



RESEARCH ARTICLE

**USING NAPIER GRASS ACCESSIONS' ORIGINS, NEIGHBOUR JOINING GROUPS AND THEIR RESPONSES TO *USTILAGO KAMERUNENSIS* TO PREDICT A PROBABLE CO-EVOLUTIONARY SCENARIO**

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ABSTRACT

Prediction of a possible co-evolutionary process is critical in a plant-pathogen interaction basing on a host plant's origin, its molecular characteristics and involved pathogen's history. Therefore, two successive screening experiments in completely randomized design were set up at Muguga-south glasshouses in Kenya. The study objective was to determine whether the resistant napier grass accessions exhibited any indications of selection preference to origin as may have been influenced by the African pathogen. Smutted tiller numbers and subsequently proportions of smutting among the accessions were determined to enable the within and across evaluation of the accessions' neighbour joining groups. The accessions exhibited observable differences with 67.9% and 32.1% smutting and not smutting respectively. Further, on across evaluation of the respective groups; the Southern Africa neighbour joining group had the most asymptomatic accessions at 35.29% whereas the USA 1 and 2 had the least at 5.88%. On within the groups' evaluation, the Southern Africa and USA 2 had the highest and least relative resistance indices of 0.2900 and -1.2606 respectively. Whereas, for the relative susceptibility indices the USA 2 had the highest and Southern Africa group the least at 0.2940 and -0.1732 respectively. The results suggest that resistance trait selection preference seems to be skewed towards accessions whose origin is from Africa especially south of Africa where the grass traces its origin at the Zambezi valley.

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INTRODUCTION

Napier grass is a fodder crop of paramount importance in Kenya, grown by over 70% of the smallholder dairy farmers (Staal *et al*, 1998; Martha *et al*, 2004; Anitha *et al*, 2006). The cultivation of the fodder crop has been on the rise in tandem with the growth of the dairy industry (Farrell *et al*, 2002; Orodho, 2006; ASARECA, 2010). Further, the crop has emerged as an important trap crop in the push-pull technology used to control maize stem borers (Midega *et al*, 2008; Khan *et al*, 2010). Despite, these uses the crop's production is constrained by a disease called napier head smut caused by *Ustilago kamerunensis* (Farrell *et al*, 2002; Orodho, 2006). The disease is an African one literally as it has not been reported elsewhere outside the continent (Farrell, 1998, ASARECA, 2010). In Kenya it is widespread in the Central region where it causes significant biomass losses of upto 46% (Farrell *et al*,

2000; Orodho, 2006). Moreover, of concern has been its continual spread to other parts of the country for instance, the Rift-valley and lower Eastern (Lukuyu *et al*, 2012).

Coupling the above spread to new areas, there has been a worrying presumption that the strain of the pathogen in Central Kenya is the most virulent in East and Central Africa basing on the high herbage yield losses witnessed in the region comparatively (Kung'u and Waller, 2001; Farrell *et al*, 2002; ASARECA, 2010). Therefore, in such a scenario where a virulent strain emerges in a region, it has been attributed to certain biotic and abiotic stresses the pathogen experiences that leads to its modification of virulence magnitude, top of the list being the intensity of resistance subjected to the pathogen by the host plant (Rauscher, 2001). This resistance can be specific or non-specific to the pathogen depending on the interaction levels of the two (Keane, 2012). A phenomenon made possible by the plants perception of the pathogen by the resistance

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gene's proteins that leads to the activation of conserved defense signaling systems either directly or indirectly through the pathogen's targeted plant proteins guarded by the resistance proteins leading to host plant resistance (Hammond *et al*, 2007; Keane, 2012). Thus, the development of this resistance in plants against a pathogen characterizes the first phase of a co-evolutionary cycle; where it's imposed by the pathogen on to the plant through natural selection, to enable the plant manage pathogen attack either completely or partially. This can occur in natural wild setting or at agricultural fields under domestication in what is considered artificial co-evolutionary cycle (Rausher, 2001).

