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DISEASE PROGRESS AND VARIATION IN PATHOGENICITY OF SUGARCANE RUSTS IN FLORIDA

By

Santosh Sanjel

December 2016

Chair: Philippe C. Rott
Major: Plant Pathology

Brown rust (caused by *Puccinia melanocephala*) and orange rust (caused by *P. kuehnii*) are two major diseases currently impacting sugarcane production in Florida. In this study, severity of sugarcane brown and orange rust was monitored biweekly during two crop seasons. In 2014 and 2015, overall disease severity was higher in cultivar CL 85-1040 (susceptible to orange rust) than in cultivar CL 90-4725 (susceptible to brown rust). Severe symptoms of orange rust were also observed for a longer period of time; therefore, orange rust is expected to have more impact on sugarcane growth and management during the crop season than brown rust. The progress of orange rust appeared to be essentially temperature dependent whereas brown rust was affected by temperature and plant age. This is because mature sugarcane did not exhibit brown rust symptoms, although temperatures were considered disease conducive. Variation in pathogenicity of the two rust pathogens was investigated by inoculating sugarcane cultivars with rust isolates collected from different susceptible sugarcane cultivars, including CP 89-2143 that has shown susceptibility to orange rust in Florida only since 2011. No variation in pathogenicity was observed among the three isolates of *P.*
*melanocephala* after inoculation of CP 96-1252, CP 78-1628 and CP 90-4725. In contrast, *P. kuehnii* collected from CP 89-2143 was more virulent than the isolates collected from CL 85-1040, CP 80-1743, and CP 88-1762 that were susceptible to rust before 2011, suggesting occurrence of at least two different populations of *P. kuehnii* in Florida.
CHAPTER 1
LITERATURE REVIEW

Understanding Host Pathogen Interactions

While studying plant diseases, pathogenicity, virulence and aggressiveness are important terms. Over the years, confusion resulted in the literature because these terms have been used in different situations or with different definitions and it is therefore very important to specify the meaning of each of them. Simply, pathogenicity is the ability of a pathogen to cause a disease. It is therefore a qualitative characteristic.

If a microorganism is able to cause a disease in a host it can be called pathogenic and, if not, it is non-pathogenic (Shaner et al. 1992). Pathogenicity can be considered as a general term which includes characteristic changes in the host or development of symptoms due to the attack of a pathogen, such as size and number of lesions, number and size of uredinia, amount of spores produced, growth of the pathogen and several other changes in host physiology (Watson 1970). Pathogens use a number of means to recognize their host, enter inside the host cell and multiply. These pathogenicity factors are encoded by one or more pathogenicity genes. A majority of pathogenicity factors include cell wall degrading enzymes and toxins that kill or injure plant cells (Keen 1988).

During the last three decades, pathogenicity has been extensively studied and, the molecular basis of pathogenicity has been unraveled for numerous plant pathogens. However, less data are currently available regarding virulence and aggressiveness (Sacristán and García-Arenal 2008).

Virulence of a pathogen is more related to a specific host. Watson (1970) defines virulence as the characteristic of a strain of a pathogen that makes it able to cause a disease because the host lacks a resistance gene against that strain. This strain has
therefore a specific virulence gene. Basically, a strain of a pathogen is more virulent than another strain when it attacks a higher number of cultivars (Watson 1970).

Aggressiveness is another term which can be used to describe a pathogen. If a strain of a pathogen produces higher amounts of symptoms, or causes a greater damage in a certain variety in comparison to other strains of same pathogen, it is considered as a more aggressive strain. Strains that are equally virulent may vary in terms of aggressiveness (Shaner et al. 1992).

**Pathogenicity and Host Resistance**

Pathogenicity and host susceptibility are both genetically determined characteristics. Pathogens deliver certain molecules encoded by pathogenicity related genes to infect and colonize host cells whereas, on the other end, plants have combinations of genes making them non-host to most pathogens but susceptible to some pathogens. The mechanisms of host resistance have been studied for years to develop resistance against pathogens. Two types of host resistance, called vertical and horizontal, and first described by Van deer Plank in 1963, are the basis of plant disease resistance (Thomas et al. 2011). Vertical resistance is a complete resistance due to a single gene and horizontal resistance is an incomplete resistance provided by multiple genes (Poland et al. 2009).

**Vertical Resistance and the Gene-for-Gene Concept**

Mechanisms of vertical resistance or qualitative resistance or R-gene mediated resistance and race specific virulence are explained by the popular “gene-for-gene” concept. This concept was first described by Flor in 1942 based on his research on flax and the rust pathogen *Melampsora lini* (Flor 1947). According to this theory of host-pathogen interaction, a gene present in the plant which confers resistance has its
counterpart in the pathogen which causes pathogenicity. Resistance was believed to be conferred by the direct interaction and recognition between the resistance (or R) protein encoded by the R gene of the resistant host and the Avr (for avirulence) protein encoded by the Avr gene of the pathogen (Thomma et al. 2011). The modern elaborated “gene-for gene concept” defines the role of new classes of molecules responsible for pathogenicity, virulence and immunity. Certain molecules encoded by pathogens are called effectors, and these effectors allow the pathogen to escape the plant’s basal defense response and to colonize the host (Ayliffe et al 2008). In turn, the host plants have evolved and have acquired R gene products which include several protein classes including kinases, receptor kinases and nucleotide-binding site-leucine-rich repeat proteins (NB-LRR). These molecules recognize the pathogen’s effectors, a phenomenon that results in effector triggered immunity (ETI) which will cease the growth of the pathogens (Thomma et al. 2011). ETI is followed by cascades of events of programmed cell death called hypersensitive reaction and activation of systemically acquired resistance to protect the host from further invasion of such recognized pathogens (Gururani et al. 2012).

**Horizontal Resistance and Minor Genes**

Horizontal resistance, also called quantitative resistance, is not specific to one pathogen and is controlled by multiple genes called minor genes which do not necessarily prevent disease but reduce disease severity (Parlevliet and Zadoks 1977). Pathogen-associated molecular patterns (PAMPs) or microbe associated molecular patterns (MAMPs) are molecules associated with groups of pathogens that are basically responsible for the fitness of a pathogen. However, plants having certain resistance genes are capable of recognizing these molecules by membrane bound receptors and
this interaction results in a basal defense response known as PAMPs triggered immunity (PTI) which controls pathogen infections (Thomma et al. 2011). Like ETI, PTI is followed by cascades of events resulting in a hypersensitive reaction (Gururani et al. 2012). Horizontal resistance provides different barriers and defense system to protect the plant against pathogens. This defense strategy includes production of phytoalexins, chemical and physical barriers, pathogenicity related proteins and hydrolytic enzymes (McDonald and Linde 2002). Quantitative resistance is relatively poorly understood and less exploited in variety development programs because of the “incomplete and inconsistent nature of phenotype conditioned by many loci of small effect”. However, the objective of current studies is to understand and exploit this means of resistance (Poland et al. 2008).

**Durability of Resistance**

Besides the high level of resistance provided by R genes and its massive use in breeding for resistance, it has limitations. R genes lack durability against pathogens having high evolutionary potential because of its specificity in recognition and simply because any mutation in an *Avr* gene will lead to a loss in recognition (Poland et al. 2008). To address this issue, a concept of durable resistance which can be achieved by pyramiding several major resistant genes in a single cultivar was formulated. Another strategy could be the rotation of major genes in time and space through mixed cultivation of varieties with different major resistance genes. Utilization of minor genes is another strategy as these genes are less likely to break down because they are not based on elicitor-receptor recognition. This concept of combining genes in a variety through various recently identified means of gene deployment is believed to be the ultimate goal for durable resistance (Boyd et al. 2013; Mundt 2014).
Variation in Pathogen Population Structure

Plant pathogen populations have evolutionary potential which results in population variation during time, and this characteristic is a serious concern for durability of resistance (Burdon 1993). In the history of plant pathology, this phenomenon came into light with many reports of resistance breakdown for major resistant genes. These breakdowns resulted solely from a change in pathogen population structure and not in the host population because, as Flor had already shown in the early 1940s, major resistant genes in a crop variety are inherited by the next generation (Flor 1971). The concept of “boom and bust cycle” is popular to explain breakdown of resistance in rusts and powdery mildews of cereals. In this concept, boom is the large proportion of the pathogen population for which the major resistance gene is effective; and bust is the new population of the pathogen which overcomes the resistance, and the cultivated variety is consequently no longer resistant (Priestley 1978). According to McDonald and Linde (2002a), major evolutionary forces that bring variation in pathogen populations are mutation, population size and random genetic drift, gene and genotype flow, reproduction and mating system, and selection imposed by a major gene. Mutation is the ultimate source of variation which causes variants in a population. Because pathogens have low rates of mutation, the probability of occurrence of mutants will be higher in the pathogens with large population sizes. Similarly, a pathogen system in which arrival of new genes and genotypes is frequent will possess higher numbers of evolutionary events. Regarding effect of mating system, pathogens having mixed types of reproduction systems have more variants in their population due to combined effect of recombination and mutation. Finally, the main force that brings variation in pathogen population is the selection due to major genes.
The wide distribution of a major resistant gene will cause an increase in the frequency of those virulent mutants which have lost their elicitor/avirulence allele (McDonald and Linde 2002).

**Races of Plant Pathogens**

Races of a plant pathogenic species are strains or isolates that are morphologically similar but physiologically and genetically different from each other (Agrios 2004). After characterization of races, host plant resistant genes against these races are searched to be used in development of resistant cultivars (Jin et al. 2007). Variation in virulence is indication of occurrence of pathogen races which are likely to overcome at least one host resistance gene (Kelly et al. 1994). There is a long history of appearance of races in wheat stem rust pathogen populations. Breakdown of genes conferring resistance to wheat stem rust are frequently overcome as illustrated by development of several sudden disease epidemics. Several races of the stem rust pathogen are currently present in nature, and *Ug 99* is one of the most evolving and important races. New resistant genes are needed in plants to withstand these new races (Jin et al. 2007).

The traditional method of race characterization is through the use of a set of differential varieties and observation of plant reactions to different isolates of the pathogen. Eriksson was the first one to show the existence of biological races in the cereal rust pathogen *Puccinia graminis* in 1894 using several wheat cultivars (Agrios 2004). Races themselves can sometimes show heterogeneity and comprise various genotypes. This property of races can be observed using additional sets of differentials (Staples 2000).
Use of differential sets for pathogen phenotyping is a popular technique but it has some limitations. If possible, both phenotyping and genotypic analysis should be done to ensure occurrence or absence of variation among isolates of a pathogen (Andrie et al. 2007). Because the use of differential varieties is time consuming, alternative methods such as biochemical and genetic analyses are also conducted for race characterization. Analyses of total cellular lipids of pathogen isolates by gas liquid chromatography resulted in successful characterization of races of *Phytophthora fragariae* (Maas 2015). Random amplified polymorphic DNA (RAPD) analysis was used to determine races of *Fusarium oxysporum* f. sp. *lycopersici*, in addition to biological characterization (Mes et al. 1999). Similarly, three races of *Fusarium oxysporum* f. sp. *vasinfectum* causing wilt of cotton were identified using a disease severity index and RAPD markers (Assigbetse et al. 1994).

**Rust Diseases in Plants**

Rusts, the basidiomycete fungi of the order *Uredinales*, are among the most destructive and notorious plant pathogens which have a brutal history of causing famines and turning down large economies (Agrios 2004). There are more than 120 genera and 6000 species of rust fungi (Duplessis et al. 2011). Rusts have been attacking mainly grain crops like wheat, oat, and barley, but they are also major pathogens of several other field crops like soybeans, coffee, beans, cotton, and sugarcane. Rusts are a serious concern for some trees and ornamental plants like pine, apple, coffee and poplar. In nature, rusts occur in more than 200 host plant families (Agrios 2004).
Life Cycle of Rust Pathogens

Rust fungi are obligate biotrophs which cannot be grown in the laboratory on artificial media. The life cycle of rust fungi is complex and it can include as many as five distinctive spore types: basidiospores, pycniospores, aeciospores, urediniospores and teliospores. Some rusts are autoecious which means they complete their life cycle on a single host. Other rusts are heteroecious and their life cycle is completed in multiple host species. Wheat stem rust is a perfect example of heteroecious rust for which the barberry plant, the alternate host of the pathogen, bears pycniospores and aeciospores (Figure 1-1). The other spores, namely urediniospores, teliospores and basidiospores, are formed in the wheat plant (Agrios 2004). Particular types of rust spores are specialized to particular hosts in case of heteroecious host (Goellner et al. 2010). Crops like soybean and sugarcane bear urediniospores but it is still unclear if these rusts have any alternate host or not, and if they have sexual cycles or not (Goellner et al. 2010; Raid and Comstock 2000). Urediniospores are repeatedly multiplying spores which invade host cells, resulting in development of numerous uredinia in the leaves and sometimes in the stem. Urediniospores are mainly dispersed from uredinia by the wind, but rain and insects also help in spore dispersal. Dispersed urediniospores land on fresh leaves where they germinate and develop a next generation of urediniospores. In the case of wheat rust, at the end of the season, teliospores are formed which are resting/overwintering spores. At the beginning of a new season, deployed teliospores undergo meiosis and basidiospores are formed. Basidiospores germinate and develop pycniospores in barberry (alternate host), which fuse and develop diploid aeciospores in barberry. Finally, aeciospores from barberry reach the wheat plants and enter the wheat stem and leaf cells through stomata. After successful infection of these cells, the fungus
ultimately causes rupture of the leaf host surface, releasing a mass of urediniospores from the rust uredinia (Petersen 1974).

The general infection process for most of the rust pathogens is similar. Urediniospores germinate generally on the leaf surface or sometimes on the stem surface of the host plant and give rise to a single germ tube. Apressoria are formed on the germ tube when they sense stomatal walls, or occasionally epidermal walls. An apressorium or penetration peg is formed to enter through the stomata or to break the cuticle and directly enter the epidermal cells. Once inside the plant cell, haustoria are formed to colonize the host cell. Finally, the rust pathogen multiplies inside the host cell and eventually bursts the epidermis to release a mass of urediniospores (Miles et al. 2003; Bhairi et al. 1989).

**Host Pathogen Interactions in Rust Diseases**

During the last decades, better understanding the mechanisms of disease development and host defense has been successful in reducing the use of huge amount of harmful pesticides by exploiting host resistance to control diseases (Keen 2000). Rust diseases have been constantly used to understand the phenomenon of host-pathogen interactions. Flor studied Flax rust and proposed the gene-for-gene concept which opened a new broader arena for host-pathogen interactions and disease resistance (Flor 1947).

Wheat rusts are among the most studied rusts and have been constantly re-evolving by forming new and different races. Consequently, wheat rust pathogens have always been threatening the long lasting rust resistances on one hand, but have allowed plant pathologists and plant breeders to study and understand host-pathogen interactions in further depth on the other hand (Roelfs et al. 1992). Wheat stem rust
resistant genes (Sr genes) and wheat leaf rust resistant genes (Lr genes) functioning under the gene-for-gene hypothesis have been successful in providing a good resistance level for a long time. Wheat rust effector molecules are hypothesized to trigger the disease or being recognized by the potential resistant gene products, but a formal demonstration has yet to be reported. Functional genomics with rust pathogens is very difficult because these pathogens cannot be grown in culture, which limits functional genomic studies. To combat the frequent appearance of new races of wheat rust, a concept of durable rust resistance strategy is thought to be an ultimate goal. Research is therefore conducted to understand the molecular basis of long lasting R genes, pyramiding of resistance genes and understanding the molecular mechanisms of effector recognition (Ayliffe et al. 2008).

Asian soybean rust caused by *Phakopsora pachyrhizi* is the major disease of soybean in Asia and an emerging new rust disease in the continental US where it was seen for the first time in 2004 (Schneider 2005). This short local disease history will help researchers to understand the evolution of pathogenicity factors and host resistance in this new pathosystem. *Phakopsora meibomiae*, another pathogen causing soybean rust, was present for a long time in the Western hemisphere where it was considered a minor pathogen until 2001 (Bonde et al. 2006). Asian soybean rust showed up in Hawaii in 1994 and in South America around 2001. It is believed that rust spores were carried from South America to North America by tropical storms and hurricanes (Schneider et al. 2005; Rupe and Sconyers 2008). *P. pachyrhizi* forms resting teliospores but the germination of these teliospores give rise to sexual spores although basidiospores have not been observed in nature. Absence of sexual reproduction of *P. pachyrhizi* in nature
is therefore limiting recombination and creation of new pathogenic strains during the sexual cycle of the pathogen. However, several races of *P. pachyrhizi* exist in nature (Rupe and Sconyers 2008). Similar observations have been made for other rusts. Using a differential set of four species of *Glycine*, six different reaction types were demonstrated in Australia, suggesting the occurrence of at least 6 races of *P. pachyrhizi* in this geographical location (Burdon and Speer 1984). Additionally, involvement of at least three resistance genes in the host plants was hypothesized (Burdon and Speer 1984). Since then, four resistance genes (*Rpp*\(_1\), *Rpp*\(_2\), *Rpp*\(_3\) and *Rpp*\(_4\)) were found to confer resistance in soybean to Asian soybean rust (Rupe and Sconyers 2008). The management of the recently appeared soybean rust in the US is currently depending on the application of strobilurin fungicides. However, breeding soybean for resistance to the different physiological rust races is expected to produce sustainable resistant varieties and reduce, if not stop, the use of fungicide treatments. Because all known resistance gene have been overcome by rust, there is a need to continue the studies on rust races and identification of new resistance sources. These resistance sources also include quantitative resistance conferred by minor genes (Hartman et al. 2005; Rupe and Sconyers 2008).

