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Verification of single nucleotide polymorphism (SNP) markers associated with maize (*Zea mays. L*) streak virus resistance in early generation maize lines

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Maize streak virus (MSV) is a devastating viral disease of maize in sub-Saharan Africa. The disease may cause up to 100% yield loss in susceptible crops. The use of molecular markers can facilitate the development of varieties resistant to the virus. The objective of this study is to assess the usefulness of Single Nucleotide Polymorphism (SNP) markers linked to MSV resistance in selecting for resistance at early generations of inbred line development in maize. A total of 160 maize lines were genotyped with three SNP markers that are linked to MSV resistance. These lines were tested for their reaction to MSV through artificial inoculation using viruliferous *Cicadulina triangular* at the three leaf stage; maize streak virus symptom was scored from 7 days after inoculation for six weeks at weekly interval on a scale of 1 to 5. MSV titer on the upper and lower leaves was determined using Direct Antigen Coating Enzyme linked Immunosorbent Assay (DAC-ELISA). One hundred and forty-two (142) of the 160 maize lines had the favourable marker allele for MSV resistance while 18 maize lines did not have the allele. Differences among the 160 maize lines for MSV symptom on upper leaves at six week after inoculation were significant *(P< 0.01)*. Favourable allele of the SNP markers was significantly associated with MSV symptom score at 6 week after inoculation and MSV titre status. The percentage of maize lines with desirable marker allele with resistance based on symptoms score and ELISA were 97.9 and 93%, respectively. The three SNP markers showed high efficiency in the identification of MSV resistant maize lines and therefore have potential for use in marker-assisted selection. The SNP markers were not effective in detecting MSV resistance in few genotypes, indicating a need to develop other markers for resistance.

Key words: ELISA, genotyping, inoculation, maize streak virus, SNPs.

INTRODUCTION

Maize (*Zea mays*. L) is among the most important cereals globally, along with wheat and rice, providing basic diet to millions of people in sub-Saharan Africa (SSA) (Gebrekidan et al., 1992). Despite its importance, the

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production and productivity are hampered by many biotic and abiotic stresses. Abiotic constraints include drought, low soil fertility, soil acidity, and expensive farm inputs, among others. The major biotic stresses include weeds such as striga, pests such as stem borers and weevils, and diseases caused by fungi and viruses, all of which contribute to substantial yield losses (Cairns et al*.,* 2012). Viral diseases have been a major threat among which "streak disease*"* or maize streak virus disease (MSVD) is by far the most important; a widespread virus responsible for high grain yield loss in SSA (Guthrie, 1978). Maize Streak Virus Disease (MSVD) is considered to be the third most important disease of maize in the world, after gray leaf spot and northern corn leaf blight (Pratt and Gordon, 2006).

MSVD, caused by the maize streak virus (MSV, genus: *Mastrevirus*, family: *Geminiviridae*) is transmitted by leafhoppers of the genus *Cicadulina.* The disease is characterized by yellow streaks which run parallel to the leaf veins. In susceptible maize lines severe infection may result in stunting, inter-veinal necrosis, chlorosis, and death of affected individuals. Streaks develop along the veins on most of the leaf lamina and, as the virus is systemic, symptoms appear only on the infected and subsequent leaves (Thottappilly et al*.,* 1993).

Similar to many insect-transmitted virus diseases, the annual incidence of MSVD in farmers' fields varies from insignificant in some years to epidemic proportions in others (Efron et al*.,* 1989). Outbreaks of the disease are often associated with drought conditions or irregular rains such as those that occurred in West Africa in 1983 and 1984 (Rossel and Thottappilly, 1985). MSVD can result in 100% yield loss in susceptible maize lines (Magenya et al*.,* 2008). Yield losses vary with the time of infection and varietal resistance (Guthrie, 1978). Field trials relying on natural infection in East Africa detected yield losses between 33 and 56% (Guthrie, 1978), whereas losses of 100% were reported in many countries in West Africa (Fajemisin et al*.,* 1986). Trials conducted between 1983 and 1985, presented by Fajemisin et al. (1986), reported a yield reduction of 71 to 93% in maize due to MSV.

It is difficult to manage MSVD due to its variability and unpredictable vector migratory patterns (Vivek et al*.,* 2010). Furthermore, there exist grasses which are host reservoirs for both the insect vector and the virus (Vivek et al*.,* 2010). Traditionally, the disease can be controlled through cultural, chemical and physical measures (Wambugu and Wafula, 2000). However, chemical sprays can only kill the insect vector found within a maize field at a given time, but since the leafhoppers are migratory insects and can travel several miles, the use of chemicals can be very ineffective especially because not all fields are sprayed at the same time. In addition, use of chemicals has been regarded as environmentally unsafe and requires continues monitoring as more insects migrate back after the chemical loses potency (Njuguna et al*.,* 1990). On the other hand, use of cultural measures such as crop rotation, early planting, intercropping is not efficient enough to control this insect transmitted virus.