According to Friedman and Baker (2007), intense and widened colonization of resistant host plants can force pathogens to evolve into virulent strains that end up breaking the resistance due to survival pressure in what is considered a second co-evolutionary phase. Currently, there is a heightened push to manage the napier head smut disease by host plant resistance approach due to its low cost and ease of adoption by farmers (Mwendia *et al*, 2007; ASARECA, 2010). As a result, promising napier grass clones collected from various native origins across the World are under trial without a clue whether they exhibit any selection bias to origin due to a likely first co-evolutionary cycle that may have occurred at their respective areas of origin. So that whatever is leading to emergence of virulent strain of this localized pathogen which has never been reported anywhere else in the world other than Africa, is narrowed down to the second co-evolutionary phase probably from the heightened adoption of resistance varieties by farmers. Therefore, this study sought to establish whether the proportions of selected resistant accessions to head smut disease exhibited any indications of selection bias to origin using molecularly characterized napier grass accessions from various parts of the world.

## MATERIALS AND METHODS

### Study area and sampled materials characteristics

This study was conducted at the Kenya Agricultural and Livestock Research Organization (KALRO-Muguga south) glasshouses, of Kiambu County in Kenya. The napier grass accessions used had been molecularly characterized into clusters of molecular similarity (Neighbour joining groups) by Lowe *et al* (2003) as illustrated on table 1 and the neighbour joining groups named basing on the origin of the majority of the accessions forming the respective molecular groups. Complementing the characterization the accessions had been acquired from different native regions of the World as indicated on table 1; by International Livestock Research Institute (ILRI) - Ethiopia germplasm bank and bulked at KALRO - Muguga South for experimentation.

### Asymptomatic/resistance accessions' identification through screening

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 *et al* (2006). The treatments comprised of the 56 ex-ILRI napier accessions (table 1) with 4 cultivars; 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 were used as positive checks against the disease due to their observed susceptibility (Farrell, 1998).

**Table 1** The 56 ex-ILRI accessions evaluated showing the origins and neighbour joining groups

Napier grass accession	Neighbour joining group	Source/Origin
18662	Unknown	Unknown
18438	Unknown	Unknown
14982	Hybrid	Unknown
14983	East Africa	Unknown
14984	USA 1	Unknown
15357	USA 1	Unknown
15743	USA 2	Cultivar Mott
16621	Miscellaneous	Namibia
16782	East Africa	Tanzania
16783	Miscellaneous	Tanzania
16785	Southern Africa	Tanzania
16786	Southern Africa	Swaziland
16787	Southern Africa	Swaziland
16788	East Africa	Swaziland
16789	Southern Africa	Swaziland
16790	USA 2	Swaziland
16791	Southern Africa	Swaziland
16792	Southern Africa	Mozambique
16793	Miscellaneous	Cuba
16794	East Africa	Mozambique
16795	Southern Africa	Zimbabwe
16796	East Africa	Zimbabwe
16797	East Africa	Zimbabwe
16798	Southern Africa	Zimbabwe
16799	Miscellaneous	Zimbabwe
16800	Southern Africa	Zimbabwe
16801	Southern Africa	Zimbabwe
16802	East Africa	Zimbabwe
16803	Southern Africa	Zimbabwe
16804	Southern Africa	USA
16805	USA 2	USA
16806	Southern Africa	USA
16807	USA 2	USA
16808	East Africa	USA
16809	East Africa	USA
16810	East Africa	USA
16811	USA 1	USA
16812	USA 2	USA
16813	USA 1	USA
16814	USA 2	USA
16815	USA 1	USA
16816	USA 2	USA
16817	USA 2	USA
16818	USA 2	USA
1026	Unknown	Unknown
16821	USA 2	Zimbabwe
16822	East Africa	Malawi
16834	Hybrid	Unknown
16835	Hybrid	Unknown
16836	Southern Africa	Unknown
16837	Miscellaneous	Unknown
16838	Hybrid	Unknown
16839	USA 2	Unknown
16840	Hybrid	Unknown
16902	Hybrid	Unknown
18448	Unknown	Tanzania

### Experimental 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).

Two inoculated canes were planted in each end of the four pots per accession giving a total of eight data points per accession

for measurement in a completely randomized experimental design.