From an economical point of view, the coffee industry is one of the most important agricultural industries in the world. At present, this agro-industry involves large scale international trade and holds huge monetary value (McCook and Vandermeer 2015). Coffee rust caused by *Hemileia vastatrix* (a major pathogen in all the world’s coffee-growing regions) and *H. coffeicola* (the predominant coffee rust in Central and Western Africa) is a major concern since the first outbreaks of the disease
in “location” in 1869. Studying the spread and intensity of the disease in a worldwide perspective is critical to control the disease (Arneson 2000; McCook and Vandermeer 2015). Coffee rust showed up for the first time on the American continent in Brazil in the late 1970s. Currently, all coffee growing areas around the world are contaminated with coffee rust (McCook and Vandermeer 2015). It is believed that coffee rust was carried to the Americas either through air currents over the Atlantic Ocean or through infected seeds and young plants during trades, or maybe both. The capacity and efficiency of this rust to spread can be illustrated by the epidemics that occurred in Central America. Initially, a massive eradication of infected plants along with healthy coffee plants in a 30 meter radius was successful. However, most likely because of wind dispersal of rust spores, the disease reestablished (Arneson 2000). Occurrence of several races of the coffee rust pathogen has been reported all around the globe. In 1975, 32 races of Hemileia vastatrix and H. coffeicola were reported from a worldwide collection (Rodrigues et al. 1975). At present, more than 40 races of coffee rust (H. vastatrix) are known to occur, and these include new races that are able to overcome some previously resistance genes. Widespread epidemics of coffee rust are more likely to occur in the Americas because most of the coffee plants grown in this part of the world are derived from the same parental lineage with a similar genetic base. Nine resistance genes to coffee rust have been identified and the major concern for the deployment of new resistant gene(s) is to maintain proper agronomic characteristics in the new varieties at the same time (Arneson 2000). In the past, several attempts to grow rust resistant coffee varieties failed because these varieties were lacking desired agronomic properties and were rejected by the growers (Rodrigues Jr et al. 1975).
Although rust races and host resistant genes have been characterized, and breeding for resistance to rust performed for numerous rust diseases, little information is available regarding detailed mechanisms of rust resistance and role of pathogen effectors. A recent experiment with poplar leaf rust (*Melampsora larici-populina*) using an effectomics pipeline resulted in new findings regarding rust effectors. Candidate effector proteins coded by *Melampsora* genes were found and these proteins are expected to interact with plant cellular proteins. Obtaining such data will result in new research areas to study host-rust interactions (Petre et al. 2015).

Because rusts have an obligate biotrophic lifestyle, they must constantly change to overcome possible barriers of infection for continuation of their life cycle (Duplessis et al. 2011). Strong dispersal capacity confers rusts the status of worldwide important pathogens in modern agriculture. With the long term goal to deploy sustainable resistance to these pathogens, the ongoing and unprecedented search for mechanisms of resistance to rusts will remain a big challenge as both the pathogen and the plant (with the help of researchers) are engaged in an armed race of uncertain issue, thus supporting the “Red queen hypothesis” of evolution (Clay and Kover 1996). This hypothesis comprises a ‘zero-sum’ assumption which implies that the fitness of a species is exactly balanced by the loss of fitness of other species (Smith 1976).

**Sugarcane**

**Sugarcane as a Crop**

Sugarcane (*Saccharum* spp.) is a tall perennial grass that is grown in tropical and sub-tropical regions around the world (Karim et al. 2002). Sugarcane is primarily grown for sugar. The importance of the sugarcane economy can easily be deducted from the fact that 70% of the world’s sugar supply comes from sugarcane. Nowadays, sugarcane
is also considered as a potential source of renewable biofuel source (Tew et al. 2008), electricity, organic chemicals and paper (Renouf et al. 2010).

Sugarcane is grown in more than 90 countries covering all five continents. In 2014, the major sugarcane producing countries were Brazil (737.15 MT), India (352.14 Mt), China (126.15 Mt), Thailand (103.69 Mt), Pakistan (67.46 Mt), Mexico (56.67 Mt), Colombia (38.15 Mt), Philippines (32.46Mt), Australia (30.51 Mt), Indonesia (28.6 Mt) and the United States of America (28 Mt) (FAO STAT 2014).

**History, Distribution and Place of Origin**

The origin of sugarcane goes back to prehistoric times, even before continents had their current shapes and locations (Cheavegatti-Gianotto et al. 2011). In 325 B.C., Alexander the Great took sugarcane from India as a treat to Europe (James 2004), but sugarcane as a crop was introduced in Europe around 100 A.D. The Persians and Greek discovered the famous “reeds that produce honey without bees” during the sixth and fourth centuries B.C. in India and started the trade (FAO, 2009). Mediterranean bordering countries grew sugarcane since 1300s. It was brought to the Western hemisphere from Spain through the Dominican Republic by Christopher Columbus in 1493. The West Indies and Cuba started growing sugarcane in 1511. In 1515, the introduction of sugarcane in Mexico started the modern sugar industry (James 2004). Sugarcane was introduced into the United States (Louisiana) in 1751 (Alfieri 1979).

Most desirable tall, thick and brightly colored canes called noble canes (*Saccharum officinarum*) travelled around half the circumference of the world (James 2004). Based on the geographical origin, phylogeny and dispersal, the most possible center of origin of the noble cane is Papua New Guinea from where three different routes of migration took place and stalks transported by man are most probably the only
source of dispersal (Artschwager and Brandes 1958). Noble canes may have originated around the equatorial region 5°S (where currently Indonesia and Papua New Guinea lie) and moved initially to regions within 21°S, and finally to the regions around 21°N (James 2004).

**Origin of Saccharum Species**

Modern varieties of sugarcane are complex interspecific hybrids of Saccharum selected after crossing various Saccharum species, especially S. officinarum and S. spontaneum (Cox et al. 2000). The noble cane Saccharum officinarum is a product of natural and complex introgression between S. spontaneum, Erianthus arundinaceus, and Miscanthus sinensis. It has been hypothesized that human domestication played a major role in the origin of S. officinarum because wild members of S. officinarum are no longer found. S. robustum possibly developed as an intermediate form during formation of S. officinarum (Daniels and Roach 1987).

**Taxonomy**

Sugarcane is a perennial monocot crop in the grass family. Taxonomic classification of sugarcane is provided by the United States Department of Agriculture, Natural Resources Conservation Services, Plants Database is as follows:

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
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<tbody>
<tr>
<td>Subkingdom</td>
<td>Tracheobionta</td>
</tr>
<tr>
<td>Superdivision</td>
<td>Spermatophyta</td>
</tr>
<tr>
<td>Division</td>
<td>Magnoliophyta</td>
</tr>
<tr>
<td>Class</td>
<td>Liliopsida</td>
</tr>
<tr>
<td>Subclass</td>
<td>Commelinidae</td>
</tr>
<tr>
<td>Order</td>
<td>Cyperales</td>
</tr>
</tbody>
</table>
Family  Poaceae/Gramineae
Genus  Saccharum L.
Species  officinarum L.

The taxonomy and phylogeny of sugarcane is complicated because this plant species shares common characteristics with a closely related inbreeding group known as the *Saccharum* complex. The members of this complex are *Saccharum, Erianthus* section *Ripidium, Miscanthus* section *Diandra, Narenga* and *Sclerostachya* (Daniels and Roach 1987). These genera have high polyploidy and frequently unbalanced chromosomes (Sreenivasan et al. 1987).

Currently, six different species of *Saccharum* have been described. Four of them are still cultivated and two are wild species (Irvine 1999) (Table 1-1).

**Botany of Sugarcane**

Sugarcane is a grass that can reach a height of 2-3 meters and it produces multiple stalks consisting of nodes and internodes. The major parts of the sugarcane plant are the stem, the leaf, the root system, the inflorescence and the seed. The stalk consists of joints known as nodes and the region between nodes are called internodes. Each node holds three parts: a leaf, a bud, and root primordia. Internodes are short near the base of the stalk and increase in length towards the tip. When seed cane (stalk pieces with multiple buds) is planted, each bud forms a primary shoot which gives rise to secondary shoots called tillers (Miller 2010). Leaves form two alternate ranks on either side of the stem. The internode consists of vascular tissue and sucrose storing parenchyma cells. The apical meristem is at the top of the stem, below which numerous short internodes are located. The leaf spindle of enclosed leaves is at the top, and 10-12 green leaves as well as several senescent leaves are at the bottom. As the plant
grows, older leaves turn senescent. New leaves emerge and expand over a period of 7-21 days depending on the growth conditions. Internodes can be as long as 30 cm (van Dillewijn 1952). Sugarcane leaves are alternate and are attached to the stalk, with one leaf per internode. The sheath consists of the sheath itself and the much smaller acropetal blade joint consisting of a leaf collar, dewlap, ligule and auricles. The shape, size and distribution of trichomes and the shape of the ligule and auricles are traits of taxonomic importance for varietal identification. Sugarcane leaves are numbered from top to bottom starting with the uppermost leaf showing a visible dewlap (TVD) which is numbered +1 (Cheavegatti-Gianotto et al. 2011) (Figure 1-2). The TVD leaf is important for the sugarcane crop as it is the most photosynthetically active leaf and very frequently used for disease, physiological and nutritional studies (Miller 2010).

Similar to other grasses, the sugarcane root system is shallow but the plant is anchored by buttress roots which can grow downward up to 5-7 meters. Buttress roots serve for water absorption during drought (Moore 1987). Two kinds of roots are found in a planted seed piece: set roots which are thin and highly branched, and shoot roots which originate from the lower root bands of shoots. Shoot roots are thick, fleshy and less branched. Set roots are temporary until the shoot roots are formed. Each new tiller will develop its own shoot root system (Miller 2010).

When the sugarcane plant reaches the mature stage, the growing point is ceased and the vegetative part changes to reproductive flowers (Miller 2010). The inflorescence/tassel of sugarcane is a ramified, conoidal, panicle with a main stem, called the rachis, which is the continuation of the last stalk internode (Cheavegatti-Gianotto et al. 2011). Each tassel is capable of producing seed that is very small (about
250 seeds per gram). After flowering, sucrose content of sugarcane is reduced and it is not a desirable trait for commercial purpose; however, flowering is crucial for sugarcane breeders (Miller 2010).

**Diseases of Sugarcane**

Sugarcane cultivation faces a big challenge from more than 100 pathogens. Bacteria, fungi, viruses, nematodes and physiological disorders can affect sugarcane growth and production. A list of sugarcane diseases reported for sugarcane all around the globe can be found on the websites of the American Phytopathological Society (APS) and the International Society of Plant Pathology (ISPP) at http://www.apsnet.org/publications/commonnames/Pages/Sugarcane.aspx and http://www.isppweb.org/names_sugarcane_common.asp, respectively. Several books and guides describing in detail numerous sugarcane diseases have been published during the last two decades, such as “Sugarcane diseases of the world. Major diseases” (Ricaud et al., 1989) and “A guide book to sugarcane diseases” (Rott et al. 2000).

**Rust Diseases**

Brown rust (formerly known as common rust) caused by *Puccinia melaocephala* and orange rust caused by *Puccinia kuehnii* are two important diseases of sugarcane that have been reported in sugarcane growing regions all around the world. Brown rust has been a problem in the Americas since 1978 (Purdy et al. 1985) but orange rust showed up for the first time in the Western hemisphere only in 2007. The first report of orange rust in the USA was in Belle Glade, Florida (Comstock et al. 2008). Up to 50% yield reductions have been reported for both orange and brown rust of sugarcane (Magarey et al. 2001; Raid and Comstock 2000).
A new sugarcane rust named Tawny rust recently developed in Africa. The first report was made from Swaziland in 2008 and shortly later, in 2009, tawny rust appeared in South Africa (Rutherford et al. 2013). The disease is currently spreading to Mozambique and Zimbabwe (Martin et al. 2015). Tawny rust can be distinguished phenotypically, molecularly and phylogenetically, from the already existing brown rust and orange rust. Differences between these two later rusts and tawny rust include color and position of uredinia (bright orange and more abundant towards the leaf tip for tawny rust) as well as color of urediniospores (bright orange to orange-red for tawny rust). A detailed taxonomic study was also conducted recently which lead to name *Macruropyxis fulva* sp. nov. the causal agent of tawny rust (Martin et al. 2015).

**Brown Rust of Sugarcane**

**Symptoms**

The characteristic symptom of brown rust are reddish-brown to brown uredinia (also called uredinia) on the abaxial surface of the sugarcane leaf (Ryan and Egan 1989). Brown rust uredinia develop parallel to venation of the sugarcane leaf and are 2-20 mm long and 1-3 mm wide. The first symptoms of brown rust are small, whitish yellow flecks which turn into reddish brown lesions after 3-4 days and, within 2 weeks, the uredinia mature and erupt exposing numerous orange brown urediniospores. The uredinia can coalesce and form larger necrotic areas on severely affected leaves. Sometimes, brown rust and orange rust uredinia can be confusing but, in general, brown rust uredinia are more dark red to brown whereas orange rust uredinia are orange in color (Raid and Comstock 2000).
Diagnosis

Both orange and brown rust of sugarcane produce similar symptoms and the causal agents also look similar. However, through microscopic examination, they are readily distinguishable. The major difference of urediniospores of both rusts is the color, size and shape of spores. *Puccinia melanocephala* forms reddish to dark reddish uredinia (orange rust uredinia are not as dark as those of brown rust) on the lower side of the sugarcane leaf surface and these uredinia contain numerous spores (Raid and Comstock 2000). *P. melanocephala* urediniospores are spherical, 21-40 x 17-27 \( \mu \)m in size, and smaller than those of *P. kuehnii* (Ryan and Egan 1989). Both rust pathogens have spines around the outer wall of urediniospores but the spines in brown rust urediniospores are spaced by 1-1.5 \( \mu \)m whereas those of *P. kuehnii* are separated by a larger space (Raid and Comstock 2000). Additionally, spores of *P. melanocephala* do not have a thickened apical wall, in contrast to those of *P. kuehnii* (Figure 1-3).

Epidemiology

Leaf wetness and temperature are two major environmental factors involved in rust development. *P. melanocephala* urediniospores can germinate from 5\(^\circ\)C to 34\(^\circ\)C (Sotomayor et al. 1983). In the fields, brown rust symptoms are observed when temperature is higher than 17\(^\circ\)C with a simultaneous leaf wetness of at least 7 hours. However, temperatures higher than 32\(^\circ\)C are unfavorable for rust development (Barrera et al. 2012), which corresponds to decrease of rust development during warmer summer months in Florida (Rott et al. 2014).

Conducive temperatures and leaf wetness will allow the development of germ tubes from spores into the sugarcane leaf. *P. melanocephala* penetrates the sugarcane
leaf by means of infection structures (germ tube, aressorium, penetration peg, sub-stomatal vesicles, and infection hypha and haustorial mother cell) through stomata. Once the germ tube receives contact stimuli from guard cells of the stomata, it forms an aressorium. At the end of aressoria where the stomata is present, a penetration peg will be formed. This penetration peg will further grow between cells and develop haustoria in the established cells and infection hypha towards new host cells. Upon maturity of the rust mycelium inside the host cell, spore mass are formed which will break the host cells' epidermis and form uredinia/uredinia. Uredinia erupt and release spores (Sotomayor et al. 1983).

The above mentioned events of a single reproductive cycle of brown rust are completed in 14 days. Because of this short reproduction cycle, during favorable times of the year when temperature and leaf wetness are optimum, rust cycles numerous times and a field with a susceptible cultivar can appear reddish-brown in just 6 weeks (Raid and Comstock 2000).

Sugarcane brown rust is mainly disseminated by wind. The mature uredinia passively release the spores which are carried by wind and disseminated over a wide range of distances, from centimeters to hundreds of kilometers. Sugarcane rust is believed to have a strong capacity of dispersal and there is evidence which suggests that brown rust travelled from Africa to the Western Hemisphere after crossing the Atlantic Ocean (Purdy et al. 1985). Dissemination by rain-splash and water can also occur but only for short distance dispersal. In contrast to some fungal diseases, seed cane does not transmit rust since it is not systemic in sugarcane, attacking only the foliage and not the stalk (Raid and Comstock 2000).
Excessive amounts of nutrients are believed to increase rust severity. Excess of phosphorus and sulfur has been strongly correlated with increased rust severity while soil organic matter, potassium and calcium can also influence rust severity (Johnson et al. 2007).

**History**

This disease is present in almost all sugarcane growing areas around the globe (Raid and Comstock 2000). Brown rust was first reported in India in 1950 (Patel et al. 1950) and then it rapidly spread to South-East Asia, Africa, Southern Asia and Australia (Glynn et al. 2010). It was absent from the Western hemisphere until a first outbreak of brown rust in the Dominican Republic in 1978 (Pressely et al. 1978). It then spread throughout the Americas. Sugarcane brown rust was not of serious concern until severe epidemics occurred in Australia, Taiwan, the Caribbean Islands and the United States in 1970s. Several sugarcane varieties showed severe symptoms of brown rust in these locations, resulting in replacement of important and valuable cultivars (Dean and Purdy 1984). In Australia, brown rust was first reported in 1978 (Ryan and Egan 1979) and it rapidly spread all over Australia and caused 30% yield reduction along with replacement of established cultivars (Taylor et al. 1991). In the USA, several economically important commercial varieties such as CL41-223, CP63-588, CP 72-1210 and CP 78-1247 had to be replaced due to their susceptibility to brown rust (Dean and Purdy 1984; Comstock et al. 2010). B4362 was one of the most popular high yielding varieties in the Caribbean Islands and it was completely eliminated from the Americas because of high susceptibility to brown rust (Raid and Comstock 2000).
Resistance to brown rust

The *Bru1* gene, first identified in cultivar R570 (Daugrois et al. 1996), is a single major resistance gene against brown rust. All sugarcane cultivars susceptible to brown rust lack *Bru1* and most, but not all, resistant cultivars around the world have been tested positive for *Bru1* (Glynn et al. 2013; Racedo et al. 2013; Costet et al. 2012). Fifty percent of the clones released during 2011-2014 by the Canal Point Sugarcane Cultivar Development Program (CP program in Florida) had the *Bru1* gene (Comstock et al. 2015). Since the increase in acreage of commercial varieties possessing *Bru1*, there has not been a report of breakdown of resistance to brown rust in Florida. The *Bru1* gene has shown durability and is still effective after 38 years of release. However, a risk of breakdown of resistance of this gene is not to be excluded, especially when large areas are covered by a single resistant gene. As proposed by the ‘boom and bust concept’, selection of virulent mutants which are not controlled by *Bru1* can evolve and break down the current resistance (Glynn et al. 2013). Therefore, identification and deployment of additional major and minor resistance genes is necessary for durability of sugarcane rust resistance (Rott et al. 2013).

