

Article

## Genotypic Variation in Seedling Tolerance to Aluminum Toxicity in Historical Maize Inbred Lines of Zambia

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**Abstract:** Maize (*Zea mays* L) is the most important food grain in sub-Saharan Africa and is mostly grown by small-scale farmers under rainfed conditions. Aluminum toxicity caused by low pH is one of the abiotic factors limiting maize production among smallholder farmers. Therefore, breeding maize hybrids that are tolerant to aluminum toxicity will sustain and increase maize production in these areas. Hence this study was undertaken to assess the genotypic variation for aluminum toxicity in maize inbred lines. Fourteen maize inbred lines of historical importance that are used in maize hybrid breeding in Zambia were studied for seedling root variation under different aluminum concentrations using hydroponic conditions. The aluminum tolerance membership index based on three traits (actual root length, relative root length and root length response) classified genotypes L3233 and L1214 as highly tolerant, L5527 and ZM421 as tolerant, and L12, L3234, and ZM521 as intermediate. The high PCV, GCV, and heritability observed for the root traits indicate that opportunities for selection and breeding for aluminum tolerance among Zambian inbred lines exist. Furthermore, the study indicated that a higher genetic gain would be expected from net root growth followed by shoot length response as selection traits, thus supporting the use of root traits for aluminum tolerance screening.

**Keywords:** maize; aluminum toxicity; seedling traits; phenotypic coefficient of variation; genotypic coefficient of variation

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## 1. Introduction

Maize is the major source of energy in sub-Saharan Africa, grown mostly by smallholder farmers [1]; as such, the crop is cultivated across a wide range of environments. Drought and low fertilizer input are two major constraints affecting maize production in addition to aluminum toxicity resulting from low pH [2,3]. Soil acidity and aluminum toxicity are common in tropical and subtropical regions [2,3]. In these areas, maize yield reductions due to toxic levels of aluminum and manganese as well as deficiencies of phosphorus, calcium, and magnesium are common [4,5]. In southern Africa, large areas of acidic soils with free aluminum ions ( $Al^{+3}$ ) exist in Zambia and Mozambique [6].

In Zambia, aluminum toxicity and low pH have been reported in high rainfall (wetter) areas of the country, *i.e.*, agro-ecological region III, which covers the northern parts of Zambia [7,8]. The soils are acidic with medium to high phosphorus (1–3 ppm) fixing capacity [9]. The aluminum saturation is mostly in the range of 53% to 80% with limited places having 80% to 100% [10]. It has been estimated that a high level of soil acidity prevails throughout 98% of Zambia's maize fields [11], reducing yield to practically nil in high acid soils [8]. In these soils, it has been observed that low pH and high concentrations of aluminum and manganese are the major factors causing soil infertility [3,12]. In view of the above, maize does not respond to fertilizer application, especially basal dressing [11]. Despite the high levels of aluminum and low pH, the northern regions of Zambia (region III) have been predicted to be the main agricultural food basket of the country as the southern and central parts of Zambia (regions I and II, respectively) become more prone to drought due to climate change [13]. Thus increasing and sustaining maize productivity in these areas implies the usage of acid-tolerant genotypes and/or amelioration of soil acidity [14]. Ameliorating factors are expensive and out of reach for most resource-poor farmers. In addition, the ameliorating factors have been known to destabilize the balance of the ecosystem [15]. Therefore, developing acid-tolerant maize genotypes is an effective and sustainable way of alleviating the impact of Al toxicity in maize production areas. Studies have shown that Al-tolerant maize genotypes outperformed the adapted local and susceptible genotypes by 13% and 61%, respectively [16]. These results suggest that growing Al-tolerant maize genotypes will ensure a high sustained maize productivity.

Screening and selection for Al tolerance in maize involves field evaluation for more than one cropping season [17]. However, the high coefficient of variation resulting from spatial variation in the soils poses a challenge to field evaluation [17] and eventually affects the reliability of the results. Therefore, laboratory hydroponic studies of root traits offer another option if the laboratory results are correlated to field performance under the same stress conditions [17–19]. In maize, laboratory studies on seedling traits have been reported to be highly correlated to field performance for low nitrogen conditions [20], nutrient and water use efficiency [21], and weak correlation for aluminum toxicity [19]. Ouma *et al.* (2013), using relative net root growth (RNRG) of maize seedling under

aluminum toxicity, were able to predict 24% and 35% of field performance. The high heritability and low coefficient of variation of root traits makes them amenable for breeding studies [22]. Hence, root traits have been effectively used for screening crops for aluminum tolerance in breeding programs [21,23]. In Zambia, most hybrids were bred for high grain yield and general adaptability, which involved multi-location and multi-season agronomic trials. This could have resulted in co-selection for aluminum tolerance in parents. Therefore, the study was conducted on historical maize inbred lines with a view to (a) quantifying the genetic variance, heritability, and expected genetic advance; and (b) identifying the traits accounting for most of the variation among the inbred lines.

