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Internal Transcribed Spacer Primers Detect Better *Ustilago kamerunensis*; A Napier Grass Head Smut Pathogen Constraining The Dairy Sector In Eastern Africa

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ABSTRACT

Two completely randomized glasshouse screening experiments in design coupled with two molecular screening assays were carried out at Kenya Agricultural Research Institute's Muguga South and Trypanosomiasis Research Centres both located at 2095m above sea level at the (1º 13' 53.0" S) and (36º 38' 1.1" E) of Kiambu County in Kenya. The aim of the studies was to determine the efficacy of four different primer pairs in the detection of *Ustilago kamerunensis* in selected asymptomatic (non-smutting) napier grass accessions' tissues at expected PCR band sizes and to investigate whether the selected accessions were completely resistant (immune) or tolerant to the pathogen. The glasshouse screening results revealed predominant differences in the smutting proportions of the accessions ranging from 0% to 90.22%. These differences were further manifested in the accession's respective neighbour joining groups where a heterogeneous pattern in response to disease challenge was observed. The molecular screening assays demonstrated that internal transcribed spacer (1 / 4) primers were better in detecting the pathogen in the asymptomatic accessions' tissues followed by β-tubulin (F1 / R2) primers at the expected band size. The molecular screening showed that ten out of eleven asymptomatic accessions and the four checks despite not smutting had the pathogen in their tissues with exception of accession 16806 which did not exhibit any in both molecular trials. The results indicated that the ten out of eleven selected asymptomatic accessions to head smut disease seemed tolerant and only 16806 seemed completely resistant (immune) to the napier head smut disease

Keywords: *Dairy Industry; Phytosanitary measures; Screening; Head smut; Primers; Africa.*

INTRODUCTION

Pennisetum purpureum Schum., (a Poaceae) popularly known as napier grass is a fodder crop of paramount importance in the tropical regions (Martha, 2004; Anitha, 2006). The forage crop is esteemed especially in Eastern and Central Africa (Valk, 1990); for its enormous biomass, ability to tolerate frequent cuttings and palatability by livestock in its leafy stage (Boonman, 1993; van de Wouw, 1999; Lowe, 2003; Nyambati, 2011). In Kenya the fodder crop is source of feed to smallholder dairy farmers where it's embraced widely through fresh harvest or as silage forms of livestock feed (Woodard, 1991; Martha, 2004; Orodho, 2006). This is clearly demonstrated by the fact that up to 40% of the smallholder dairy farmers use the grass in comparison with other sources of feed (McLeod, 2002). These smallholder dairy farmers supply 80% of the total marketed milk nationally (Omore, 1999). As a result, its cultivation has been on the rise in tandem with the dairy industry's growth (Farrell,

2002a; Orodho, 2006; ASARECA, 2010). Despite, its importance as a fodder crop in boosting of the milk industry in terms of feed provision and the emergence of other uses; example in the push and pull technology as a trap crop to control cereals' stem borers (Midega, 2008; Khan, 2010), the crop faces weighty production constraints mainly pests and diseases as the most prevalent (Farrell, 2002b; Mwendia, 2007). Currently, napier head smut, napier stunt and snow white mould are the most common diseases (Farrell, 2002b; Orodho, 2006). Among the three, napier head smut and stunt diseases cause the most yield losses in napier grass (Orodho, 2006).

Napier head smut, is caused by *Ustilago kamerunensis* (P. & H. Sydow) a hemibiotrophic pathogen (Farrell, 1998; Farrell, 2000; Orodho, 2006; NAFIS, 2012). The disease firstly manifests itself in susceptible hosts through induced premature flowering covered in a black mass of ustilospores commonly referred to as the smut as shown on figure 1. This occurs even in plants that are below 1.5metres in height which is not usually the case in health plants that usually flower at heights above 1.5 to 8 metres depending on the variety of the grass (Farrell, 1998). This visual sign is later compounded by other severe symptoms up on first harvest and regrowth, influenced largely by the levels of susceptibility of the grass type including; slow regrowth after cutting, withering and chlorosis setting in with gradual browning towards drying and death of the entire stool of the crop within the subsequent 2-3 cuttings in severe cases (ASARECA, 2010; NAFIS, 2012). Besides the above primary signs other secondary characteristics of the disease like; induced dwarfing (stems are thinner and shorter than normal less than 1.5m in height) has been observed in serious cases, characterized by short internodes with distorted leaves in shape that are reduced in number and size on stools, with an increased tillering scenario and eventually the total dry matter of the affected crop reduces massively (Farrell ., 2002b; Mwendia, 2007; NAFIS, 2012).

