ETIOLOGY OF LEAF RUST CAUSING FUNGI INFECTING SELECTED BRACHIARIA (*Brachiaria mutica*; *Forssk)* GENOTYPES IN KENYA

BY

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DECLARATION

DECLARATION BY THE CANDIDATE

I, Cherunya Jeruto Angela, do hereby declare that this thesis is my original work and has not been presented for a degree in any other University.

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SC/PGB/040/14 Date

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DEDICATION

This work is dedicated to my father Michael Cherunya, my mother Jane Chepkemboi Sawe my brother Henry Kipyesang and my Sisters, Rose, Emaculate, Hellen, Lilian, and Vivian and all my nephews and nieces.

ABSTRACT

These rust diseases causing pathogens are also of importance to many crops, hence the need to clarify the species delimitations in the natural lineages infecting brachiaria grass. The classification of yellow leaf rust fungi on brachiaria grass of the family *Poaceae* has experienced a long history of controversy and uncertainty due to the reduced morphological characteristics available for taxonomy. Therefore, this research was conducted to identify rust causing pathogens in brachiaria grass in selected regions of Kenya based on pathological, morphological and molecular techniques. Identification of rust causing pathogen was determined through pathogenicity on brachiaria genotypes and further sequenced. All brachiaria genotypes MG4, Piata, Xareas and Marandu were susceptible to the rust diseases in the field and also when inoculated under controlled environment. In the field the disease incidence was 100% during 2016 and 2017. The disease severity was higher in MG4 during 2016 (7.5) and 2017 (8.5) in the disease scale of 1-9 (least severe to highly severe respectively), but least severe in Piata (2.5) in 2016 and in Marandu during 2017. MG4 proved more susceptible with a disease score of 6.5 and the least was Piata (1.7) under glass house, but under growth chamber Xareas was the most affected at severity of 6.5. On pathological characterization the isolates from MG4 were more virulent on MG4 than isolates obtained from other brachiaria genotypes followed by isolates from Marandu with the disease severity of 6.8 and 5.6. When the isolates were subjected to different conditions it was established that relative humidity of 75% and temperature of between 20^{o}C – 25^{o}C was optimal and ideal for germ tube germination. Morphologically the isolates varied from spore size of 20µm to79µm and spore shape was oval and globose but all were yellow in colour. BLAST analysis identified distinct phylogenetic lineages within the complex of brachiaria leaf rust fungi. *Uromyces japonicas* (E-value 92.44% (574/580 base pairs) was predominant at 18% of isolates followed by *Kweilingia divina* (15%) and *Puccinia graminis f. sp. tritici* (14%) *Cronartium coleosporioides* (9%), *Puccinia amari, Puccinia coronata var and Cronartium comandrae* (6%) each, *Pleurotus djamor strain, Kuehneola uredinis* and *Puccinia nakanishikii* were 3% each, as the fungal community responsible for leaf rust disease in Kenya. The yellow leaf rust fungus (*Uromyces japonicas)* grouped alone as a strongly supported clade based on the phylogenetic affinities of five type specimens and aecial host associations. All brachiaria genotypes were susceptible with varying levels of susceptibility.

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LIST OF ABBREVIATIONS

µM	Micrometer
ASALS	Arid and Semi-Arid Lands
BecA	Biosciences eastern and central Africa
BF	Bright field (BF)
Blast	Basic Local Alignment Search Tool
CIAT	International Centre for Tropical Agriculture
DIC	Differential interference contrast (DIC)
DM	Dry Matter
DNA	Deoxyribonucleic acid
ILRI	International Livestock Research Institute
ITS	Internal Transcribed Spacer
KALRO	Kenya Agricultural and Livestock Research Organization
KARI	Kenya Agricultural Research Institute
MoLD	Ministry of Livestock Development
PCR	Polymerase Chain Reaction
RH	Relative humidity
Sem	Standard error of mean
SIDA	Swedish International Development Cooperation Agency

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CHAPTER ONE

INTRODUCTION

1.1 Background

Brachiaria grass a warm-season (C4) perennial grass native to Africa. The valuations of *Brachiaria* species for pasture improvement started during the 1950s. Moreover, these were not widespread because of ample communal grazing lands; limited roles of sown pasture in the livestock production systems, livestock feed system, (Rao *et al*., 1996). Recently, the mounting demand for livestock products in Africa have renewed interest of livestock farmers, researchers, development agencies and government organizations on forages, particularly in species with good adaptability to climate change such as brachiaria grass which have improved forage availability and livestock productivity in Kenya (Mutimura and Everson, 2012*)*. These have revealed brachiaria as an ideal forage option for livestock farmers in East Africa. Despite high popularity of brachiaria the acreage in Africa is low and relies on a few varieties of brachiaria that were developed for tropical Americas and Australia (Ghimire *et al*., 2015).Some of these varieties have shown susceptibility to pests and diseases this is within a short period of introduction of brachiaria grass to its native land, (Ondabu *et al.,* 2016).

1.2 Constraints to brachiaria production

Currently over 20,000 farmers' grow brachiaria in Kenya and Rwanda (BecA Hub report 2017). However forage quality, seed yields and biomass of brachiaria are negatively affected by pest and diseases (Nzioki *et al.,* 2016).

Grass diseases may limit effective utilization of the grass species and affect herbage quality, digestibility, tillering and root growth (Djikeng *et al*., 2014). Among the

fungal diseases leaf rust disease complex have been reported to cause yield loss worldwide (Phaikaew *et al*., 1979). Management of these diseases is primarily dependent on deployment of tolerant varieties, but in many crops and pasture such as brachiaria grass, resistant varieties are not available for leaf rust which is attributed to lack of sources of resistance to the pathogens. (Baker *et al.,* 1970). It has also been reported in Australia, (Lenne, *et al.,* 1990b).This has also been reported in Brazil, (Fernandes *et al.,* 1992).Leaf Rust, caused by *Uromyces setariae-italicae* is a serious pathogen of the brachiaria grass in humid regions. It produces cream-coloured pustules containing yellow urediniospores on both leaf surfaces, resulting in leaf necrosis. The pathogen can inflict substantial foliar damage on susceptible genotypes on a wide range crops and plant species, including various tropical and subtropical crops, (Yang *et al.,* 1990).

1.3 Statement of the problem

Brachiaria originated from Africa, and it is within the same continent particularly in Kenya, that pasture supply in terms of quality and quantity is now inadequate. Against the foregoing background, efforts towards reintroducing it back 'home' are being made especially in the agro pastoral and pastoral systems in order to arrest the escalating forage crisis. Diseases are among the most important biotic constraints of brachiaria grass in Kenya. Among the diseases leaf rust associated with a number of fungal species causes great loss to the brachiaria production in quality and quantity of even up to 100%. No information is available on the causal agent of different brachiaria rust disease in Kenya, the vital information necessary to formulate effective disease management plan. Therefore this study aims to identify causal agent and confirm the association of the pathogen with disease symptoms.

1.4 Justification

Diseases of brachiaria include several fungal and viral diseases that hamper their normal development. Most of the diseases reported occur in Africa (Lenne, *et al*., 1990b), which is the centre- of diversity of brachiaria, but in the last few years, some diseases have also acquired importance such as leaf rust, leaf sport, leaf blight, ergot and physiological leaf disorder (Nzioki *el al*., 2016). Reliable methods of identification of leaf rust causing fungus using molecular technique and confirm pathogenicity to determine relationships on host plant is very useful for any meaningful studies and development of management strategies of brachiaria grass in Kenya. Therefore, this study was to confirm the pathogen associated with symptoms and identify the causal agent of brachiaria rust diseases using morphological, pathological and molecular techniques.

No information is available on the causal agent of different fungal, bacterial and viral pathogens in Kenya, the vital information necessary to formulate effective disease management plan. Therefore this study aims to identify causal agent and confirm the association of the pathogen with disease symptoms and management strategy for surveillance of endemic and re-emerging diseases through farmers' participatory breeding of brachiaria grasses.

Cultivation of improved Brachiaria grasses increase supply of quality forages enabling smallholder farmers of Kenya to increase livestock productivity and generate extra income. The proposed action fulfils the increasing demand for the climate resilient forage technologies.

Moreover, the action will strengthen interactions and collaboration within and between partner institutions and improve resources mobilization and knowledge sharing. Expected major outputs of the action includes expansion of current Brachiaria germplasm collections, identification of major pest and diseases and identification of

drought tolerant and pests and disease resistance resources, In long term these outputs contribute to enhanced livestock production and productivity, and improved food and nutrition security and improved livelihood of Kenya smallholder farmers.

1.5 Objectives

1.5.1 Broad objective

To characterize and identify leaf rust causing fungi infecting brachiaria grass genotypes in Kenya.

1.5.2 Specific objectives

i. To determine the occurrence of leaf rust disease in selected brachiaria genotypes.
ii. To determine the reaction of selected brachiaria genotypes to isolates of rust fungi
iii. To characterize morphologically and determine growth conditions of leaf rust pathogen of brachiaria grass in Kenya.
iv. To identify using molecular techniques the pathogens responsible for rust disease in brachiaria grass.