## 2. Material and Methods

### 2.1. Plant Materials

The response of maize inbred lines to aluminum stress was determined in the tissue culture laboratory of the University of Zambia in the school of Plant Sciences, using test tubes. The 11 maize inbreds and three open maize pollinated varieties used in the study were supplied by the Zambia Agricultural Research Institute (ZARI).

### 2.2. Hydroponic Experiment

The seeds were first pre-germinated on filter paper in petri dishes. After germination, the roots of the seedlings were allowed to elongate to at least a uniform length. Thereafter, the seedlings were transferred to a test tube containing a diluted nutrient solution, after measuring the initial root length. The nutrient solution used for plant growth had the following composition (in milligrams per liter, mg/L): 48.1 Ca, 14.6 Mg, 42.61 N, 23.5 K, 0.02 Na, 0.03 Cl, 0.03 Mn, 0.06 Cu, 0.03 Mo, 0.16 Zn, 1.67 Fe (added as  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ), and 0.32 B [24]. Different aluminum concentrations in the nutrient solution were achieved by dissolving 0, 4, 8, 12, 16, and 20 milligrams per liter (mg/L) of aluminum sulfate ( $\text{Al}_2[\text{SO}_4]_3$ ). The pH of the nutrient solution was then adjusted to 4.2 and left unadjusted thereafter. Each treatment was replicated four times.

The plants were grown at room temperature (28 °C) with an average day length of 13 h for 10 days. After 10 days, the plants were harvested and separated into roots and shoots. The traits (Table 1) were recorded. The root and shoot dry mass was determined by drying the respective plant parts in an oven for 4 days at 70 °C.

In addition, the following traits were derived:

$$\% \text{ response} = \frac{(\text{Growth Parameters in Control}) - (\text{Growth Parameters in Al treatments})}{\text{Growth Parameters in Control}} \times 100 \quad (1)$$

[25].

$$\text{Relative Root Length (RRL)\%} = \frac{\text{root length in Al solution}}{\text{root length in control}} \times 100 \quad [25,26]. \quad (2)$$

$$\text{Specific Root Length (SRL)} = \frac{\text{Total Root Length}}{\text{Root biomass}} \quad [27]. \quad (3)$$

$$\text{Actual root length (ARL)} = \text{Final root length (FRL)} - \text{Initial root length (IRL)}. \quad (4)$$

$$F_{ij} = \frac{X_{ij} - X_{min}}{X_{max} - X_{min}} \text{ and } F_i = \text{average of } F_{ij} \quad [28], \text{ where:} \quad (5)$$

$F_{ij}$  = membership index value of the  $i$ th inbred line,  $j$ th trait

$X_{ij}$  = ratio of the  $i$ th inbred line,  $j$ th trait

$X_{\min}$  = minimum ratio of the trait

$X_{\max}$  = maximum ratio of the trait

$F_i$  = membership index averaged over  $n$  traits of the  $i$ th accession

**Table 1.** Maize root seedling characteristics measured in the study.

Trait	Details
Initial root length (IRL)	Length of roots recorded before the seedlings were transferred into the nutrient solution
Final root length (FRL)	Root length recorded after seedlings have been exposed to nutrient solution
Number of roots (NOR)	Number of roots formed after the seedlings have been exposed to aluminum stress
Shoot or Root dry matter (SDM or RDM)	The weight of root or shoot growth parts
Total dry matter (TDM)	The weight of shoot and root growth parts (Sum of root and shoot biomass)
Shoot Length (SL)	Length of the shoot measured after seedlings have been exposed to aluminum stress
Shoot length response or root length response	This was calculated by using Equation (1).
Net root growth (NRG)	This was calculated by subtracting the root length of maize seedlings in aluminum stress from the control.
Shoot length: shoot dry matter ratio (SLSDMratio)	Estimated by dividing shoot dry matter by shoot length
Shoot: root dry matter ratio (SRDMratio)	Estimated by dividing shoot dry matter by root dry matter
Shoot: root length ratio (SLRLratio)	Estimated by dividing shoot length by root length

For each trait and concentration, the membership index was calculated, and then the membership index averaged over all the traits. The mean overall membership index was then used as an indicator for Al tolerance. The rank of each inbred line to aluminum stress was then classified as Table 2:

**Table 2.** Aluminum tolerance membership index.

<b>Rank 1: <math>F_i \geq 0.8</math></b>	<b>(highly tolerant)</b>
Rank 2: $0.6 \geq F_i < 0.8$	(tolerant)
Rank 3: $0.4 \geq F_i < 0.6$	(intermediate tolerance)
Rank 4: $0.2 \geq F_i < 0.4$	(susceptible)
Rank 5: $F_i < 0.2$	(highly susceptible)