#### **Inoculum preparation, standardization and inoculation**

Napier planting materials and preparation of the inoculum was done following the procedure described by Mwendia *et al.* (2006). The inoculum of *Ustilago kamerunensis* ustilospores was prepared and standardized using haemocytometric techniques as per (Kinyua, 2004). The concentration target was  $5 \times 10^6$  spores/ml as used previously in the screening of Kakamega 1 (Farrell, 1998). Accessions inoculation was by dipping method as described by Mwendia *et al.* (2006) and Farrell, (1998). The inoculated canes were then planted in plastic pots of 27 cm diameter filled with potting mixture. The canes were planted at an angle with one third of the cane above the soil (Boonman, 1993). The potting mixture comprised of non-sterile forest 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 *et al.*, 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. 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) as the reinoculated asymptomatic accessions were monitored whether they could succumb to the disease by smutting for eighty eight weeks (eleven ratoons; equivalent to eleven harvesting times of the mature grass by cutting and subsequent regrowths of the same by a consumer).

#### **Estimating neighbour joining groups performance and trend in resisting the disease**

This was to estimate the within group relative performance of the respective neighbour joining groups against the overall performance of all the neighbour joining groups cumulatively regardless of each neighbour joining group's accessions size/number becoming an intervening factor. Since, the number of accessions forming each group in the study sample was unequal. The respective proportions of the resistant and smutted (susceptible) accessions within each neighbour joining groups were evaluated against the entire resistant and susceptible pool of accessions identified from the sample studied. The obtained respective proportions were subjected to a modified relative logarithmic functions (formula 1 and 2) as explained by (Parry, 1990; Andrivon *et al.*, 2006) to obtain each neighbour joining group's relative resistance index and relative susceptibility index that estimate the magnitude of each group to resist the disease or be diseased respectively. These values complimented the assessment of the resistance selection trend.

#### **Formula 1**

$$\text{Relative Resistance Index (RRI)} = \frac{\ln(r\%) - \ln(R\%)}{t}$$

Where:  $\ln(r\%)$  is the natural logarithm of the proportion of resistant accessions within respective neighbour joining group over the total accessions evaluated belonging to that respective group. Whereas,  $\ln(D\%)$  is the natural logarithm of the proportion of all resistant accessions from all the neighbour joining groups evaluated over the total sample accessions studied. And (t) is a unit (1) value since it describes the period under which the accessions were screened. Since, it is equal across all the accessions therefore; it has no effect on the final indices magnitude.

#### **Formula 2**

$$\text{Relative Susceptibility Index (RSI)} = \frac{\ln(d\%) - \ln(D\%)}{t}$$

Where:  $\ln(d\%)$  is the natural logarithm of the proportion of smutted accessions within respective neighbour joining group over the total accessions evaluated belonging to that respective group. Whereas,  $\ln(D\%)$  is the natural logarithm of the proportion of all smutted accessions from all the neighbour joining groups evaluated over the total sample accessions studied. And (t) is a unit (1) value since it describes the period under which the accessions were screened. Since, it is equal across all the accessions therefore; it has no effect on the final indices magnitude.

The maximum possible relative resistance index (RRI) a neighbour joining group could have; is if all its accessions were all resistant ( $r\% = 100\%$ ). And the maximum relative

susceptibility index (RSI) a group could have is if all its accessions succumbed to head smut, so that (d% = 100%). Therefore, if these values are substituted to the formulas above it will derive expected maximum possible values of the same in terms of R% and D% obtained from the experiment.