Figure 1. A smutted napier crop head

The disease has only been reported in African and in Eastern Africa region it's widespread in the Central region of Kenya where over 70% of the smallholder dairy farmers grow the crop (Bayer, 1990; Mwangi, 1994; Staal, 1998; Farrell, 1998; Mwendia, 2007). This region is a high potential market oriented dairy zone second to Rift-valley zone in Kenya (Owango ., 1998; Omore, 1999). Moreover, of concern is its continual spread to other parts of the country and the region like the Rift-valley and lower Eastern (Lukuyu ., 2012). Two ways have been identified through which the disease spreads to new areas; they include the production of spores from smutted heads of susceptible cultivars and the cuttings exchange and transfer between farmers to unaffected areas as seed of susceptible clones unaware the systemic intercellular pathogen is being carried along within the tissues (Mwendia ., 2007, ASARECA, 2010, NAFIS, 2012).

Therefore, to mitigate this disease's spread via the mentioned ways; non-smutting cultivars amidst the disease challenge have been developed like Kakamega 1 and 2. However, the two are over relied upon and are highly susceptible to napier stunt disease (Arocha, 2009). Moreover, these two varieties provide a narrow range of resistance genes to the head smut pathogen with an imminent threat of a likely evolving pathogen (NAFIS, 2012). Efforts have also been made so far to develop an accurate molecular based diagnostic strategy by Arocha . (2009), to determine the status of unknown clones' tissues before transfer as a phytosanitary measure to be undertaken by mandated institutions as regards their transfer within the region so as to effectively manage the disease's spread via susceptible clones cuttings. However, the molecular approach since its development it has not been tested on its efficacy at consumer level in detecting *U. kamerunensis* in plant tissues and lay a platform for quick adoption in the African setting by focusing the region to one reliable primer pair instead of all the four to cut on acquisition and optimization cost. The present study sought to establish these using four different primer pair combinations in selected asymptomatic (non -smutting) napier grass accessions' tissues at expected PCR band sizes and to investigate whether the selected accessions were completely resistant (immune) or tolerant to the pathogen. Thereby providing a rapid diagnostic strategy of napier tissues by mandated institutions before transfer to other regions and potential napier germplasm candidates for breeding programs for superior varieties resistant to head smut disease.

MATERIALS AND METHODS

The study of selecting test materials was conducted at Kenya Agricultural Research Institute-Muguga South in a continuum of two glasshouses screening experiments coupled with the molecular diagnostic assays at Trypanosomiasis Research Centre

laboratories of also Kenya Agricultural Research Institute. The initial fifty six ex-ILRI napier grass accessions screened had been molecularly characterized into clusters of molecular similarity (Neighbour joining groups) by Lowe . (2003). The accessions had been acquired from various regions of the world by ILRI germplasm bank and bulked at KARI -Muguga South for experimentation.

Experiment one: Asymptomatic accessions' identification

The selection of asymptomatic accessions was determined through screening of the napier accessions. The methodology used was as described by Farrell (1998) but as modified by Mwendia . (2006). The treatments comprised of the fifty six ex-ILRI napier accessions with; Kakamega 1 and Kakamega 2 being used as negative checks against the disease since they have been validated as resistant (Mwendia, 2007). Farmer Bana and Clone 13 varieties being used as positive checks against the disease due to their observed susceptibility (Farrell, 1998).

Experimental design and planting material preparation

Eight canes per accession were cut at three internode length and sheaths removed to expose two live buds at the nodes in all canes (Farrell, 1998). The two inoculated canes were to be planted in each end of the four pots per accession to obtain a total of eight data points per accession in a completely randomized design.