1.6 Hypotheses

i. There is no incidence of leaf rust disease in selected brachiaria cultivars in Kenya.
ii. There is no difference in the reaction of brachiaria genotypes to isolates of rust fungi.
iii. There is no difference in morphology of leaf rust pathogen of brachiaria grass in Kenya.
iv. There is only one pathogen responsible for rust disease in selected brachiaria genotypes in Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Distribution of brachiaria

Brachiaria grasses are adaptable in Kenya and have been planted in a few sections of Kenya this has help to grab shortage of pasture and fodder and is well improve livestock production and reduce shortage of fodder and pasture, (Renvoize *at al*., 1996).

2.2 Importance of brachiaria

It has high nutritious herbage produces and biomass production potential. Livestock herders and farmers practicing mixed crop livestock farming system: they need pests and disease resistance, high quality and productive forages and knowledge on improved forage production and utilization. They will benefits from improved forages and knowledge for improving their livestock production and productivity, and as a results improve their food and nutritional security and livelihood. By the proposed work target women and youths in reducing burden of collecting livestock feeds and moving with livestock in search of feeds.

This will facilitates youths to engage in education and increase women contribution in improving food and nutrition and livelihood at household levels. Livestock product industries: Among the pressing needs are both domestic and international demand for livestock milk products out striped the supply and trend of per capita consumption of livestock product increased but livestock production and productivity didn’t increase.

They will benefits from increased livestock production activities due to improved forages.

By the proposed action targeting potential source of surplus livestock for investment, particularly foreign exchange, through the export of dairy product, meat and other products, this will increase export earnings of partner countries. Livestock product consumers: due to increase per capita consumption of livestock product and population growth, consumers demand for livestock products out striped the supply. They will benefits from increased livestock numbers and their products entering the markets. Environmentalists: the proposed action contributes to sustainable method for maintaining soil fertility, quality, and water retention. They will benefits from maintenance of the environment sustainable from improved forages.

2.4 Production of brachiaria grass

Brachiaria grass have been adapted to the different climate conditions, therefore supporting a highly vibrant beef industry and dairy animals. (Rodrigues *et al*., 2014).) The development of high yielding and nutritious grasses with high biomass has been release of several genotypes from *Brachiaria* genus.

2.5 Diseases constraining of brachiaria production

Diseases and pest are major constrain to livestock herders and farmers practicing mixed crop livestock farming system: they need pests and disease resistance, high quality and productive forages and knowledge on improved forage production and utilization. They will benefits from improved forages and knowledge for improving their livestock production and productivity, and as a results improve their food and nutritional security and livelihood.

By the proposed work target women and youths in reducing burden of collecting livestock feeds and moving with livestock in search of feeds. This will facilitates youths to engage in education and increase women contribution in improving food and nutrition and livelihood at household levels.

2.6 Reproduction in rust pathogen

Reproduction is generally by means of spores that are specialized unicellular or multicellular propagative bodies. Fungus spores may be formed asexually or by the sexual process. Spores are microscopic in size, ranging from about 5 to 100 micrometers in length, depending on the species. They are dispersed by wind, splashing or flowing water, animal vectors and machinery. Although infections of plants are often initiated from germinating spores in some species infection may be initiated from mycelia from specialized vegetative resting structures such as small seed-like sclerotia (Cummins *et al,*. 1991). They produce from one to five distinct spore forms. Autoecious rusts produce their spores on a single host or on two closely related hosts and the heteroecious rusts produce spores on two unrelated hosts.

2.7 Life cycle

(Plate 1) (Agrios 2005). This can be contrasted with an **autoecious** fungus spore which can complete the life cycle on a single host species of rust.

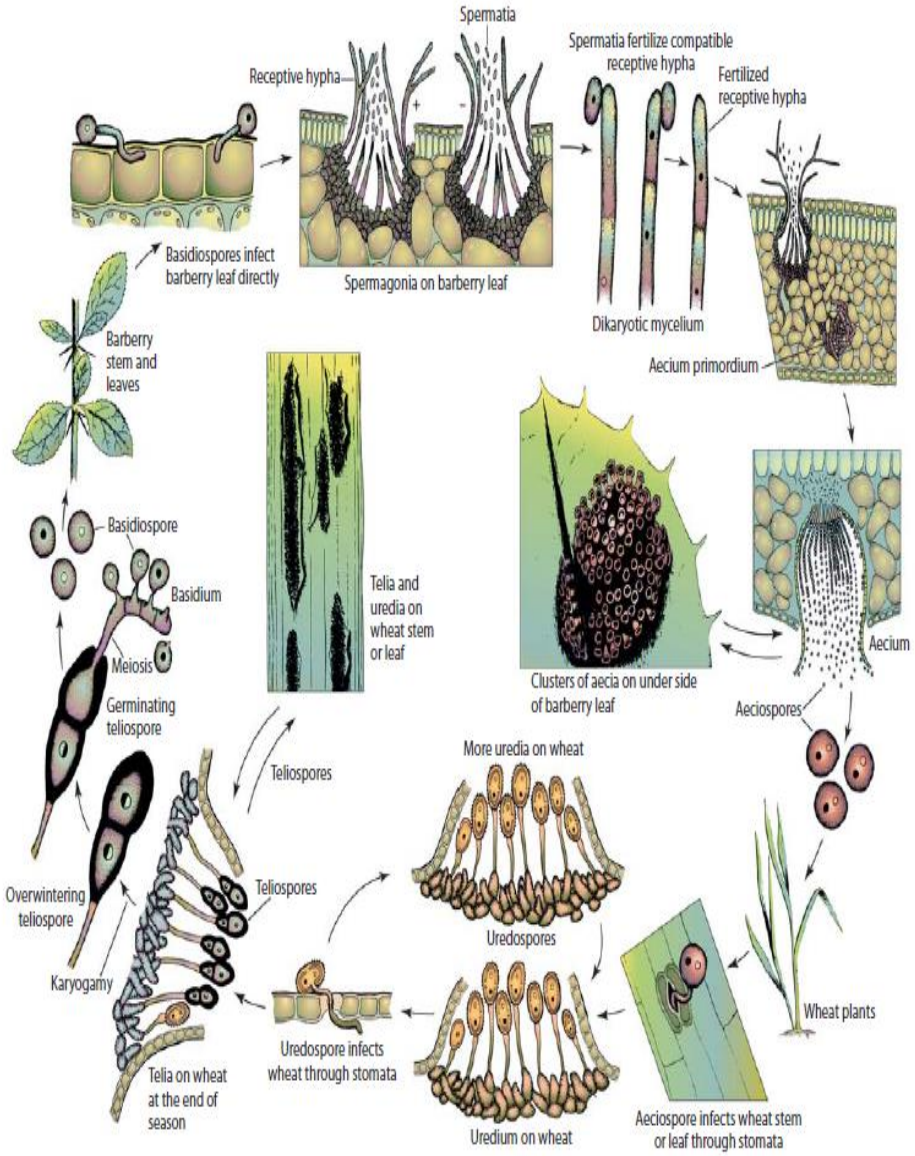

Plate 1: Life cycle of rust (Source: Agrios 2005)

2.7.1. Infection process and spread of rust fungus

The fungus can survive for a long time in the soil or in infected plant debris as sclerotia, which are first seen as white masses on infected tissues. As these slerotia mature, they become brown and loosely attached and fall to the soil, forming the primary source of inoculums for the next plant infection (Agrios 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Determination of incidence and severity of rust fungus on brachiaria grass

Field surveys were conducted on the brachiaria fields of Biosciences Eastern and Central Africa-International Livestock Research Institute hub, (BecA ILRI Hub) trials field. These BecA ILRI Hub contained brachiaria genotypes collected from selected regions of Kenya that had farmers preferred genotypes and were infected with rust under natural conditions.

In the field three quadrants (2M X 2M) were randomly drawn across the four brachiaria genotypes namely Piata, MG4, Marandu and Xereas plots. The disease incidence was recorded by counting number of plants showing rust disease signs and symptoms against the total plants in each quadrant.The disease severity for genotypes were visually rated on a scale of 1 to 9, as follows; 1 = 0% to 10%, 2= 10% to 20%, 3 = 20% to 30%, 4 = 30% to 40%, 5 = 40% to 50%, 6 = 50% to 65%, 7= 65% to 75%, 8 = 75% to 90% and 9= 90% to 100% leaf area exhibiting signs of foliar infection as described by Cobby (1995).

3.1.1 Collection of rust urediniospores

Fresh urediniospores were collected using artist air brush from brachiaria plants infected by the rust. The samples were obtained from genotypes of brachiaria namely Piata, MG4, Marandu and Xereas in 2016 and 2017 (Plate 2). The collected uredidospore were taken to the the Biosciences Eastern and Central Africa- International Livestock Research Institute hub laboratory and were maintained at -20^{0}C until analysis.

Plate 2: Brachiaria at the BECA ILRI HUB field infected with rust

(Source: Author, 2017)

3.1.2 Data analysis

Data on incidence of rust in brachiaria grass was analysed by (ANOVA) procedure using the GenStat computer Software 14th Edition, release 14.10.5943, 2013 (VSN International Ltd). Means separation was by Fishers unprotected least significant difference (LSD) at 0.05.