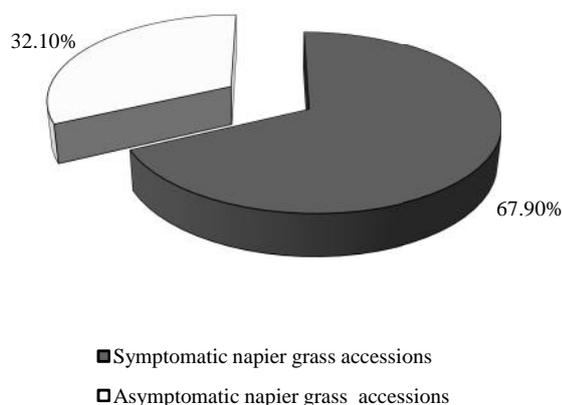
**Data analysis**

The study was largely a descriptive statistical study however; one sample tail test was used to test for presence or absence of significant differences on the accessions smutting proportions. Also, on the smutting and non-smutting proportions within the neighbour joining groups the one sample t-test was used to test for significant differences before relative resistance and susceptibility indices were generated using statistical analysis software (SAS) version 9.0.

**RESULTS**

**The response patterns of the screened napier grass accessions**

The screening experiment revealed the existence of observable differences in the levels of smutting as indicated on table 4. A total of 38 accessions smutted within the first 24 weeks without cutting back accounting for 67.9% of the total accessions sampled. On the other hand, 18 accessions did not smut accounting for the remaining 32.1% as demonstrated on figure 1 at the end of the screening trials.



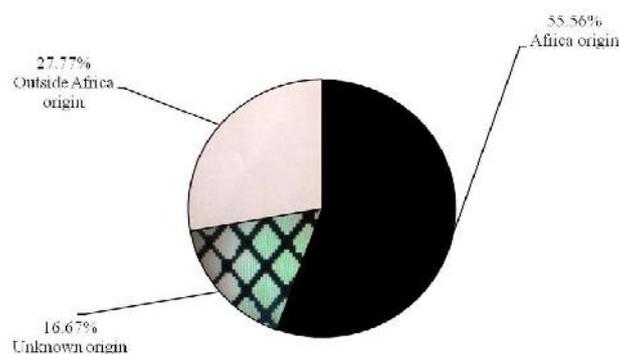
**Figure 1** Showing the respective proportions of the 56 evaluated sample accessions' response to napier head smut disease challenge with 67.90% (D %) becoming diseased (susceptible) and 32.1% (R %) not expressing the disease. The values were used in the generation of the relative resistance and susceptibility indices.

The observed response patterns of the accessions to the disease challenge across the neighbour joining groups revealed that the USA 1 and USA 2 had the most smutted accessions; which were accessions 14984 and 16821 at 90.22% and 85.45% proportions respectively as summarized on table 4. In addition, these groups had the top four most smutted accessions with a combined mean of 84.34% ± 4.49%. The most smutted ten accessions of the 56 evaluated USA 1 and 2 groups accounted for 60% of the same. Whereas, for the least smutted ten accessions of the same (table 4) USA 1 and 2 neighbour joining groups' accessions accounted for 20%. Further, significant differences (P < 0.05) were observed upon performing a one

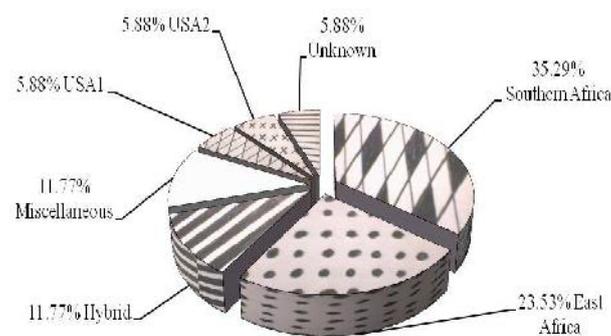
sample tail test on the within group analysis of the neighbour joining groups' proportions of smutting and non-smutting shown on table 2.

**Table 2** Showing the proportions of the selected resistant (r %) and the diseased/susceptible (d %) accessions within each neighbor joining group that were used to generate the relative resistance and susceptible indices.