The development and deployment of resistant varieties is a more appropriate and cost- effective approach to controlling MSVD. Significant progress has been made in breeding maize for resistance to MSVD through conventional methods. These methods involve crossing of the best plants possessing the most desirable traits such as high yield, disease resistance, or any other character that is preferred by farmers. With this method, it may take over eight years to produce a variety that has acceptable levels of resistance, and there is usually no guarantee that the resistance will hold for different virus strains. In most cases, resistance has been reported to break down in different environments, partly due to difference in the strains of the pathogen and mode of inheritance of the resistance, and partly due to maize line-by environment interactions (Njuguna et al*.,* 1990).

Development and application of marker assisted selection (MAS) in crop improvement has become a useful technique for breeders. Since MSVD resistance trait has a high heritability and is controlled by a few genes (Welz et al*.,* 1998), the application of markers in transferring gene for resistance is quite possible and quicker to assay than in conventional breeding. Molecular markers can help to select individuals carrying target genes in a segregating population based on patterns of tightly linked markers rather than on their phenotypes.

The Quantitative Trait Loci (QTLs) for resistance to MSVD have been identified and mapped in maize. Welz et al*.* (1998) detected a major QTL on chromosome 1 and minor QTL on chromosome 2, 3 and 4. The presence of a major QTL for resistance to MSVD on the short arm of chromosome 1 has been replicated by other authors (Lagat et al*.,* 2008); this QTL explains 50 to 60% of phenotypic variation, although other minor QTLs have also been implicated. Alleles at this locus were additive or partially dominant depending on the resistance source (Redinbaugh et al*.,* 2004). Due to the availability of high-throughput single nucleotide polymorphism (SNP) detection and validation technologies, the development of SNP markers is becoming a routine process, especially in crops with reference genome.

Identification of candidate genes linked markers can be used for forward breeding for MSV resistance in maize. Since the germplasms developed at IITA (International Institute of Tropical Agriculture) have recovery type of resistance, that is, plants which upon virus infection develop severity symptoms but the symptom severity is reduced in the leaves that develop subsequent to virus infection, thereby allowing plant to recover from infection. However, other QTLs may confer resistance. Consequently, there is a need to investigate the effectiveness of the known SNP markers in maize germplasm having recovery type of resistance for MSVD resistance. The

objective of this study was to assess the usefulness of SNP markers in selecting MSV resistance in early generation maize line development.

MATERIALS AND METHODS

Maize germplasm and insect vector sources

The experiment was carried out in the laboratories and screen house at the International Institute of Tropical Agriculture (IITA), Ibadan (190 m; Latitude 7° 29'11.99"N, longitude 3° 54'2.88"E), Nigeria. The location has mean and minimum temperatures of 26.5 and 21.4°C, respectively, a mean relative humidity of 74.6%. The maize lines used for the experiment were obtained from IITA Maize Breeding Unit. A total of 160 maize lines with known pedigree were used in the experiment. Of the 160, 151 lines were selected from 3003 S_1 lines developed from nine different bi-parental crosses; 9 inbred lines which were resistant to MSV were included in the experiment. A highly MSV-sensitive maize Line (Pool 16) was used as MSV susceptible check. All the maize lines were genotyped using three SNP markers (snpZM0020-PZE101093951, snpZM0021-PZE0186065237 and snpZM0022-PZE0186365075) linked to the gene for MSV resistance. The markers which were developed at CIMMYT (International Maize and Wheat Improvement Center, 1985) detect the presence or absence of favorable allele for MSV resistance gene on Chromosome 1 (Nair et al., 2015). Leafhopper cultures and MSV isolates used in this study were obtained from the IITA Virology and Molecular Diagnostic Unit at Ibadan. The insects were bred artificially in cages on pearl millet *(Pennisetum typhoides)* seedlings, and all the young adults used were able to transmit MSV following their feeding on infected host plants.

Experimental design

The 160 maize lines were arranged in a completely randomized design (CRD) with two replications. Each maize line was considered a treatment. Eight seeds of each maize line and four seeds of the susceptible maize line (control) were sown per pot on March, 2018. After germination the control line was thinned to one, each pot had 1 plant of pool 16 as a susceptible check.

MSV inoculation and disease assessment

Plants of the test entries and susceptible controls were inoculated under screen house condition using viruliferous leafhoppers. Leafhopper colonies reared on pear millet seedlings were transferred onto MSV-infected maize maintained in cages for a virus acquisition access period (AAP) of 48 h. Viruliferous leafhoppers from maize were subsequently transferred onto one week old seedlings of test lines maintained in cages and allowed 48 h inoculation access period (IAP) to facilitate virus inoculation of test plants. This procedure allowed uniform inoculation of all the maize lines. The inoculated plants were removed from the cage followed by insecticide spray to kill insect vectors. The inoculated plants were transferred to an insect-proof screen house for observations. MSV infection and symptom severity score was assessed based on visual observation of inoculated plants for six weeks at weekly intervals, beginning from seventh day after inoculation. A 1 to 5 rating scale was used as described by Beyene et al. (2012) where 1 = < 10% of the leaf area covered with streaks, 2 = 11-25% of the leaf area covered with streaks, 3 = 26-50% of the leaf area covered with streaks, 4 = 51-75% of the leaf area covered with streaks and 5 = >75% of the leaf area covered with streaks. The scores were used to define resistance categories as follows: 0=Immune, 1.0-2.0= resistant, 2.1-3.0 = moderately resistant, 3.1-4.0 = susceptible and 4.1-5.0 =highly susceptible.