3.2 Preparation of rust inocula

Four brachiaria genotypes, MG4, Piata, Marandu and Xereas were selected for use in the greenhouse-based inoculation trials. Before inoculation experiments, urediniospores were removed from -20^0 C storage and were allowed to acclimate to room temperature for 30 minutes to one hour. Urediniospore germination was tested and observed using microscope to see the germ tube, Xereas isolate from the field collected in 2017 was selected and used as it showed sufficient germination.

3.2.1 Pathogenicity test of leaf rust fungus infecting brachiaria

The pathogenicity was performed under glass house and growth chamber with seedlings germinated from glass house using split cuttings of brachiaria. The brachiaria genotypes were seeded (3 seed per pot) in a 10cm diameter pots filled with sterile soil and a post germination plant was also placed in 10cm diameter pots. No fungicides or fertilizers were applied. The same isolates of rust spores collected from fields were used for artificial inoculation under growth chamber conditions and glass house for comparison (Plate 3). Each isolate was inoculated to the genotypes it was isolated from.

Plate 3: The glasshouse and growth chamber with 2-month-old inoculated brachiaria plants (Source: Author, 2017)

Plate 4: Custom –built growth chamber having brachiaria under study

(**Source: Author, 2017)**

3.2.2 Inoculation and assessment of reaction of genotype

All brachiaria genotypes were inoculated separately with one isolate that was collected from MG4, Marandu, Piata and Xereas genotype. The inoculum was of approximately 0.10 g of urediniospore. Tapping method was used by applying adhesive tape segments (2 × 5 cm) bearing leaf rust spores to the central part of the leaves. The tape segments were previously 'inoculated' in a settling tower with 6mg of a mixture (1:6) of spores and talc powder. Immediately after spore deposition, the tape segments were transported to the glasshouse/growth chamber and placed on the leaves. The thin air layer between the tape and the leaf surface was rapidly saturated with humidity, ensuring proper conditions for infection. The tapes were put into place after 17:00 hours and covered with polythene bags for 72 hours in a glass house, as show in (Plate 3) and (Plate 4). Plants previously inoculated with rust spores were kept in the metallic bench top in the greenhouse until pustules developed. All plants were watered at the base to avoid wetting the inoculated foliage. Inoculated plants were monitored until rust pustules developed.

3.3 Reaction of brachiaria genotypes to rust fungus

To test the reaction of brachaiaria grass to rust causing pathogens, all Brachiaria cultivars were inoculated separately with one isolate of each rust species. The inoculum consisted of approximately 0.10 g of urediniospores. The mineral oil suspension was sprayed onto the foliage of 5 week post-emergent plants in a fume hood using a Badger 150 airbrush paint sprayer (Franklin Park, IL) with a bottom feed head adapter. Non inoculated plants were sprayed with water. Inoculated plants were allowed to dry for 1 hour in the fume hood and then transferred to a custom-made plexi-glass misting chamber. A humidifier (Herrmidifier, Phoenix,AZ) provided the source of mist in the chamber. Inoculated plants were incubated in the misting chamber for 10 hours overnight, with misting/dry cycles alternating every 30 min.

The air temperature and relative humidity inside the misting chamber fluctuated between 18 and 20^0C and 88% and 99%, respectively. After 10 hours, the misting chamber door was opened and plants were allowed to acclimate for 1 to 2 hours before moving to the greenhouse in a custom-built glass house -framed incubation chamber cubes, measuring 10m to 10m on all sides. Three of the six sides of the incubation chambers were covered with 4 mm translucent plastic (Film-Gard, Inc.) to keep plants separated, while still allowing exposure to light. Plants previously inoculated with rust spores were kept in the wood framed bench top incubation chamber cubes in the greenhouse until pustules developed. Daytime temperatures were maintained between 19 and 21^0C beginning 2 hours after dawn and ending 1 hour before dusk.

Night temperatures were maintained between 18 and 20^0C. All the cultivars were scored for the reaction of rust, Cultivars were visually rated on a scale of 1 to 9, where 1 represented the most disease and 9 the least disease. More specifically, 1 = 90% to

100%, 2 = 75% to 90%, 3 = 65% to 75%, 4 = 50% to 65%, 5 = 40% to 50%, 6 = 30% to 40%, 7 = 20% to 30%, 8 = 10% to 20%, and 9 = 0% to 10% leaf area exhibiting signs of foliar infection.

3.4 Pathological characterization of leaf rust fungi infecting brachiaria

All the isolates collected in section 3.1 above were inoculated to the most susceptible brachiaria genotype M4G to determine their virulence. The plants were inoculated under glasshouse and growth chamber, the rust postulates were inoculated using spraying method and condition maintained as explained in section 3.3 above.

3.5 Determining the effect of temperature, relative humidity and incubation time on the germ tube germination

3.5.1 Determining effect of temperature on germ tube germination

The uredospore collected from Xareas genotype which showed high germination as tested in section 3.2 above in triplicate was inoculated into a cavity to determine optimal temperature and the the length of germ tube. The cavity slides containing spore suspension were kept at different temperatures ,After 24 hours the cavity slide was removed and observed for the development/germination of the germ tube under microscope, while noting the presence and absence of germ tube and the length.

3.5.2 Determining the effect of relative humidity (RH) on germ tube germination

The humidity for spore germination and length of germ tube was determined. Cavity slides were inoculated with rust spores collected from Xereas genotype. The cavities containing spore suspension were kept in desiccators having different relative humidity level. Four relative humidity levels i.e. 20, 50, 75 and 100 per cent at 20^{o}C were

maintained in the convenor chamber. After 24 hours the cavity slides were removed and observed for the development/germination of the germ tube under microscope.

3.5.3 Determining the effect of incubation on rust fungus

Observations were made to find out the incubation period required for maximum uredospore germination and germ tube length. Cavity slides containing spore suspension were incubated at 20^0C for 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hours. After every 2 hours the cavity slide were removed and observed for the development/germination. The final reading was read at 24 hours for the development/germination of the germ tube under microscope and noting the presence/absence and the length of the germ tube.

3.6 Morphological Characterization of rust causing fungi

Morphological characterization was done to establish the size of urediniospore, size shape, colour and the number of germ tubes using protocol of Cummins *et al.,* (1971). Uredospores collected from different genotypes were used to determine their size by measuring the length and width plus shape under compound microscope, with the help of ocular micrometer fitted with Lucida digital camera image.

Germ pores were stained using glycerine and visualized using the microscope (Optika microscope Italy model IM-3FL/3FL4). Prior to this, calibration for ocular micrometer reading with that of stage micrometer was standardized by using the following formula:

$$\text{One division of ocular micrometer } (\mu m) = \frac{\text{No of division of stage micrometer}}{\text{No of division of ocular micrometer}} \times 100$$

Size = Calibration factor x number of division in ocular micrometer

3.6.1 Data analysis

The spore was measured using the digital image and Student's t-test. Using these statistical analyses all samples were put in different groups.

3.7 Molecular identification of rust causing fungi

3.7.1 Collection of urediniospore

The urediniospore collected from the four brachiaria genotypes were used for the identification of the pathogens responsible for leaf rust.

3.7.2 Genomic DNA Extraction

Approximately 50mg of urediniospores of each isolate was taken and crashed using mortar and Pestle in liquid nitrogen and transferred to a new tube and DNA extracted using the extraction kit (ZR Plant/Seed DNA MiniPrep kit Catalog NO. D6020, Scientific industries, Genie® Inc., Bohemia, New York USA. 2014). The crashed 50mg of rust postulates sample was added to a ZR Bashing Bead™ lysis tube and vortex at maximum speed of 10,000rpm for 10 minute then 400µl of the supernatant was transferred to a Zymo-Spin™ IV.

Spin Filter in a collection tube and was centrifuged at 7,000 rpm for 1 minute, 1,200µl binding buffer was added to collection tube and then 800µl of the mixture was transferred from the Zymo-Spin™ II and centrifuged at 10,000 rpm for 1 minute. The flow was discarded and 500µl DNA wash buffer was added to the Zymo -Spin™ II column in a new collection tube and centrifuged at 10,000 x g for 1 minute. The DNA was transferred to a clean 1.5 ml micro centrifuge tube and 50µl DNA elution buffer was added directly to the column matrix which was centrifuged at 10,000 x g for 30 seconds to elute the DNA. The final product was confirmed using nano drop reading and agarose gel electrophoresis

3.7.3 DNA Amplification using Specific fungal primer.

The polymerase chain reaction amplification have been done using the rust specific fungal primer sets ITS1rust F10d (TGAACCTGCAGAAGGATCATTA) and Rust 1 (GCTTACTGCCTTCCTCAATC) (Kropp and Bruns 2007).

3.7.4 DNA sequencing of leaf rust isolates

The leaf rust sample was identified through sequence analysis of the internal transcriber spacer (ITS) region (Schoch et al., 2012). Identification was done with blastin (http://blast.ncbi.nlm.nih.gov) against all Gene bank using the complete ITS1 and ITS4 region and phylogenetic analysis was done to confirm the blast results.

3.7.5 Phylogenetic analysis

The results of all the sequences were checked using SeqMan™II 5.07 (1989-2003 DNASTAR. Inc.)

