be used for specific identification. The ITS region was selected as the potential species-specific conserved sequence for primer design. Forty-four ITS region sequences were aligned by FASTA multiple alignment. Thirty-five of these sequences were generated from *Ug* strains by S. Kenaley and G. Bergstrom (Cornell University, Ithaca, NY). The remaining 9 sequences were those of *Puccinia* species (*P. emaculata, P. asparagi, P. andropogonis, P. sorghi, P. horiana, P. graminis, P. striiformis* and *P. coronata* f.sp. *avenae*) from NCBI database. The NCBI primer designing tool was used to generate a *Ug* specific primer set. The forward and reverse primers were named UgF and UgR, respectively (Table 3.1).

3.2.2 Verification of specificity of *Ug*-specific primers

DNA isolated from individual telia of *Ug* and *Pe* was used to check the specificity of UgF and UgR primer set. Individual telia with attached leaf tissue were excised from infected leaves. DNA was extracted from each sample using NucleoSpin Tissue kit (740952.50). Five *Ug* single telium samples were used, two from leaf samples collected from Mead, NE; two from Lincoln, NE (Pioneers Park); and one from Arlington, WI. There were three *Pe* single telium samples, one collected from Mead, NE and two from Arlington, WI. The leaves were among those collected and used in the study described in Chapter 2. The DNA samples were amplified with UgF and UgR under different annealing temperatures. Amplification products were

subjected to gel electrophoresis and the gels examined for predicted bands.

3.2.3 Design of PCR system for identify rust species on switchgrass

Three sets of primers were used to identify rust species on switchgrass: *Ug*-specific primers UgF & UgR; *Pe*-specific primer SGR-SP1- FW & SGR-SP1- RV (Uppalapati et al., 2013); and common rust primers ITS1rustF10d & RUST1 (Uppalapati et al., 2013). Primer sequence information is shown in Table 3.2. The primer sets for *Ug* and *Pe* were used for detecting DNA of the respective rust species, while common rust primer set was intended as a control, i.e. verify the presence of a rust fungus DNA. A total of 25 rust infected leaves were used for evaluating the accuracy of this PCR system. The leaves were part of field collections made in 2014 for the study described in Chapter 2. The rust species in each sample was identified previously on the basis of teliospore morphology, and then the samples was stored at -20℃ for over 10 months. Leaves used in this test were examined to verify the identity of the rust species. DNA was extracted from 0.08g of infected leaves using Qiagen DNeasy Plant Mini Kit (no. 69104). DNA samples were then amplified by PCR with each primer set separately. For every amplification, the reaction mixture contained 2 µl (~1 ng) of diluted DNA template, 1× Takara Ex Taq buffer, 250 µM dNTP, 0.5 µM of each primer, and 0.5 U Takara Ex Taq Polymerase (Takara, Kyoto, Japan) in a total volume of 20 µl. PCR amplification conditions were different for each primer set.

For UgF & UgR, there was 5 min of denaturation at 95 °C, followed by 35 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min and a final extension step

of 72 °C for 10 min. For SGR-SP1-FW and SGR-SP1-RV, there was 5 min of denaturation at 95 °C, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min and a final extension step of 72 °C for 10 min. For ITS1rustF10d & RUST1, there was 5 min of denaturation at 95 °C, followed by 35 cycles of 95 °C for 30 s, 56 °C for 90s, and 72 °C for 1 min and a final extension step of 72 °C for 10 min. A sample of 3.5 µl of product from each PCR amplification was resolved by agarose gel electrophoresis, and gels were examined for diagnostic bands.