Diversity in *Puccinia melanocephala* populations

Rusts, by nature, are pathogens with high evolutionary potential. Therefore, diversity exists in rust populations in the form of races or variants (McDonald and Linde 2002a). Occurrence of races of *P. melanocephala* has been reported at different time periods, and pathogenic specialization and physiological races of the brown rust pathogen were reported in India (Srinivasan et al. 1965) and in Florida, USA. In 1984, Dean and Purdy reported existence of at least two races of *P. melanocephala* in Florida (Dean and Purdy 1984). A decade later, in 2005, four physiological races of *P.
melanocephala were described (Shine et al. 2005). The breakdown of brown rust resistance in commercial sugarcane varieties during different time periods is considered evidence for changes in *P. melanocephala* populations (Rott et al. 2013). It is assumed that changes in rust races are affected by changes in varieties grown in a given area (Shine et al. 2005). Events of breakdown of brown rust resistance in commercial sugarcane varieties in Florida are summarized in the Table 1-2.

Similarly, in Louisiana, cultivar LCP85-384 showed resistance breakdown when its acreage reached 85%, and a similar event was reported in Columbia with varieties CC85-92 and CC84-75 when their acreage reached 80% (Comstock et al. 2010). All these reports of changes in variety resistance level are evidence for changes in pathogenicity of *P. melanocephala* during different time periods. However, these changes in pathogenicity remain to be proven and characterized (Raïd et al. 1991; Shine et al 2005; Comstock et al 2010, Rott et al. 2013).

If outbreaks of brown rust epidemics are often associated with development of new races of *P. melanocephala*, other factors may be involved in these epidemics. A particular case of brown rust epidemics in Australia during the early 1980s was not linked to breakdown of resistance, but to a sudden change of climatic conditions favoring rust development (Taylor 1992). Therefore, both population structures and environmental conditions need to be considered while studying breakdown of resistance to sugarcane rust in a given location.

**Orange Rust of Sugarcane**

**Symptoms**

As discussed in the brown rust section, orange rust and brown rust show similar symptoms. For both diseases, rust uredinia develop on the sugarcane leaves and the
coalesced uredinia form necrotic areas. Typical orange rust symptoms are oval shaped uredinia that are 2-10 mm long and 1-3 mm wide. They appear on the abaxial surface of the leaf and extend parallel to the venation of the sugarcane leaf (Rott et al. 2014). Initial symptoms start with small elongated yellow to white flecks which turn into orange brown uredinia upon maturity. Orange rust uredinia occur in groups which are more closely spaced than uredinia of brown rust (Magarey et al. 2000). Uredinia are the major cause of damage as they impair photosynthesis (Zhao et al. 2011), and the disease causes increased number of suckers which results in reduction of sugar content of millable stalks (Rott et al. 2014).

**Diagnosis**

The causal agent of orange rust of sugarcane is *Puccinia kuehnii*. The main diagnostic features that differentiate this pathogen from *P. melanocephala* are color, size, and shape of urediniospores. Urediniospores of *P. kuehnii* are larger than those of *P. melanocephala*, and a prominent thickening of the apical wall can be seen in orange rust spores which is absent in brown rust spores (figure 1-3). *P. kuehnii* urediniospores are mostly ovoid to pyriform or broadly ellipsoidal, measuring 32-45 × 25-30 μm. Spines are present around the outer wall of urediniospores and spaced 2 to 5 μm apart (wider than that of *P. melanocephala*). The wall of urediniospores is orange-to-light cinnamon brown and 1-2.5 μm thick (Chavarría et al. 2009; Comstock et al. 2008; Flores et al. 2009; Ovalle et al. 2008).

Besides visual characterization of orange rust spores, the pathogen can also be diagnosed using molecular methods. PCR assays based on rust specific primers have been developed to identify *P. kuehnii* (Glynn et al. 2010).
Epidemiology

Relative humidity and temperature are considered key factors for orange rust progress. Studies performed in Australia have shown that maximum germination of spores of *P. kuehnii* occurs at 25°C and 97% relative humidity (Magarey et al. 2004). Field observations in Florida/USA also suggested that leaf wetness and temperature play major roles in orange rust progress. In contrast to brown rust, development of orange rust is not as reduced when temperatures exceed 30°C. Consequently, orange rust symptoms can be seen all year round except during cold periods in winter, or after frost events that damage the leaf canopy and reduce the leaf area for fungal sporulation (Rott et al. 2014).

Orange rust is mainly spread by wind. The mature uredinia open and passively discharge urediniospores to the environment where they are picked up by wind and disseminated from field periphery to hundreds of kilometers. Water, insects, and even human activities can help to disperse orange rust spores over short distances, but the major force for long distance dissemination is the wind (Magarey 2000).

History

Before the year 2000, orange rust was considered a minor disease of sugarcane existing only in Asia and Australia (Magarey 2000). However, during 1999-2001, a severe orange rust epidemic occurred in Australia causing yield losses of up to 50%, and an estimated monetary loss of 200 million Australian dollars (Magarey et al. 2001a). In 2007, the USA reported a first outbreak of orange rust which was also the first case of this disease in the Western hemisphere (Comstock et al. 2008). During the initial introduction of orange rust, as with brown rust, it was hypothesized that spores were carried from Africa to America through transoceanic transportation of *P. kuehnii*
urediniospores (Rott et al, 2014). Later on, most of the sugarcane growing regions in South and Central America reported the presence of orange rust. Belize, Brazil, Columbia, Costa Rica, Cuba, Dominican Republic, Ecuador, El Salvador, Guatemala, Jamaica, Mexico, Nicaragua, and Panama have confirmed the presence of orange rust (Chavarría et al. 2009; Comstock et al. 2008; Ovalle et al. 2008; Flores et al. 2009; Barbasso et al. 2010; Pérez-Vicente et al. 2010; Rott et al. 2014). It has been hypothesized that the outbreaks reported in western hemisphere were due to a single event of introduction of *P. kuehnii* because the orange rust populations in those regions did not show any genetic variation (Glynn et al. 2010; Rott et al. 2014).

**Diversity in *Puccinia kuehnii* populations**

Knowledge of diversity of pathogen populations is crucial for varietal screening and breeding for resistance, and diversity in *P. kuehnii* populations has been suspected to occur. The huge orange rust epidemic in Australia during the 1999-2001 period has been attributed to the development of a new race of *P. kuehnii*. Recent studies in Brazil also showed genotypic and phenotypic diversity in the orange rust pathogen. Based on sporulation capacity of *P. kuehnii* isolates on different varieties, two physiological races of the orange rust pathogen were reported in this country (Urashima et al. 2015).

In Florida, no races of *P. kuehnii* have been reported yet, although there is strong evidence for variation in pathogenicity and virulence among *P. kuehnii* isolates (Comstock et al. 2010, Rott et al. 2014). Variety CP80-1762, that was symptomless when orange rust first occurred in 2007, showed severe orange rust symptoms in 2009 (Comstock et al. 2010). Similarly, during 2010-2012, another variety (CP80-2143) showed a shift from resistance to susceptibility (BASF, unpublished data) suggesting breakdown of rust resistance and appearance of a new race of the pathogen. Recent
inoculation studies with \textit{P. kuehnii} isolates collected from sugarcane cultivars CP85-1040 and CP-89-2143 resulted in additional evidence of variation in pathogenicity of the orange rust pathogen in Florida (Hincapie et al. 2015).

**Hypotheses and Objectives**

The aim of the research project was to define and compare the disease progress of brown and orange rust of sugarcane and to analyze the population structure of \textit{P. melanocephala} (causal agent of brown rust of sugarcane) and \textit{P. kuehnii} (causal agent of orange rust of sugarcane). Although races of the brown rust pathogen \textit{P. melanocephala} have been described in the past, extensive use of the brown rust resistance gene \textit{Bru 1} may have resulted in selection of strains with reduced virulence. We hypothesize that there is either uniformity or low variation in \textit{P. melanocephala}. Based on recent shifts of sugarcane resistance to orange rust, we hypothesize that pathogenic variation currently occurs among \textit{P. kuehnii} isolates in Florida. We assume that the field population of \textit{P. kuehnii} is formed by a mixture of races or strains. We also hypothesize that the varieties that were susceptible when rust initially broke out in Florida (CP 85-1040, etc.) are infected by former races and possibly also by new races, but that the recently susceptible cultivars (CP 89-2143) are infected by only the new race. The objectives of this work were to: I) determine the disease progress of sugarcane rusts in Florida; II) identify the variation of pathogenicity in the current population of sugarcane brown rust (\textit{P. melanocephala}); and III) identify the variation of pathogenicity in the current population of sugarcane orange rust (\textit{P. kuehnii}).
<table>
<thead>
<tr>
<th>Species</th>
<th>Chromosome number</th>
<th>Region of cultivation/occurrence</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharum officinarum</em></td>
<td>2n = 80</td>
<td>South East Asia</td>
<td>Thick juicy canes with high sucrose and low fiber contents</td>
</tr>
<tr>
<td><em>S. barberi</em></td>
<td>2n = 111-120</td>
<td>North India</td>
<td>Thin, short, cylindrical internodes</td>
</tr>
<tr>
<td><em>S. sinense</em></td>
<td>2n = 81-124</td>
<td>China</td>
<td>Tall plants, broad leaves, and bobbin shaped internodes</td>
</tr>
<tr>
<td><em>S. edule</em></td>
<td>2n = 60-80</td>
<td>New Guinea to Fiji</td>
<td>Edible inflorescence</td>
</tr>
<tr>
<td><em>S. robustum</em></td>
<td>2n = 60-80</td>
<td>Indonesia, Papua New Guinea</td>
<td>Wild species</td>
</tr>
<tr>
<td><em>S. spontaneum</em></td>
<td>2n = 40-128</td>
<td>Papua New Guinea to Africa and Mediterranean basin</td>
<td>Wild species</td>
</tr>
</tbody>
</table>
Table 1-2. Breakdown of brown rust resistance in commercial sugarcane varieties during different time points

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Event</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1984</td>
<td>Florida, USA</td>
<td>Originally resistant CP79-1580 was found susceptible</td>
<td>The variety was found susceptible for the first time in some field test sites</td>
<td>Dean and Purdy (1984)</td>
</tr>
<tr>
<td>1987</td>
<td>Florida, USA</td>
<td>Variety CP72-1210 lost brown rust resistance</td>
<td>The variety was initially resistant when released in 1980 but in 1987, 39% yield reduction due to brown rust was estimated in this variety.</td>
<td>Raid et al. (1991)</td>
</tr>
<tr>
<td>1988</td>
<td>Florida, USA</td>
<td>Disease severity increased in CP78-1247</td>
<td>Symptoms of brown rust observed was minimal for 2 years but amount of disease increased drastically</td>
<td>Raid (1989)</td>
</tr>
<tr>
<td>Since 1989</td>
<td>Florida, USA</td>
<td>CP74-2005, CL73-239, CP70-113, and CP78-1628 were found susceptible to brown rust at different times</td>
<td>These varieties were resistant when released and were used to replace susceptible varieties</td>
<td>Shine et al. (2005); Comstock et al. (2010)</td>
</tr>
</tbody>
</table>

Figure 1-3. Comparison of symptoms and causal agents of sugarcane brown and orange rust. Photo courtesy of Jack Comstock and Lisa Castlebury.
CHAPTER 2
PROGRESS OF SUGARCANE BROWN RUST AND ORANGE RUST DURING TWO CROP SEASONS IN FLORIDA

Introduction

Brown rust caused by *Puccinia melanocephala* and orange rust caused by *Puccinia kuehnii* are two major diseases of sugarcane (*Saccharum* interspecific hybrids). Brown rust is present in almost all sugarcane growing areas around the world (Raid and Comstock 2000). India was the first country to report brown rust in 1950 (Patel et al. 1950) and then it spread to South-East Asia, Africa, Southern Asia and Australia (Glynn 2010). Brown rust epidemics occurred in Australia, Taiwan, the Caribbean islands and the United States during 1978-1979 (Dean and Purdy 1984). Ten to 50% yield reductions due to brown rust were reported in several sugarcane cultivars in Florida and Louisiana (Purdy et al. 1983; Comstock and Shine 1992; Raid and Comstock 2006; Hoy and Hollier 2009).

Orange rust was considered a minor disease before a severe epidemic of this disease occurred in Australia during 1999-2001. Yield losses reached up to 50%, and a loss of 200 million Australian dollars was attributed to this epidemic (Magarey et al. 2001a). Orange rust was first reported in the Western Hemisphere in 2007, when an outbreak of the disease occurred in the USA, in Florida (Comstock et al. 2008). Within a few years, the disease was found in sugarcane growing regions in South and Central America and the Caribbean (Comstock et al. 2008; Ovalle et al. 2008; Chavarría et al. 2009; Flores et al. 2009; Barbasso et al. 2010; Pérez-Vicente et al. 2010; Rott et al. 2014). In Florida, both brown and orange rust of sugarcane have been posing constant threat to the sugarcane production (Comstock 2010). More than 90% of sugarcane
cultivars grown during the 2015-2016 crop season in Florida were susceptible to either brown or orange rust (Raid et al. 2015).

Brown rust and orange rust produce similar symptoms in sugarcane leaves (Figure 2-1). They form uredinia on the lower side of the sugarcane leaf, and these uredinia contain numerous spores called urediniospores. However, *P. melanocephala* forms reddish to dark reddish uredinia whereas orange rust uredinia are not as dark as those of brown rust (Raid and Comstock 2000). The fungal signs of the two rusts also look similar to the unaided eye. However, through microscopic examination, they are readily distinguishable (Figure 2-1). The major differences between urediniospores of these two rusts are color, size and shape of spores. Urediniospores of both rusts are spherical but *P. melanocephala* urediniospores are smaller (21-40 x 17-27 μm) than urediniospores of *P. kuehnii* (32-45 x 25-30μm) (Ryan and Egan 1989). Both rust pathogens have spines around the outer wall of urediniospores but the spines of *P. melanocephala* uredinispores are spaced by 1-1.5 μm whereas those of *P. kuehnii* are separated by a larger space (3-4 μm) (Raid and Comstock 2000). Additionally, spores of *P. kuehnii* have a 1-2.5 μm thickened apical wall, with this thickening being absent in *P. melanocephala* (Chavarría et al. 2009; Flores et al. 2009; Ovalle et al. 2008). Beside visual characterization of their spores, both pathogens can also be differentiated molecularly using PCR assays based on specific diagnostic primers (Glynn et al. 2010).

Disease progress in both rusts is mostly determined by the host genotype and by environmental factors such as temperature and leaf wetness (Purdy et al. 1983; Sandoval et al. 1983; Comstock and Ferreira 1986; Irey 1987; Victora et al. 1990; Raid and Comstock 2000; Barrera et al. 2012). Plant age has also been described as a
limiting factor for brown rust progress. Brown rust severity is mostly observed in 2-6 month old sugarcane and, after 8 months of growth, a gradual decline of disease symptoms is observed in the field in Florida and in Cuba (Sandoval et al. 1983; Comstock and Ferreira 1986). However, this effect of plant age has not been reported for orange rust, for which symptoms can be seen from early season until harvest (Rott et al. 2014).

Germination of brown rust urediniospores is favored by temperatures between 15°C to 30°C, and temperatures above 30°C greatly reduce germination capacity of these spores (Purdy et al. 1983; Sotomayor et al. 1983; Raid and Comstock 2000). At least 8 hours of leaf wetness are considered to be needed for brown rust development in Florida (Raid and Comstock 2000), with the highest levels of brown rust infection being observed after 14 hours of leaf wetness in Australia (Ryan and Egan 1989).

For orange rust, at least 97% relative humidity and temperatures between 17°C and 24°C are the most conducive conditions in Australia (Magarey et al. 2004). In Florida, temperatures between 20°C and 25°C and more than 8 hours of leaf wetness were found to be necessary for development of sugarcane orange rust (Martins et al. 2010).

Although general trends of sugarcane brown and orange rust progress during the crop season have been reported in Florida (Raid and Comstock 2006; Rott et al., 2014), variation of disease progress based on numerical data has not been reported so far. These data are needed to better understand epidemiology of both brown and orange rust and to improve control of these two diseases of sugarcane. We hypothesize that brown and orange rust progress will vary during the crop season and according to
weather conditions. The objectives of this study were therefore to: 1) regularly monitor, quantify and compare severity of brown and orange rust during two consecutive crop seasons; 2) identify the periods with highest disease levels; 3) identify the favorable and unfavorable temperatures for in vitro germination of rust spores; and 4) compare the disease progress with the temperature variables to identify the most conducive temperatures for progress of the two rusts in Florida.

**Materials and Methods**

**Sugarcane Cultivars Monitored in the Field**

Two sugarcane cultivars were used: CL 90-4725, which is highly susceptible to brown rust, and CL 85-1040, which is highly susceptible to orange rust. In 2013, a randomized block design experiment was set up at the Everglades Research and Education Center (EREC), Belle Glade, USA in which five commercial sugarcane cultivars were planted at three different dates in four blocks. Each plot was bordered with a single row of CL 90-4725 on the east side and a single row of CL 85-1040 on the west side. The border rows planted on November 12, 2013 were used to investigate rust progress in 2014 (2013-2014 crop season). To monitor progress of rust severity in 2015 (2014-2015 crop season), two separate commercial fields were set up at EREC: one field was planted with cultivar CL 90-4725 and was formed by 27 sugarcane rows (each 900 feet long); the second field was planted with cultivar CL 85-1040 and was composed of 39 rows (each 500 feet long). Both sugarcane cultivars were planted on November 14, 2014.