CHAPTER FOUR

RESULTS

4.1 The incidence and severity of rust on brachiaria in the field during the two seasons

Findings showed that Brachiaria genotypes MG4, Piata, Xareas and Marandu were infected by the rust diseases in the field with percent disease incidence of 100%, while disease severity ranged between 2.5 and 7.5 in a scale of 1to 9 (Table 4.1). Results further indicated that MG4 had significant highest susceptibility with severity of 7.5 and 8.5 for leaf rust during 2016 and 2017 respectively. Xareas and Marandu were moderately infected with the disease severities of 5.6; 4.6 and 4.0; 3.0 in 2016 and 2017 respectively. However, Piata recorded significantly ($p \leq 0.05$) lowest severity due to the disease (2.5 and 3.5) during both seasons.

Table 4.1: Incidence and Severity of rust on brachiaria grass in the field during 2016 and 2017

	Season One (2016)		**Season Two (2017)**	
Genotype	**Severity**	**Incidence (%)**	**Severity**	**Incidence (%)**
MG4	7.5±0.23d	100	8.5±0.19d	100
Piata	2.5±0.41a	100	3.5±0.37a	100
Xareas	5.6±0.12c	100	4.6±0.17c	100
Marandu	4.0±0.37b	100	3.0±0.21b	100
LSD	3.7		4.9	

Means followed by different letters along the column are significantly different ($P \leq 0.05$)

4.2 Severity and pathological susceptibility of brachiaria genotypes in the glasshouse

Comparison of the disease severity among genotypes under controlled environment was determined in the two seasons. Results indicated that upon inoculation of brachiaria genotypes the severity varied among the genotypes. Higher occurrence was recorded in 2016 than 2017. During the first season of the study (2016), disease severity was highest in MG4 (6.5), followed by Marandu (5.5), Xareas (4.0) and Piata (3.5) (Fig. 4.1; Plate 5). Similarly, during the second season, disease severity of 5.5 was reported in MG4, followed by Marandu (5), Xareas (3.5) with Piata recording less severity of 1.5. However, it was noted that during inoculation in 2017 the disease severity was lower for all the brachiaria genotypes as compared with severity levels of 2016.

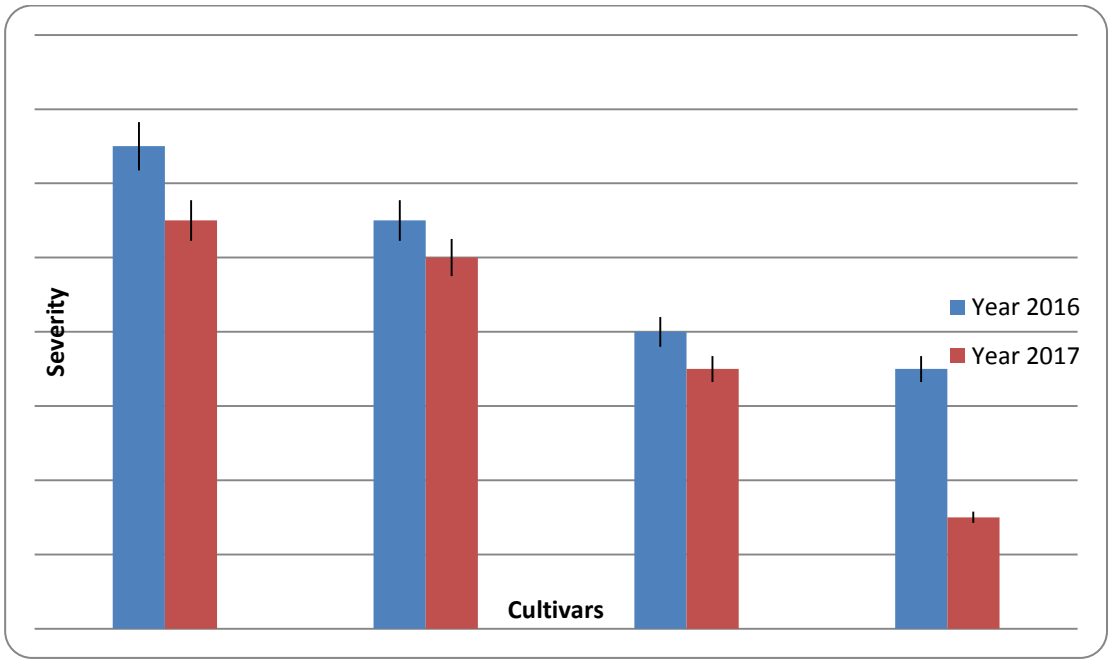

Figure 4.1: Severity of rust disease on brachiaria genotypes under glasshouse condition

MG4 **Xareas** **Marandu** **Piata**

Plate 5: Brachiaria genotypes showing infection of fugal rust pathogen

(Source: Author,2017)

4.3 Disease severity on brachiaria under growth chamber

Results in Figure 4.2 showed that genotypes recorded significant difference in disease severity during 2017 when inoculation under growth chamber condition. Significant highest severity of 6.5 was observed in MG4 genotype, followed by Xareas with 6.1 disease score. lowest severity of 2.7 was reported in Piata genotype followed by Marandu (4.2).

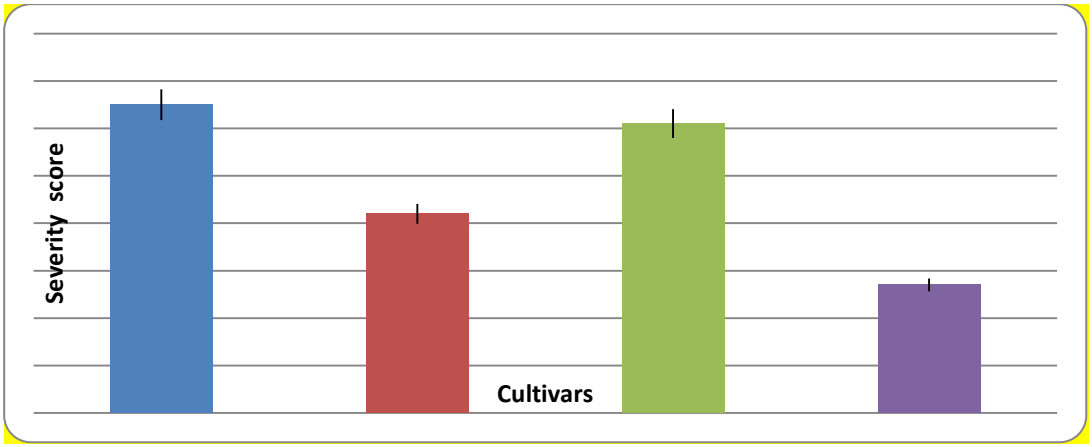

Figure 4.2 Severity of rust fungi on brachiaria genotypes under growth chamber

4.4 Comparison of severity on brachiaria genotypes under different conditions

Higher rust disease severity was recorded in growth chamber than glasshouse (Figure 4.3). However, there was no significant difference between the two conditions (glasshouse and growth chamber).

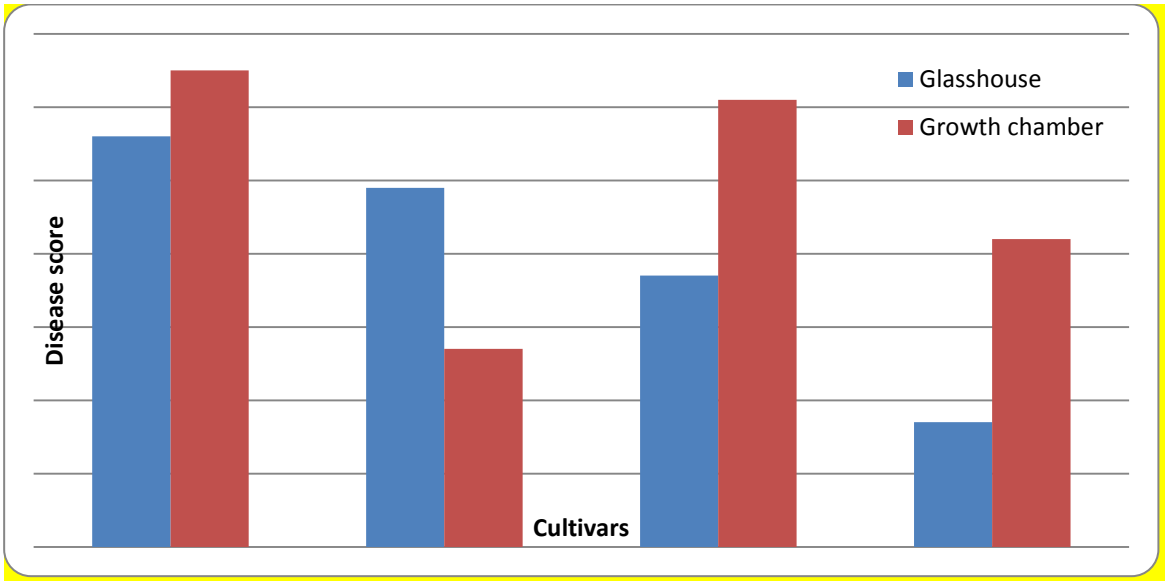

Figure 4.3 Disease score on brachiaria genotypes under different environmental conditions

4.5. Pathological characterization

The pathological characterization of rust isolates were determined by inoculation of the rust isolates collected from the different genotypes on most susceptible genotype MG4. The findings showed that isolates from MG4 were more severe on MG4, which is the original host genotype with mean severity of 6.8 followed by isolates from Marandu (5.6) but the least severe isolates were those collected from Piata which caused disease severity of (2.5) (Fig.4.4).