3.3 RESULTS

3.3.1 PCR primers for *U. graminicola*

Alignment of the ITS sequences from *Ug* and *Puccinia* species revealed a 7 bp distinction between *Ug* sequences and *Puccinia* sequences at about 530bp position in the multiple alignment. Taking advantage of this difference, a 25 bp reverse primer, UgR, was designed for *Ug*-specific amplification. The last two bps of UgR match the *Ug* sequences but do not match the *Puccinia* spp. sequences (Fig. 3.1). The forward primer UgF locates at about 300 bp position in the multiple alignment result and is shared by all *Ug* and *Puccinia* spp. sequences. Thus, this primer pair is designed to specifically amplify a 260 fragment from *Ug* genome DNA (Fig. 3.2).

When tested with DNA extracted from single telium samples, the primer set UgF & UgR amplified a 260 bp fragment from four out of five samples of *Ug* DNA (80% success rate), but not from any of the three *Pe* DNA samples (Fig. 3.3). The reciprocal results were found when the *Pe*-specific primer set was applied against the same DNA samples. When the common rust primers were used, however, *Pe* DNA was amplified but not *Ug* DNA.

The highest amplification efficiency with UgF & UgR occurred using 61 °C annealing temperature. Specific amplification of *Ug* DNA also occurs at 56 °C annealing temperature, but amplifications are not as strong as those under 61 °C annealing condition (Fig. 3.4). Temperatures lower than 56 °C were also tested, but there were no significant differences from 56 °C.

3.3.2 Validation of the PCR system for identifying rust species on switchgrass

When the PCR system involving the 3 primer sets was tested against 25 field samples, the system was found to be unreliable, with the PCR system agreeing with morphological identification only in 5 samples (20%) that included 3 non-infected samples. The disagreements were primarily in the PCR system failing to detect the expected rust species, i.e. false negative amplification results. Out of 22 rust-infected samples, 8 (36%) had no positive amplification by any primer set (Table 3.4). Amplification using the common rust primer set yielded the highest frequency of false negatives (16/22, 73%). The *Ug* and *Pe* primer sets yielded 46% (6 of 13 samples) and 47% (8 of 17 samples) false negatives, respectively. All of these cases of false negatives also were negative with the common rust primers. The *Ug* and *Pe* primer sets also yielded false positives in 3 of 11 samples (27%) and 2 of 8 samples (25%), respectively. All of these false positives occurred in rust-infected leaf samples in which amplifications with the common rust primers and the primer set corresponding to the visually-identified rust species were successful. The DNA extracts from the samples having false positive reactions were retested and the same results were obtained. The leaves remaining from the samples that were false positive for *Ug* were examined microscopically, and scattered teliospores of *Ug* and also some unclassified urediniospores were found.

3.4 DISCUSSION

The primer set UgR & UgF developed in this study for *Ug* provides specific identification of that species when used in amplifying DNA from individual telial pustules. The specificity of the primer set was not verified on uredinial pustules of *Ug* and *Pe* because such samples were unavailable. Nevertheless, the primers ought to have the same species-specificity when tested on uredinia.

Used along with the *Pe*-primer set developed by Uppalapati et al. (2013), the *Ug*-primers could be useful for identifying the rust species in individual uredinia, the stage at which *P. emaculata* and *U. graminicola* cannot be distinguished by morphology. The primers also could be used as a means to identify rust fungi within individual aecia and, thus, could be important in verifying reports of different species in the Euphorbiaceae being aecial hosts of the switchgrass rusts. The PCR system developed for identifying rust species on stretches of switchgrass leaves however, did not prove to be an effective system. The primary issue was missed detection of rust in an infected leaf. One possible explanation for this type of error is that the rust DNA extract from the leaves was too low in concentration and/or quality for effective amplification. There could have been several reasons why the extraction did not yield sufficient fungal DNA. First, samples from which the DNA had been stored in -20℃ for more than ten months and thawed and refrozen several times, ;thus, there was a great chance that both leaf and rust DNA had started to degrade. Second, the amount of leaf tissue used in extracting DNA was standardized according to weight. The leaf samples, however, were frozen after collection without further drying and so the samples varied in water content. Wetter leaf samples, therefore, would have yielded