### 2.3. Statistical Analysis

The Analysis Of Variance (ANOVA), variance components, and broad sense heritability ( $H_{bs}$ ) were calculated using a web based software, the PBSTAT [29]. Heritability, genotypic, and phenotypic variances (GCV and PCV, respectively) were estimated as suggested by Singh and Chaudhary [30]. The PCV and GCV were calculated as:

$$PCV(\%) = \frac{\sqrt{V_p}}{X_{mean}} \times 100; V_p = \text{phenotypic variance} \quad (6)$$

$$PCV(\%) = \frac{\sqrt{V_g}}{X_{mean}} \times 100; V_g = \text{phenotypic variance, where} \quad (7)$$

PCV = Phenotypic Coefficient of Variability

GCV = Genotypic Coefficient of Variability,

$X_{mean}$  = mean of the character

Heritability in the broad sense, genetic advance at 5% intensity, and genetic gain as a percentage of trait mean were calculated as:

$$H_{bs} = \frac{V_g}{V_p} \times 100; H_{bs} = \text{Heritability (broad sense)} \quad (8)$$

$$\text{Genetic advance (GA)} = \frac{V_g}{V_p} \times \sqrt{V_p} \times k; \quad (9)$$

$$\text{Genetic gain (\%)} = \frac{GA}{X_{mean}} \times 100, \text{ where:} \quad (10)$$

$K$  = selection differential at 5% selection intensity. The value of  $k = 2.06$ .

The genetic advance as percent of mean (GG), genotypic coefficient of variability (GCV), and phenotypic coefficients of variability (PCV) were categorized as low (0%–10%), moderate (10–20%), or high (>20%) [31,32], while heritability was categorized as low (0%–30%), moderate (30%–60%), or high (>60%) [33]. The principal component analysis, cluster analysis, and association among traits were calculated using Minitab version 16 software.

## 3. Results

### 3.1. Analysis of Variance (ANOVA) for Seedling Traits

The interaction between aluminum concentration and genotypes was highly significant ( $p < 0.01$ ) for all the traits except for shoot length response (SLR), number of roots formed (NOR), and Shoot Length-Root Length Ratio (SLRLratio) (Table 3). This indicates that the genotypic performance was not the same across aluminum concentrations for these traits. The main effects (genotype and environment) were also significant for most of the traits, indicating that aluminum concentration had different effects while superior genotypes can be found (Table 3). The mean performance of each of the genotype is shown in Table 3. The superior performance of the genotypes in aluminum toxic solutions is exhibited in RRL, SRL, and NRG traits, with some genotypes exhibiting negative net root growth (impaired root growth) compared to the control (Table 3).

**Table 3.** Mean performance of maize genotypes averaged across all aluminum concentrations, and probability of main effects and their interaction.

Genotype	IRL	FRL	ARL	SRL	RRL	RLR	SL	SLR	SLSDM Ratio	NOR	RDM	SDM	TDM	SRDM Ratio	SLRL Ratio	NRG
POOL 16	1.81	16.47	14.66	692.04	101.49	-0.63	6.17	-11.35	172.09	3.38	0.03	0.04	0.08	1.83	0.45	1.20
L12	2.33	7.81	5.49	521.96	113.39	21.50	3.21	2.32	156.95	2.00	0.02	0.03	0.04	2.72	0.41	-6.04
L143	1.13	10.13	8.99	491.29	100.26	2.68	6.06	0.24	216.46	3.92	0.02	0.03	0.06	1.64	0.89	1.99
L710	1.13	5.70	4.56	996.63	102.21	-0.62	12.25	-9.04	661.89	1.17	0.01	0.02	0.03	4.92	2.97	-1.57
L911	1.45	6.53	4.68	1039.71	94.41	-23.79	5.96	-1.70	199.25	1.54	0.01	0.04	0.05	7.73	1.25	-0.02
L913	2.68	14.17	11.49	504.79	93.29	-1.71	5.71	-16.59	133.72	3.71	0.03	0.05	0.08	2.08	0.69	4.25
L917	2.35	8.18	5.83	149.13	76.64	-27.19	5.85	-5.92	176.08	3.54	0.04	0.04	0.08	1.08	1.45	-3.56
L1214	1.89	12.17	10.28	529.29	150.21	89.03	5.88	-14.31	111.20	2.96	0.03	0.06	0.08	3.74	0.69	3.51
L3233	1.70	11.56	9.87	1152.00	149.25	85.50	5.79	-3.48	308.92	2.63	0.01	0.03	0.04	3.50	1.13	-0.14
L3234	3.05	17.40	14.34	1129.04	127.58	44.96	5.63	23.95	168.29	1.92	0.02	0.04	0.06	2.54	0.45	3.13
ZM421	2.19	16.50	14.31	323.08	146.23	68.85	6.83	-5.45	155.00	3.21	0.06	0.05	0.10	0.99	0.55	-0.29
ZM521	2.17	15.47	13.30	311.54	123.18	45.60	6.52	-1.83	206.21	5.08	0.05	0.03	0.08	0.77	0.55	-1.54
L5522	2.06	9.90	7.84	844.00	92.02	-11.78	5.10	2.67	159.95	1.71	0.02	0.04	0.07	2.79	0.63	-3.20
L5527	2.10	13.54	11.44	936.29	123.36	45.58	7.48	32.09	136.48	2.17	0.03	0.06	0.10	5.37	0.83	3.46
<b>Mean</b>	<b>2.003</b>	<b>11.824</b>	<b>9.791</b>	<b>687.199</b>	<b>113.823</b>	<b>24.141</b>	<b>6.317</b>	<b>-0.600</b>	<b>211.606</b>	<b>2.781</b>	<b>0.027</b>	<b>0.040</b>	<b>0.068</b>	<b>2.979</b>	<b>0.924</b>	<b>0.084</b>
<b>Probability of main effects and their interactions</b>																
<b>Genotype</b>	0.000	0.033	0.049	0.065	0.007	0.027	0.287	0.869	0.002	0.167	0.092	0.000	0.000	0.034	0.317	0.756
<b>Environment</b>	0.426	0.898	0.785	0.497	0.519	0.346	0.572	0.815	0.933	0.557	0.942	0.603	0.842	0.515	0.605	0.573
<b>interaction</b>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