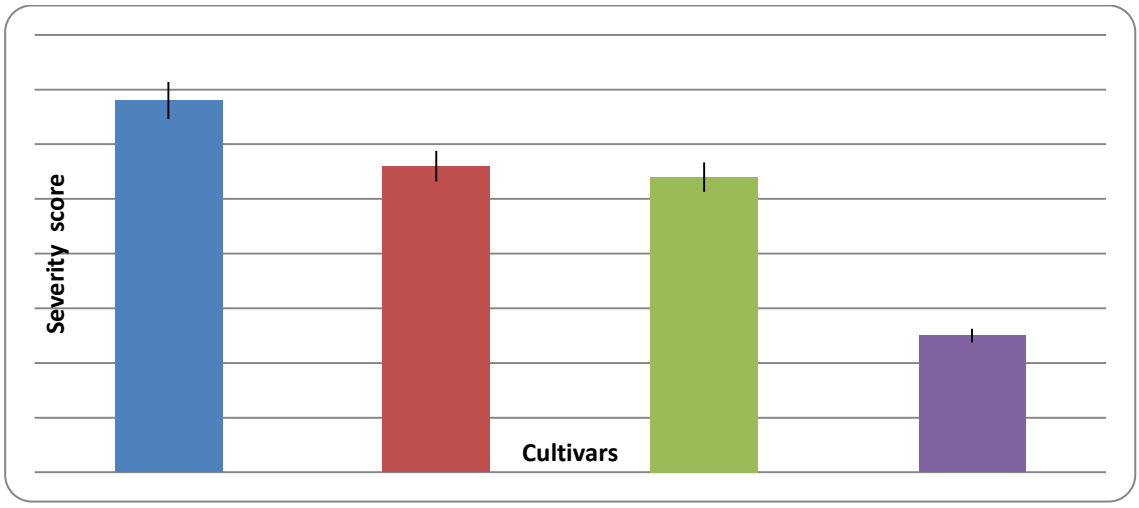

Figure 4.4. Pathological characterization of rust isolate on susceptible genotypes MG4

4.6. The influence of various factors on spore germination and germ tube length of Uredospores under growth chamber

4.6.1 The effect of temperature on the germination of germ tube of uredospore

The spore germination and germ tube length varied significantly ($P<0.05$) when spores were incubated at different temperatures from 5 to 50^0C. Significant highest uredospore germination of 90.3% was recorded at 20^0C, followed by 25^0C (13.3%).

While significant lowest uredospore germination of 6.7% was recorded at 15^0C. Similarly, maximum germ tube length of 51.6 µm was recorded at 20^0C, followed by 25^0C (31.4 µm) with significant shortest length of 28.7 µm reported at 15^0C.

Table: 4.2. Effect of temperature on spore germination and germ tube length of uredospores

Temperature (^{0}C)	Spore germination (%)	Germ tube length (µm)
5	0.00 (0.57)	0.00
10	0.00 (0.57)	0.00
15	6.7 (14.8)	28.7
20	90.3 (72.3)	51.6
25	13.3 (21.3)	31.4
30	0.00 (0.57)	0.00
35	0.00 (0.57)	0.00
40	0.00 (0.57)	0.00
45	0.00 (0.57)	0.00
50	0.00 (0.57)	0
CD (5%)	**3.5**	**0.2**

4.6.2 Effect of relative humidity on the germination and length of germ tube

The percentage of spore germination of brachiaria rust causing fungi ranged from 20.1 to 79.2%, when subjected to various levels of relative humidity (RH). Significant highest spore germination of 79.2% was recorded at 75 per cent relative humidity, followed by 50 per cent relative humidity (32%)

However, this was not significantly different ($p \leq 0.05$) from 22.5% and 20.1% at RH of 100 and 25 % respectively (Table 4.3). Similarly, significant longest germ tube length was observed at 75 per cent RH (47.9 µm), followed by 50 per cent (42.0µm) which was not significantly different from 41.0µm at 100 % RH. Significant shortest germ-tube length of 34.2 µm was observed at 25 % RH.

Table 4.3. Effect of relative humidity on uredospore germination and germ tube development

Relative Humidity (%)	Spore germination (%)	Germ tube length (µm)
25	20.1	34.2
50	32.5	42.0
75	79.2	47.9
100	22.5	41.0
CD (5%)	**8.760**	**2.40**

4.6.3 Period to spore germination and germ tube formation

The uredospore germination and growth of germ tube under 20^0C and 75% RH, started after 8 hours of incubation (17.76 µm) which gradually increased to 54.28 µm at 22 hours after which it remained constant even after 24 hours (Table 4.4). This indicated that at 20^oC, the spores of the rust fungus germinates and thereby can penetrate host cell just after 8hours and reaches its maximum length after 22 hours, which can allow it to reach the stomatal openings of the plant leaf.

Table 4.4: The duration of incubation to uredospore germination and germ tube development

Hours	Spore germination (%)	Germ tube length (µm)
2	0.00	0.00
4	0.00	0.00
6	0.00	0.00
8	18.33	17.76
10	21.66	21.88
12	36.66	24.60
14	51.66	32.80
16	90.00	43.76
18	90.00	49.96
20	90.00	54.16
22	90.00	54.28
24	90.00	54.28

4.7 Morphological characterization

4.7.1. Morphological characterization of rust causing fungi collected from the field

The uredospores collected from the four different genotypes MG4, Piata, Xareas and Marandu were compared for morphological variations. The isolates cock tail showed variation from mostly ellipsoid, yellowish to orange and scattered germ pores. In microscopic examinations, shape of uredospores was found similar in all genotypes but slight variation was noticed in size.

Out of four different genotypes, the uredospores collected from the four genotype showed mean lengths and widths which did not significantly vary i.e. MG4 (25.7µm), Piata (25.9µm) and Xareas (25.3µm), whereas (26.4µm) was measured on Marandu which had larger sized uredospores (Table 4.5).

Table 4.5 Morphological variation in rust causal agent infecting genotypes of brachiaria

Genotype	Length (µm)	Width (µm)
MG4	25.7	22.7
Piata	25.9	23.0
Xareas	26.4	22.6
Marandu	25.3	21.8
LSD	19.4	16.9

4.7.2 Morphological characterization of isolates collected after artificial incubation

Under the growth chamber the isolates were collected from each plant in the four replicates and characterized morphologically for sizes, colour, shape and the presence of germ tube. It was established that isolates from MG4 ranged in size of 34µm to 68µm and were globose to oval and yellow in colour with two isolates showing the presence of germ tube. Piata isolates ranged between 20µm to 34µm with two being oval and globose in shape, three showed the absence of germ tube. Xareas had the largest spore size ranging from 52µm to 79µm and were oval in shape and yellow in colour with two having presence of germ tube. Marandu isolates had a spore size range between 45µm to

65µm and they were all yellow in colour and germ tube was observed in two isolates as show in the Table 4.6 below.

Table 4.6: Morphological characterization of rust causing fungi in brachiaria

	Spore		**Spore**	
Isolate number	**Size**	**Spore Shape**	**Colour**	**Germ tube**
Isolate-1 Piata	23 µm	oval	Yellow	absent
Isolate-1 Piata	34 µm	oval	Yellow	absent
Isolate-1 Piata	20 µm	globose	Yellow	absent
Isolate-1 Piata	30 µm	Globose	Yellow	present
Isolate-2 MG4	34 µm	Oval	Yellow	present
Isolate-2 MG4	55 µm	Oval	Yellow	present
Isolate-2 MG4	35 µm	Globose	Yellow	absent
Isolate-2 MG4	68 µm	Globose	Yellow	absent
Isolate-3 Xareas	79 µm	Oval	Yellow	Absent
Isolate-3 Xareas	52 µm	Oval	Yellow	Present
Isolate-3 Xareas	60 µm	Oval	Yellow	Present
Isolate-3 Xareas	53 µm	Oval	Yellow	Absent
Isolate-4 Marandu	65 µm	Oval	Yellow	Absent
Isolate-4 Marandu	45 µm	Oval	Yellow	Present
Isolate-4 Marandu	60 µm	Oval	Yellow	Present
Isolate-4 Marandu	50 µm	Globose	Yellow	Absent

4.7 Molecular identification of leaf rust isolates

The BLAST analysis identified the brachiaria leaf rust causing fungi as *Uromyces japonicus* genes; TNS: F:61987 (E-value 92.44% (574/580 base pairs) sequence identity (LC203759.1) of most sample send for sanger sequence and it had the highest E- Value among the samples. Other rust fungi associated with the brachiaria leaf rust were *Kweilingia divina* E-value 92.7% (547/548 base pairs) sequence identity [EF192212.1], *Kuehneola uredinis* E-value (80.3%) (264/376 base pairs) sequence identity (MF158087.1), *Puccinia coronata* HSZ0760, E-value (79.9%) (219/542 base pairs) sequence identity [DQ355447.1] and *Puccinia graminis f. sp. tritici* E-value (80.1%) (190/257 base pairs), sequence identity. (Table, 4.7).