Collection of smutted heads, inoculum preparation and standardization

Napier planting materials and preparation of the innoculum was done following the procedure described by Mwendia . (2006). Inoculum was prepared using *Ustilago kamerunensis* ustilospores which were collected from affected farmers' fields in Murang'a and Kiambu districts. The spores were collected by cutting the smutted heads using a pair of scissors and putting them in collection bags which were then manually shaken to remove the spores from the inflorescence. Fifteen grams of the spores were weighed using an electronic balance and put in a plastic bucket containing 10 litres of distilled water and stirred using a glass rod until the spores were mixed with water. The standardization of the inoculum was done by pipetting 1 millilitre from the mixture, which was then placed on haemocytometer mounted on a light microscope and viewed at a lower then high magnification. The spores were counted on a 12 square grids and the mean (18.5) of each square count was obtained. This mean was used to calculate the concentration using the formula $A/4 \times 10^6$ spores per ml where; A denotes the mean indicated above from the grids (Kinyua, 2004). The concentration target was 5×10^6 spores/ml as used previously in the screening of Kakamega 1 (Farrell, 1998).

Artificial inoculation of the accessions, planting and observation

The fifty six accessions inoculation was by dipping method as described by Mwendia . (2006) and Farrell, (1998). The inoculated canes were then planted in plastic pots of between 27 cm diameter filled with potting mixture. The canes were planted at an angle with one third of the cane above the soil. The potting mixture comprised of non-sterile soil, gravel and cattle manure at a ratio of 4: 1: 0.75 respectively. The watering was once a day at 6p.m and after emergence of shoots, daily examination was conducted from the 8th week; a recommended harvesting interval for napier grass (Muyekho ., 1999). The monitoring was without cutting back of the accessions so as to avoid introducing cutting stress that may have influenced the resistance of the accessions in case it was polygenic. The number of smutted tillers and non-smutted tillers was determined for each accession to enable determination of the proportions of smutting later for each respective accession as an indicator of disease severity levels among susceptible accessions. This allowed monitoring of the disease visually by how fast the accessions succumbed to the disease by expressing the first symptom of the disease that is premature smutted inflorescence.

The appearance of smutted heads marked the elimination point of that respective accession as susceptible from the glasshouse and its smutting levels determined weekly from then by counting the number of smutted tillers and total number of tillers each week to aid in the accessions' smutting proportion determination. The elimination of the susceptible accessions to a secondary glasshouse was to avoid altering the disease intensity at the primary glasshouse and it continued until (24 week period) when the disease was expected to have developed enough pressure undisturbed, when the first harvest was conducted. Fresh sample weights and respective dry sample weights of the accessions that had not smutted were determined to aid in their percentage dry matter determination as described by AOAC (1994) using the formula:

$$Dry\ matter\ \% = \frac{Sample\ Dry\ weight}{Sample\ fresh\ weight} \times 100$$

After this harvest the remaining asymptomatic accessions continued to be monitored and eliminated accordingly until a relatively stationary period characterized by asymptomatic accessions only of more than 8 weeks from the last elimination was attained.

Experiment two: selected asymptomatic accessions' reinoculation

This experiment was conducted on the selected asymptomatic (non-smutted) accessions only from experiment one to ensure no escapes whatsoever could be mistaken as asymptomatic to the disease. The respective asymptomatic accessions' fresh canes from experiment one trials were harvested at the 36th week exactly eight weeks from the last elimination of a smutted accession. They were prepared and reinoculated with head smut pathogen spores as per experiment one in also a completely randomized design. Two reinoculated canes were planted per pot to give a total of eight data points per accession's treatments. The reinoculated accessions were watered once in the evenings at 6 p.m in a one day interval and harvested after every eight week interval to mimic the cutting stress the plants undergo on harvest by farmers at field conditions. The cutting continued for eleven ratoons (where one ratoon was equivalent to eight weeks of growth then harvested in readiness for subsequent regrowth) as the reinoculated asymptomatic accessions were monitored whether they could succumb to the disease by smutting for eighty eight weeks (eleven ratoons).