IRL = Initial root length, FRL = Final root length, ARL = Actual root length, SRL = specific root length, RRL = relative root length, RLR = Root length response, SL = shoot length, SLR = Shoot length response, SLSDMratio = Shoot length-shoot dry matter ratio, NOR = Number of roots, RDM = root dry matter, SDM = shoot dry matter, TDM = total dry matter, SRDM ratio = shoot-root dry matter ratio, SLRL Ratio = shoot root length ratio, and NRG = net root growth.

### 3.2. Phenotypic and Genotypic Coefficients of Variation, Heritability, and Genetic Advance as Percent of the Mean

The genetic variance ( $V_G$ ) was greater than the interaction variance ( $V_{G \times En}$ ) for FRL, ARL, RRL, RLR, SL, SLR, SLSDMratio, NOR, RDM, TDM, and SLRLratio (Table 4). Therefore, aluminum concentration had less influence on these traits. The broad sense heritability ranged from 48.1% for SRL to 94.0% for SLSDMratio with all the traits having high heritability (>60%), except for SRL. The PCV and GCV ratios for most of the traits were almost equal. The phenotypic variance ( $V_P$ ) was higher than the genotypic variance ( $V_G$ ) for all the traits, except for RDM and TDM. All the traits studied had high GG, with the highest (5097.4) observed for NRG, followed by SLR (3313.6). The least GG was observed for RR (31.9%).

**Table 4.** Estimates of variance components, heritability, genetic gain, and genetic advance as percent of mean, phenotypic, and genotypic coefficients of variability for 14 inbreds tested in six aluminum concentrations \*.

Variable	Grand Mean	$V_G$	$V_{G \times En}$	$V_P$	$h^2_{bs}$ (%)	PCV	GCV	GA	GG
IRL	2.00	0.2031	0.4339	0.2947	68.9	27.1	22.5	0.77	38.5
FRL	11.82	13.716	5.553	15.2272	90.1	33.0	31.3	7.24	61.2
ARL	9.79	11.6236	7.4616	13.4773	86.3	37.5	34.8	6.52	66.6
SRL	687.20	52,020.44	158,743.45	108,080.2273	48.1	47.8	33.2	325.96	47.4
RRL	113.82	412.6663	351.6464	548.0072	75.3	20.6	17.8	36.31	31.9
RLR	24.14	1224.88	672.9026	1531.7734	80.0	162.1	145.0	64.47	267.1
SL	6.32	3.511	1.2157	3.8265	91.8	31.0	29.7	3.70	58.5
SLR	-0.60	130.6968	79.2851	183.4006	71.3	2257.1	1905.4	19.88	3313.5
SLSDMratio	211.61	17,912.03	3212.58	19,060.2367	94.0	65.2	63.2	267.27	126.3
NOR	2.78	1.0852	0.0806	1.2036	90.2	39.4	37.5	2.04	73.3
RDM	0.03	0.0002	0.0001	0.0002	90.3	52.1	52.1	0.03	107.3
SDM	0.04	0.0001	0.0001	0.0002	88.3	35.4	25.0	0.01	36.4
TDM	0.07	0.0005	0.0002	0.0005	87.8	33.0	33.0	0.05	67.9
SRDMratio	2.98	2.5002	3.6912	3.8111	65.6	65.5	53.1	2.64	88.6
SLRLRATIO	0.92	0.3902	0	0.4806	81.2	75.0	67.6	1.16	125.4
NRG	0.08	6.3832	11.5206	9.3671	68.2	3631.2	2997.5	4.30	5097.4