less DNA than drier samples. Third, the number of pustules on each leaf sample was not the same; samples with few lesions would have yielded low quantities of rust DNA. In order to solve the false negative problem, the following procedures are suggested for use in future experiments: 1) use fresh green leaf tissue and avoid long term storage as much as possible; 2) select more heavily-infected leaves for extraction; 3) extract DNA from spores scraped off leaf surfaces rather than from sections of leaves. The common rust primer set was included in the identification system as a "check" to verify that detection of *Pe* or *Ug* in a sample is associated with the presence of a rust fungus. Such a check is not needed when analyzing leaves with obvious rust pustules, but it would be more critical if the species-specific primers were used to detect and identify the rust fungi in leaves exhibiting possible early symptoms of rust infection, e.g., yellow spots or dark elongated lesions.

There were several rust infected samples that could not be amplified using the common rust primers but were successfully amplified by the *Pe* or/and *Ug* specific primer sets. Based on results from the specificity experiment (Fig. 3.3) the rust common primer seems to be inconsistent in detection of rust fungi when dealing with low quality or lows quantity of DNA. This may be in part due to low amplification efficiency of the large target fragment (1250bp), but also high level of salt, protein, or PCR inhibitor contamination from degraded plant tissue.

Pe and *Ug* specific primers also gave several false positives in the test with infected leaf tissue. Those samples which showed false positive detection of *Ug* later proved to contain teliospores of *U. graminicola.* Thus, there is the possibility that the false positives were actually correct amplifications of DNA from telia that were not

seen during in the microscopic examination prior to extraction or from teliospores that contaminated the leaf sections that were extracted. Further testing of the PCR system using greenhouse grown switchgrass plants inoculated with known rust species is needed to verify the specificity of the system.

Further development and testing of the PCR system is needed before it can be considered a reliable tool for detecting the rust fungi in relative large amounts of switchgrass tissue. Future testing also should include leaves at earlier stages of infection when rust is not yet manifested as pustules. At the early stages, the amount of extractable fungal DNA also might be too small for reliable detection. In order to solve the problem of low DNA quantity or quality, the following procedures are suggested: 1) use fresh green leaf tissue and avoid long term storage as much as possible; 2) select leaf portions that are heavily infected (i.e. high number of lesions per area) for DNA extraction; 3) extract DNA from spores scraped off of leaf surfaces rather than from sections of leaves.

Once this PCR-based identification method is perfected and validated, it will be a valuable tool in the selection and breeding of switchgrass for resistance to both rust pathogen and in answering critical epidemiological questions.

Tables and Figures

Table 3.1 Primer set used in second part of the study for identify *Ug*

Name	Sequence (5'->3')	Length	Tm	GC%
UgF	CACCTTGCGCCTTTTGGTATT	21	59.73	47.62
UgR	AGTCTCTTGCTCAACAACAAAATAA	25	57.67	32.00

Table 3.2 Primers used for polymerase chain reaction in this study

Sequence	Target species	Amp Dl
TGCAGAAGGATCATTA-3′	All rust	
ACTGCCTTCCTCAATC-3′	All rust	
CCCCTTTTATTCTTAAA-3′	*Pe*	
TCTCAACAACAAAATTTTAC-3′	*Pe*	
TGCGCCTTTTGGTATT-3′	*Ug*	
GCTCAACAACAAAATAA-3′	*Ug*	

Table 3.3 PCR amplification of switchgrass leaves infected with *Ug*, *Pe*, both species, or neither species using primer sets for common rust, *Pe*, and *Ug*s. Yellow highlighted results indicate false negatives; red highlighted results indicated false positives