Extraction of total DNA from the asymptomatic napier grass accessions

The determination of the presence or absence of *Ustilago kamerunensis* in the selected asymptomatic accessions was done on the napier grass' accessions regrowth after the eleventh ratoon's harvest. Approximately eighty eight weeks after reinoculation of the accessions as described in experiment two. Total DNA was extracted from the napier grass accessions using a modified Bioline® Isolate II Genomic DNA extraction kit. Before the extraction lysis buffer G3, wash buffer GW2 and proteinase K were prepared as per the kit manual dictation. Then 75 mg of the asymptomatic Napier grass accessions' young leaves collected from the screening glasshouses were thoroughly ground using a different mortar and pestle for each accession in 2 ml extraction buffer due to the high fibre content in napier grass. Then the extract was resuspended in 180 µl lysis buffer GL and 25 µl proteinase K solution and vortexed vigorously. The mixture was incubated at 56°C for 1 hour 30 minutes. The samples were then lysed by vortexing briefly and added 200 µl lysis buffer G3 then vortexed vigorously and incubated at 70°C for 10 minutes. After the incubation the extracts were vortexed briefly and 200 µl of ethanol (96-100%) was added to the sample followed by a vigorous vortexing.

Then for each sample it was placed in ISOLATE II Genomic DNA spin column into a collection tube. The entire sample was added to the column and centrifuged for 1 minute at 11,000 gravity to bind the total DNA. The flow through was discarded and the each of the collection tube reused as per the kit instructions. The centrifugation was repeated at a higher gravity force for those whose samples had not completely filtered through the matrix. Then 500 µl wash buffer GW1 was added and centrifuged for 1 minute at 11,000 gravity. The flow through was discarded and the collection tube reused. This was followed by addition of 600 µl wash buffer GW2 to the column and centrifugation for 1 minute at 11,000 gravity. The flow through was discarded and collection tube reused. The resultant product was centrifuged at 11,000 gravity to remove residual ethanol and placed the ISOLATE II Genomic DNA spin column in a 1.5 ml microcentrifuge tube. Finally, the DNA was eluted by adding 30 µl of preheated elution buffer G at 70°C directly onto silica membrane and incubated at room temperature for 1 minute. The samples were then centrifuged at 11,000 gravity for 1 minute then repeated by repassing the 30 µl through the silica membrane centrifuging again before finally topping up the final volume to 60 µl to ensure the limited *Ustilago kamerunensis* DNA was not diluted extensively.

Amplification of Ustilago kamerunensis DNA using different primer combinations

The accessions' DNA samples with a positive and negative control (has no DNA sample put in this treatment but other PCR reaction reagents are involved) were then amplified using a modified methodology of Arocha . (2009). Four different pairs of primer combinations reconstituted by Bio-NEER of the ILRI –BECAHUB in Nairobi Kenya as described by Arocha . (2009) were used. First set of primers entailed those targeting the internal transcribed spacer region of the fungal species (ITS1/4) whose sequences are shown on table 1. Those primers targeting the β-tubulin gene sequence of *Ustilago kamerunensis* entailed: F1 / R2 primers, F2/R1 primers and β-tubulin Fa/ Rb primers as shown on table 1 adapted from Arocha . (2009). For the extracted total DNA 3.0 µl of the same was subjected to PCR in a total volume of 10 µl per unit reaction per treatment. The unit reaction mix per treatment consisted of 2 µl of 5× My-Taq buffer, 0.2 µl of 10 mM dNTPs, 0.5 µl of each primer, 0.2 µl of My-*Taq* DNA polymerase and 3 µl of PCR water. Reactions were run in an Eppendorf Mastercycler under the following thermal cycling conditions: ITS (1 / 4) primers; 94 ºC for 2 minutes, followed by 40 cycles of denaturation at 94 ºC for 15 seconds; annealing at 53 ºC for 30 seconds and extension at 72 ºC for 1 minute 30 seconds, followed by a final extension step at 72 ºC for 9 minutes. For the three β-tubulin primers; 94 ºC for 2 minutes, followed by 40 cycles of denaturation at 94 ºC for 15 seconds; annealing at 55 ºC for 30 seconds and extension at 72 ºC for 1 minute 30 seconds, followed also by a final extension step at 72 ºC for 9 minutes.