\* En = Environment, related to different aluminum concentrations; IRL = Initial root length, FRL = Final root length, ARL = Actual root length, SRL = specific root length, RRL = relative root length, RLR = Root length response, SL = shoot length, SLR = Shoot length response, SLSDMratio = Shoot length-shoot dry matter ratio, RDM = root dry matter, SDM = shoot dry matter, TDM = total dry matter, SRDM ratio = shoot-root dry matter ratio, NOR = Number of roots, SLRL Ratio = shoot root length ratio, and NRG = net root growth.

### 3.3. Associations among Seedling Traits

The correlation coefficients with  $0 \leq r^2 \leq 0.5$ ,  $0.5 < r^2 \leq 0.8$  and  $0.8 < r^2$ , were classified as weak, moderate, and strong, respectively [34]. SL was significantly ( $p < 0.01$ ) and positively correlated to SLSDMratio and SLRLratio (Table 5). FRL was positively and perfectly correlated ( $r = 0.99$ ) to ARL. Both ARL and FRL were moderately and positively correlated to RDM and NRG, while being negatively correlated to SLRLratio. The magnitudes of the correlation coefficient was similar in each of the traits for both ARL and FRL. NOR was moderately and negatively correlated to SRDMratio and SRL, while positively and moderately correlated to RDM only.

**Table 5.** Pearson correlation coefficients among 15 seedling traits of 14 maize inbred lines in six aluminum concentrations ( $n = 84$ ).

Trait	IRL	FRL	ARL	SRL	RRL	RLR	SL	SLR	SLSDM Ratio	NOR	RDM	SDM	TDM	SRDM Ratio	SLRL Ratio
FRL <sup>2</sup>	0.56 <sup>1</sup> *														
ARL	0.45	0.99**													
SRL	-0.18	-0.11	-0.10												
RRL	0.09	0.46	0.49	0.16											
RLR	0.16	0.49	0.51	0.09	0.98**										
SL	-0.48	-0.18	-0.12	0.24	-0.02	-0.05									
SLR	0.29	0.19	0.16	0.43	0.14	0.14	-0.07								
SLSDM ratio	-0.57*	-0.48	-0.42	0.36	-0.09	-0.14	0.82**	-0.18							
NOR	0.14	0.48	0.50	-0.73**	0.05	0.14	-0.22	-0.33	-0.36						
RDM	0.39	0.57*	0.56*	-0.76**	0.20	0.25	-0.09	-0.11	-0.43	0.67**					
SDM	0.39	0.43	0.40	-0.14	0.27	0.31	-0.23	0.17	-0.67**	0.07	0.40				
TDM	0.39	0.64*	0.62*	-0.50	0.12	0.18	-0.14	0.12	-0.64**	0.50	0.81**	0.78**			
SRDM ratio	-0.40	-0.50	-0.49	0.70**	-0.02	-0.10	0.28	0.23	0.27	-0.72**	-0.65**	0.09	-0.37		
SLRL ratio	-0.57*	-0.66**	-0.62*	0.26	-0.28	-0.30	0.82**	-0.20	0.89**	-0.39	-0.40	-0.45	-0.50	0.41	
NRG	0.08	0.53*	0.56*	0.24	0.29	0.31	0.15	0.13	-0.22	0.16	0.01	0.57*	0.38	0.15	-0.18

<sup>1</sup>,\* = significant at  $p=0.05$ , \*\* = significant at  $p = 0.01$ ; <sup>2</sup> IRL = Initial root length, FRL = Final root length, ARL = Actual root length, SRL = specific root length, RRL = relative root length, RLR = Root length response, SL = shoot length, SLR = Shoot length response, SLSDMratio = Shoot length-shoot dry matter ratio, RDM = root dry matter, SDM = shoot dry matter, TDM = total dry matter, SRDM ratio = shoot-root dry matter ratio, NOR = number of roots, SLRL Ratio = shoot root length ratio, and NRG = net root growth.