Accessions' neighbour joining groups	Proportion (r %)	Proportion (d %)
Southern Africa	42.9%	57.1%
East Africa	36.3%	63.7%
Hybrid	33.3%	66.7%
USA 1	20.0%	80.0%
USA 2	9.1%	90.9%
Miscellaneous	40.0%	60.0%
Unknown	25.0%	75.0%



**Figure 2** Potential effects of co-evolutionary bias to Africa region on the selected asymptomatic (resistant) napier grass accessions to the African head smut pathogen.



**Figure 3** Proportions of asymptomatic accessions out of the total selected per neighbour joining group showing the selection orientation towards some groups in having the largest number of accessions expressing resistance against the head smut disease.

Further, on generation of relative resistance indices the southern Africa neighbour joining group had the highest index of 0.2900 followed by the miscellaneous group at 0.2200 as shown on table 3. Whereas, USA 1 and USA 2 groups had the least relative resistance indices of -0.4731 and -1.2606 respectively as demonstrated on table 3. On the other hand, the USA 2 and USA 1 exhibited the highest relative susceptibility indices of 0.2917 and 0.1640 respectively with Southern Africa and Miscellaneous groups exhibiting the least relative

susceptibility indices of -0.1732 and -0.1237 respectively. Coupling the above observations, majority of all the selected asymptomatic (non-smutted) accessions accounting for 55.56% during the screening had their origin from Africa as shown in figure 2. This was followed at a far second by those from outside the African continent that accounted for 27.77%. Moreover, a further analysis of the accessions within the neighbour joining groups, a selection bias was observed. The Southern Africa neighbour joining group had majority of its member accessions selected as asymptomatic against the disease at 35.29% of the total asymptomatic accessions selected as summarized on figure 3.

**Table 3** Relative resistance and susceptibility indices of the respective neighbour joining groups

Neighbour joining groups	Relative resistance indices	Rank	Relative susceptibility indices	Rank
Southern Africa	0.2900	1	-0.1732	7
Miscellaneous	0.2200	2	-0.1237	6
East Africa	0.1230	3	-0.0639	5
Hybrid	0.0367	4	-0.0178	4
Unknown	-0.2500	5	0.0995	3
USA 1	-0.4731	6	0.1640	2
USA 2	-1.2606	7	0.2917	1

**Table 4** Proportions of smutting of the 38 accessions that succumbed to head smut

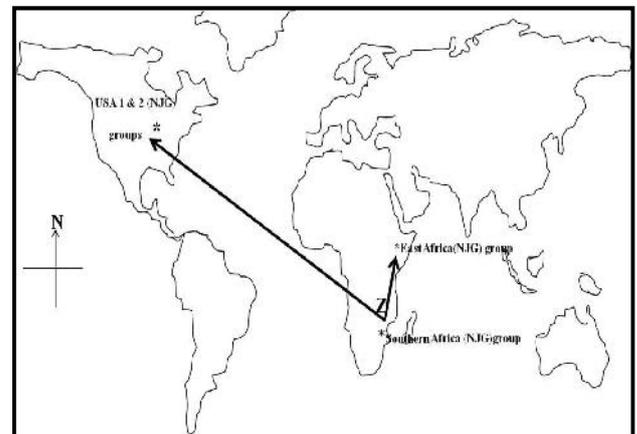
Napier grass Accession	Smutted Tillers	Total Tiller Number	Smutting Levels
14984	83	92	90.22%
16821	47	55	85.45%
15743	73	90	81.11%
16807	83	103	80.58%
16621	39	51	76.47%
16798	33	44	75.00%
16818	32	44	72.73%
16810	52	72	72.22%
14983	33	47	70.21%
15357	36	52	69.23%
18662	18	27	66.67%
16834	28	43	65.12%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

The Southern Africa group was followed by East Africa group at 23.53% and the USA 1 and 2 groups exhibited the least

asymptomatic accessions selected at 5.88% each as illustrated on figure 3. Moreover, within each neighbour joining group still the Southern Africa group exhibited the highest proportions of asymptomatic accessions at 42.9% as shown on table 2, whereas the USA 1 and 2 had the highest proportions of smutted accessions within their neighbour joining groups (table 2).