Table 1. The different primer combinations tested in this study to detect *Ustilago kamerunensis*

Type of primer	Primer sequence
Internal Transcribed Spacer	Forward 1; 5′-TCTGTAGGTGAACCTGCGG-3′
(ITS 1 / 4) Primers	Reverse 4; 5′-TCCTCCGCTTATTGATATGC -3′
β-tubulin (F1 / R2) primers	Forward 1(F1); 5′ -CACAACCGCCAACATGCGTGAGAT-3′
	Reverse 2 (R2); 5′ -CGTACCGCGCTCGAGATCCGACGAG-3′
β-tubulin (F2 / R1) primers	Forward 2 (F2); 5′- GCTCGAGCGCATGAACGTCTACTT-3′
	Reverse 1 (R1); 5′- GCATCTGGTCCTCGACTCCTTCAT-3′
β-tubulin (Fa / Rb) primers	Forward a; 5′- CTCGTCGATCTCGAGCGCGGTACG-3′
	Reverse b; 5′-ATGAAGGAGTCGAGGACCAGATGC-3′

Detection of Ustilago kamerunensis in selected asymptomatic napier grass accessions

The detection of the presence or absence of *Ustilago kamerunensis* in the accessions' tissues was achieved by analyzing the PCR products through electrophoresis in 2% agarose gel in 10× tris-acetate EDTA (TAE) buffer's working solution generated from 50× stock solution of TAE buffer. The method utilized the ultraviolet-induced fluorescence emitted by ethidium bromide molecules that intercalate into DNA to observe PCR product. The 50× electrophoresis TAE buffer stock solution was prepared by dissolving 242g of tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA in 1 litre of distilled water at pH 8. For the analysis of total DNA preparations from plants, standard 2% agarose gels prepared in TAE electrophoresis buffer was used. Agarose powder was added to a TAE buffer (2% w/v) and microwaved for 2 min to dissolve the powder. To the cooling solution, 1.5 µl of Ethidium bromide stock was added to the solution swirled and subsequently poured into a tray in which a comb was inserted to form sample slots. The agarose gel was allowed to solidify for approximately 30 minutes before the comb was removed and the gel immersed in the electrophoresis tank containing the TAE buffer. Then 5µl of the respective PCR product samples mixed with1 µl of 1× loading dye (bromophenol blue) were added and the total volume (6 µl) loaded into a slot in the gel. The gel was run at 120 volts and maximum current for 45 minutes before being viewed under UV light trans illuminator and photographed. The expected sizes of *Ustilago kamerunensis* DNA in the PCR product by primer combination ITS (1 / 4) was at 760 bp and that of β-tubulin primers was at 860 bp (Arocha ., 2009). The New England BioLabs® *Inc* 1 kb DNA ladder (N3232L) was used to check for the expected sizes of the PCR product. The best two primers that produced a good PCR product on the agarose gel from the first run were used again on a repeat diagnosis test using different fresh samples of the same accessions harvested from the glasshouse to validate the performance of the two primers and observed results using their total DNA extracted, amplified and electrophoresed as described.

RESULTS AND DISCUSSION

The experiment one's screening revealed the existence of predominant differences in the levels of smutting as indicated on table 2. A total of 38 accessions smutted within the first 24 weeks without cutting back with a mean smutting level of 51.61% ± 20.7%. The smutting proportions ranged from 3.13% to 90.22% for the most and least smutted accessions 14984 and 16838 of the USA 1 and hybrid neighbour joining groups respectively. However, differences were observed in another group of 18 ex-ILRI accessions on table 3 which did not smut at all (0% smutting proportion) by the end of the 24 week screening period. In addition, differences were also revealed between the smutted and non-smutted accessions in their tillering number at the 24th week's harvest point. The smutted accessions had a mean tillering number of 44 ± 20, whereas the non-smutted accessions had 18 ± 8 tillers. The results from screening experiment one, suggested that phenotypic differences in the reponses of the napier grass accessions to head smut disease challenge existed. These differences were observed when a group of accessions listed on table 3 did not smut at all, whereas those that smutted as listed on table 2 did so in continuum manner. An observation which is a characteristic of genetically controlled resistance that breaks down in such a continuous manner in a sample population (Freeman and Beattie, 2008). In addition, the observed scenario of non-smutting and continuous smutting by some of the accessions, amidst the removal of physical barriers like leaves and bud scales from all the accessions before they were inoculated and still the differences emerged, could be attributed to the varying capacities of the internal resistance mechanisms controlled by genes of the accessions in resisting the Ustilago kamerunensis establishment in vivo. These genotypic variations have been reported by Lowe . (2003) and Anitha . (2006) among the napier grass accessions. The observed increased tillering capacity among susceptible (smutting) accessions phenomenon depicts a seemingly survival strategy by the grasses where they try to compensate for the damage caused by disease on their tissues by tillering more. A similar case having been reported in sugarcane infected by smut pathogen Sporisorium scitaminae (Dalvi ., 2012).