Sample collection and MSV detection by DAC-ELISA

Leaf samples were collected 42-days after inoculation from the oldest leaves available at the lower portion of the plants for leaf symptoms and virus titer in early formed leaves; and the youngest leaves at the upper portion of the plants were sampled for symptoms and virus titer in newly formed leaf, which were used for virus detection using enzyme-linked immunosorbent assay (ELISA). The samples collected were immediately wrapped in aluminum foils to prevent dehydration and placed in labelled transparent polythene zip lock bags. Thereafter, the samples were transported to laboratory on ice. Samples were tested by Direct Antigen Coating (DAC)-ELISA as described by Peterschmitt et al. (1992).

The DAC-ELISA was the serological virus detection method used to determine the relative titer of MSV in each tested maize lines. DAC-ELISA, also referred as Antigen Coated Plate (ACP)-ELISA involved coating of viral antigen to the ELISA plate surface and the antigen was used for virus detection with primary antibody (anti-MSV polyclonal antibody, sourced from IITA); the antigen-antibody complex was detected by the enzyme-labeled secondary antibody (anti-rabbit antibody) tagged with an enzyme (alkaline phosphate). The reaction of antigen and antibody was detected using chromogenic substrate p-nitrophenoyl phosphate and the color intensity was measured at an absorbance of 405 nm using a 96-well spectrophotometer. About 100 mg of maize leaf sample from the lower and upper leaves obtained from the maize lines were ground with 0.5ml of coating buffer (1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, 1 L of distilled water pH 9.6). 100 µl of the ground sample was dispensed into each well of a new ELISA plate and kept in a refrigerator for overnight incubation at 4°C. The plate was washed three times with PBS-Tween [2.38 g of Na_2HPO_4, 0.4 g of KH_2PO_4, 0.4 g of KCl, 16.0 g of NaCl, 2 L of Distilled water and Tween -20 to 0.2% (v/v) pH 7.4] allowing 3 min interval between washes. The MSV polyclonal antibody cross adsorbed with healthy maize extract was prepared and used at a final dilution of 1/5000 (v/v) and 100 µl was dispensed into wells of the ELISA plate. The plate was covered and placed in a humid chamber and incubated at 37 °C for 1 h.

After incubation, the plate was washed three time with-PBS-Tween. 1 µl of anti-rabbit enzyme with 15 ml conjugate buffer (PBS-TPO) at a ratio of 1/15000 (v/v) was prepared and 100 µl of the solution dispensed into each well of the ELISA plate and incubated at 37°c for 1 h. The plate was washed again three times with PBS-T and 100 µl of substrate was dispensed to each well of the plate to detect the positive reactions. Washing with PBS was done at each stage of the ELISA procedure after incubation for 1 h at 37 °c allowing three minutes interval between two washes to remove non-binded materials and to avoid contamination of sample. MSV-diseased, healthy and buffer samples were placed in each ELISA plate as control at the middle and edge wells of the plate. Each sample was tested in duplicate wells, and each plate included positive (sap extracted from known MSV infected tissue) and negative (healthy plant) controls from stock maintained at IITA.

Absorbance at 405 nm was measured after 30, 60, 90 and 120 min with microplate reader after the addition of substrate. An overnight reading was also done after keeping the microplate in refrigerator (4°C) overnight. Absorbance values were considered positive when the optical density readings were at least twice that of the mean of the negative control.

Disease severity assessment by image analysis

For accurate quantification of streak symptoms induced by MSV, leaf image captured by a digital camera was assessed using Leaf Doctor Software as described by Martin and Rybicki (1998). For

Table 1. Analysis of variance for the mean symptom severity score for tested maize lines over the five weeks of assessment after inoculation.

Source of variation	DF	Sum of squares	Mean square	F Value	Pr>F
Line	159	569.35	3.58	116.28**	<0.01
Week	4	598.32	149.58	4857.17**	<0.01
Line * Week	636	215.81	0.34	11.02**	<0.01
Error	800	24.64	0.03		
$LSD_{(0.05)}$	0.19				
CV	5.4				

DF= degree of freedom, CV= coefficient of variation.

each maize line, about 12 cm long portion at the middle part of the 5th leaf was captured. Scaled black cardboard background was used to maintain the size and quality of the picture. The Leaf Doctor software provided percentage of the diseased (streak area) and healthy portion (asymptomatic area) of the camera-captured portion of the leaf.