**DISCUSSION**

Heightened susceptibility of the napier grass accessions belonging to the USA 1 and USA 2 neighbour joining groups was observed, basing on the relative susceptibility indices (RSI) generated to the high smutting levels of respective individual accessions belonging to the groups. This was in contrast with those accessions that belonged to the Southern and East Africa neighbour joining groups which drew most of their origins from Africa. According to Lowe *et al* (2003), the origin of most of the accessions belonging to the USA 1 and USA 2, molecular groups is in North America. Therefore, considering that this disease (napier head smut) caused by *Ustilago kamerunensis* has not been reported anywhere else outside Africa (Farrell, 1998; ASARECA, 2010), since its first route of spread was mapped in the 1930’s from West Africa to the Eastern Africa region (Farrell *et al*, 2002). This scenario then could point to the effects of co-evolutionary modifications on the African accessions that have co-existed with the African disease in their native establishment longer than those accessions from other continents. As a result, through natural selection the accessions nucleic material has been modified to withstand the pathogen pressure that is expressed as resistance in the accessions over time in a bid to limit forced extinction in a ‘survival for the fittest scenario’ (Rauscher, 2001; Friedman and Baker, 2007).



**Figure 4** Global chart showing the Z- region (Zambezi valley) where napier grass is indigenous and the proximity of each neighbour joining group (NJG) to the region in terms of majority member accessions’ origin. The closest groups as indicated had their highest asymptomatic accessions selected: Southern Africa group leading with 35.29% followed by East Africa group with 23.53%. The furthest USA 1 and 2 groups with the least each had 5.88% proportion.

In addition, this co-evolutionary scenario is affirmed further by the position on the globe of the place where napier grass is presumed to have originated from; which is the Zambezi valley located at the South of Africa (Boonman, 1993). Majority of the accessions from the Southern Africa neighbour joining group were selected as asymptomatic (resistant) besides

exhibiting a high resistance index and low susceptibility index in a seemingly co-evolutionary modification of the accessions to withstand the napier head smut disease challenge, which might have started in their natural habitat at the Zambezi valley. Therefore, through a longer co-existence of the disease with the grass at the South of Africa and adaptation to the unpredictable environmental modifying factors of resistance existing there, most accessions whose origin is from the continent developed mechanisms to handle the disease challenge in a skewed manner from the Zambezi valley as shown on figure 4 towards other areas in a decreasing trend, like the USA that received their napier grass crop later on through germ plasm transfers (Boonman, 1993). Hence, with time the new areas have had their own significant and unpredictable environmental effects on the accessions genotypes and subsequently their resistance (Pratt *et al.*, 2003). The theory on the trend in figure 4 is further supported by the East Africa neighbour joining group coming second after the Southern Africa in majority resistance accessions contribution, whereas USA 1 and 2 which are far away from the Zambezi region had the lowest numbers of resistant accessions selected (figure 3). This scenario of a likely involvement of co-evolutionary process in the induction of resistance in the accessions is worrying as this can lead to the natural selection of the pathogen (*U. kamerunensis*) into a more virulent form in a second phase's cycle triggered by the widespread use of selected highly resistant accessions against the pathogen (Rauscher, 2001).

## CONCLUSION AND RECOMMENDATIONS

Co-evolutionary process seems to play a role in imposing the asymptomatic (resistance) trait in a skewed manner among napier grass accessions whose origin is from Africa and especially those from the South of Africa. Therefore, if this process is involved as it seems there is need for farmers to adopt a farming system strategy which emphasizes on planting varieties of mixed resistance levels in farms in an integrated pest management approach to slow the likely natural selection of *Ustilago kamerunensis* pathogen into a more virulent strain due to resistance pressure in cases where only limited and highly resistant accessions are adopted by farmers. Hence, sustain the identified resistance genes in the fodder crop to head smut disease.

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