**Disease Scoring of Naturally Infected Sugarcane Plants**

The two plant cane crops were exposed in the field to natural rust infections, and disease severity data were collected every two weeks: from January to December 2014.
and 2015, following planting of sugarcane in November 2013 and 2014, respectively. Thirty two stalks per variety were assessed for rust at each scoring date: 8 stalks from a 175 feet row in each of the four blocks of the randomized field trial in 2014, and 8 stalks from each of four different locations separated by at least 150 feet in the commercial fields in 2015.

Rust development was determined on the top visible dewlap (TVD) leaf and on leaf L+5 (fourth leaf below the TVD) of each scored sugarcane stalk (Figure 2-2). The TVD leaf is the most recent fully expanded leaf of a sugarcane stalk. As TVD leaves open every 7-10 days (van Dillewijin, 1952), rust symptoms that develop on TVD leaves represent most recently developed symptoms at a given observation date. In contrast, L+5 leaves are much older leaves that have been exposed to rust for at least 3-4 weeks. Therefore, they reflect an accumulation of disease pressure that sugarcane endured during its growth. Only the upper third area was considered for the TVD leaf whereas the entire leaf surface of L+5 was scored. For each leaf, sporulation intensity (rust reaction) was measured on a 0-5 scale (described in Table 2-1) and the percentage of rust affected area was assessed visually using a scale of simulated diseased leaf surface developed by Purdy and Dean (1981) (Figure 2-3).

**In Vitro Germination Assay of Brown and Orange Rust Spores**

The effect of temperature on germination capacity of urediniospores of both rusts was determined in vitro. Leaves of sugarcane cultivars CL 90-4725 and CL 85-1040 exhibiting rust uredinia were collected in the field at Belle Glade. Urediniospores of *P. melanocephala* and *P. kuehnii* were collected in the laboratory by vacuuming the lower side of the sampled leaves. Urediniospores were spread on a glass slide covered with a one mm thick layer of 1% water agar and kept overnight (18-20 hours) at dark, and at
different temperatures ranging from 22°C to 33°C. One 50 μl drop of deionized water supplemented with 0.002% nonanol and 0.1% tween 20 were added on top of the agar and spores were spread evenly with a glass rod. After 18-20 hours of incubation, percentage of germination was determined by counting four sets of one hundred spores from two slides under a microscope (400x magnification). Urediniospores producing germ tubes larger than the size of the urediniospores were considered as germinated spores.

Comparison of Field Temperatures and Disease Progress Curves

Daily temperature data for Belle Glade, Florida were retrieved from the website of Florida Automated Weather Network (http://fawn.ifas.ufl.edu/). Daily average, maximum and minimum temperatures of each week of 2014 and 2015 were averaged to calculate weekly average temperature, weekly maximum temperature and weekly minimum temperature. Disease progress curves were compared with the temperature curves for both crop seasons to determine the conducive temperatures for brown and orange rust.

Data Analyses

Data of rust reaction and percent rust affected area were averaged for each scoring date (average of four sets of eight stalks). Data for TVD leaf and L+5 leaf were used to construct disease progress curves using SigmaPlot for Windows, Version 13.0. Areas under disease progress curve (AUDPC) were calculated for the percent of rust affected area data using the formula \( A_k = \sum_{i=1}^{N} \left( \frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i) \) where \( A_k \) = AUDPC, \( N \) = Total number of observations, \( i \) = observation points, \( y_i \) = sample disease value, \( t_i \) = sample time points. Analysis of variance (ANOVA) was run for AUDPC values and the
means were separated using least significant difference (LSD) at $P=0.05$, using 
Agricola package in the statistical software R (Mendiburu 2016; R Core Team 2016).

For the in vitro germination test, the means, standard deviation and standard 
errors were calculated using MS excel 2013. Mean germination percent at different 
temperatures were differentiated using LSD at $P=0.05$ using statistical software R, 
Agricola Package.

Results

Progress of Sugarcane Brown Rust in Cultivar CL 90-4725

In 2014, rust reaction of the TVD leaf of cultivar CL 90-4725 gradually increased 
from a score of 2 in January to a score of 4 in mid-March (Figure 2-4A). After mid-
March, rust reaction progressively increased from a score of 4 and reached a high score 
of 5 at mid-May. From that date on, it progressively declined and reached a score of 4 
at the end of July. This period from mid-March to late July, when rust reaction was 
between 4 and 5, was considered as a period of active sporulation because a score of 4 
was given when 10-50 open rust uredinia were observed, and a score of 5 was given 
when more than 50 uredinia were observed in the upper third portion of the TVD leaf. 
From the beginning of August, rust reaction gradually decreased, and scores were less 
than 3 by late September. Although rust reaction slightly increased again from October 
to December, these late scores were always below 4. The following year, in 2015, rust 
reaction of the TVD leaf showed a similar progression pattern but with a major 
difference: the onset of intense sporulation (score 4) was reached in mid-May, i.e. more 
than one and a half months later than in 2014.

In 2014, rust affected area of the TVD leaf was below 5% until mid-March (Figure 
2-4B). Diseased leaf surface then progressively increased and reached maximum
values of 16-17% from mid-May to mid-June. After mid-June, rust affected area declined progressively and was below 5% at the end of July. It remained below this value until the end of year. In 2015, when intense sporulation started in mid-May, rust affected area reached values of 5% and above also only at that time of the year. Rust affected area reached maximum values (11-13%) from end of May 2015 to mid-July. As in 2014, values were below 5% in August and remained below that level until the end of the year.

In 2014, medium (score 4) to intense sporulation (score 5) was observed on the L+5 leaf of cultivar CL 90-4725 from mid-March to the end of August, with highest scores from mid-April to late July (Figure 2-4C). During these periods, sporulation was much higher on the lower leaf (L+5) than on the upper leaf (TDV) of the sugarcane canopy. The period of intense sporulation on leaf L+5 was much shorter in 2015 than in 2014. In 2015, scores of 5 for rust reaction were only seen in May and values declined rapidly starting in the month of July.

In 2014, rust affected area of leaf L+5 was above 5% at the first scoring date beginning of March (note: this leaf was not available on the sugarcane plant before that date) (Figure 2-4D). From March to the beginning of May, rust affected area progressively increased and reached maximum values (approx. 20%) in May. Although values decreased after that date, they remained above 10% until the end of July 2014. However, in 2015, rust affected area of leaf L+5 was above 5% only in mid-April (one and a half months later than in 2014) and the period of values above 10% was shorter (from May to late July). In both 2014 and 2015, the rust affected area of leaf L+5 started
to decline after mid-July and was no longer above 5% from the end of August until the end of the year.

**Progress of Sugarcane Orange Rust in Cultivar CL 85-1040**

In 2014, intensity of orange rust sporulation on TVD leaf of cultivar CL 85-1040 was low (rust reaction score 2-3) from January to beginning of April (Figure 2-5A). In April 2014, rust reaction gradually increased from a score of 3 and reached a score of 5 (high sporulation) in July. Sporulation intensity started to decrease one month later, in August, but increased again around mid-September. High sporulation intensity (score 5) on the TVD leaf was observed again in November 2014, at the end of the crop season. The following year, in 2015, rust reaction of the TVD leaf showed a similar progression pattern but with a major difference: high intensity of orange rust sporulation (score 5) was already observed mid-May whereas high sporulation intensity was only scored two months later (mid-July) in 2014. The duration of high sporulation intensity on TVD leaf was therefore longer in 2015 than in 2014.

In 2014, rust affected area of the TVD leaf was below 5% until May (Figure 2-5B). Diseased leaf surface then rapidly increased and reached maximum values above 20% from June to mid-July. In July 2014, rust affected area declined rapidly and was below 5% at the end of August. Rust severity started to increase again at the end of September and was above 10% at the beginning of December 2014. In 2015, the progress pattern of rust affected area on the TVD was very similar to the one observed in 2014. However, rust affected area at the end of the year was higher in 2015 than in 2014: 24% versus 11%.

In 2014, sporulation intensity was low (score 3) on the L+5 leaf of cultivar CL 85-1040 at the first scoring date beginning in March (Figure 2-5C). However, it
progressively increased from that month and intense sporulation (score 5) was observed from June until the end of the year, with a small reduction for two weeks beginning of September. During these periods, intensity of sporulation was much higher on the lower leaf (L+5) than on the upper leaf (TDV) of the sugarcane canopy. The period of intense sporulation on leaf L+5 was one month longer in 2015 than in 2014. In 2015, a score of 5 for rust reaction was already present in May.

In 2014, rust affected area of leaf L+5 was above 5% at the first scoring date beginning of March (note: this leaf was not available on the sugarcane plant before that date) (Figure 2-5D). From March to beginning of May, rust affected area progressively increased and reached approx. 15% in May. Rust severity then rapidly increased and values above 20% were obtained from end of May to mid-August, with peak values above 40% in June and July 2014. Rust affected area of leaf L+5 rapidly decreased from mid-July to the beginning of September when only 5% of the leaf area showed rust symptoms. Rust severity increased again from mid-September (5%) to early December (40%). In 2015, rust affected area of leaf L+5 was above 5% only at the beginning of April (one month later than in 2014), but the rapid increase and decrease of rust severity during the May to mid-August period was the same as in 2014. However, at the end of 2015, rust affected area of leaf L+5 was always higher than 10%, and disease severity values from September to December 2015 were always higher in 2015 than in 2014.

Comparison of Disease Progress between Brown Rust in CL 90-4725 and Orange Rust in CL 85-1040

In 2014, high values of rust reaction (sporulation intensity with score 5) were observed on TVD and leaf L+5 two months earlier for brown rust than for orange rust: April-May for brown rust vs. June-July for orange rust (Figure 2-6A and C). Similarly,
rust severity (percent of rust affected leaf area) was higher than 5% on TVD in March for brown rust but only in May for orange rust (Figure 2-6B). Once peak values were reached for orange rust, they remained higher than those of brown rust until the end of the crop season. In contrast, in 2015, rust reaction and disease severity values of TVD and leaf L+5 were similar at the beginning of the year (from February to end of May). Starting in June 2015, these values were always higher for orange rust than for brown rust.

Based on data of sporulation intensity and percent affected leaf area of TVD of 2014 and 2015, there was a single period of severe disease expression of brown rust from April to end of July (Figure 2-6A and B). In contrast, two periods of active disease expression were observed for orange rust: the first one from May to August and the second one at the end of the year (November). On the lower leaf (L+5), sporulation intensity started to decrease in June-July for brown rust and it remained low for the rest of the year (Figure 2-6C). In contrast, significant sporulation (more than 50 uredinia per leaf) was seen for orange rust until the end of the year. Overall, and for both 2014 and 2015, rust symptom accumulation on leaf L+5 was much higher for orange rust than for brown rust (Figure 2-6D). The highest average percent of damaged area for leaf L+5 was 23% for brown rust, whereas it was 47% for orange rust.

Areas Under Disease Progress Curves (AUDPCs) were determined with percent rust of TVD and L+5, respectively, and for 2014 and 2015 data (Figure 2-7). AUDPC of brown rust on the TVD leaf was not different in 2014 and in 2015. Similarly, no difference was found between AUDPCs of the TVD leaf for orange rust in 2014 and in 2015. However, AUDPC of TVD leaf was larger for orange rust compared to the one of
brown rust, thus confirming that orange rust symptoms cover a larger leaf area than those of brown rust during the crop season. AUDPC of brown rust on leaf L+5 was also not different in 2014 and in 2015. However, AUDPC of orange rust leaf L+5 was larger in 2015 than in 2014, indicating more severe symptoms or similar diseased area over a longer period in 2015 than in 2014. As with the TVD leaf, AUDPC of orange rust on the lower leaf (L+5) was higher than that for brown rust.

**In Vitro Germination of Urediniospores of *Puccinia melanocephala* and *Puccinia kuehnii* at Different Temperatures**

Urediniospores of *P. melanocephala* (brown rust) germinated in vitro at temperatures from 20°C to 32°C, whereas urediniospores of *P. kuehnii* (orange rust) germinated from 20°C to 30°C (Figure 2-8). More than 50% of urediniospores of both fungal species germinated between 20°C and 29°C. Germination of spores of *P. kuehnii* dropped from 65% at 29°C to 18% at 30°C, and was nil at 31°C (Figure 2-9). At 31°C, 40% of spores of *P. melanocephala* still germinated and spores of the brown rust pathogen only stopped germinating at 33°C.

**Relationship between Temperature in the Sugarcane Field, Disease Severity and In Vitro Sporulation Capacity of Sugarcane Rusts**

In 2014, the average weekly temperature varied from 22°C to 25°C during active progress of brown rust (Figure 2-10). Before the onset of active brown rust sporulation and area damage on TVD (Jan –March), average weekly temperatures were below 20°C and weekly minimum temperature were below 15°C during that period. During the first active period of orange rust progress (mid –May to end of July), the average weekly temperature varied from 22°C to 26°C. The average weekly minimum temperatures before the onset of the first peak of orange rust severity were below 18°C. During the second active progress of orange rust in TVD (October – December), the average
weekly temperature varied from 19°C to 25°C. The average weekly temperature and maximum weekly temperature were higher than 25°C and 32°C respectively during August-September when sporulation and disease affected area of TVD were low. (Figure 2-10).

In 2015, average weekly temperature varied from 24°C to 26°C during active progress of brown rust (Figure 2-11). Before the onset of active brown rust sporulation and damaged leaf areas greater than 5% on the TVD leaf (during mid-April to June), the average weekly temperatures were below 22°C and the weekly minimum temperatures were below 18°C during that period. During the first active period of orange rust progress (May-July), the average weekly temperature varied from 25°C to 27°C. The average weekly minimum temperature before the onset of the first peak of orange rust severity was below 18°C for most of the time (Figure 2-11). During the second active progress of orange rust on TVD (October-December), the average weekly temperature varied from 21°C to 25°C. The average weekly temperature and maximum weekly temperature were higher than 25°C and 32°C respectively during August-September when sporulation and disease affected area of TVD were low (Figure 2-11).

The decline of in vitro germination of *P. kuehni* urediniospores (orange rust spores) at temperatures above 30°C was consistent with the field observation as the higher temperatures restrict rust progress during August-September. With the decrease of temperatures in October, orange rust symptoms increased again from mid-October to December. Although, *P. melanocephala* urediniospores have the capacity to germinate in vitro at higher temperatures (up to 32°C), brown rust progress decreased in the field during the July-August period and prior to the decrease of orange rust. Brown rust
symptoms did not develop again in October to December although temperatures were favorable for spore germination.

Discussion

Brown and orange rust showed two different progress patterns in Florida in sugarcane cultivars CL90-4725 and CL85-1040, respectively. For brown rust, intensity of sporulation and disease severity on leaves increased for 3-4 months at the beginning of the year, peaked for a few weeks in “spring”, and then decreased in June-July. Later on, the disease remained at low levels through the end of the year, when sugarcane was mature and ready to be harvested. Initial progress of orange rust was similar to brown rust: intensity of sporulation and disease severity on leaves increased for 3-4 months at the beginning of the year and peaked from mid-May to July. However, the duration of high disease levels was longer for orange rust than for brown rust, as orange rust started to decline only in August -September. Additionally, and in contrast to brown rust, orange rust sporulation intensity and severity increased again at the end of the year. Similar trends of progress of brown rust during spring and orange rust in summer were reported in Australia (Magarey et al. 2001b).

Temperature and leaf wetness are considered to be key factors for development of sugarcane rusts (Raid and Comstock 2000; Magarey et al 2004. However, in Florida, leaf wetness duration appears to be sufficient all year long for rust development (Rott et al. 2014), and it should therefore not be a major limiting factor. Large variations in temperature occur from January to December in Florida, with very hot summer temperatures (average weekly maximum summer temperatures of 32-34°C). Based on growth chamber studies, Martins et al (2010) reported that temperatures between 20°C to 25°C are the most favorable temperatures for orange rust development.
Consequently, temperatures above 30°C appear to be too high for orange rust development during August-September. Additionally, temperatures in the field are not constant during the day and daily variation in temperature most likely also accounts for progress of fungal foliar diseases (Huber and Gillespie 1992).

The in vitro germination assays conducted herein differed somewhat from data obtained with orange rust in Australia (Magarey et al., 2004). In Australia, temperatures around 20°C (17-23°C) were the most favorable for spore germination whereas temperatures between 20-29°C all resulted herein in similar and high percentages of spore germination. Different strains of the pathogen or different spore germination conditions such as the use of agar media for spore germination in our experiments versus the use of only free water in Magarey’s experiments may explain these differences.

In vitro, brown rust spores are able to germinate at higher temperatures than those of orange rust. Therefore, in vitro germination data do not support the effect of temperature on decline of brown rust before decline of orange rust in the field. It cannot be excluded that high temperatures also affect other characteristics of the infection process such as lesion/uredinia development, including sporulation. However, if we assume that high summer temperatures are mainly responsible for orange rust decline, then brown rust decline should occur later (and not earlier) than orange rust. Additionally, brown rust should re-increase at the end of the year when temperatures are once again favorable for disease development. Therefore, brown rust decline during the crop season is more related to other factors than climate factors, such as plant physiology or plant age for example. Brown rust appears to be a disease of young
sugarcane (2-6 months) (Comstock and Ferreira 1989; Raid and Comstock 2000), which would explain why brown rust is absent in the fall and winter when temperatures are lower than in summer and similar to those in spring. Similar age related disease resistance was reported in other plants such as in wheat against *Puccinia recondita* f.sp. *tritici* (Pretorius et al. 1988), in tobacco against *Peronospora tabacina* (Reuveni et al. 1986), and in arabidopsis against *Pseudomonas syringae* (Kus et al. 2002). In contrast, orange rust can be observed at two different periods of the year covering young and mature sugarcane, and plant age does not appear to be a main factor for disease progress. Similar observations were made in Australia where the effect of relative humidity and temperature on in vitro spore germination of orange rust spores was consistent with field observations (Magarey et al. 2004). Additional experiments such as planting sugarcane in the field at different times of the year need to be undertaken to validate this hypothesis.