Data analysis

Analysis of variance was carried out on data collected symptoms severity on the leaves of inoculated plants, percentage of diseased portion of the leaf from leaf image analysis and ELISA test result using (PROC GLM in SAS program). Correlation analysis was carried out using PROC CORR in SAS. The area under disease progress curve (AUDPC) values was calculated for the disease severity scores using the formula of Wilcoxson et al., (1975) as follows:

$$AUDPC = \Sigma \left(\frac{x_i + x_{i+1}}{2}\right).(t_{i+1} - t_i)$$

Where, x_i is the disease rating on date i and t_i is the time (in calendar days) on which x_i recorded; i= 1, 2…5

RESULTS

Genotyping for maize streak virus resistance with SNP markers

The maize lines showed variation in marker allele. Majority of the tested maize lines had favorable allele for MSV resistance. Of the total 160 maize lines tested, 142 had favorable allele for MSV resistance while 18 lines did not have the favorable allele after SNP genotyping; the 18 lines originated from SW5-S-C6-18-2-1-B-TZISTR1248, SW5-S-C6-18-3-1-B-TZISTR1248 and KS27-S-C3-2-6-2-B-1-TZISTR1262 bi-parental crosses.

MSV symptom scores of 160 maize lines and relationship with alleles of SNP markers

Artificial inoculation of MSV successfully induced disease development in the maize lines. Continuous, narrow chlorotic streaks appeared on secondary and tertiary leaf vein of the plants within 3 to 5 days after inoculation in all inoculated maize lines as well as Pool 16, the control. The mean symptom severity scores at weeks 2, 3, 4, 5 and 6 weeks after inoculations were 4, 3.8, 3.2, 2.8 and 2.3, respectively. Thus, there was a reduction in disease symptoms over time. In general, the disease symptoms reduced from the lower leaves to the upper leaves. Significant differences (*P<0.01*) in MSV symptoms severity were obtained among the 160 maize lines at six week after inoculation (Table 1).

On the basis of mean symptom score assessed at 42 days after inoculation, 61 of the lines had symptoms scores between 1.0 -2.0 (resistant), 88 had scores between 2.1-3.0 (moderate resistant), 8 were rated 3.1-4.0 (susceptible) and 3 had scores of 4.1-5.0 (highly susceptible) (Table 2). Of the 142 maize lines that had the favorable allele for MSV resistance, 61 lines had MSV symptoms scores in the range 1.0 - 2.0, and 78 had scores in the range 2.1-3.0 while 3 had scores in the range 3.1-4.0 (Table 2). None of the 18 maize lines without favorable allele for resistance to MSV had symptoms scores in the range 1.0- 2.0. Ten of the maize lines had MSV symptoms scores between 2.1 and 3.0; 5 had scores in the range 3.1-4.0, and 3 had scores in the range 4.1-5.0 (Table 2). It was interesting to observe that 10 of the 18 lines that did not have the favorable allele for resistance to MSV were moderately resistant. None of the maize lines were immune to MSV. For the susceptible control, 93.1 % of the plants tested in this study had symptoms score of 4.1-5.0 (highly susceptible) while 6.9 % had scores of 3.1-4.0 (susceptible). This distribution observed for Pool-16 was within expectation and indicates the effectiveness of the inoculation procedure, using *C.triangular*. The very low scores observed for some of the lines indicate the availability of promising germplasms that can be used to improve resistance to MSV in breeding programmes in Africa.

A Chi-square value of 52.25 obtained from the test of association between allele of the SNP markers and MSV symptoms was significant *(P<0.01)* indicating that the marker allele was associated with resistance to MSV in

Table 2. Distribution of maize lines reaction to maize streak virus based on MSV score at 6 week after inoculation and desirable alleles of SNP markers.

Parameter	Total no. of maize lines		Classification based on SNP markers			
			No favorable allele		Favorable allele	
MSV severity score class	Frequency	%	Frequency	%	Frequency	%
1.0 - 2.0	61	38.1	0	0.0	61	43.0
2.1 -3.0	88	55.0	10	55.5	78	54.9
3.1 - 4.0	8	5.0	5	27.8	3	2.1
4.1 -5.0	3	1.9	3	16.7	0	0.0
Total	160	100	18	100	142	100

Table 3. Distribution of percentage recovery of 160 maize lines tested under artificial inoculation of MSV.

Symptom severity score	% Recovery			
	No. of lines	Range	Mean	%
1.0 - 2.0	61	33.1-77.2	59.4	38.1
2.1 -3.0	88	0.0-52.9	33.6	55
3.1 - 4.0	8	0.0-36.7	22.2	5
4.1 -5.0	3	0.0-10.0	5.0	1.9
Total	**160**			**100**

the population studied. Majority of the maize lines tested were moderately resistant to MSV following successful inoculation. Of the 142, maize lines with the favorable allele for resistance, 139 were resistant, a success rate of 79.9%. However, the success rate in identifying susceptible maize lines was 44.4% (8 out of 18). These results indicate that the SNP markers were useful in identifying majority of resistant maize lines; however, they were not able to identify all maize lines resistant to MSV. Ten maize lines that did not have the favorable allele for resistance and which on the basis of the marker allele would have been regarded as susceptible, had some moderate level of resistance.