### 3.4. Principal Component Analysis

Principal component analysis was carried out to identify the main trait that could be used in selection for aluminum tolerance or the trait that explained much of the variation observed in maize inbred lines. The first five principal components had eigenvalues greater than 1 and were thus important in explaining the variation observed (Table 6). The first principal component explained 40.40% of the variation followed by the second component (18.3%). The third, fourth, and fifth components explained 13.0%, 9.2%, and 6.6% respectively. Cumulatively, all five factors explained 87.7%, with the first and second component accounting for 58.7% together. The traits FRL, ARL, SLSDMratio, RDM, TDM, and SLRLratio loaded highest ( $>0.30$ ) on the first component. On the second component, SRL, RRL, RLR, SLR, NOR, SRDMratio, and SLRLratio loaded highest ( $>0.30$ ). The root components (FRL, IRL, FRL, NOR, RDM) were important in the first component. The second component was largely influenced by SRL ( $-0.46$ ) followed by RRL ( $-0.35$ ). It was observed that some variables that had positive loadings in PC1 had negative loadings in PC2, except for NOR, RDM, and TDM. The first PC separated the inbreds on six traits (traits with values  $\geq 0.30$ ). The second PC separated the inbreds on seven traits. Amongst all the traits, RRL and RLR appeared three times in the first five components.

**Table 6.** Principal component analysis of 14 maize genotypes, eigenvalues, proportion, and cumulative variance for the first six components for root morphological characters in six aluminum concentrations ( $n = 84$ ).

Value	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Eigenvalue	6.462	2.932	2.100	1.477	1.061	0.892
Proportion	0.404	0.183	0.131	0.092	0.066	0.056
Cumulative	0.404	0.587	0.718	0.811	0.877	0.933
Component loadings *						
IRL	0.25	-0.02	0.27	0.09	<b>-0.34</b>	0.23
FRL	<b>0.34</b>	-0.16	-0.14	0.04	<b>-0.31</b>	-0.16
ARL	<b>0.33</b>	-0.16	-0.20	0.04	-0.29	-0.20
SRL	-0.19	<b>-0.46</b>	0.10	0.07	-0.21	-0.18
RRL	0.15	<b>-0.35</b>	-0.27	<b>0.37</b>	<b>0.32</b>	0.17
RLR	0.18	<b>-0.32</b>	-0.27	<b>0.36</b>	<b>0.31</b>	0.18
SL	-0.19	-0.10	<b>-0.49</b>	<b>-0.31</b>	-0.21	0.20
SLR	0.04	<b>-0.32</b>	0.26	-0.01	<b>-0.43</b>	0.40
SLSDM ratio	<b>-0.31</b>	-0.02	<b>-0.39</b>	0.06	-0.21	0.08
NOR	0.25	<b>0.30</b>	-0.24	0.02	0.02	-0.29
RDM	<b>0.30</b>	0.22	-0.21	-0.10	0.01	0.40
SDM	0.25	-0.20	0.13	<b>-0.44</b>	<b>0.34</b>	0.15
TDM	<b>0.32</b>	0.03	-0.04	<b>-0.41</b>	0.03	0.22
SRDMratio	-0.24	<b>-0.33</b>	0.16	-0.25	0.27	0.03
SLRLratio	<b>-0.32</b>	0.03	<b>-0.30</b>	-0.20	-0.05	0.19
NRG	0.15	<b>-0.33</b>	-0.15	<b>-0.38</b>	-0.01	-0.49

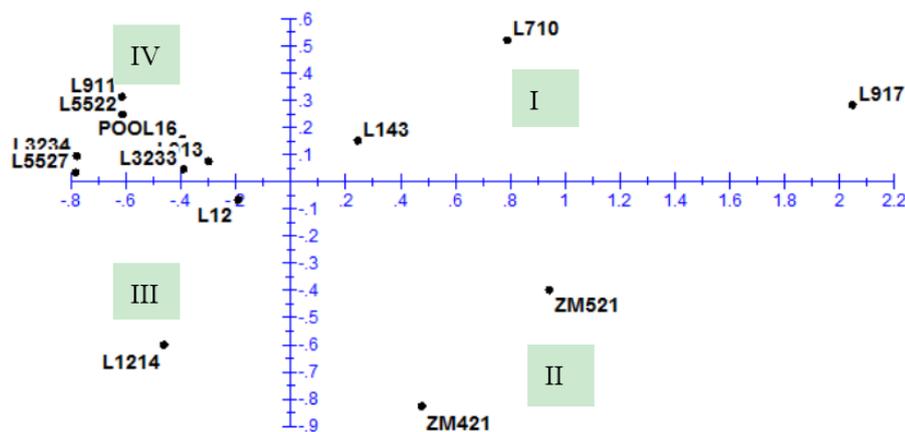
\* values  $\geq 0.30$  are presented in bold face and indicates traits important for PC definition; IRL = Initial root length, FRL = Final root length, ARL = Actual root length, SRL = specific root length, RRL = relative root length, RLR = Root length response, SL = shoot length, SLR = Shoot length response, SLSDMratio = Shoot length-shoot dry matter ratio, RDM = root dry matter, SDM = shoot dry matter, TDM = total dry matter, SRDMratio = shoot-root dry matter ratio, NOR = number of roots, SLRL Ratio = shoot root length ratio, and NRG = net root growth.