Occurrence of severe brown rust and temperature ranging from 15°C to 30°C during the same period in April-May 2014 are in accordance with previous reports of development conditions of brown rust (Purdy et al. 1983; Sandoval et al. 1983; Sotomayor et al. 1983; Raid and Comstock 2000; Magarey et al. 2004; Raid and Comstock 2006; Barrera et al. 2012). The onset of severe brown rust symptoms was delayed until mid-May in 2015 which was almost a month and half later than in 2014. Comparison of the weather data showed that the month of February was cooler in 2015 than that of 2014 and frost damage was seen during the first week of March 2015 in the fields. Cooler winter and reduced healthy leaf canopy due to frost may have reduced the inoculum levels and thus delayed the onset of brown rust symptoms in 2015. A similar
case of two and half month delay of onset of brown rust was reported in Florida in 1985 due to freeze associated damage (Irey 1987). Similarly, delayed starts of brown rust epidemics have been attributed to winter freezes in Louisiana (Hoy and Hollier 2009).

In both cultivars CL 90-4725 and CL 85-1040, the lower leaf (L+5) had more sporulation and damaged area due to rust than the TVD leaf. In addition to the fact that L+5 is an older leaf and thus exposed to rust for a longer time than the TVD leaf, we hypothesize that the closed canopy maintained by the lower leaves in the sugarcane field may have provided longer leaf wetness hours which favored better sporulation.

Although the yield losses due to rust in the two sugarcane cultivars used in this experiment are yet not known, higher sporulation intensity and longer duration of epidemics of orange rust than brown suggests that the yield losses due to orange rust might be higher than those of brown rust.

Because the orange rust epidemic occurs over a longer period than the brown rust epidemic, orange rust management needs to be applied for a longer period of time in the field. Currently, more foliar fungicide applications are performed to control orange rust than brown rust in Florida (Philippe Rott personal communication). Further information is needed about these diseases, such as severity data from other sugarcane varieties susceptible to rust diseases and planted at different periods of the year, in order to develop a rust prediction system based on weather data.
Table 2-1. Scoring scale of sugarcane brown and orange rust based on symptom type and sporulation intensity

<table>
<thead>
<tr>
<th>Score</th>
<th>Reaction/symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No visible reaction</td>
</tr>
<tr>
<td>1</td>
<td>Chlorotic flecks only (no sporulation)</td>
</tr>
<tr>
<td>2</td>
<td>Chlorotic flecks with red or brown/necrotic centers (no sporulation)</td>
</tr>
<tr>
<td>3</td>
<td>Variable lesion size and red to brown lesions may coalesce; a few lesions (&lt;10) produce uredia/uredinia</td>
</tr>
<tr>
<td>4</td>
<td>Variable lesion size and red to brown lesions may coalesce; several lesions (10-50) produce uredia/uredinia</td>
</tr>
<tr>
<td>5</td>
<td>Variable lesion size and red to brown lesions may coalesce; numerous (&gt;50) lesions produce uredia/uredinia</td>
</tr>
</tbody>
</table>
Figure 2-1. Leaf symptoms of sugarcane brown and orange rust and urediniospores of the causal agents. Photo courtesy of Jack Comstock and Lisa Castlebury.
Figure 2-2. Location of the top visible dewlap (TVD) leaf and the fourth leaf below the TVD leaf (L+5) on a sugarcane stalk. Photo courtesy of author.

Figure 2-3. Simulated percentages of sugarcane leaf surface affected by rust (from Dean and Purdy, 1981).
Figure 2-4. Progress of sugarcane brown rust in cultivar CL 90-4725. A) Progress of rust reaction on the top visible dewlap (TVD) leaf. B) Progress of percent rust affected area of the TVD leaf. C) Progress of rust reaction on the fourth leaf below TVD (leaf L+5). D) Progress of percent rust affected area of leaf L+5. A-D) Solid lines correspond to rust progress in 2014 and broken lines correspond to rust progress in 2015. Each point at each scoring date represents the average value of four sets of 8 stalks. The mean coefficients of variation of rust reaction on the TVD leaf, percent rust of the TVD leaf, rust reaction on leaf L+5 and percent rust of leaf L+5 for the crop season 2013/2014 were 5.9, 34.2, 2.4, and 20.4%, respectively. The mean coefficients of variation of rust reaction on the TVD leaf, percent rust of the TVD leaf, rust reaction on leaf L+5 and percent rust of leaf L+5 for the crop season 2014/2015 were 4.2, 19.7, 1.1, and 16.5%, respectively.
Figure 2-5. Progress of sugarcane brown rust in cultivar CL 85-1040. A) Progress of rust reaction on the top visible dewlap (TVD) leaf. B) Progress of percent rust affected area of the TVD leaf. C) Progress of rust reaction on the fourth leaf below TVD (leaf L+5). D) Progress of percent rust affected area of leaf L+5. A-D) Solid lines correspond to rust progress in 2014 and broken lines correspond to rust progress in 2015. Each point at each scoring date represents the average value of four sets of 8 stalks. The mean coefficients of variation of rust reaction on the TVD leaf, percent rust of the TVD leaf, rust reaction on leaf L+5 and percent rust of leaf L+5 for the crop season 2013/2014 were 5.9, 34.2, 2.4, and 20.4%, respectively. The mean coefficients of variation of rust reaction on the TVD leaf, percent rust of the TVD leaf, rust reaction on leaf L+5 and percent rust of leaf L+5 for the crop season 2014/2015 were 4.2, 19.7, 1.1, and 16.5%, respectively.
Figure 2-6. Comparison of progress of brown and orange rust in CL 90-4725 and CL 85-1040, respectively. A) Progress of rust reaction on the top visible dewlap (TVD) leaf. B) Progress of percent rust affected area of the TVD leaf. C) Progress of rust reaction on the fourth leaf below TVD leaf (leaf L+5). D) Progress of percent rust affected area of leaf L+5. A-D) Solid lines joining black circles represent orange rust progress in 2014; broken lines joining white circles represent brown rust progress in 2014; solid lines joining black triangles represent orange rust progress in 2015; broken lines joining white triangles represent brown rust progress in 2015. Each point at each scoring date represents the average value of four sets of 8 stalks.
Figure 2-7. Comparison of area under the disease progress curve (AUDPC) values of percent rust affected area of the top visible dewlap (TVD) leaf and the fourth leaf below TVD leaf (L+5) of sugarcane varieties CL 90-4725 (susceptible to brown rust) and CL 85-1040 (susceptible to orange rust). Columns with the same letter are not different at $P = 0.05$ according to the Least significant difference test. The lines at the top of each column represents the standard error of the mean.

Figure 2-8. Percent germination of urediniospores of *Puccinia melanocephala* (brown rust) and *P. kuehnii* (orange rust) at eight different incubation temperatures. Each column represents average data of three independent experiments performed separately for each temperature. The lines at the top of each column represents the standard error of the mean.
Figure 2-9. Percent germination of urediniospores of *Puccinia melanocephala* (brown rust) and *P. kuehnii* (orange rust) at four different temperatures. Each column represents average data of three independent experiments. Sub-samples of each spore sample were submitted at the same time to the four different temperatures. Columns with the same letter are not different at $P = 0.05$ according to the Least significant difference test. The lines at the top of each column represents standard error of the mean.
Figure 2-10. Average weekly temperatures in 2014 at Belle Glade. The grey rectangle corresponds to the period of maximum brown rust sporulation on the TVD leaf of sugarcane cultivar CL 90-4725; the two orange rectangles correspond to the two periods of maximum orange rust sporulation on the TVD leaf of sugarcane cultivar CL 85-1040.
Figure 2-11. Average weekly temperatures in 2015 at Belle Glade. The grey rectangle corresponds to the period of maximum brown rust sporulation on the TVD leaf of sugarcane cultivar CL 90-4725; the two orange rectangles correspond to the two periods of maximum orange rust sporulation on the TVD leaf of sugarcane cultivar CL 85-1040.
CHAPTER 3
VARIATION IN PATHOGENICITY OF *PUCCINIA MELANOCEPHALA*, THE CAUSAL AGENT OF SUGARCANE BROWN RUST

Introduction

Brown rust caused by *Puccinia melanocephala* is an important fungal disease of sugarcane. The disease has been reported to cause 10 to 50 percent yield losses in susceptible sugarcane cultivars (Purdy et al. 1983; Comstock and Shine 1992; Taylor 1992; Raid and Comstock 2000; Hoy and Hollier 2009). *P. melanocephala* was reported in sugarcane in India as early as 1949 (Patel et al. 1950), and worldwide epidemics of brown rust occurred in the Caribbean, the USA, Taiwan and Australia during the late 1970s (Presley et al. 1978; Ryan and Egan 1979; Dean and Purdy 1984). In 1978, the first case of sugarcane brown rust in the Western Hemisphere was reported in Dominican Republic and soon after the pathogen spread all over the Americas (Purdy et al. 1983). It is believed that *P. melanocephala* spores came to the Americas through a transoceanic route from Africa (Purdy et al. 1985). Since then, the disease has constantly affected the sugarcane production of this region (Raid and Comstock 2006).

The best means of brown rust control is the use of resistant sugarcane cultivars (Raid and Comstock 2006; Rott et al. 2013). However, maintaining durability of resistance to this disease has been difficult. In Florida, during different time periods, several cultivars which were resistant to brown rust after their release became susceptible within a few years of commercial cultivation, and had to be withdrawn (Dean and Purdy 1984; Raid 1989; Shine et al. 2005; Comstock et al. 2010). Similar cases of resistance breakdown of previously resistant cultivars were observed in Louisiana (Hoy et al. 2005; Hoy et al. 2014). The major cause of breakdown of brown rust resistance is attributed to the genetic variability occurring in the *P. melanocephala* population (Raid
and Comstock 2000). Occurrence of six pathogenic races of *P. melanocephala* has been reported in India in 1965 (Srinivasan and Mutaiyan 1965; Muthaiyan et al. 1966). In Florida, presence of at least two and four pathogenic races of *P. melanocephala* were described in 1984 and 2005, respectively (Dean and Purdy 1984; Shine et al. 2005).

The exploitation of host resistance in sugarcane has been difficult because of the complexity of the genetic structure of the sugarcane genome. Modern sugarcane cultivar are interspecific hybrids of *Saccharum* species with high ploidy levels (2n = 100-130) (D’Hont et al. 1996). Although mechanisms of resistance to brown rust are poorly understood, several sugarcane genotypes resistant to the disease have been identified after artificial or natural infection of sugarcane with *P. melanocephala*. Currently, two major brown rust resistant genes, *Bru1* and *Bru2*, have been identified in sugarcane (Asnaghi et al. 2001; Raboin et al. 2006). The *Bru1* gene is the gene that is the most frequently found in brown rust resistant sugarcane cultivars (Costet et al. 2012; Glynn et al. 2013).

Almost all brown rust resistant sugarcane cultivars grown in Florida contain *Bru1*, and the Canal Point Sugarcane Breeding Program heavily relies on the use of this resistance gene. Cultivar R570, the cultivar in which *Bru1* was originally described, is resistant to strains of *P. melanocephala* from around the world (Asnaghi et al. 2001). Additionally, this cultivar has been in commercial cultivation for several decades and is present in germplasm collections around the world. It has never been reported susceptible to brown rust, suggesting that although *Bru1* is a single resistance gene, it is highly sustainable. However, history has shown that brown rust resistance governed by a single major gene is vulnerable to changes in pathogenicity of *P. melanocephala*.
Glynn et al. 2013, Rott et al 2013). Sugarcane cultivar CP 96-1252 was the most grown sugarcane cultivar in 2015 in Florida (Van Weelden et al 2016). This cultivar which is highly susceptible to brown rust does not possess \textit{Bru1} and has no history of breakdown of rust resistance. Management of brown rust for this cultivar by commercial growers relies on the use of fungicidal products. As mentioned above, at least four strains of the brown rust pathogen were present over a decade ago (Shine et al. 2005), but the current situation is unknown. Since the extensive use of brown rust resistance gene \textit{Bru1}, there has not been any report of breakdown of brown rust resistance in commercial cultivation. We hypothesize that there is a low or no pathogenic variation within the brown rust population in Florida in 2016. The objective of this research was to investigate the current variation in pathogenicity of \textit{P. melanocephala} populations in Florida.

**Materials and Methods**

**Sugarcane Cultivars and Rust Spores**

Single node stalk cuttings of three sugarcane cultivars susceptible to brown rust were planted in 1.7 gallon pots in December 2015: CP 96-1252, CP 78-1628, and CP 90-4725. Plants were grown in an organic soil also known as Lauderhill Muck (Euic, hyperthermic Lithic Haplosaprists) (USDA-NRCS Soil Survey 2016) that was collected from nearby fields at the Everglades Research and Education Center (EREC) in Belle Glade, Florida. Plants were watered by drip irrigation in a closed greenhouse to prevent rust infection (Figure 3-1A). Fifteen grams of slow release fertilizer (Osmocote, The Scotts Company, N-P-K: 15-9-12) and 5 grams of granular insecticide (Tree & Shrub Protect & Feed, Bayers Advance, 0.55% Imidacloprid and 0.275% Clothianidin) were added to each pot when plants were two months old. After three months of growth, the
foliage of the sugarcane plants was also sprayed with essential minerals using 15ml/gallon of Chelated Palm Nutritional (Manganese 2.5%, Magnesium 1.5%, Iron 1%, Combined Sulphur 3.3%) (Southern Agricultural Insecticides, Inc.). When plants were 4-6 months old, healthy top visible dewlap (TVD) leaves were collected and inoculated with rust in the laboratory using a detached leaf bioassay (see below).

During peak brown rust epidemics in April-June 2016, 10-15 diseased leaves were collected per inoculation experiment from each of three brown rust susceptible sugarcane cultivars: CP 96-1252, CP 90-4725, and HoCP 96-540. These cultivars were growing in the fields of the Everglades Research and Education Center at Belle Glade, Florida (Figure 3-1B). Urediniospores of *P. melanocephala* were collected from these leaves in the laboratory using a vacuum pump (Figure 3-1C). Each sample of freshly collected spores from each cultivar was air dried overnight in the dark at 21°C. To determine viability of spores, a sub-sample (1-2 mg) of one hour dried spores from each cultivar were spread on a one mm thick layer of 1% water agar (Bacto® Agar, Becton, Dickinson & Company) made on a microscope slide. The microscope slide with agar and spores was stored overnight in the dark and inside an incubator at 20 ± 1°C. After 18-20 hours, urediniospores were observed under a microscope (400x magnification) and four sets of one hundred spores were counted on two microscope slides to determine the percent of germination. Spores producing germ tubes longer than the length of spores were considered as germinated spores. Only urediniospores samples with more than 20% germinated spores were used for leaf inoculation experiments. Once spore viability was determined, overnight air dried spores from each cultivar were added to sterile deionized water supplemented with 0.1% Tween 20 and 0.002%
nonanol. A hemocytometer was used to calibrate the spore suspension at $10^5$ spores/ml. All leaf inoculations were performed the day following collection of the rust spores in the field.

**Leaf Inoculation Protocol**

A detached leaf bioassay was developed to inoculate sugarcane leaves with rust urediniospores. This bioassay was based on previously published methods (Braithwaite 2005; Liu 1982; Taylor 1992). TVD leaves of disease-free sugarcane stalks were harvested in the greenhouse and two 10 cm-long pieces were cut from the middle section of the leaf. The leaf pieces were thoroughly rinsed with deionized water. The lower side of each leaf piece was then brush inoculated with 100 μl of the spore suspension (Figure 3-1D). Control leaves were brushed with 100 μl water-nonanol-tween 20. Inoculated leaf pieces were placed horizontally, with the abaxial surface up, in a humidity box for 15-20 hours in the dark at 20 ± 1°C (Figure 3-1E). Each inoculated leaf fragment was then transferred to a transparent 77 mm x 77 mm x 97 mm Magenta™ box (Sigma-Aldrich) containing 40 ml sterile deionized water, and kept vertically with the lower section of the leaf immersed in the water (Figure 3-1F). The Magenta™ boxes were placed in an incubator with 8 hours of fluorescent light at 25 ± 0.5°C and 16 hours of dark at 21 ± 0.5°C (Figure 3-1G). In each experiment, six leaf fragments were inoculated per sugarcane cultivar and rust isolate, and two leaf fragments of each cultivar were used as negative controls. Two weeks after inoculation, the number of brown rust uredinia that developed in each inoculated leaf piece was determined using a dissecting microscope (10x magnification).
Statistical Analyses

Three sugarcane cultivars were inoculated with three brown rust isolates in three separate and independent experiments. The data (number of rust uredinia) were collected from a total of 18 leaf pieces for each isolate-cultivar combination. Data were analyzed using one way ANOVA and treatment (isolate-cultivar) means were separated using the least significant difference (LSD) at $P=0.05$ using the Agricolae package in statistical program R (version 3.2.3) (R core team 2016; Mendiburu 2016). Mean numbers of uredinia produced for each isolate in three cultivars were analyzed separately.

Disease resistance of each cultivar inoculated with a given rust isolate was determined after comparison with the compatible interaction (isolate A x cultivar A) that was considered susceptible (S).

Results

Brown rust uredinia were visible on leaf fragments 14 days post inoculation (Figure 3-2). Control fragments inoculated with water remained free of brown rust symptoms and had a greener color than rust inoculated leaf pieces.

The overall mean number of brown rust uredinia per leaf fragment varied between 17 and 24. No significant differences were found between the three sugarcane cultivars, each inoculated with three different rust isolates (Table 3-1 and 3-2). All cultivar x rust isolate interactions were therefore rated as susceptible.