Table 2. Smutting proportions of the various screened susceptible napier grass accessions that smutted in experiment one and were not included in the subsequent evaluation in experiment two

Accession	Neighbour Joining Group	Total Tiller Number	Number of Smutted Tillers	% Smutting	Rank
14984	USA 1	92	83	90.22%	1
16821	USA 2	55	47	85.45%	2
15743	USA 2	90	73	81.11%	3
16807	USA 2	103	83	80.58%	4
16621	Miscellaneous	51	39	76.47%	5
16798	Southern Africa	44	33	75.00%	6
16818	USA 2	44	32	72.73%	7
16810	East Africa	72	52	72.22%	8
14983	East Africa	47	33	70.21%	9
15357	USA 1	52	36	69.23%	10
18662	Unknown	27	18	66.67%	11
16834	Hybrid	43	28	65.12%	12
18438	Unknown	31	20	64.52%	13
16801	Southern Africa	58	36	62.07%	14
16804	Southern Africa	74	45	60.81%	15
16794	East Africa	40	24	60.00%	16
16840	Hybrid	28	16	57.16%	17
16813	USA 1	27	15	55.56%	18
16822	East Africa	63	33	52.38%	19
16788	East Africa	41	20	48.78%	20
16792	Southern Africa	35	17	48.57%	21
16790	USA 2	25	12	48.00%	22
16802	East Africa	29	13	44.83%	23
16814	USA 2	39	17	43.59%	24
16815	USA 1	41	17	41.46%	25
16839	USA 2	33	13	39.39%	26
16817	USA 2	28	11	39.29%	27
14982	Hybrid	34	13	38.24%	28
16812	USA 2	29	11	37.93%	29
16799	Miscellaneous	22	8	36.36%	30
16791	Southern Africa	42	14	33.33%	31
16809	East Africa	19	6	31.58%	32
16803	Southern Africa	29	9	31.03%	33
16816	USA 2	33	10	30.30%	34
1026	Unknown	54	16	29.63%	35
16795	Southern Africa	18	3	16.67%	36
16837	Miscellaneous	33	5	15.15%	37
16838	Hybrid	32	1	3.13%	38

Table 3. Selected eighteen asymptomatic (non-smutted) accessions from experiment one's screening as at the third ratoon without cutting back (24 weeks after planting). However, the seven marked by the asterisk symbol smutted on reinoculation within the first ratoon in experiment two leaving only eleven non-smutted accessions that were molecularly screened.

Napier accessions	Neighbour joining groups	Number of tillers	Dry matter%
*16797	East Africa	42	21.14%
*16808	East Africa	28	19.88%
16902	Hybrid	26	24.10%
*16836	Southern Africa	25	21.08%
*16805	USA 2	25	22.61%
*16787	Southern Africa	23	21.69%
*16785	Southern Africa	21	27.85%
16806	Southern Africa	17	20.08%
16783	Miscellaneous	15	23.83%
*18448	Unknown	15	23.33%
16782	East Africa	14	22.31%
16796	East Africa	14	21.67%
16789	Southern Africa	12	23.78%
16811	USA 1	12	24.68%
16800	Southern Africa	11	20.51%
16786	Southern Africa	10	22.76%
16793	Miscellaneous	10	20.56%
16835	Hybrid	8	22.23%

Focusing on the responses pattern after the screening of the accessions against the head smut disease challenge within each neighbour joining group, a heterogeneous pattern was observed as shown on figure 2. The responses were heterogeneous in a sense that each neighbour joining group had some of its accessions succumb to the disease and some remained asymptomatic (non-smutted). This was converse of what was expected as these groups are constituted of individual accessions that exhibit very similar molecular patterns upon multiple sequence alignment that creates the neighbour joining groups. Hence, expected to exhibit a similar response in each of the group against the disease challenge. Therefore, the converse having been observed the phenomenon could be explained by the seemingly quantitative nature of the resistance observed that is associated with polygenes. This polygenic resistance is controlled by multiple genes that are involved in various processes of growth as influenced by pathogen antagonism (Keane, 2012). Furthermore, due to the involvement of these genes in the general growth of the plant, there are unpredictable effects from the environment that influence the growth and hence the resistance leading to variations in responses despite having similar molecular characteristics (Pratt ., 2003). These polygenic resistance genes are non-Mendelian inherited converse to the major gene resistance (oligogenic resistance) that is inherited in a characteristic Mendelian fashion across the plant's progeny (Keane, 2012). Hence, explaining why the accessions in the respective neighbour joining groups expressed a heterogeneous and not the expected homogeneous response to the disease challenge.