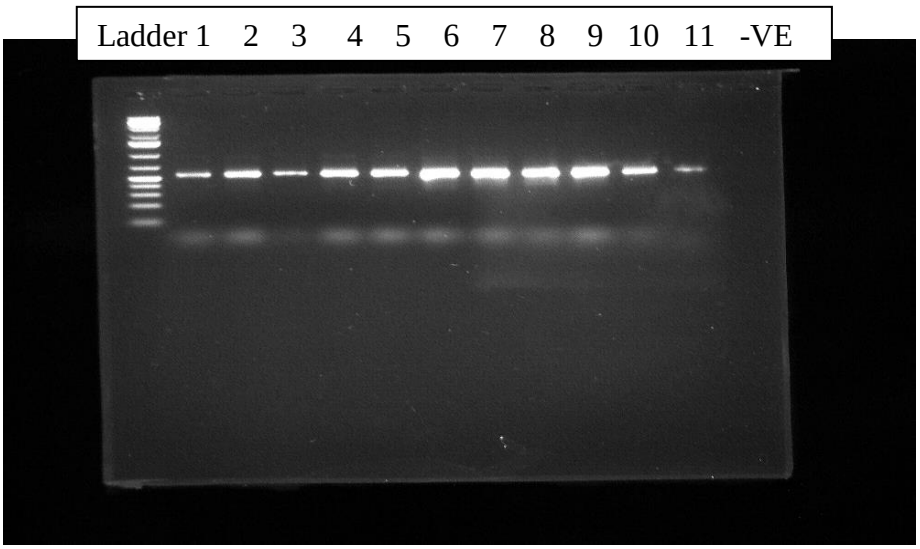

Figure 4.5 Representative gel image from rust specific primers

(Source: Author, 2017)

Table 4.7 Molecular identity of rust pathogenic fungus in brachiaria

Molecular subject sequence reference			Morphological characteristics			
No of isolate	**Sample identity**	**Subject sequence identity**	**Spore Size (µm)**	**Spore Shape**	**Spore Colour**	**Germ tube**
***Uromyces japonicus genes :*LC203759.1**	R13-RUST-1	LC203759.1	65 µm	oval	Yellow	present
***Kweilingia divina* EF192212.1**	R2-ITS-RUST-10d-F	JQ688941.1	34 µm	oval	Yellow	Present
***Puccinia amari* KX190837.1**	R3-ITS-RUST-10d-F	EU113215.1	20 µm	globose	Yellow	absent
***Puccinia graminis f. sp. tritici* JX424532.1**	R4-ITS-RUST-10d-F	JX424532.1	30 µm	globose	Yellow	Present
***Puccinia graminis strain* JQ688941.1**	R5-ITS-RUST-10d-F	KU163393.1	34 µm	oval	Yellow	absent
***Puccinia coronata var.* KT827286.1**	R6-ITS-RUST-10d-F	DQ355447.1	55 µm	oval	Yellow	Present
***Cronartium comandrae* JN943211.1**	R7-ITS-RUST-10d-F	MF158087.1	35 µm	globose	Yellow	absent
***Cronartium coleosporioides* JN943235.1**	R8-ITS-RUST-10d-F	EF192212.1	68 µm	globose	Yellow	absent
***Pleurotus djamor strain* MF574728.1**	R9-ITS-RUST-10d-F	FJ644697.1	79 µm	oval	Yellow	absent

***Kuehneola uredinis* DQ354551.1**	R10-ITS-RUST-10d-F	JX424532.1	32 µm	oval	Yellow	Present
***Puccinia nakanishikii* GU058002.1**	R11-ITS-RUST-10d-F	KU163393.1	31 µm	oval	Yellow	Present
***Phellinus igniarius strain* KC590327.1**	R12-ITS-RUST-10d-F	EU113215.1	53 µm	globose	Yellow	absent

4.8.2 The identification of brachiaria rust

Phylogenetic analysis of leaf rust pathogen was with a mean coverage of 22,027. *Uromyces japonicus* genes and *Puccinia graminis f. sp. Tritici* (Figure 4.5). The two leaf rust specimens grouped with both little bootstrap supports (< 75%) within the clade.

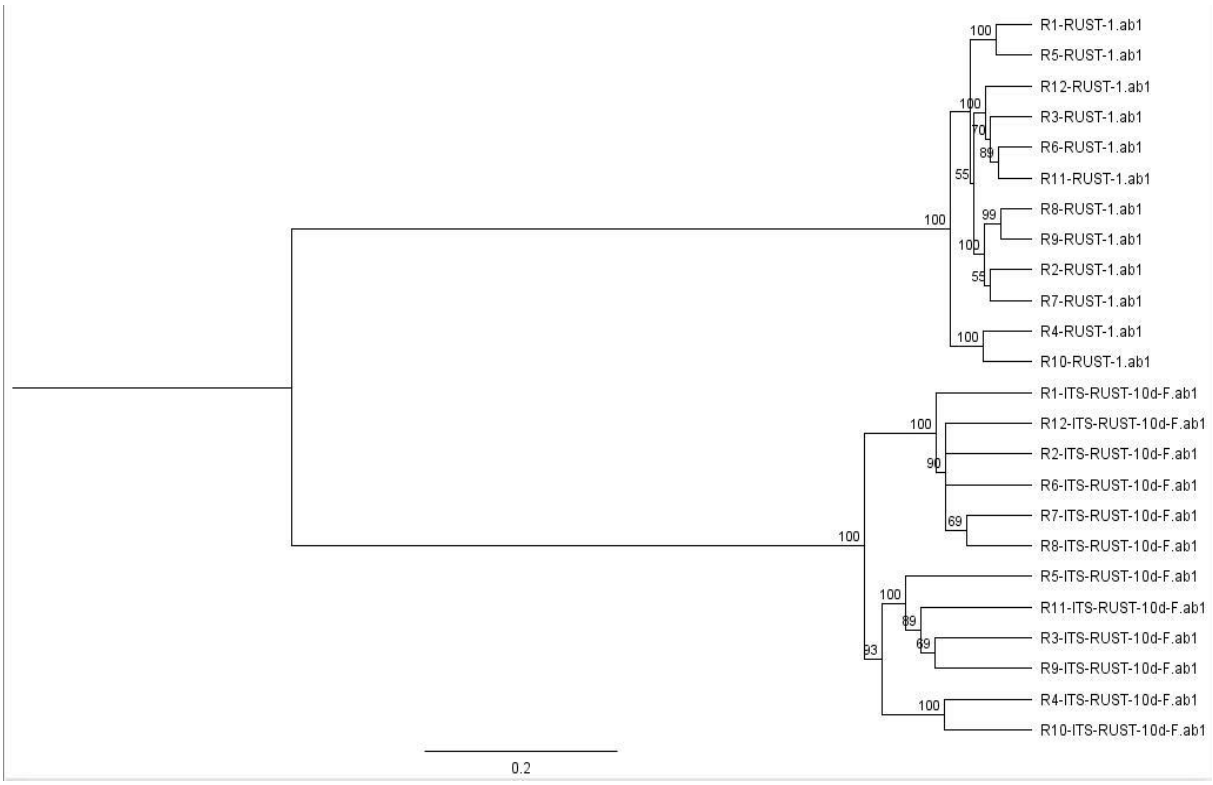

Figure 4.6 Phylogenetic tree of brachiaria leaf rust pathogens

4.8.3 Frequency of the occurrence of rust causing fungi in brachiaria grass

Isolation of fungi pathogen of brachiaria genotypes showed the association of a wide range of rust fungi was done usingi molecular markers of fungal species and same of the fungal pathogen of rust was foung as the the following rust pathogenof fungal species, *Uromyces japonicas* (18%) *Kweilingia divina (*15%) *Puccinia graminis* f. sp. *tritici (*14%), *Puccinia graminis strain* (12%), *Cronartium coleosporioides* (9%), *Puccinia amari, Puccinia coronata var and Cronartium comandrae* (6%) each, *Pleurotus djamor strain, Kuehneola uredinis* and *Puccinia nakanishikii* at (3%) each as the fungal community responsible for leaf rust disease complex infecting selected brachiaria genotypes in Kenya (Fig. 4.6).