3.5. Cluster Analysis and Similarity between Inbred Lines

The similarity of the inbred lines based on the seedling traits is shown in Table 7. The inbred line L5522 and L911 were the closest (0.08) and the furthest (2.85) was between L3234 and L917. The principal coordinate analysis based on the Euclidean distance was used to visualize the genotypes (Figure 1). The maize genotypes were clustered in all four quadrants, with most of them in quadrant IV. The two maize populations were in quadrant II, while L12 and L1214 were in quadrant III. L710, L143, and L917 were in quadrant I, while the rest were in quadrant IV.

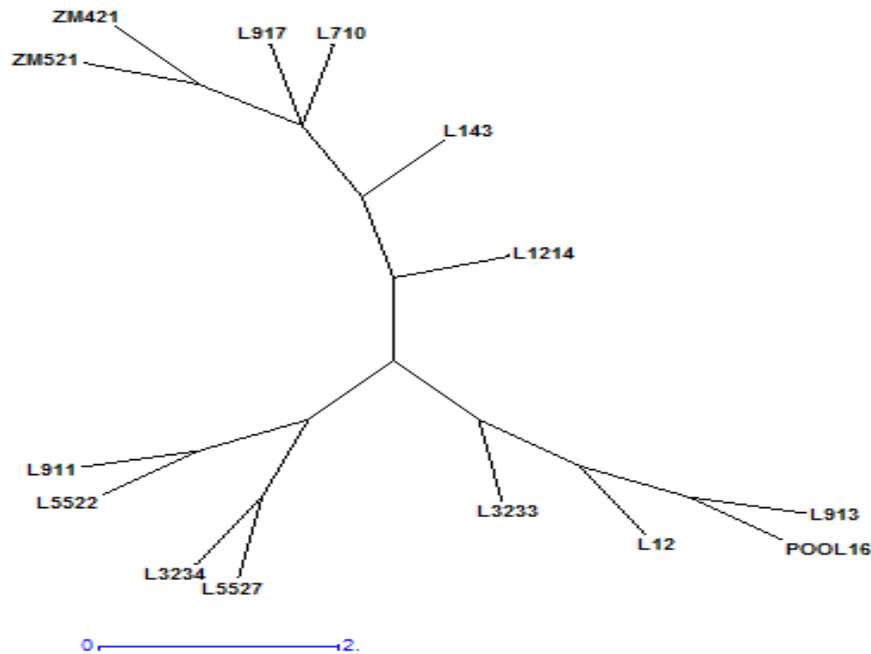
**Table 7.** Dissimilarity matrix of 14 maize genotypes based on Euclidean distance of seedling traits.

Genotype	POOL16	L12	L143	L710	L911	L913	L917	L1214	L3233	L3234	ZM421	ZM521	L5522	L5527
POOL16	0.00													
L12	0.33	0.00												
L143	0.66	0.50	0.00											
L710	1.34	1.24	0.78	0.00										
L911	0.29	0.58	0.90	1.50	0.00									
L913	0.16	0.26	0.60	1.32	0.43	0.00								
L917	2.46	2.29	1.86	1.49	2.68	2.38	0.00							
L1214	0.78	0.63	1.06	1.74	0.93	0.73	2.68	0.00						
L3233	0.30	0.35	0.68	1.29	0.43	0.38	2.50	0.68	0.00					
L3234	0.44	0.64	1.05	1.69	0.30	0.56	2.85	0.78	0.46	0.00				
ZM421	1.32	1.02	1.01	1.44	1.58	1.21	1.96	0.97	1.24	1.56	0.00			
ZM521	1.47	1.20	0.90	0.97	1.73	1.37	1.40	1.44	1.41	1.81	0.65	0.00		
L5522	0.26	0.54	0.89	1.52	0.08	0.39	2.67	0.87	0.41	0.26	1.54	1.70	0.00	
L5527	0.47	0.63	1.07	1.72	0.36	0.57	2.86	0.74	0.48	0.08	1.54	1.80	0.31	0.00



**Figure 1.** A plot of principal coordinate analysis of 14 genotypes based on the Euclidean distance of eight seedling traits.

The consensus dendrogram based on the Euclidean distance of quantitative traits (Figure 2) clustered maize genotypes into three major groups with a coefficient of 0.858. Cluster 1 consisted of L911, L5522, L3234, and L5527. Cluster 2 consisted of L3233, L12, Pool16, and L913. Cluster 3 consisted of L1214, L143, L710, L917, ZM421, and ZM521.