Discussion

No pathogenic specialization was observed among the brown rust isolates used in this study: All inoculated cultivars showed a similar disease reaction (susceptibility), regardless of the isolate of $P.\ melanocephala$. This data suggests no difference in
virulence within the *P. melanocephala* isolates at Belle Glade during 2016. The three rust isolates were collected from the only sugarcane cultivars that showed severe rust symptoms during the 2016 disease epidemic at EREC. All plants in the sampled plots showed symptoms, and there was no evidence of development of a new race as it was observed by Dean and Purdy (1984) in their study. This result is also consistent with the absence of any report of breakdown of resistance in commercial cultivation in Florida in recent years, the last report being in at least a decade ago (Raid 1989; Comstock et al. 2010).

However, Shine and collaborators (2005) described occurrence of several strains/races of *P. melanocephala* after inoculation of six sugarcane cultivars (CP 72-1210, CP 78-1247, CP 74-2005, B4362, CL 41-223, and CL 73-239) with rust isolates collected from different locations in Florida, including the Sugarcane Breeding Station at Canal Point. This station hosts a large germplasm collection and, if the brown rust strains identified over a decade ago are still existing, they are most likely present at Canal Point (located 15 miles from Belle Glade). Therefore, the detached leaf bioassay developed herein will be very useful for future investigations of variation in the populations of *P. melanocephala* in Florida. A similar in vitro bioassay was successfully used to demonstrate absence of variation of the brown rust pathogen in Australia, in the 1980s, after a severe rust outbreak (Taylor 1992). Alternatively, inoculation of young sugarcane plants in a controlled environment may also be performed. Using this method, occurrence of different strains of the brown rust pathogen has been recently reported in Louisiana (Hoy et al. 2014).
The majority of cultivars grown commercially in Florida and the parental germplasm at Canal Point now contain \textit{Bru1}, a major brown rust resistant gene (Glynn et al. 2012). Interestingly, none of the sugarcane cultivars used by Purdy and Dean in 1985 and Shine and collaborators in 2005 to identify pathogenic strains of \textit{P. melanocephala} in Florida contained \textit{Bru1}, and none of these cultivars are currently grown in Florida (Rott et al. 2013; van Weelden et al. 2016). Large distribution of this major and sustainable resistance gene may have also contributed to reduce diversity of the brown rust pathogen in Florida. However, recent increase in Florida of cultivation of large areas with brown rust susceptible sugarcane cultivars (such as CP 96-1252; VanWeelden et al. 2015) will certainly increase disease pressure on resistant cultivars with a potential breakdown of resistance due to mutation or introduction of a new race. Additionally, any \textit{P. melanocephala} strain overcoming the \textit{Bru1} gene could easily establish because sugarcane is a perennial crop with continuous infection and rust has short infection cycles.

The \textit{Bru1} gene has been providing a durable resistance to brown rust in cultivar R570 since its release in 1978 and no resistance breakdown has been reported (Asnaghi et al. 2001; Rott et al. 2013). \textit{Bru1} appears to be highly effective and durable, but as this resistance is maintained by a single major gene, it always poses a threat of evolution of more virulent races of \textit{P. melanocephala} as described by the theory of boom and bust cycle (Priestly 1978). The boom occurs when a single major resistant gene covering a huge acreage will exert an extreme pressure on a pathogen to overcome the resistance. As a result, the pathogen gains virulence (bust) against the existing resistance and develops as a new race. The potential breakdown of \textit{Bru1} can
be speculated through a typical boom and bust case. Resistance to wheat stem rust (	extit{Puccinia graminis f. sp.tritici}) was conferred by gene Sr31 for almost 20 years, from the 1980s to mid-1990s. This highly effective resistance was broken by an aggressive new race, Ug99, which evolved in Uganda (Africa) in the late 1990s. Later on, Ug99 rapidly migrated to neighboring wheat growing regions and dominated the previously existing races (Schumann and Leonard 2000). Monitoring population diversity of 	extit{P. melanocephala} is therefore vital for the effective screening for brown rust resistance, and the detached leaf assay used herein will be an effective method to reach this objective. Additionally, identification and deployment of other sources of host resistance is essential for efficient management of sugarcane brown rust in Florida, but also in other countries where the disease occurs.
Table 3-1. Symptom severity of three sugarcane cultivars inoculated with three isolates of *Puccinia melanocepha* collected in 2016

<table>
<thead>
<tr>
<th>Experiment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rust isolate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Inoculated sugarcane cultivar</th>
<th>Mean number of uredinia&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Reaction&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>i1252_16</td>
<td>CP 96-1252</td>
<td>28 a</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 78-1628</td>
<td>21 a</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL 90-4725</td>
<td>22 a</td>
<td>S</td>
</tr>
<tr>
<td>B</td>
<td>i4725_16</td>
<td>CP 96-1252</td>
<td>21 a</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 78-1628</td>
<td>18 a</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL 90-4725</td>
<td>21 a</td>
<td>S</td>
</tr>
<tr>
<td>C</td>
<td>i540_16</td>
<td>CP 96-1252</td>
<td>25 a</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 78-1628</td>
<td>27 a</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL 90-4725</td>
<td>27 a</td>
<td>S</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each experiment (inoculation of six leaf sections per cultivar) was performed separately three times (total of 18 leaves inoculated per cultivar). Replicated experiments were performed with different collections of spores from the same cultivar, but in each experiment the same spore sample was used to inoculate the three sugarcane cultivars.

<sup>b</sup>Isolates were named based on the sugarcane cultivar from which they were collected and expected to be the best adapted.

<sup>c</sup>Mean numbers of uredinia (mean of 18 leaves per cultivar) were analyzed separately for each isolate. Numbers followed by the same letter are not significantly different at P = 0.01 according to the Least Significant Difference test (Table 3-2).

<sup>d</sup>Disease resistance of each cultivar inoculated with a given rust isolate was determined after comparison with the compatible interaction (isolate A x cultivar A) that was considered susceptible (S).

Table 3-2. Symptom severity data analysis of three sugarcane cultivars inoculated with three isolates of *Puccinia melanocepha* collected in 2016

<table>
<thead>
<tr>
<th>Experiment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rust isolate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mean square error (MSE)</th>
<th>Least significant difference (LSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>i1252_16</td>
<td>165.40</td>
<td>8.60</td>
</tr>
<tr>
<td>B</td>
<td>i4725_16</td>
<td>202.80</td>
<td>9.52</td>
</tr>
<tr>
<td>C</td>
<td>i540_16</td>
<td>238.51</td>
<td>10.33</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each experiment (inoculation of six leaf sections per cultivar) was performed separately three times (total of 18 leaves inoculated per cultivar). Replicated experiments were performed with different collections of spores from the same cultivar, but in each experiment the same spore sample was used to inoculate the three sugarcane cultivars. One-way ANOVA and LSD test for each experiment were analyzed separately.

<sup>b</sup>Isolates were named based on the sugarcane cultivar from which they were collected and expected to be the best adapted.
Figure 3-1. Detached leaf inoculation assay for brown rust. A) Healthy sugarcane plants grown in a greenhouse. B) Selection of symptomatic leaves in the field. C) Collection of urediniospores in the laboratory using a vacuum pump. D) Brush inoculation of detached leaf pieces using a spore suspension prepared in deionized water supplemented with 0.002% nonanol and 0.1% tween 20; the spore suspension has been calibrated at $10^5$ spores/ml. E) Overnight incubation in the dark of inoculated leaves in a humidity box placed in an incubator at 20 ± 1°C. F) Detached leaf transferred into a transparent Magenta™ box containing 40 ml of sterile deionized water. G) Magenta boxes™ placed in a growth chamber with 8 hours of fluorescent light at 25 ± 0.5°C and 16 hours of dark at 21 ± 0.5°C. Photo courtesy of author.
Figure 3-2. Development of brown rust uredinia on a leaf fragment inoculated with *Puccinia melanocephala* using a detached leaf inoculation. A) Entire detached leaf with uredinia. B) Close-up (10x magnification) of sporulating uredinia of brown rust on a detached leaf. Photo courtesy of Philippe Rott.
CHAPTER 4
VARIATION IN PATHOGENICITY OF *PUCCINIA KUEHNII*, THE CAUSAL AGENT OF ORANGE RUST OF SUGARCANE IN FLORIDA

Introduction

During the 20th century, orange rust caused by *Puccinia kuehnii* was considered a minor disease of the Asian-Oceania region (Magarey 2000). However, this perception changed in 2000 when a major orange rust epidemic occurred in Australia. In that year, sugarcane cultivar Q124, which occupied over 90% of the cultivated area in Queensland, showed severe disease symptoms. Yield losses up to 40% were reported, as well as an estimated monetary loss of about 200 million Australian Dollars (Magarey et al. 2001a). Because *P. kuehnii* was present in Australia over 100 years, this sudden outbreak of orange rust was attributed to the development of new race of this pathogen (Magarey et al. 2001b).

Sugarcane orange rust had never been reported in the Western Hemisphere until 2007 when *P. kuehnii* was detected for the first time in Florida, USA (Comstock et al. 2008). Soon after, orange rust was found in several countries in Central and South America: Argentina, Belize, Brazil, Colombia, Costa Rica, Cuba, Dominican Republic, Ecuador, El Salvador, Guatemala, Jamaica, Mexico, Nicaragua and Panama (Barbasso et al. 2010; Cadavid et al. 2012; Chavarria et al. 2009; Comstock et al. 2008; Flores et al. 2009; Funes et al. 2016; Garces et al. 2014; Ovalle et al. 2008; Perez-Vincente et al. 2010). In Florida, yield losses of cultivar CP 80-1743 during the 2008 orange rust epidemic were estimated at 40% (Comstock et al. 2010). As was the case for sugarcane brown rust, it is generally believed that the first introduction of *P. kuehnii* spores to the Americas was through transoceanic atmospheric transportation from Africa (Rott et al. 2014).
Since its arrival in 2007, orange rust posed a constant threat to the sugarcane production in the Americas (Comstock et al. 2010). The preferred method of disease control is the use of resistant cultivars. Although the genetic mechanisms of resistance are not known, orange rust resistant sugarcane clones have been identified using several screening techniques (Rott et al. 2013). In Florida, the Canal Point Sugarcane Cultivar Development Program (CP program) has been screening for rust resistance, and new varieties resistant to orange rust are being released (Rott et al. 2014). However, durability of rust resistance in sugarcane has been challenged by the potential change of virulence in the rust pathogen. Since the first occurrence of orange rust in Florida during 2007, two sugarcane cultivars shifted from resistant to susceptible. For example, the cultivar CP 88-1762 has no orange rust infections occur during the 2007 epidemic. However, severe orange rust symptoms were observed in the same variety only two years later in 2009 (Comstock et al. 2010). Similarly, cultivar CP 89-2143 was considered resistant to the disease until 2011 when this cultivar started to show symptoms in Florida scouting program surveys (BASF, unpublished data). In 2012, orange rust severity of CP 89-2143 was similar to disease severity of susceptible cultivars CP 88-1762 and CP 80-1743, suggesting breakdown of resistance in CP 89-2143 and pathogenic changes in the P. kuehnii population. Therefore, to maintain durability of rust resistance, an efficient cultivar screening process needs to comprise careful monitoring of pathogenic changes in P. kuehnii (Comstock et al. 2010).

Several techniques have been used for determining differential reactions of sugarcane cultivars to rust isolates, such as detached leaf inoculation, leaf whorl inoculation, and inoculation of young plants in controlled environments (Asnaghi et al.
2001; Sood et al. 2009; Hoy et al. 2014). Disease symptoms are phenotypic data that can reflect pathogenic specialization and variation in pathogenicity of the fungus. Ultimately, the genetic support of pathogenicity and variation among rust isolates can be determined using molecular techniques (Rott et al. 2013).

In Florida, shifts from rust resistance to susceptibility of certain sugarcane cultivars are strong evidence for changes in pathogenicity of *P. kuehni* populations. However, no formal demonstration of this variation in *P. kuehni* has been reported so far. We hypothesize that the breakdown of resistance to orange rust in commercial cultivar CP 89-2143 is linked to the development of a new race or a more aggressive strain of *P. kuehni*. The objective of this study was to demonstrate occurrence of pathogenic specialization among rust isolates collected from sugarcane cultivars susceptible to orange rust in Florida.

**Materials and methods**

**Sugarcane Cultivars Grown in a Greenhouse**

Four sugarcane cultivars (CL 85-1040, CP 80-1743, CP 88-1762, and CP 89-2143) with different histories of resistance to orange rust were selected. CL 85-1040 and CP 80-1743 were susceptible to orange rust since the first orange rust epidemic in 2007 (Comstock et al. 2010). CP 88-1762 was considered moderately susceptible until 2009 but became susceptible in 2012 (Rice et al. 2013). CP 89-2143 was resistant to orange rust until 2010, but became susceptible to the disease in 2011-2012 (BASF, unpublished data). In December 2015, single node stalk cuttings of sugarcane cultivars CL 85-1040, CP 80-1743, CP 88-1762 and CP 89-2143 were planted in 1.7 gallon pots containing organic soil also known as Lauderhill Muck (euic, hyperthermic Lithic Haplosaprist) (USDA-NRCS Soil Survey 2016) that was collected from nearby fields at
the Everglades Research and Education Center (EREC) in Belle Glade, Florida. Plants were watered by drip irrigation in a closed greenhouse to prevent rust infection (Figure 4-1A). Fifteen grams of slow release fertilizer (Osmocote, The Scotts Company, N-P-K: 15-9-12) and 5 grams of granular insecticide (Tree & Shrub Protect & Feed, Bayers Advance, 0.55% Imidacloprid and 0.275% Clothianidin) were added to each pot when plants were two months old. After three months of growth, the foliage of the sugarcane plants was also sprayed with essential minerals using 15ml/gallon of Chelated Palm Nutritional (Manganese 2.5%, Magnesium 1.5%, Iron 1%, Combined Sulphur 3.3%) (Southern Agricultural Insecticides, Inc.).

**Orange Rust Inoculum**

Urediniospores of *P. kuehnii* were collected from the same four cultivars mentioned above and grown in the field at the UF/IFAS Everglades Research and Education center in Belle Glade, Florida: CL 85-1040, CP 80-1743, CP 88-1762 and CP 89-2143. A laboratory vacuum pump was used to collect the urediniospores of *P. kuehnii* from the diseased leaves of each cultivar (Figure 4-1B and C). Each sample of freshly collected spores from each cultivar was air dried overnight in the dark at 21°C. To determine viability of spores, a sub-sample (1-2 mg) of one hour dried spores from each cultivar were spread on a one mm thick layer of 1% water agar (Bacto® Agar, Becton, Dickinson & Company) made on a microscope slide. The microscope slide with agar and spores was stored overnight in the dark and inside an incubator at 20 ± 1°C. Eighteen to 20 hours after spreading on agar, urediniospores were observed under a microscope (400x magnification) and four sets of one hundred spores were counted on two microscope slides to determine the percent of germination. Spores producing germ tubes longer than spore length were considered as germinated spores. Only
urediniospores samples with more than 20% germinated spores were used for leaf inoculation experiments. Once spore viability was determined, overnight air dried spores from each cultivar were added to sterile deionized water supplemented with 0.1% Tween 20 and 0.002% nonanol. A hemocytometer was used to calibrate the spore suspension at $10^5$ spores/ml. All leaf inoculations were performed the day following collection of the rust spores in the field.

**Detached Leaf Inoculation**

This detached leaf bioassay was based on previously published methods (Braithwaite 2005; Liu 1982; Taylor 1992). In December 2015, single node stalk cuttings of sugarcane cultivars CL 85-1040, CP 80-1743, CP 88-1762 and CP 89-2143 were planted in pots inside a greenhouse (Figure 4-1 C). When sugarcane plants were 4-6 month old, the top visible dewlap (TVD) leaves were collected. In the laboratory, two 10 cm-long pieces were cut from the middle section of each leaf. The leaf pieces were thoroughly rinsed with deionized water. Each leaf piece was then brush inoculated with a *P. kuehnii* spore suspension calibrated at $10^5$ spores per ml (Figure 4-1 D). In each experiment, two leaf pieces of each cultivar were also brushed with 100 µl of water-tween20-nonanol and used as a negative control. Further treatment of leaves differed according to protocols #1 and #2, as described below. A single experiment consisted of inoculation of a given rust isolate on six leaf pieces of each of four susceptible sugarcane cultivars. Each inoculation experiment was carried out separately four times and inoculation was always performed one day after collecting the spores in the sugarcane field.
**Detached Leaf Inoculation Protocol #1**

Brush-inoculated leaf pieces were placed horizontally with abaxial side up in a humidity box, for 15-20 hours, in the dark at 20 ± 1°C (Figure 4-1 E). Each leaf piece was then transferred to a transparent 77 mm x 77 mm x 99 mm Magenta™ box (Sigma-Aldrich) containing 40 ml sterile of deionized water. The lower cut section of the inoculated leaf piece (i.e. the section that was closest to the stalk when collected) was immersed in the deionized water (Figure 4-1 F). The Magenta™ boxes were kept in an incubator with 8 hours fluorescent light at 25 ± 0.5°C and 16 hours of dark at 21 ± 0.5°C (Figure 4-1 G). Two weeks after inoculation, the number of orange rust uredinia that developed on each leaf piece was determined using a dissecting microscope (10x magnification).

**Detached Leaf Inoculation Protocol #2**

Some modifications were made to protocol #1 in order to improve symptom expression in detached leaves. First, nitrogen was added to the sugarcane plants in the greenhouse: 10 g of ammonium nitrate per 1.7 gallon pot one month before sampling the leaves to be inoculated. Nitrogen is known to increase susceptibility to diseases, including sugarcane brown rust (Anderson et al. 1990; Johnson et al. 2007; Kiraly 1964). Second, after brush inoculation, the leaf pieces were sprayed with a fine mist of deionized water using a sprayer. Leaf pieces were then not kept horizontally in a humidity box but were directly transferred to the Magenta™ boxes. As in protocol #1, inoculated leaf pieces were placed vertically with the lower end of the inoculated leaf piece (i.e. the section that was closest to the stalk when collected) immersed in 40 ml of deionized water (Figure 4-1E). The Magenta boxes were kept in an incubator with 8 hours of fluorescent light at 25 ± 0.5°C and 16 hours of dark at 21 ± 0.5°C (Figure 4-1
Two weeks after inoculation, the number of all orange rust uredinia that developed on each leaf piece was determined using a dissecting microscope (10x magnification) (Figure 4-2).