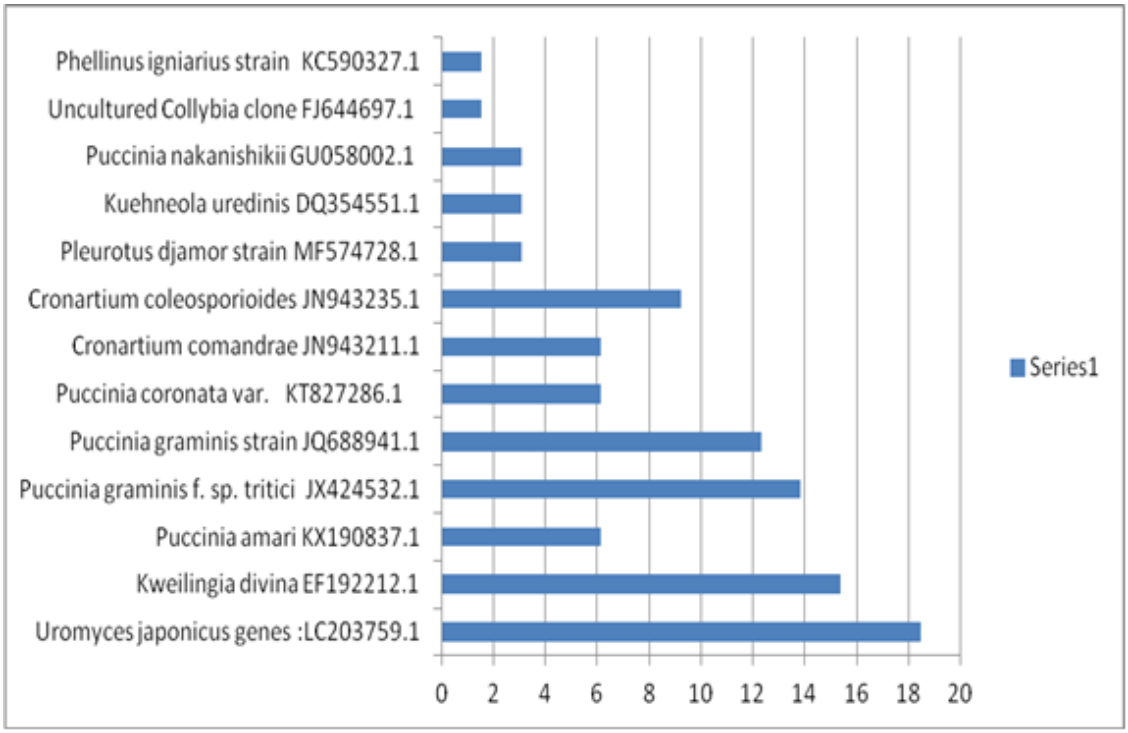

Figure 4.7 Frequency percentage of occurrence of rust causing species in brachiaria grass

CHAPTER FIVE

DISCUSSION

5.1 The occurrence of rust fungus on brachiaria grass

Four brachiaria genotypes were found susceptible to leaf rust disease in Kenya. MG4 genotype under field, glasshouse and growth chamber conditions were found to be the most susceptible genotype and Piata was found to be more tolerant when compared with the four genotypes studied. These results indicate high level of tolerance of Piata genotype to leaf rust causing fungi; therefore it can be a possible source of resistance genes. Similar results were observed by Gravert; *et al*., (2002) in their screening of genotypes which can offer potential sources of genetic exploitation for rust management. This result are also in tandem with Nzioki *et al.,* (2016), though they reported that Xareas was most infected under growth chamber but in the current study MG4 was the most severely infected followed by Xareas. Similarly, under field conditions MG4 was also severely infected indicating the high susceptibility of this genotype to rust disease this implies therefore that the genotype need not be grown under rust prone environment.

5.2 Pathological characterization of leaf rust fungi and growth condition of rust

The results showed that isolates from MG4 were more virulent on MG4 genotype than isolates obtained from other brachiaria genotypes followed by isolates from Xareas which caused high disease severity on MG4, this indicate that isolates tend to be more virulent to the plant where they were initially parasitizing.

This phenomenon could be due to the high presence of germ tube producing isolates from these brachiaria genotypes and also the high growth rate shown by these isolates. Isolates from Piata showed low germtube presence and shorter germ tube length, thus can explain the low virulence shown in the current study and was also done by Lenné and Trutmann, (1994). The growth conditions of rust pustules were determined by exposing into different regimes of relative humidity, temperature and time to germtube development were established under rust ideal environmental conditions. All brachiaria cultivars tested (MG4, Piata, Xareas and Marandu) were susceptible to rust diseases in the field as well as in controlled environment. In the field the percent disease incidence of 100% was recorded while disease severity was higher in MG4 (7.5 and 8.5) in 2016 and 2017, respectively in a disease severity scale of 0 to 9. The disease was least severe in Piata in 2016 but in 2017 Marandu was least infected. This result could be due the tolerance levels of the two brachiaria genotypes as was earlier reported by Nzioki *et al*., (2016).

5.3 Morphological Characterization of rust fungus

Pustules of leaf rust were observed on all inoculated genotypes from 10 to 28-day post inoculation. Pustules of the leaf rust fungus were bright yellow-orange colour, formed on the adaxial leaf surface, and were slightly oblong to round in shape. Uredinia were randomly distributed from mid leaf to the leaf tips of inoculated genotypes was also reported by Emeran *et al*., (2005), which is in agreement with the present findings. Uredospores of brachiaria rust required 6-8 hours for germination in the presence of water droplets as was previously observed by Patil and Thirumalachar, (1969b) and Imhoff *et al*., (1981). In the present study at 75% relative humidity and at 20^{o}C maximum

germination was observed after 8 hours, which was similar to reported by Lenné and Trutmann, (1994). On the contrary Hasan *et al.,* (2002) recorded the germination after 18-24 hrs of incubation which was similar to what was reported by Zeng *et al*., (1999). In the current study the uredospores required higher relative humidity for germination and growth of germ tube which are in agreement with findings on other rust causing fungi < 85% RH for *U. appendiculatus* and 95-100% RH for *U. fabae* as reported by Zeng *et al*., (1999).

Results on morphological characteristics (spore size, spore shape, spore colour and germ tube) of rust pathogenic fungus in brachiaria indicated that the spore size ranged between 20µm and 79µm while spore shape was categorized into two oval and globose. Spore colour was predominantly yellow. Similar pattern was recorded in relation with the results of Emeran *et al*., (2005) and Negussie *et al*., (2005).

5.4 Molecular Identification of rust isolates

This study shows that *Uromyces japonicas, Kweilingia divina, Puccinia graminis f. sp. tritici, Puccinia graminis strain, Cronartium coleosporioides, Puccinia amari, Puccinia coronata var. Cronartium comandrae,* respectively *, Pleurotus djamor strain, Kuehneola uredinis and Puccinia nakanishikii, Phellinus igniarius strain* and *Collybia clone* as some other fungal community isolated from diseased brachiaria genotypes. This result is the first to report the occurrence of a complex of fungal pathogens causing leaf rust disease in brachiaria in Kenya, therefore illustrates the importance of the diseases in Kenya. Similar pattern was recorded in relation with the results of Kago *et al*., (2016) and Nzioki *et al.,* (2016. However, the high frequency of the occurrence of *Puccinia species,*

(*Puccinia graminis* f. sp. *tritici* ,*Puccinia graminis strain, Puccinia amari* and *Puccinia coronata*) may indicate a threat to brachiaria grass and wheat when they are grown in the field as they may exchange the pathogen propagules and thereby increase the fungal inocula over time. This is the first report for identification of rust in brachiaria grass and linked the rust symptoms shown to *Uromyces japonicas* and *Puccinia species* which also agrees with reports by Gardes *et al*., (1993) and Lenne *et al*., (1990).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

There was high incidence of rust disease in all the four brachiaria genotypes collected from the different regions of Kenya and were planted at BECA- ILRI trial plots. Similarly the disease severity was also high in all the brachiaria genotypes. MG4 genotype under field, glasshouse and growth chamber conditions were found to be the most susceptible genotype, but Piata was found to be more tolerant when compared with the four genotypes studied.

On pathological characterization the isolates from MG4 were more virulent on MG4 than isolates obtained from other brachiaria genotypes followed by isolates from Xareas and Marandu but isolates from Piata were least virulent.

The relative humidity of 75% and temperature of between $20^{o}C$ – $25^{o}C$ was optimal and ideal for spore germination and germ tube development. Morphologically the isolates varied from spore size of 20µm to79µm and spore shape was oval and globose but all were yellow in colour. BLAST analysis identified two phylogenetic lineages within the complex of brachiaria leaf rust fungi, *Uromyces japonicas* was predominant followed by *Kweilingia divina* and *Puccinia graminis f. sp. Tritici, Cronartium coleosporioides, Puccinia amari, Puccinia coronata var and Cronartium comandrae, Pleurotus djamor strain, Kuehneola uredinis* and *Puccinia nakanishikii* as the fungal community responsible for brachiaria leaf rust disease in Kenya.

6.2 Recommendations

i. Future rust surveys should include all brachiaria population to show and monitor rust for disease management.

ii. The temperature of 20^0C and 75% RH is ideal for the development of rust disease, therefore whenever these conditions prevail in the field, farmers should apply control measures to reduce disease occurrence.

iii. There is need to avoid the cultivation of MG4 genotype as was found more susceptible instead farmers should adopt cultivation of Piata which proved tolerant.