Percentage recovery from disease and area under disease progress curve

Some lines had high percentage recovery while recovery for other lines was low. Percent recovery varied from 0 to 77.2% (Table 3). Amongst the 160 tested lines, 58 lines had above 50% recovery percentage whereas 102 lines had lower than 50% recovery percentage. Of the 58 lines with high percentage recovery only one line did not carry the favorable allele for MSV resistance markers. Among the maize lines with the favorable marker allele for resistance, 43% showed high recovery percentage (33.1-77.2), 54.9% showed moderate recovery (23.9-52.9) while 2.1% did not show appreciable recovery (4.5-23.3).

There were significant differences ($P<0.01$) among the maize lines for AUDPC value. Low AUDPC value indicates high resistance while high AUDPC value infers the susceptibility. Entries 152,153,158, 17 and 145 had the lowest AUDPC values of 43.8, 50.1, 53.2, 54.7 and 55.2 respectively. These lines showed high resistance to MSV, with a considerable decline in symptoms severity scores (Figure 1). Entries 115, 105, 112,140 and 110 had the highest AUDPC values of 139.1, 137.1 135.6, 126.7 and 122.5 respectively, and were therefore regarded as susceptible (Figure 2). In effect, the trends in symptoms severity scores of highly resistant lines were different from the trends observed for the highly susceptible lines. This difference provides unique information on the nature of resistance in the lines studied, a type of resistance in which host plants limit the spread and development of symptoms with growth.

The MSV damage of lines from the bi-parental crosses also showed considerable variation. The number of lines developed from each of the nine bi-parental crosses varied from 2 to 47. Among the bi-parental crosses, the lowest mean MSV damage score was observed for lines developed from IITATZi1715/TZISTR1262 (score = 2.8), followed by lines derived from BBB/KU1409/SC55-KU1409)-S2-19-1-BBB-17-B-3-B-TZiSTR1262 (score = 2.9). Lines from these crosses are potential sources of resistance to MSV in Africa. Among the inbred lines, TZiSTR1100 had the lowest MSV symptom score of 1.9. Maize lines developed from SW 5 (S) C6-18-2-1-B/TZiSTR1248 and KS 27 (S) C3-2-6-2-B-1/TZiSTR1262

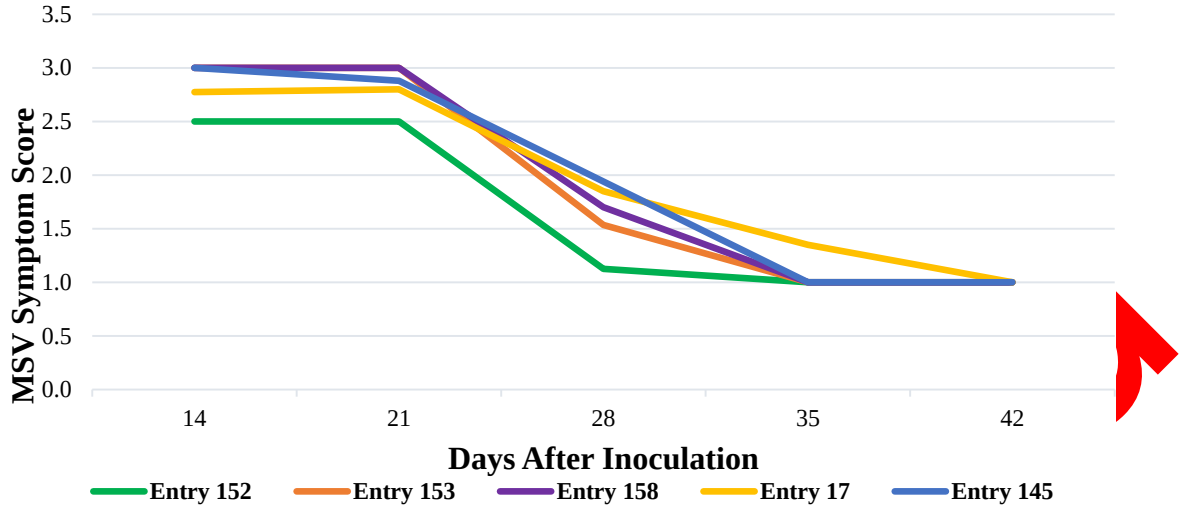

Figure 1. Trends in MSV symptom score of five maize lines with the lowest AUDPC value.