Rust species by microscopic inspection; total number of samples tested	PCR results (+ = detection; - = no detection)				Interpretation of PCR results
	Common rust primers	*Pe* primers	*Ug* primers	Number of samples exhibiting result	
Ug only; 5 samples	+	-	+	0	Expected result
	+	+	+	2	False positive
	-	-	-	3	False negative
Pe only; 9 samples	+	+	-	0	Expected result
	+	+	+	3	False positive
	-	+	-	4	False negative
	-	-	-	2	False negative
Ug and *Pe*; 8 samples	+	+	+	2	Expected result
	-	+	+	1	False positive
	-	-	+	2	False negative
	-	-	-	3	False negative
None; 3 samples	-	-	-	3	Expected result

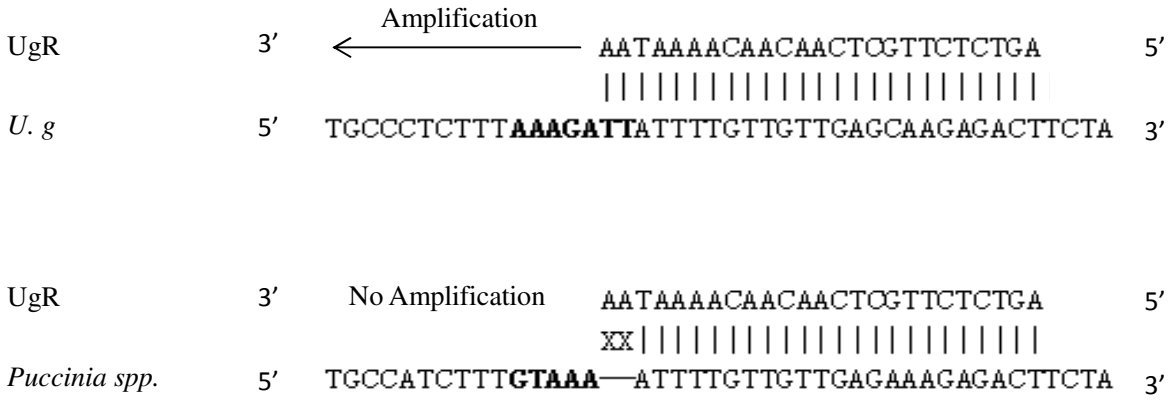

Figure 3.1 *Uromyces graminicola* specific reverse primer design strategy.

*Blacked letters are nucleotides distinct between *Ug* and *Puccinia* spp. sequences

Figure 3.2 A schematic showing the ITS and locations of the primer set UgF&UgR used for amplification of *Uromyces graminicola*