**Leaf Whorl Inoculation of Young Sugarcane Plants**

In July 2015, single-node cuttings of the four sugarcane cultivars (CL 85-140, CP 80-1743, CP 88-1762 and CP 89-2143) were planted in trays (55 X 35 X 10 cm) containing organic soil (muck soil). Each tray contained a row of five plants of each cultivar (Figure 4-3 A). Plants were grown in a greenhouse and plant height was maintained at one to one and half feet by regular trimming. Leaf whorl inoculation of these plants was performed in May-June 2016 according to the method described by Sood and collaborators (2009). Inoculum consisted of a spore suspension of *P. kuehnii* adjusted at $10^4$ spore/ml of deionized water supplemented with 0.002% 1nonol and 0.01% tween 20. Using a micropipette, 200 $\mu$l of the spore suspension was deposited inside the leaf whorl of the sugarcane plants in the greenhouse (Figure 4-3 B). In a single experiment, 15 plants per variety (distributed in three trays) were inoculated with each rust isolate. Three to four weeks post inoculation, orange rust symptoms appeared as a band of uredinia on the leaf blade that had emerged from the whorl (Figure 4-3 C and D). The number of orange rust uredinia that developed on each leaf blade was determined using a hand lens (10x magnification). Each experiment was carried out separately three times with freshly collected spores of the pathogen.

**Statistical Analyses**

Regardless of the inoculation technique, results obtained for each rust isolate were analyzed using one-way ANOVA and treatment (cultivar) means were separated
using the least significant difference (LSD) using Agaricolae package in statistical program R version 3.2.3 (R core team 2016; Mendiburu 2016).

ANOVA results were also used to attribute a resistance level to each sugarcane cultivar inoculated with a given rust isolate. In this analysis, the reaction (based on pustule number) resulting from the interaction of sugarcane cultivar A x isolate A (= rust isolate collected from cultivar A) was considered susceptible (S). Disease resistance was rated highly susceptible (HS) when the number of uredinia was significantly higher than for the S interaction, moderately resistant (MR) when significantly lower than the S interaction, and resistant (R) when the number of uredinia was very low (8-10 times less than the S interaction).

Results
Detached Leaf Inoculation Protocol #1

All four orange rust isolates (i1040_16, i1743_16, i1762_16, and i2143_16) produced rust uredinia in all four inoculated cultivars (CL 85-1040, CP 80-1743, CP 88-1762 and CP89-2143) (Table 4-1 and Table 4-2, Figure 4-2). Isolate i1040_16 (collected from cultivar CL 85-1040 in 2016) produced an average of 104 uredinia per leaf fragment in cultivar CL 85-1040, and this was the highest number of uredinia among all four inoculated cultivars. The number of uredinia that developed in cultivar CL 80-1743 and CL 88-1762 after inoculation with i1040_16 was lower (64 and 44, respectively vs. 104 uredinia) than in CL 85-1040. In contrast, the average number of uredinia produced by this rust isolate in cultivar CP 89-2143 was much lower (8 vs. 104 uredinia) (Table 4-1).

Orange rust isolates i1743_16 (collected from cultivar CP 80-1743 in 2016) and i1762_16 (collected from cultivar CP 88-1762 in 2016) produced a similar and large
number of uredinia in cultivars CL 85-1040, CP 80-1743 and CP 88 1762: 56-80 uredinia for \textit{i1743\_16} and 82-102 uredinia for \textit{i1762\_16} (Table 4-1). In contrast, cultivar CP 89-2143 inoculated with these isolates showed much less rust uredinia than the other three cultivars: 5 vs. 56-80 uredinia for \textit{i1743\_16} and 9 vs. 82-102 for \textit{i1762\_16}.

Orange rust isolate \textit{i2143\_16} (collected from cultivar CP 89-2143 in 2016) produced more leaf uredinia than any of the other three isolates of the pathogen, regardless of the sugarcane cultivar inoculated (Table 4-1). This difference in average pustule production was highest in sugarcane cultivar CP 89-2143: 85 uredinia for \textit{i2143\_16} and only 12, 5, and 9 uredinia for isolates \textit{i1040\_16}, \textit{i1743\_16}, and \textit{i1762\_16}, respectively.

Data obtained for all sugarcane cultivar x rust isolate combinations were also summarized based on disease resistance level of each sugarcane cultivar towards each rust isolate (Table 4-3). Cultivars CL 85-1040, CL 80-1743 and CL 88-1762 were susceptible to moderately susceptible when inoculated with orange rust isolates \textit{i1040\_16}, \textit{i1743\_16}, and \textit{i1762\_16} that were collected from these cultivars. In contrast, cultivar CP 89-2143 was moderately resistant to resistant when inoculated with these three isolates of the pathogen. All four inoculated sugarcane cultivars were susceptible to highly susceptible to orange rust isolate \textit{i2143\_16} collected from cultivar CP 89-2143.

**Detached Leaf Inoculation Protocol #2**

The reaction of the sugarcane cultivars to the different rust isolates using protocol #2 was similar to the reaction described above with protocol #1. However, the number of uredinia produced on the leaves was three times higher when leaves were inoculated with protocol #2 (Table 4-4 and Table 4-5). Sugarcane cultivars CL 85-1040, CP 80-1743 and CP 88-1762 showed an average of 243-365 rust uredinia per leaf
section when inoculated with orange rust isolates *i1040_16*, *i1743_16*, and *i1762_16*. The fourth cultivar, CP 89-2143, showed only 47-58 rust uredinia when inoculated with *i1040_16*, *i1743_16*, and *i1762_16*. The fourth rust isolate, *i2143_16* collected from CP 89-2143, produced large numbers of rust uredinia (236-334) on all four sugarcane cultivars (Table 4-4). Cultivars CL 85-1040 and CP 80-1743 appeared to be the most susceptible cultivars against this isolate as they showed 325-334 uredinia, whereas CP 88-1762 and CP 89-2143 showed a lower number of uredinia (236-251).

Data obtained for all sugarcane cultivar x rust isolate combinations were also summarized based on disease resistance level of each sugarcane cultivar towards each rust isolate (Table 4-3). As for protocol #1, cultivars CL 85-1040, CL 80-1743 and CL 88-1762 were susceptible to moderately susceptible when inoculated with orange rust isolates *i1040_16*, *i1743_16*, and *i1762_16* that were collected from these cultivars. Cultivar CP 89-2143 was moderately resistant to resistant when inoculated with these three isolates of the pathogen. All four inoculated sugarcane cultivars were susceptible to highly susceptible to orange rust isolate *i2143_16* collected from cultivar CP 89-2143.

**Leaf Whorl Inoculation of Young Plants**

All four orange rust isolates (*i1040_16*, *i1743_16*, *i1762_16*, and *i2143_16*) produced rust uredinia in all four inoculated cultivars (CL 85-1040, CP 80-1743, CP 88-1762 and CP89-2143). Additionally, as observed previously with the detached leaf inoculation protocols, the number of uredinia that developed in each cultivar varied according to the rust isolates (Table 4-6 and Table 4-7). Cultivars CL 85-1040, CP 80-1743, and CP 88-1762 showed more rust uredinia per leaf section after inoculation with their respective rust isolates (*i1040_16*, *i1743_16*, and *i1762_16*) than after inoculation with isolate *i2143_16* collected from CP 89-2143: 37-63 vs. 24-28. Isolate *i2143_16*
produced large numbers of rust uredinia (47-58) on all four sugarcane cultivars (Table 4-6).

Disease resistance level of the four sugarcane cultivars against the four rust isolates varied from moderately resistant (MR) to highly susceptible (HS) (Table 4-3). Cultivars CL 85-1040, CP 80-1743, and CP 88-1762 were moderately susceptible (MS) to highly susceptible (HS) depending on the rust isolate. Cultivar CP 89-2143 was moderately resistant to three isolates of the pathogen (i1040_16, i1743_16, and i1762_16), and only isolate i2143_16 induced a susceptible to highly susceptible disease reaction in all four sugarcane cultivars.

Discussion

Both the detached leaf and leaf whorl inoculation techniques used during this research resulted in expression of differential host reactions to *P. kuehnii* isolates. Isolate i2143_16 collected from sugarcane cultivar CP 89-2143 was more virulent than the isolates collected from sugarcane cultivars that were already susceptible in 2011, when resistance of CP 89-2143 broke down. These results support the recent shift of CP 89-2143 cultivar resistance, and are strong evidence of existence of at least two virulence groups within the orange rust population in Florida. These differences in virulence of the pathogen also support pathogenic specialization of *P. kuehnii*, as it was also shown to occur recently in Brazil where the presence of two populations of *P. kuehnii* varying in virulence was reported (Urashima et al. 2015).

Isolates of *P. kuehnii* used in this study are bulk or population isolates collected from several leaves of each cultivar in a given field. These isolates are likely not clonal but rather a genetic mixture, especially if variation occurs in the pathogen population.
A few orange rust uredinia developed in CP 89-2143 when this cultivar was inoculated with rust isolates collected from cultivars known to be susceptible before the breakdown of CP 89-2143. It is most likely that cultivars CL 85-1040, CP 80-1743, and CP 88-1762, that were susceptible to orange rust before the breakdown of CP 89-2143, host a mixture of at least two pathotypes of *P. kuehnii*: the former pathotype that was introduced in 2007 and the new pathotype that developed on CP 89-2143 in 2011-2012. Cultivar CP 89-2143 exhibits much more rust pustules when inoculated with isolate *i2143_16* than with isolates *i1040_16, i1743_16,* and *i1762_16* collected from sugarcane cultivars CL 85-1040, CP 80-1743, and CP 88-1762, respectively. Therefore, isolates *i1040_16, i1743_16,* and *i1762_16* are not well adapted to cultivar CP 89-2143 and most likely belong to the original orange rust pathotype that was introduced into Florida in 2007. Consequently, the new orange rust pathotype hosted by cultivar CP 89-2143 did not outcompete the former pathotype on all sugarcane varieties susceptible to rust in Florida.

The detached-leaf inoculation assay was more discriminative than the leaf whorl inoculation assay to show pathogenic differences between rust isolates. Significant differences were observed between the high numbers of uredinia produced in susceptible interactions in comparison to the low numbers of uredinia observed in resistant interactions in detached leaf assays. In contrast, although differences between susceptible and resistant reactions were mostly significant using leaf whorl inoculation, numbers of produced uredinia were not as discriminative. A major technical concern during the inoculation of the leaf whorl was the deposition of the spores into the leaf whorl. Although the same amount of inoculum was deposited in each leaf whorl, it
cannot be excluded that unequal amounts of inoculum remained in the whorl as shown by the leakage of liquid from some whorls during inoculation. Additionally, the accumulation of large number of spores in a small confined whorl can reduce the germination capacity of rust spores due to crowding effect (Staples 2000). The leaf whorl inoculation technique has been successfully used for screening sugarcane varieties for resistance to rust (Comstock et al. 2010). Since the clonal screening requires qualitative results of susceptibility/resistance rather than quantitative variation of the symptom expression, the leaf whorl technique is useful for screening large number of plants in a short time. However, this technique needs to be improved (to avoid the drip issue for example) before it can be more reliably used for assessing variation among rust isolates.

In a few studies, detached-leaf assays have been reported not to represent complete field conditions for resistance or susceptibility of sugarcane cultivars (Hsiang et al. 2004; Sood et al. 2009). However, this technique was successfully used in other investigations to observe resistance of sugarcane cultivars to brown rust isolates from several geographical locations (Asnaghi et al. 2001). In our experiments, the detached-leaf inoculation assay was very useful to identify variation in virulence of P. kuehnii. Additionally, data obtained after inoculation of entire plants by the leaf whorl inoculation technique confirmed detached-leaf inoculation data.

Detached-leaf inoculation protocol #1 already produced a fair amount of disease on detached leaves but it appeared that only 1.5% of inoculum was able to produce rust uredinia (up to 6000 uredinia were expected to develop if all inoculated germinating spores were able to infect the leaf section; tentative 60% germination was expected in
each detached leaf after inoculation of 100 µl of 10^5 spore/ml. Therefore, a new protocol (#2) was designed in an attempt to improve disease expression. As excess nutrients are believed to increase rust severity (Johnson et al 2007), an additional dose of nitrogen fertilizer was added to the sugarcane plant grown in the greenhouse, one month prior to inoculation. Leaf sections were also sprayed with water to ensure best hydration of leaf sections. These two modifications resulted in overall increase of rust symptoms as expressed by the number of uredinia produced in the detached leaf assay. Additionally, because disease resistance reactions of the sugarcane cultivars to the different rust isolates were similar regardless the inoculation protocol, protocol #2 can be considered as an improved version of the detached-leaf inoculation assay.

*Puccinia kuehnii* was considered a minor pathogen in Australia for decades until 2000 when this pathogen caused a devastating epidemic in sugarcane cultivar Q124. This cultivar was grown over large areas (45%) in Queensland when its resistance to orange rust broke down. The cause of this breakdown was attributed to the emergence of a new pathogenic race of *P. kuehnii* (Magarey et al. 2001a), and a genetic variation study concluded that a single dominant genotype of *P. kuehnii* was present in Australia in the early 2000s (Braithwaite 2005). Investigations also concluded that the orange rust epidemic in 2000 was not due to introduction of any foreign virulent race of *P. kuehnii* but to a single gene mutation in the existing pathogen. This event of orange rust in Australia appeared to be different than the sugarcane brown rust epidemic which occurred in the same country in the late 1970s, and which was attributed to changes in environmental conditions (Taylor 1992).
Even though orange rust resistance breakdown in Australia and Florida can both be attributed to changes in virulence of the pathogen, the situation in Florida appears to have unique features as *P. kuehnii* was not present in the Western Hemisphere until 2007. Orange rust was present in Australia for almost a hundred years until it became a major disease in sugarcane in 2000. In contrast, this disease was devastating in Florida sugarcane and other geographical locations in Central and South America immediately upon its arrival. One could hypothesize that the highly virulent race of *P. kuehnii* occurring in Australia was transported through transoceanic winds to the Western Hemisphere as it was suggested for emergence of sugarcane brown rust in the Caribbean in 1960s (Purdy et al. 1985). This hypothesis is supported by the recent reports of occurrence of *P. kuehnii* in African countries such as Cameroon and Ivory Coast (Saumtally et al. 2011).

This new strain of orange rust may have the propensity of rapid variation or adaptation to new cultivars, which may explain why a new pathotype of *P. kuehnii* developed on cultivar CP 89-2143 less than 4 years upon the emergence of orange rust in Florida. Similarly, phenotypic and genotypic variation was reported in the populations of *P. kuehnii* that emerged recently in Brazil (Urashima et al. 2015). On the other hand, it cannot be excluded that orange rust was introduced into the Western Hemisphere as a mixture of isolates that progressively adapted to different cultivars as they were produced or until they were grown by the industry over large areas. In this context of rapid evolution or changes of the orange rust populations, a close eye should be kept on the development of orange rust in the Western Hemisphere. Detached leaf inoculation will be a useful method for identifying variation among rust isolates. Knowing
and monitoring variation of a pathogen is essential in sugarcane breeding programs to develop cultivars resistant to diseases, and newly released cultivars should be resistant to all known strains of a pathogen (Rott et al. 2013). Increasing the number of sugarcane cultivars resistant to rust diseases in Florida will contribute to reduce chemical control and benefit the sugarcane industry.
Table 4-1. Symptom severity of four sugarcane cultivars inoculated using detached-leaf inoculation protocol #1 with four isolates of *Puccinia kuehnii* collected in 2016

<table>
<thead>
<tr>
<th>Experiment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rust isolate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Inoculated sugarcane cultivar</th>
<th>Mean number of uredinia&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>i1040_16</em></td>
<td>CL 85-1040</td>
<td>104&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 80-1743</td>
<td>64&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 88-1762</td>
<td>44&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td><em>i1743_16</em></td>
<td>CL 85-1040</td>
<td>80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
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<td>CP 80-1743</td>
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<td>CP 88-1762</td>
<td>70&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td><em>i1762_16</em></td>
<td>CL 85-1040</td>
<td>82&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 80-1743</td>
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<td>CP 88-1762</td>
<td>102&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>D</td>
<td><em>i2143_16</em></td>
<td>CL 85-1040</td>
<td>150&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>CP 88-1762</td>
<td>122&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each experiment (inoculation of six leaf sections per cultivar) was performed separately two (experiments B and C) or three times (experiments A and D) (total of 12-18 leaves inoculated per cultivar). Replicated experiments were performed with different collections of spores from the same cultivar, but in each experiment the same spore sample was used to inoculate the four sugarcane cultivars.

<sup>b</sup>Isolates were named based on the sugarcane cultivar from which they were collected and expected to be the best adapted.

<sup>c</sup>Mean numbers of uredinia (mean of 12-18 leaves per cultivar) were analyzed separately for each experiment. Numbers followed by the same letter are not significantly different at $P = 0.05$ according to the Least Significant Difference test (Table 4-2).
Table 4-2. Symptom severity data analysis of four sugarcane cultivars inoculated using detached-leaf inoculation protocol #1 with four isolates of *Puccinia kuehnii* collected in 2016

<table>
<thead>
<tr>
<th>Experiment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rust isolate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mean square error (MSE)</th>
<th>Least significant difference (LSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>i1040_16</em></td>
<td>2799.96</td>
<td>35.19</td>
</tr>
<tr>
<td>B</td>
<td><em>i1743_16</em></td>
<td>2671.99</td>
<td>42.53</td>
</tr>
<tr>
<td>C</td>
<td><em>i1762_16</em></td>
<td>1510.10</td>
<td>31.97</td>
</tr>
<tr>
<td>D</td>
<td><em>i2143_16</em></td>
<td>4835.45</td>
<td>46.25</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each experiment (inoculation of six leaf sections per cultivar) was performed separately two (experiments B and C) or three times (experiments A and D) (total of 12-18 leaves inoculated per cultivar). Replicated experiments were performed with different collections of spores from the same cultivar, but in each experiment the same spore sample was used to inoculate the four sugarcane cultivars. One-way ANOVA and LSD test for each experiment were analyzed separately.