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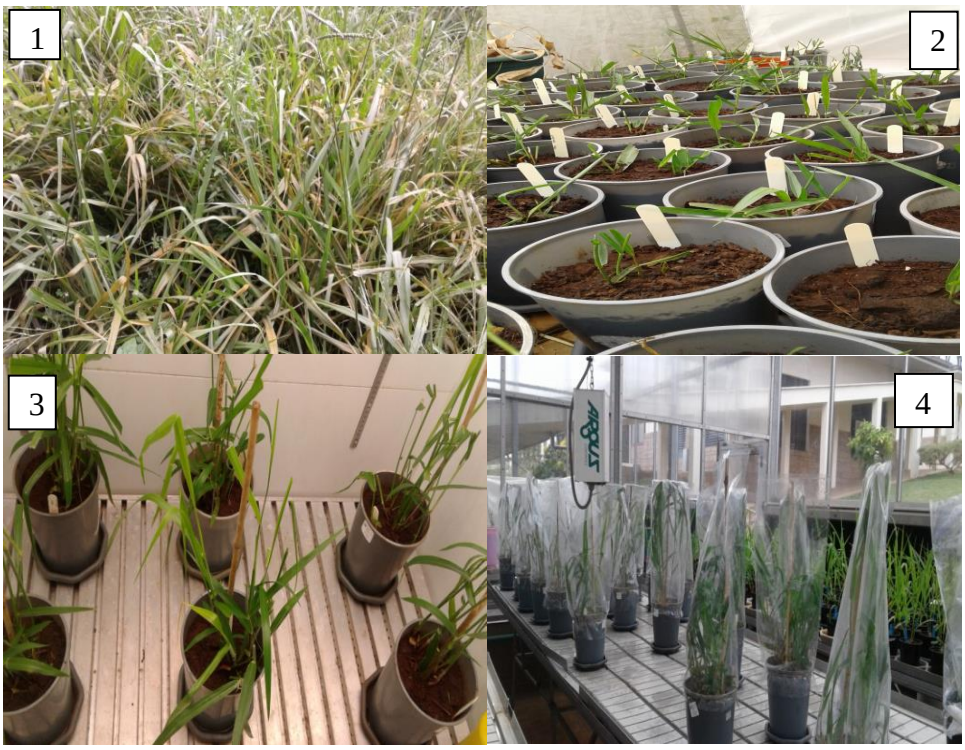
APPENDICES

Appendix I: Representative image of brachiaria plants raised in pots

Representative image for the pathological characterization of the rust pathogen the plant was raised on pots as below.

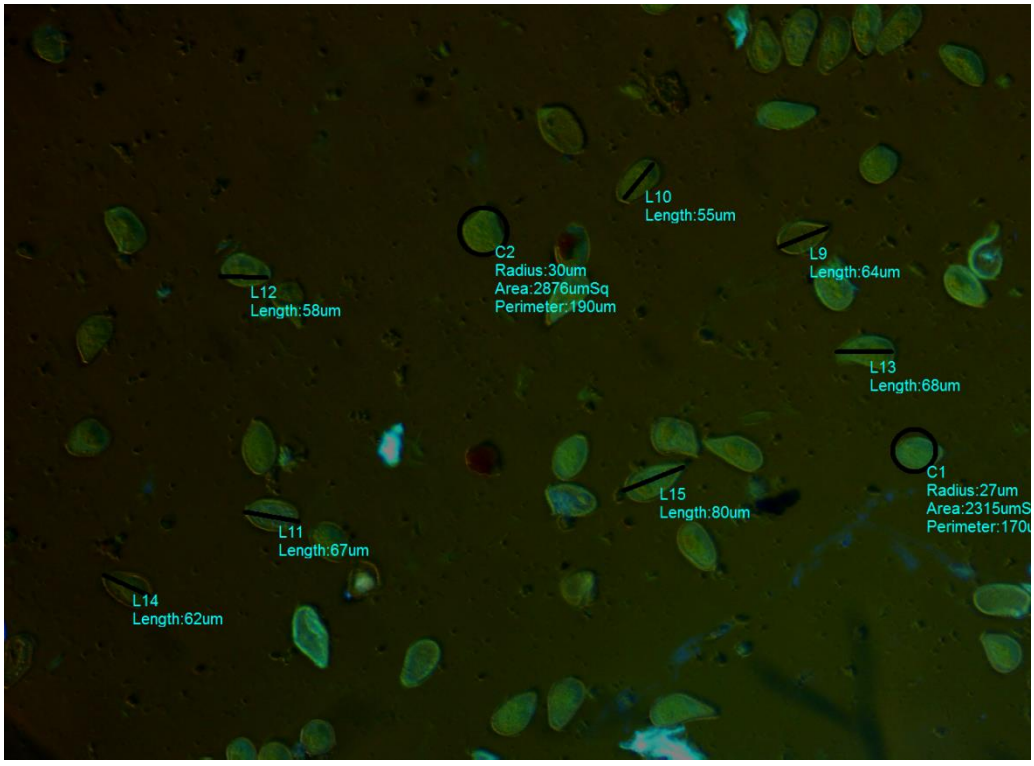


Representative Field affected by rust in the farm. 2. Brachiaria spits in pots.3 growth chamber experiments.4 Glass house experiments (Source: Author)

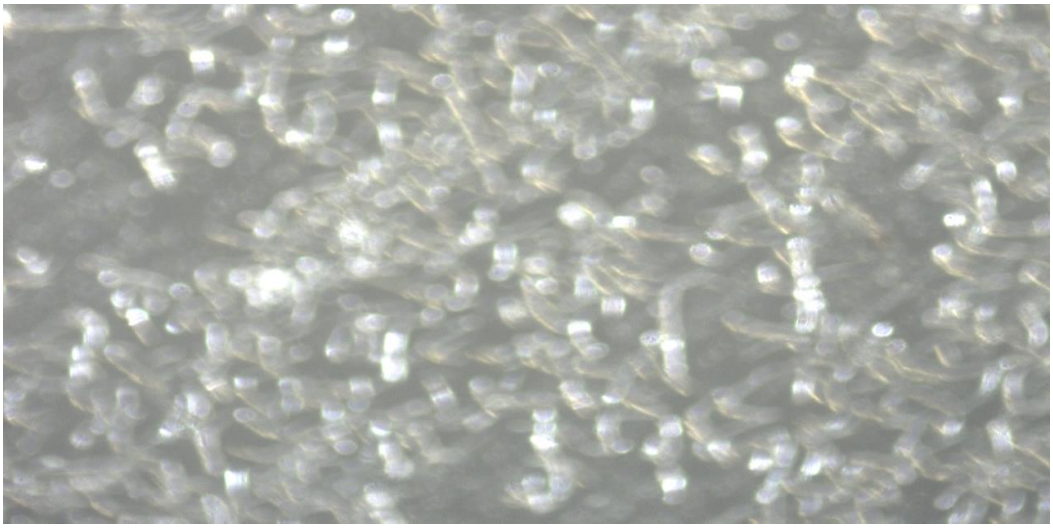
Appendix II: Above is a representative image of Rust infection

Appendix III: Uredinia of the rust fungus on inoculated brachiaria plants and urediniospores germinating urediniospore on genotypes (Source: Author, 2017)

Appendix IV: Measurement of rust spore (Source: Author, 2017)



Appendix V: Spore of rust having germ tube (Source: Author, 2017)



Appendix VI: Fungi associated with symptomatic rust fungi in brachiaria genotypes in Kenya (Source: Author, 2017)

Fungal taxa	Frequency of occurrence (%)	Number of isolate
***Uromyces japonicus genes :*LC203759.1**	18	12
***Kweilingia divina* EF192212.1**	15	10
***Puccinia amari* KX190837.1**	6	4
***Puccinia graminis f. sp. tritici* JX424532.1**	14	9
***Puccinia graminis strain* JQ688941.1**	12	8
***Puccinia coronata var.* KT827286.1**	6	4
***Cronartium comandrae* JN943211.1**	6	4
***Cronartium coleosporioides* JN943235.1**	9	6
***Pleurotus djamor strain* MF574728.1**	3	2
***Kuehneola uredinis* DQ354551.1**	3	2
***Puccinia nakanishikii* GU058002.1**	3	2
***Uncultured Collybia clone* FJ644697.1**	2	1
***Phellinus igniarius strain* KC590327.1**	2	1

Appendix VII: Purified product Nano drop reading for rust fungus (Source: Author)

	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
T45	54.4	ng/µl	1.087	0.606	1.8	1.79	DNA	50
T46	60.6	ng/µl	1.211	0.662	1.83	2.16	DNA	50
T47	64.9	ng/µl	1.298	0.725	1.79	1.13	DNA	50
T48	23.4	ng/µl	0.468	0.212	2.21	2.5	DNA	50
T49	67.6	ng/µl	1.352	0.735	1.84	2.25	DNA	50
T50	66.8	ng/µl	1.336	0.741	1.8	1.69	DNA	50
T51	49	ng/µl	0.981	0.541	1.81	1.95	DNA	50
T52	52	ng/µl	1.039	0.591	1.76	1.61	DNA	50
T53	51.3	ng/µl	1.026	0.565	1.82	2.4	DNA	50
T54	60.5	ng/µl	1.21	0.66	1.83	1.84	DNA	50
T55	159.4	ng/µl	3.188	1.711	1.86	2.3	DNA	50
T56	89.6	ng/µl	1.791	0.982	1.82	1.54	DNA	50
T57	54.8	ng/µl	1.096	0.618	1.77	1.16	DNA	50
T58	64.1	ng/µl	1.281	0.702	1.83	1.79	DNA	50
T59	57	ng/µl	1.139	0.624	1.83	2.31	DNA	50
T60	66.2	ng/µl	1.323	0.734	1.8	2.02	DNA	50
T61	26.9	ng/µl	0.538	0.296	1.82	2.47	DNA	50
T62	35.7	ng/µl	0.715	0.397	1.8	1.41	DNA	50
T63	25.7	ng/µl	0.513	0.272	1.89	2.01	DNA	50
T64	71.7	ng/µl	1.435	0.786	1.83	2.42	DNA	50