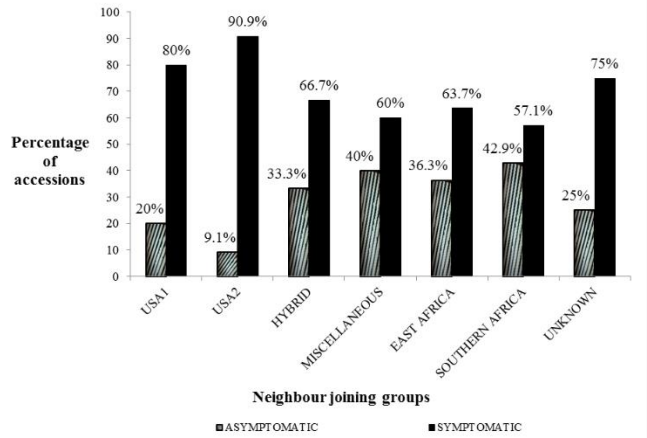

Figure 2. Percentage of napier grass accessions that smutted versus the non-smutted per neighbour joining group (dendogram of jacard's similarity estimates) after experiment one of screening without cutting back exhibiting their observed heterogeneity in symptom expression

The molecular screening conducted on the asymptomatic accessions using internal transcribed spacer primers (1 / 4) revealed the presence of the pathogen with clarity in all the asymptomatic accessions inclusive of the positive controls with exception of accession 16806 at the expected PCR band size of 760 bp for primer combination ITS (1 / 4) and 860 bp for β-tubulin primers as described by Arocha . (2009) (as shown on figures 3 and 4) with aid of the 1 kb DNA ladder. Whereas, the negative control of all the other PCR products revealed absence of an amplified band as expected. This was not the case with the other primer combinations whose products were not clear and specific in amplification as shown on the gels of figure 3 and 5. The detection of the pathogen in the asymptomatic accessions despite the accessions not smutting under the two glasshouse screening experiments upon artificial inoculation, could be due to internal resistance mechanisms that do not favour the aggressive establishment of the pathogen (U. kamerunensis) in these accessions unlike in the susceptible accessions in a classic case of polygenic (quantitative) resistance (Keane, 2012). Example of such internal mechanisms have been reported in sugarcane crop a member of grass family (poaceae) attacked by the head smut Ustilago scitaminea, where the crop produced increased levels of glycoproteins with cytoagglutination properties as a defense against the pathogen's proliferation (Blanca ., 2002; Ana-Maria ., 2005). Further, in the pearl millet (Pennisetum glaucum) a very close member to napier grass; similar biochemical defenses have been observed against downy mildew infection (Niranjan ., 2012), considering some of the accessions in this study like 16902, are hybrids of pearl millet and napier grass (Lowe ., 2003). For accession 16806 that had no pathogen detected in its tissues can be attributed to complete resistance or immunity which is the top most level of resistance that is characterized by complete absence of the pathogen and disease (Van der Plank, 1975).

This study concludes that internal transcribed spacer primers if optimized can be used to detect Ustilago kamerunensis in napier grass tissues effectively. Moreover, the possibility of unearthing more resistant and tolerant accessions to head smut disease exist and the selected accessions should be evaluated further on the various fodder quality parameters besides their tolerance to the disease and set a platform for a breeding program eventually. The β-tubulin primers amplification conditions needs to be optimized through several laboratory trials.