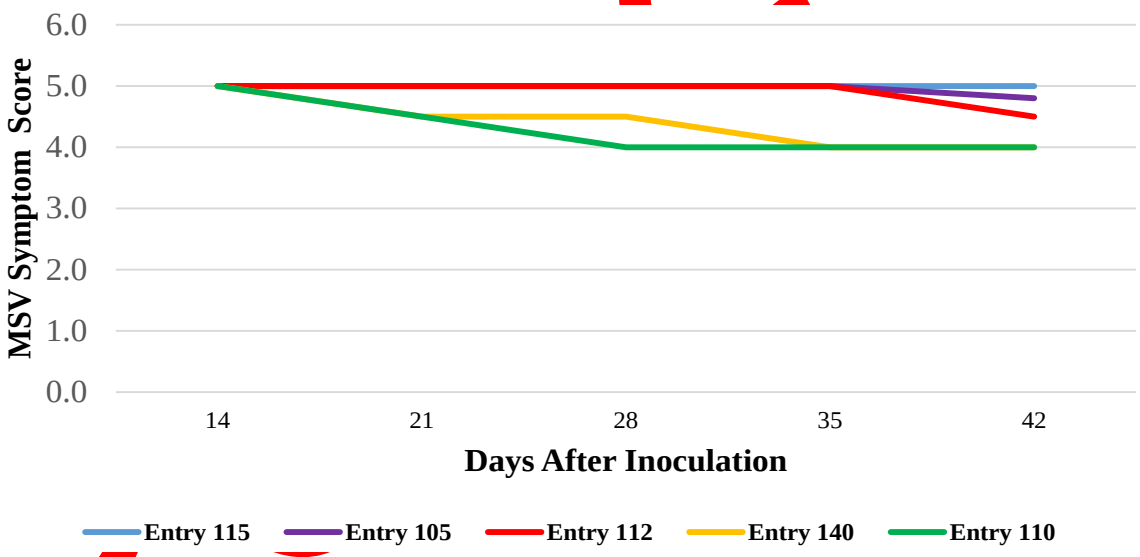

Figure 2. Trends in MSV symptom score of five maize lines with the highest AUDPC values.

bi-parental crosses had the highest average symptoms severity scores value (4.2), indicating highest susceptibility to MSV. Lines developed from IITATZi1715/TZiSTR1262 had better recovery resistance compared to the other maize lines. Amongst the 32 lines developed from IITATZi1715/TZiSTR1262 bi-parental crosses, 20 had symptoms severity score values less than or equal to 2. As resistant plants grew, resulting in an improvement in the level of resistance, the symptom gradually decreased from the lower to the upper leaf, especially on the resistant maize lines.

Maize streak virus detection through enzyme-linked immunosorbent assay

The virus titer determined from DAC-ELISA ranged from 0.3 to 8.5 ng/mg for the upper leaves and 0.4 to 12.1 ng/mg for the lower leaves. Thus, the MSV titer value for the lower leaves was in general higher than the titer value for the upper leaves. The mean concentration of the MSV negative control was 0.15 ng/mg. MSV was detected on the lower leaves in 90 of the 160 maize lines while the remaining 70 maize lines were negative for MSV, using the negative control as reference. The trend was different

for the upper leaves; MSV was not detected on a total of 134 lines while 26 lines were MSV positive.

All the 18 maize lines without the favorable allele of the SNP markers were positive for MSV in their lower leaves based on the ELISA test. In the upper leaves, however, 16 lines were positive while 2 were negative. For the 142 maize lines that carried the favorable allele, 72 were positive for MSV in the lower leaf while 70 were negative for MSV. In the upper leaf, the maize lines with favorable allele and which were positive for MSV reduced to 7 while the maize lines which were negative increased to 132. For both the lower and the upper leaves the association between MSV titer response and the SNP markers allele was significant (*P<0.01)* with chi-square value of 78.6 for the upper leaf and 13.6 for the lower leaf.

The 61 maize lines that had symptom scores considered resistant (1-2) on the MSV resistance scoring scale had the favorable allele and did not have MSV detected in their upper leaf by ELISA. Of this, 24 had MSV detected in their lower leaves while it was not detected in the upper leaves of the remaining 37 maize lines. All the lines in this group had higher MSV titer values in the lower than the upper leaves, except five lines which had the same value for leaves from the two positions. MSV was not detected in the upper and lower leaves of five lines as the titer values were low.

Of the 88 maize lines with MSV symptom scores considered as moderately resistant, 10 did not carry the favorable allele while the remaining 78 had favorable alleles. The ten lines were derived from KS27/TZISTR1205 (1 line) and SW5/TZISTR1248 (9 lines). Eight of the 10 lines had MSV detected in their lower and upper leaves while MSV was not detected in the upper leaves of two lines derived from SW/TZISTR1428. The MSV titer values of the lower leaves for the two lines were 4.7 and 4.2 while for the upper leaves the value were 2.3 and 3.1 respectively. These results suggest that the resistance in the two lines were different from that linked to the three markers. MSV was detected by ELISA in the lower leaves of 56 of the 88 maize lines; 18 of the 88 lines had MSV detected in their upper leaves.