<sup>b</sup>Isolates were named based on the sugarcane cultivar from which they were collected and expected to be the best adapted.

Table 4-3. Disease resistance of four sugarcane cultivars inoculated using three inoculation techniques with four isolates of *Puccinia kuehnii* collected in 2016

<table>
<thead>
<tr>
<th>Inoculation technique</th>
<th>Rust Isolate</th>
<th>Inoculated sugarcane cultivar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CL 85-1040</td>
</tr>
<tr>
<td>Detached leaf</td>
<td><em>i1040_16</em></td>
<td>S</td>
</tr>
<tr>
<td></td>
<td><em>i1743_16</em></td>
<td>S</td>
</tr>
<tr>
<td></td>
<td><em>i1762_16</em></td>
<td>S</td>
</tr>
<tr>
<td></td>
<td><em>i2143_16</em></td>
<td>HS</td>
</tr>
<tr>
<td>Detached leaf</td>
<td><em>i1040_16</em></td>
<td>S</td>
</tr>
<tr>
<td></td>
<td><em>i1743_16</em></td>
<td>S</td>
</tr>
<tr>
<td></td>
<td><em>i1762_16</em></td>
<td>S</td>
</tr>
<tr>
<td></td>
<td><em>i2143_16</em></td>
<td>HS</td>
</tr>
<tr>
<td>Leaf whorl inoculation</td>
<td><em>i1040_16</em></td>
<td>S</td>
</tr>
<tr>
<td></td>
<td><em>i1743_16</em></td>
<td>S</td>
</tr>
<tr>
<td></td>
<td><em>i1762_16</em></td>
<td>HS</td>
</tr>
<tr>
<td></td>
<td><em>i2143_16</em></td>
<td>HS</td>
</tr>
</tbody>
</table>

Disease resistance was determined after comparison with the compatible interaction (isolate A x cultivar A) that was considered susceptible (S). HS= highly susceptible when the number of uredinia was significantly higher than for the S reaction. MS= moderately susceptible when significantly lower than S, MR= moderately resistant when significantly lower than the MS reaction, and R= resistant when the number of uredinia was very low.
Table 4-4. Symptom severity of four sugarcane cultivars inoculated using detached-leaf inoculation protocol #2 with four isolates of *Puccinia kuehnii* collected in 2016

<table>
<thead>
<tr>
<th>Experiment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rust isolate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Inoculate sugarcane cultivar</th>
<th>Mean number of uredinia&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>i1040_16</em></td>
<td>CL 85-1040</td>
<td>338 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 80-1743</td>
<td>287 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 88-1762</td>
<td>243 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>54 c</td>
</tr>
<tr>
<td>B</td>
<td><em>i1743_16</em></td>
<td>CL 85-1040</td>
<td>354 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 80-1743</td>
<td>365 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 88-1762</td>
<td>320 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>58 b</td>
</tr>
<tr>
<td>C</td>
<td><em>i1762_16</em></td>
<td>CL 85-1040</td>
<td>262 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 80-1743</td>
<td>282 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 88-1762</td>
<td>248 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>47 b</td>
</tr>
<tr>
<td>D</td>
<td><em>i2143_16</em></td>
<td>CL 85-1040</td>
<td>334 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 80-1743</td>
<td>325 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 88-1762</td>
<td>251 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>236 b</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each experiment (inoculation of six leaf sections per cultivar) was performed separately four times (total of 24 leaves inoculated per cultivar). Replicated experiments were performed with different collections of spores from the same cultivar, but in each experiment the same spore sample was used to inoculate the four sugarcane cultivars.

<sup>b</sup>Isolates were named based on the sugarcane cultivar from which they were collected and expected to be the best adapted.

<sup>c</sup>Mean numbers of uredinia (mean of 24 leaves per cultivar) were analyzed separately for each experiment. Numbers followed by the same letter are not significantly different at *P* = 0.05 according to the Least Significant Difference test (Table 4-5).
Table 4-5. Symptom severity data analysis of four sugarcane cultivars inoculated using detached-leaf inoculation protocol #2 with four isolates of *Puccinia kuehnii* collected in 2016

<table>
<thead>
<tr>
<th>Experiment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rust isolate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mean square error (MSE)</th>
<th>Least significant difference (LSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>i1040_16</em></td>
<td>10334.27</td>
<td>58.28</td>
</tr>
<tr>
<td>B</td>
<td><em>i1743_16</em></td>
<td>15213.01</td>
<td>70.71</td>
</tr>
<tr>
<td>C</td>
<td><em>i1762_16</em></td>
<td>13235.24</td>
<td>65.95</td>
</tr>
<tr>
<td>D</td>
<td><em>i2143_16</em></td>
<td>14330.79</td>
<td>68.63</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each experiment (inoculation of six leaf sections per cultivar) was performed separately four times (total of 24 leaves inoculated per cultivar). Replicated experiments were performed with different collections of spores from the same cultivar, but in each experiment the same spore sample was used to inoculate the four sugarcane cultivars. One-way ANOVA and LSD test for each experiment were analyzed separately.

<sup>b</sup>Isolates were named based on the sugarcane cultivar from which they were collected and expected to be the best adapted.
Table 4-6. Symptom severity of four sugarcane cultivars inoculated using the leaf whorl inoculation protocol with four isolates of *Puccinia kuehnii* collected in 2016

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Rust isolate</th>
<th>Inoculate sugarcane cultivar</th>
<th>Mean number of uredinia &lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>i1040_16</td>
<td>CL 85-1040</td>
<td>63 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 80-1743</td>
<td>53 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 88-1762</td>
<td>40 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>28 c</td>
</tr>
<tr>
<td>B</td>
<td>i1743_16</td>
<td>CL 85-1040</td>
<td>64 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 80-1743</td>
<td>60 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 88-1762</td>
<td>40 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>24 c</td>
</tr>
<tr>
<td>C</td>
<td>i1762_16</td>
<td>CL 85-1040</td>
<td>65 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 80-1743</td>
<td>61 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 88-1762</td>
<td>37 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>24 c</td>
</tr>
<tr>
<td>D</td>
<td>i2143_16</td>
<td>CL 85-1040</td>
<td>58 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 80-1743</td>
<td>53 ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 88-1762</td>
<td>50 ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CP 89-2143</td>
<td>47 b</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each experiment (inoculation of 15 plants per cultivar) was performed separately three times (total of 45 plants inoculated per cultivar). Replicated experiments were performed with different collections of spores from the same cultivar, but in each experiment the same spore sample was used to inoculate the four sugarcane cultivars.

<sup>b</sup>Isolates were named based on the sugarcane cultivar from which they were collected and expected to be the best adapted.

<sup>c</sup>Mean numbers of uredinia (mean of 45 plants per cultivar) were analyzed separately for each experiment. Numbers followed by the same letter are not significantly different at $P = 0.05$ according to the Least Significant Difference test (Table 4-7).
Table 4-7. Symptom severity data analysis of four sugarcane cultivars inoculated using the leaf whorl inoculation protocol with four isolates of *Puccinia kuehnii* collected in 2016

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Rust isolate</th>
<th>Mean square error (MSE)</th>
<th>Least significant difference (LSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>i1040_16</em></td>
<td>539.78</td>
<td>10.23</td>
</tr>
<tr>
<td>B</td>
<td><em>i1743_16</em></td>
<td>531.24</td>
<td>9.85</td>
</tr>
<tr>
<td>C</td>
<td><em>i1762_16</em></td>
<td>517.44</td>
<td>9.28</td>
</tr>
<tr>
<td>D</td>
<td><em>i2143_16</em></td>
<td>640.98</td>
<td>11.14</td>
</tr>
</tbody>
</table>

*a* Each experiment (inoculation of 15 plants per cultivar) was performed separately three times (total of 45 plants inoculated per cultivar). Replicated experiments were performed with different collections of spores from the same cultivar, but in each experiment the same spore sample was used to inoculate the four sugarcane cultivars. One-way ANOVA and LSD test for each experiment were analyzed separately.

*b* Isolates were named based on the sugarcane cultivar from which they were collected and expected to be the best adapted.
Figure 4-1: Detached leaf inoculation assay for brown rust. A) Healthy sugarcane plants grown in a greenhouse. B) Selection of symptomatic leaves in the field. C) Collection of urediniospores in the laboratory using a vacuum pump. D) Brush inoculation of detached leaf pieces using a spore suspension prepared in deionized water supplemented with 0.002% nonanol and 0.1% tween 20; the spore suspension has been calibrated at $10^5$ spores/ml. E) Overnight incubation in the dark of inoculated leaves in a humidity box placed in an incubator at $20 \pm 1 ^\circ C$. F) Detached leaf transferred into a transparent Magenta™ box containing 40 ml of sterile deionized water. G) Magenta™ boxes placed in a growth chamber with 8 hours of fluorescent light at $25 \pm 0.5 ^\circ C$ and 16 hours of dark at $21 \pm 0.5 ^\circ C$. Photo courtesy of author.
Figure 4-2: Sugarcane leaf fragments showing rust uredinia after inoculation with *Puccinia kuehnii* using detached-leaf inoculation protocol #2. A) Isolate *i2143_16* inoculated on cultivar CL 85-1040. B) Isolate *i2143_16* inoculated on cultivar CP 89-2143. C) Isolate *i1040_16* inoculated on cultivar CL 85-1040. D) Isolate *i1040_16* inoculated on cultivar CP 89-2143. Photo courtesy of author.
Figure 4-3: Leaf whorl inoculation assay for sugarcane orange rust. A) Tray with four sugarcane cultivars grown in four different rows. B) Inoculation of the sugarcane leaf whorl with a suspension of spores of *Puccinia kuehnii*. C) Orange rust symptoms that developed as bands of uredinia three weeks after inoculation. D) Sugarcane leaf showing a band of rust uredinia that developed three weeks after inoculation. Photo courtesy of author.
Brown rust (caused by *Puccinia melanocephala*) and orange rust (caused by *P. kuehnii*) are currently the most damaging fungal diseases of sugarcane in Florida (Comstock et al. 2010). Application of fungicides and use of resistant cultivars are the main approaches to manage these two diseases (Raid and Comstock 2000; Rott et al. 2014). Better knowledge of rust epidemiology will help guide the timing of chemical control of sugarcane rusts, and the first objective of this work was therefore to determine disease progress in the field of both brown and orange rust of sugarcane across multiple years.

Disease severity was monitored for sugarcane brown rust and orange rust in susceptible sugarcane cultivars on a biweekly basis throughout two crop seasons: 2014-2015 and 2015-2016. Brown rust symptoms were mainly observed during mid-March to mid-July in 2014 and from mid-April to mid-August in 2015. In contrast, orange rust symptoms were observed all year round, but sporulation and percent diseased leaf area peaked during the mid-May to early August. Both rusts slowed down after August but orange rust severity increased again later during mid-October until the end of December. This late season increase of disease severity was not observed for sugarcane brown rust.

As Florida’s climate provides sufficient leaf moisture all year long, temperature was considered to be the most important factor for development of sugarcane rust (Raid and Comstock 2000; Rott et al. 2014). Therefore, in vitro germination tests were carried out to observe the effect of temperature on germination capacity of urediniospores of *P. melanocephala* and *P. kuehnii*. Urediniospores of *P. melanocephala* were able to
germinate in vitro at higher temperatures than urediniospores of *P. kuehnii*. This result was not in agreement with field data that indicated that orange rust was able to develop at higher temperatures than brown rust: Comparison of weekly average temperature curves and disease progress curves showed that 15°C to 30°C temperatures were conducive for brown rust whereas temperatures between 20°C and 32°C were conducive for orange rust. Severity of orange rust was lower than 5% of affected leaf area when temperature exceeded 33°C. Disease field ratings, climate (temperature) and spore germination data suggested that orange rust progress was strictly temperature dependent in south Florida. In contrast, progress of brown rust appeared not only to be temperature dependent but also plant age dependent: after 5-6 months of growth in plant cane, sugarcane was no longer susceptible to brown rust, as it was already previously reported in Florida (Raid and Comstock 2000). Physiological characteristics of the plant most likely contribute to resistance (or non-susceptibility) of mature sugarcane to brown rust, a phenomenon called age-related resistance that has been observed in several plants such as in wheat against *Puccinia recondite* f.sp. tritici (Pretorius et al. 1988), in tobacco against *Peronospora tabacina* (Reuveni et al. 1986), and in arabidopsis against *Pseudomonas syringae* (Kus et al 2002).

During both years of rust monitoring, disease severity was higher for orange rust than for brown rust, and for a longer period of time. Therefore, orange rust can impact sugarcane growth for a longer period of time during the crop season. These observations are in agreement with the industry’s disease control data, as more fungicide applications are performed in commercial fields to control orange rust than to control brown rust (Philippe Rott personal communication). Detailed conducive
environmental conditions for disease progress of both sugarcane rusts remain to be determined. Once available, these data will be very useful for prediction modelling and development of a disease forecasting system for improved timing of fungicide applications in Florida.

If application of fungicides is currently efficient to control sugarcane rusts, the long term objective for controlling these diseases is the cultivation of sugarcane cultivars exhibiting sustainable resistance. The second objective of this study was to develop a leaf inoculation technique and to determine the variation in pathogenicity of the brown and orange rust pathogens in Florida. Breakdown of sugarcane rust resistance in commercial cultivars can be attributed to evolution or selection of virulent strains of *P. melanocephala* and *P. kuehnii*. Therefore, knowing diversity of the populations of these two pathogens is essential for successful screening of new sugarcane clones for rust resistance. A detached leaf assay was used to determine the variation in pathogenicity within a population of the brown rust pathogen but no significant differences were observed among the three cultivars or the three rust isolates.

Occurrence of different strains or races of *P. melanocephala* has been reported in 1983 and 2005 in Florida (Dean and Purdy 1984; Shine et al. 2005). Since the sugarcane cultivars used in these former experiments are no longer grown, the brown rust isolates tested in our experiments were collected from cultivars currently exhibiting brown rust symptoms. No variation in virulence was found among the brown rust isolates collected in 2016, and only a single race/strain of the pathogen appears to be present at the sampling location (Belle Glade). Several hypotheses can be formulated
to explain this result. A single dominant brown rust resistant gene, *Bru1*, was recently found in high frequency in Florida sugarcane as well as in the parental stock at the Canal Point Cultivar Development Program (Glynn et al. 2013). The high frequency of such a single major resistance gene may result in selection pressure on the pathogen population and reduction of its variability. There have been no reports of breakdown of brown rust resistance in a commercial cultivar during the last decade (Richard Raid Personal communication). This supports the hypothesis of reduced variation in the brown rust pathogen in south Florida. However, incomplete representation of the diversity of *P. melanocephala* in our study cannot be ruled out. Further work with more isolates collected during several years from various locations in Florida, including the sugarcane germplasm collection of the Sugar Cane Field Station of USDA at Canal Point, FL, should result in a much better picture of the diversity of sugarcane brown rust in Florida.

Pathogenic diversity of the orange rust pathogen was investigated by inoculating four isolates of *P. kuehni* on four sugarcane cultivars, and using two different inoculation techniques (detached leaf inoculation and leaf whorl inoculation). Regardless of the inoculation technique, the rust isolate collected from recently susceptible sugarcane cultivar CP 89-2143 was more virulent than the other three rust isolates. This is the first formal report of occurrence of at least two different virulence groups of *P. kuehni* in Florida. These data also confirm that breakdown of orange rust resistance CP 89-2143 is associated with a new and more virulent strain of *P. kuehni*. The genetic support of the variation in pathogenicity of the orange rust pathogen is currently unknown and not investigable, mainly because of a lack of genomic data for *P.*
kuehnii. Sequencing the orange rust genome should therefore significantly help in identifying markers linked to variation in pathogenicity of *P. kuehnii*. Ultimately, such markers would be very useful to identify the different pathogenic variants of the orange rust pathogen, and to use the most virulent ones in breeding programs and screening sugarcane clones for rust resistance.

Interestingly, breakdown of resistance of sugarcane to orange rust has only been observed since 2000. The disease was present in Australia for a century before the pathogen evolved and a new race developed (Magarey et al. 2001). In Florida, a new race developed within only four years of the initial disease outbreak in the Western Hemisphere in 2007. Analyzing the reasons for this recent change in behavior of *P. kuehnii* would also contribute to the understanding of evolution of plant pathogens. The detached leaf inoculation assay successfully developed in this study will also be very helpful to investigate pathogenicity of sugarcane rust pathogens in controlled environments.
LIST OF REFERENCES


Braithwaite, K.S. 2005. Assessing the impact that pathogen variation has on the sugarcane breeding program. SRDC Final Report for Project BSS258. BSES Publ. SD05018.


BIOGRAPHICAL SKETCH

Santosh Sanjel was born in Lalitpur, Nepal, in 1987. He graduated from high school in 2006 from Caspian College, Kathmandu. He completed his undergraduate degree in agriculture (B. Sc. Ag) with plant pathology major from the Institute of Agricultural and Animal Sciences, Tribhuvan University, Nepal. In August 2014, he joined the Plant Pathology Department at the University of Florida, Gainesville as a master’s student under the guidance of Dr. Philippe C. Rott. At the Everglades Research and Education Center in Belle Glade, he conducted research on the progress of rust diseases of sugarcane, and variation in pathogen populations of the sugarcane rusts.