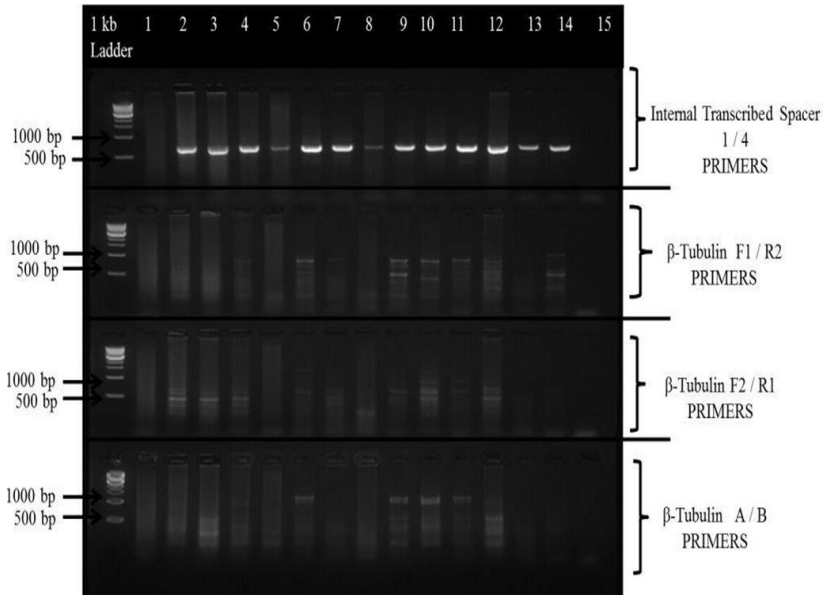

Figure 3. First trial's molecular screening showing the various respective PCR products as generated using the four different primer combinations in the initial trial that selected the best two for a repeat screening whose results shown on figure 2 and 3. 1 kb DNA ladder then Lanes 1- 15, in that order is respective total DNA PCR amplifications for accessions; 16806, 16783, 16789, 16786, Kakamega II, 16796, 16793, Kakamega I, 16902, 16800, 16811, 16805, 16782, Clone 13 (positive control) and negative control

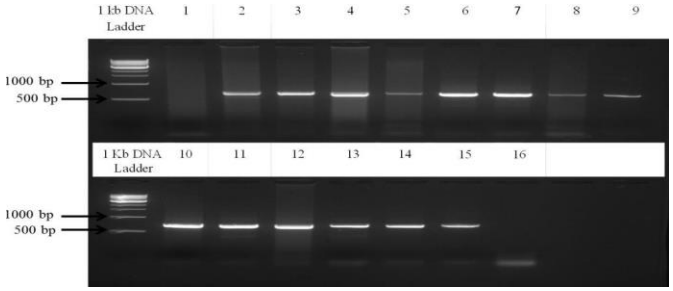

Figure 4. Second trial's PCR product of internal transcribed spacer (ITS) Primers targeting fungal species as used to diagnose Ustilago kamerunensis presence or absence in sixteen napier grass accessions in the repeated diagnosis. 1 kb DNA ladder then Lanes 1- 16, in that order is respective total DNA PCR amplifications for accessions; 16806, 16783, 16789, 16786, Kakamega II, 16796, 16793, Kakamega I, 16902, Bana, 16800, 16811, 16805, 16782, Clone 13 (positive control) and negative control

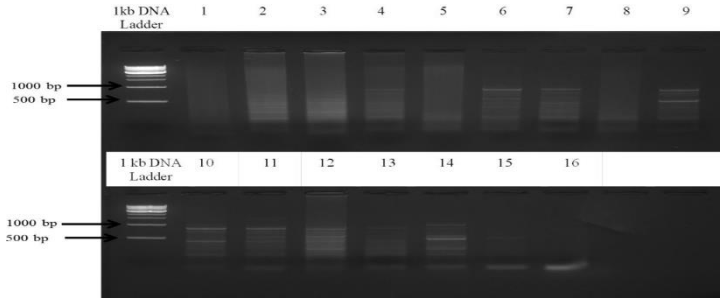

Figure 5. Second trial's PCR product of using selected β-tubulin (F1/R2) primers targeting fungal species as used to diagnose Ustilago kamerunensis presence or absence in sixteen napier grass accessions in the repeated diagnosis. 1 kb DNA ladder then Lanes 1- 16, in that order is respective total DNA PCR amplifications for accessions; 16806, 16783, 16789, 16786, Kakamega II, 16796, 16793, Kakamega I, 16902, Bana, 16800, 16811, 16805, 16782, Clone 13 (positive control) and negative control

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