Of the 11 lines that had MSV scores considered as susceptible, three carried the favorable SNP marker allele while the remaining eight lines did not. MSV was detected by ELISA on the lower leaves of the 11 lines with titer values ranging from 4.1 to 8.9. However, MSV was not detected in the upper leaves of the three lines with the favorable alleles; titer values for these ranged from 2.4 to 2.7. MSV was detected in the upper leaves of the remaining eight lines for which titer values ranged between 4.0 and 5.3. These results suggest the effectiveness of the marker allele in detecting resistance. However, the resistance does not include infection (of the lower leaves), but it did not allow the virus to spread to the upper leaves.

Chi-square analysis of the relationship between resistance/susceptible class (resistance, moderate resistance and susceptible) and ELISA MSV status showed significant *(P<0.01)* association for the lower (χ^2 =17.62, df=2) and upper leaves (χ^2 =38.75, df=2). Thus the association was stronger for the upper than the lower leaf. The percentage recovery of maize lines based on virus titer on upper leaves relative to the lower leaves varied considerably among the maize lines. The percentage recovery value ranged from -12.2% (no recovery) to 74.1% (high recovery). The negative percentage recovery value showed that the titer of the virus was higher on upper leaf compared to the lower leaf. Of the 160 tested lines, sixty-four (40%) had percent recovery greater than or equal to 50 % and ninety-six (60%) had below 50% recovery. Of the 64 high recovery resistance maize lines, only one did not carry the favorable allele for MSV resistance. The percentage recovery of phenotypically resistant lines ranged from 0 to 74.1%, for moderately resistant lines it ranged was from 0.0 to 70.6% while for the phenotypically susceptible lines range was from -12.2 to 49%.

There was significant association at six week after inoculation between the type of SNP allele carried by the maize lines and titer of the virus. Significant association was detected for both the lower and upper leaves. More maize lines than expected (in the calculation of the Chi-square value) without the allele of the SNP markers had MSV detected in their leaf tissue. The higher Chi-square value obtained for the upper leaf (78.63 vs 13.40) indicate the upper leaf at week 6 after inoculation to be more effective in assaying response to MSV by ELISA due to the resistance gene detected by the SNP markers. Of the 142 maize lines with the favorable allele of the marker, 132 (93.0%) were negative for MSV in their upper leaf, a much higher amount than the 70 (49.3%) that were negative for the virus in their lower leaves.

MSV symptoms assessment by image analysis

Significant differences *(P<0.01)* among the maize lines were observed on the percentage of disease severity on the sampled portion of the leaf. The mean disease severity on the fifth leaf ranged from 4.6 to 81.2% for the most resistant and susceptible maize lines respectively. In maize lines with the favorable allele for resistant, disease severity ranged from 4.6 to 66.4% while without the favorable allele it ranged from 29.8 to 81.2%. The mean disease severity for maize lines that had the favorable allele for MSV resistance and those that did not have favorable allele were 28.9 and 43.3% respectively. Entries 155,158 and 159 had the lowest disease severity values of 4.6, 6.7 and 10.7% respectively while entries 105, 140 and 117 had the highest disease severity values of 81.2, 66.4 and 63.3% respectively. The result obtained from image analysis significantly correlated with the

symptom score with correlation coefficient of 0.67 and ELASA test result 0.23 and 0.24 with the lower and upper leave test result respectively.

DISCUSSION

The level of resistance of the maize lines with the favorable allele indicated by the three SNP markers linked to MSV resistance varied with the source. These markers are linked to MSV resistance gene on Chromosomes 1. Molecular markers that are associated with MSV resistance in a range of genetic backgrounds could potentially enable pre-selection of genomic regions in tropical germplasm developed within and outside SSA. This can facilitate accelerated genetic gains.The SNP markers used in this study were reported to have an MSV reaction prediction efficiency of 0.94 (Nair et al*.,* 2015). Inheritance of MSV resistance in maize is complex, involving at least three major genes and a number of minor genes. A major QTL (*Msv1*) for MSV resistance on Chromosome bin 1.05 had been reported in several studies on MSV resistance (Kyetere et al*.* 1999). Due to their high prediction efficiency, kompititive allele specific PCR (KASP) assay was developed for the three SNP markers that mapped to this chromosomes location.

In this study all the maize lines evaluated and the Pool 16 developed sever symptom which confirm the success of the artificial inoculation technique. MSV resistance score of the maize line evaluated ranged between highly resistant (score = 1) to highly susceptible (score = 5). Majority of the maize lines tested were moderately resistant to MSV following successful inoculation of the virus using *C. triangular* as vector. In similar studies Markham et al. (1984), reported that *C. triangular* had 60 to 100 % efficiency in transmitting MSV after acquiring the virus. In the present study, the inoculated plants develop symptoms 3 to 5 days after inoculation. These results are consistent with the report of Mesfin et al. (1995) that symptoms of MSV appear faster in younger maize plants, usually 3 to 5 days in one week old plants and 7 to 9 days in 9 week old plants. Viral symptoms observed in this study did not differ from the symptoms described in the literature (Asanzi et al., 1994; Bosque-Perez, 2000).