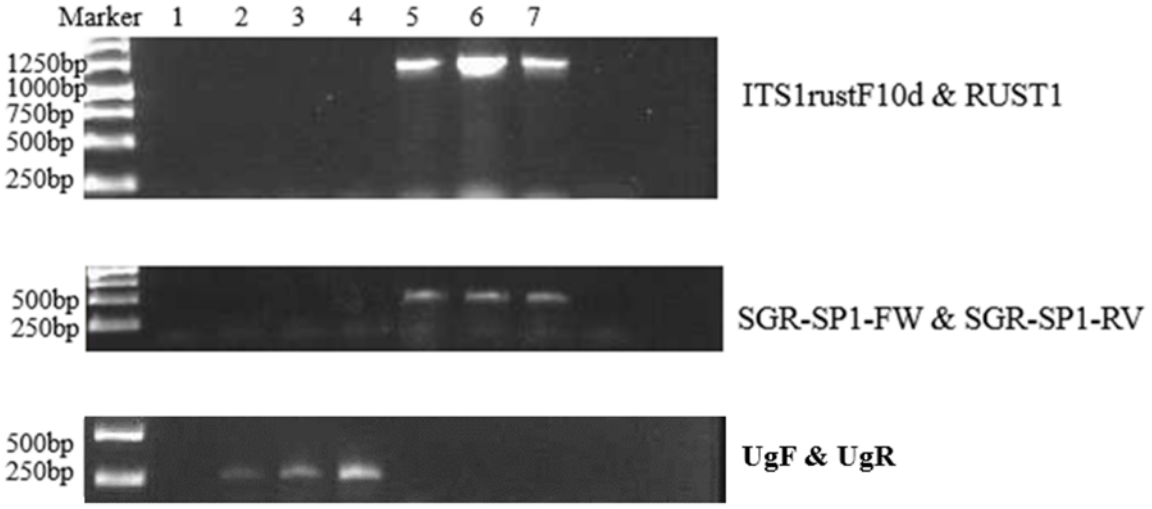

Fig 3.3 Amplification of internal transcribed spacer (ITS) primers in rust DNA.
Lane 1-4: *U. graminicola*, lane 5-7: *P. emaculata*

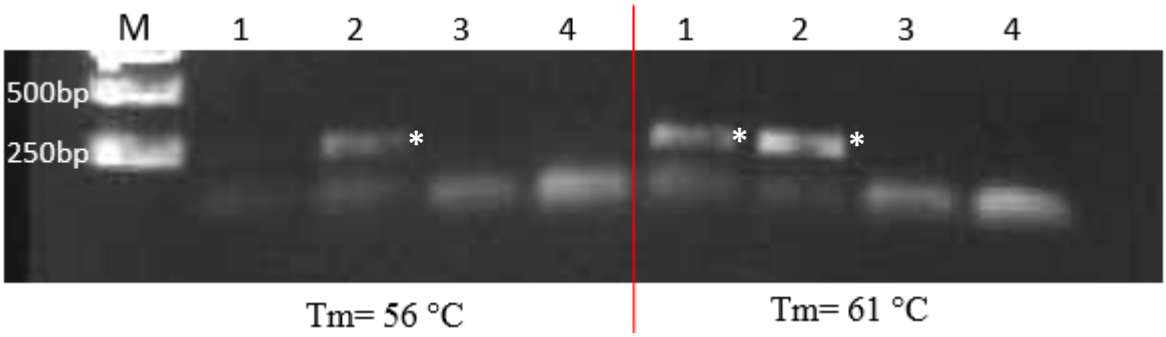

Figure 3.4 An ultraviolet fluorescent agarose gel showing the specific PCR amplification (* marked bands) of *U. graminicola* genome DNA using UgF&UgR primer set. Lane 1&2: *Ug* DNA. Lane 3&4: *Pe* DNA

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Chapter 4 Conclusion and General Discussion

The earliest research on switchgrass rust pathogens conducted over 100 years ago were done on wild grass populations. Then there followed about a 50 year gap before studies of rust pathogen on switchgrass were renewed, but this time is on switchgrasses first being developed for use in pastures and soil conservation (Cornelius & Johnston, 1941; Aikman & Robert, 1943; Riegel, 1947). *U. graminicola* was reported at that time to be found in numerous states and to be the most commonly observed rust pathogen on switchgrass in the central United States (Cornelius & Johnston, 1941; Farr et al., 1989). But little emphasis was put on switchgrass rust disease research subsequently because of its limited usage and importance.

In the later part of the 20th century, the increasing interest of using switchgrass as a biofuel feedstock promoted the study of diseases in switchgrass again and rust disease again was recognized as a threat to switchgrass. But research conducted in the southern states found *P. emaculata* to be the only causal agent of rust on switchgrass instead of *U. graminicola* (Zale et al., 2008; Hirsch et al., 2010; Frazier et al., 2013; Gilley et al., 2013; Uppalapati, et al., 2013) while little was known in the North Central states as to the etiology of rust. In Chapter 2 of this study, I report the finding of *U. graminicola* being a rust pathogen in Nebraska and Wisconsin while *P. emaculata* was found to be the major pathogen in all states surveyed (NE, IL, WI, IN and MO). The identification of *U. graminicola* in WI and *P. emaculata* in IL, IN and MO constitutes the first reports of these pathogen on switchgrass in those states. Thus, this study contributed new information as to the distribution of rust fungi on switchgrass. From analyzing a selection of switchgrass strain for the proportion of