Among the maize lines with the favorable marker allele for resistant, 43% showed a significance recovery, 54.9% showed moderate recovery while 2.1% did not show significant recovery. As resistant plants grew, resulting in an improvement in the level of resistance, the symptom gradually decreases from the lower to the upper leaf, especially on the resistant maize lines. This pattern of resistance is referred to as mature plant resistance (Mesfin et al., 1995). Mature plant resistance has been reported for resistance to MSV and MLN in maize (Bosque-Perez, 2000; Sitta et al*.,* 2017). The highly susceptible lines had less vigour compared to the resistant maize lines. Other author reported that affected maize plants are shorter and has less vigour especially when the infection is early (Okoth et al*.,* 1988).

MSV has been reported to infect all cell types of the host plant, with streak symptoms manifest only on inoculated leaves or on leaves produced after infection of the plant (Thottappilly et al*.,* 1993). Bosque-Perez (2000) reported that the streak pattern is a result of the failure of chloroplasts to develop tissues surrounding the vascular bundles. The basal region of the maize leaf laminae expresses the symptoms of MSV disease first, and this gradually spreads towards the leaf apex.

The favorable allele of the SNP markers was significantly associated with resistance to MSV inferred from symptoms of the disease of the host plants. The three SNP markers were successful in identifying 139 of the 142 maize lines (97.9%) as resistant. The susceptibility of three lines carrying the favorable allele for resistance may be due to broken-down linkage between the SNP markers and the gene for resistance to MSV in the maize lines and/or the influence of different genetic backgrounds. However, the successful rate in identifying susceptible maize lines was 44.4% (8 out of 18). These results indicate that the SNP markers are useful for identifying many resistant maize lines, but are not able to identify all maize lines resistant to MSV. Reliance on the three SNP markers alone may result in discarding resistant maize lines that would otherwise be useful in breeding programmes.

In the present study, the ELISA serological viral detection method further confirmed the results obtained from phenotyping following artificial inoculation. The ELISA technique, in addition to offering insight on the nature of resistance in the genetic materials studied and also provided information on the distribution of MSV antigen in the leaves of infected plants. The titer values of the virus obtained from the upper and the lower leaves were significantly different among maize lines. The differences in virus titer between the susceptible and resistant maize lines were related with the symptom severity on the leaves. In this study, higher virus titer was associated with the more severe streak symptoms and vice versa. Peterschmitt et al. (1991) showed that virus concentration in leaves increases with increase in density of chlorotic streaks.

In this study, the MSV titer ranged from 0.3 to 12.1 ng/mg. A study conducted by Peterschmitt et al. (1991) reported MSV concentration value of 4.0 ng/mg on maize leaves 15 days after inoculation using indirect ELISA serological viral detection method. The titer of the leaf sample is a function of the level of resistance/ susceptibility of the maize line, the time (days/weeks) after infection when leaves were assayed and the position on the plant of leaf assayed. The alleles of the SNP markers strongly associated with the leaf virus titer response for the upper leaf. These results are in

agreement with the observation and inference on the mature plant type resistance (based on symptom severity score) to MSV earlier made for the lines used in the study.

In the present study, the digital imaging of the 5th leaves of the maize lines processed using the Leaf Doctor Software provided estimates of symptoms severity in agreement with the genotype information provided by the SNP markers, visual symptoms scores and ELISA result. The software has a potential for use in severity assessment of MSV disease. Sanjay and Shrikant (2011) reported that similar digital image processing achieved an accuracy of 98.6% in the estimation of brown spot disease severity in sugar cane *(Saccharum officinarum)* leaves. The approach has been credited with improvement in accuracy, precision, and reliability of estimates of plant disease severity over visual score (Bardsley and Ngugi, 2013). Assessment of disease severity visually has drawbacks which include rater fatigue, the decrease in accuracy and precision of rater estimates over time due to the repetitiveness of task and physically tiring nature of assessment task (Sanjay and Shrikant, 2011).

Martin and Rybicki (1998) used digital image processing to estimate disease severity of MSV and compared the results obtained with visual assessment, using commercial software package as well as an in-house customized package. They concluded that the commercial and customized software packages had approximately the same performance, and both computer-based methods achieved better accuracy and precision than the visual method.

CONCLUSION

The three SNP markers were useful in identifying maize lines with resistance to MSV. The MSV symptoms scores were higher on the older leaves and reduced over time on the upper leaves. The severity of symptoms displayed was dependent on the level of resistance of the maize lines. Resistant maize lines had reduced symptom on the upper leaves at six week after inoculation at which time a total of 139 out of 142 (97.9%) maize lines with the favorable marker allele for resistant to MSV showed some resistance based on symptoms scores on the upper leaves; 61 of these were resistant while the remaining 78 were moderately resistant. A total of 10 maize lines out of the 18 that did not have the favorable marker allele for resistance to MSV had moderate resistant to the virus. This result suggests that the existence of other resistance gene(s) not linked to the marker allele among the lines.

The three SNP markers have potential for use in marker assisted selection for the development of MSV resistant varieties. However, there is need to develop additional markers that can be used to identify other genes responsible for resistance in some of the lines used in the study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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