rust infection caused by *U. graminicola* and *P. emaculata*, it was found that the relative virulence of each species was dependent on the switchgrass strain. The difference in rust resistance between lowland type and upland type switchgrass found in this study is consistent with previous studies. The unique finding from this study is that switchgrass strains differ in their susceptibility to each pathogen. The immediate conclusion that can be drawn from these results is that, in contrast to the switchgrass rust picture in the southern US where it appears that only *P. emaculata* is of importance, switchgrass rust in northern areas such as Nebraska can also involve *U. graminicola.* The broader implications is that it will be necessary to focus on both *U. graminicola* and *P. emaculata* in the breeding and selection of biofuel switchgrasses, particularly for varieties targeted for use in the North Central states.

A big challenge we will face in such efforts is the lack of efficient methods to identify *U. graminicola* and *P. emaculata.* Unlike the various rust diseases of wheat and other cereals, the two species of switchgrass rust cause identical symptoms on switchgrass. Furthermore the two species cannot be distinguished morphologically until the end of the switchgrass growing season. Therefore, molecular identification methods are greatly needed. Chapter 3 of this thesis provides a prototype diagnostic method for two rust species *P. emaculata* and *U. graminicola* in switchgrass based on PCR with a set of *Ug*-specific primers designed in this study in conjunction with previously described primers for *P. emaculata* and all rust fungi in general. However, the PCR system tested in this study did not prove to be very effective in identifying the rust fungi in switchgrass leaves stored for long periods due to low sample quality. Therefore, the accuracy and reliability of the system needs to be evaluated on fresh

plant samples. Furthermore, the sensitivity level of all the primers should be tested using different concentrations and ratios of *U. graminicola* and *P. emaculata* DNA. In Chapter 3, I provide suggestions for other modifications of the PCR system that might improve its effectiveness. Single nucleotide polymorphisms (SNPs) have been found in *Fusarium* ITS regions for identifying *Fusarium* complex causing wheat head blight (Chung et al., 2008). Quantitative identification of *Fusarium* species has been accomplished through competitive PCR or quantitative PCR (Edwards et al., 2001; Waalwijk et al., 2004). Specific primers of *U. graminicola* and *P. emaculata* could be useful for quantifying rust population on switchgrass in future studies. As other sources of nucleotide polymorphisms, other commonly used genes like β-tubulin, TEF1-α can be sequenced and analyzed for *U. graminicola* and *P. emaculata* (Fernández-Ortuño et al., 2010; Matny et al., 2012).

If the PCR system is developed further to improve sensitivity and reliability, the method would make it possible to detect the rust pathogens in a more timely manner and to distinguish the two rust species in all developmental stages. Thus, it could be a powerful tool for evaluating switchgrass lines for resistance to either or both pathogens. The PCR system could also be a valuable tool for epidemiological investigations. There are many critical questions regarding the epidemiology of switchgrass rust diseases that requires effective molecular tools to distinguish the two rust species. These questions include the alternate uredinial and aecial host range for each rust species, sources of the primary inoculum that initiates epidemics in switchgrass, the ability of inoculum of each species to disperse long distances, and the environmental conditions that favor infection and disease development by each

rust species.

As rust pathogens, *U. graminicola* and *P. emaculata* have the potential to be destructive on switchgrass when biofuel switchgrasses are grown as monocultures over wide geographic areas. Although host resistance appears to be a promising strategy for managing rust diseases in switchgrass, there is also the potential for rust resistance to decline over time given the ability of rust fungi in general to change in response to selection pressure and the effects of environmental stress on the ability of plants to express resistance. Therefore, a more practive effort must be made towards studying the epidemiology of the switchgrass rust diseases, understanding interactions between switchgrass and the pathogens, and elucidating interspecies and intraspecies diversity of switchgrass rust populations.

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