**Figure 2.** Dendrogram showing clustering of 14 maize genotypes.

The characteristics of the clusters are shown in Table 8. Genotypes in Clusters 1 and 3 had generally experienced reduced root net growth, while genotypes in Cluster 2 had increased net root growth. Genotypes in Cluster 2 had high SRL and high dry matter partitioning (SRDMratio) compared to those in Clusters 1 and 3. Based on these characteristics, it is apparent that genotypes in Cluster 2 possess good traits that can be used in breeding.

The root tolerance index (RTI) has been recommended as the trait to use for identifying Al-tolerant cereals in hydroponics [35]. The RTI is specific and removes the effect of genes controlling root vigor by taking the relative growth of the genotype in Al solution compared to the one without Al [35]. Therefore, ARL, RRL, and RLR were used to identify Al tolerant genotypes (Table 9). The three root traits, on average, classified seven genotypes as susceptible to Al toxicity (HS and S classes), with four genotypes being identified as tolerant (HT and T classes) and only three genotypes being intermediate (I class). Some genotypes were consistently classified in the same group, regardless of the trait used (Table 9).

**Table 8.** Cluster membership, mean, maximum, minimum, and range of seedling characteristics of each cluster.\*

Cluster	Parameters	IRL	FRL	ARL	SRL	RRL	RLR	SL	SLR	SLSDM Ratio	NOR	RDM	SDM	TDM	SRDM Ratio	SLRL Ratio	NRG
<b>Cluster 1</b> Pool16, L12, L913, L3233	Mean	2.13	12.50	10.38	717.70	114.36	26.17	5.22	-7.28	192.92	2.93	0.02	0.04	0.06	2.53	0.67	-0.18
	Max	2.68	16.47	14.66	1152.00	149.25	85.50	6.17	2.32	308.92	3.71	0.03	0.05	0.08	3.50	1.13	4.25
	Min	1.70	7.81	5.49	504.79	93.29	-1.71	3.21	-16.59	133.72	2.00	0.01	0.03	0.04	1.83	0.41	-6.04
	Range	0.98	8.66	9.17	647.21	55.96	87.21	2.96	18.91	175.20	1.71	0.02	0.02	0.04	1.67	0.72	10.29
<b>Cluster 2</b> L911, L3234, L5522, L5527	Mean	2.17	11.84	9.58	987.26	109.34	13.74	6.04	14.25	165.99	1.84	0.02	0.05	0.07	4.61	0.79	0.84
	Max	3.05	17.40	14.34	1129.04	127.58	45.58	7.48	32.09	199.25	2.17	0.03	0.06	0.10	7.73	1.25	3.46
	Min	1.45	6.53	4.68	844.00	92.02	-23.79	5.10	-1.70	136.48	1.54	0.01	0.04	0.05	2.54	0.45	-3.20
	Range	1.60	10.87	9.66	285.04	35.56	69.37	2.38	33.79	62.77	0.63	0.02	0.02	0.05	5.19	0.80	6.66
<b>Cluster 3</b> L143, L710, L917, L1214, ZM421, ZM521	Mean	1.81	11.36	9.55	466.83	116.46	29.73	7.23	-6.05	254.47	3.31	0.04	0.04	0.07	2.19	1.18	-0.24
	Max	2.35	16.50	14.31	996.63	150.21	89.03	12.25	0.24	661.89	5.08	0.06	0.06	0.10	4.92	2.97	3.51
	Min	1.13	5.70	4.56	149.13	76.64	-27.19	5.85	-14.31	111.20	1.17	0.01	0.02	0.03	0.77	0.55	-3.56
	Range	1.22	10.80	9.75	847.50	73.57	116.22	6.40	14.55	550.69	3.91	0.05	0.04	0.07	4.15	2.42	7.07

\* IRL = Initial root length, FRL = Final root length, ARL = Actual root length, SRL = specific root length, RRL = relative root length, RLR = Root length response, SL = shoot length, SLR = Shoot length response, SLSDMratio = Shoot length-shoot dry matter ratio, RDM = root dry matter, SDM = shoot dry matter, TDM = total dry matter, SRDM ratio = shoot-root dry matter ratio, NOR = number of roots, SLRL Ratio = shoot root length ratio, and NRG = net root growth.

**Table 9.** Membership index for the 14 maize genotypes based on three selected root traits.\*

Genotype	ARL		RRL		RLR		Mean	
	Score	Class	Score	Class	Score	Class	Score	Class
POOL16	0.32	S	0.34	S	0.29	S	0.32	S
L12	0.52	I	0.51	I	0.49	I	0.51	I
L143	0.38	S	0.37	S	0.34	S	0.36	S
L710	0.30	S	0.34	S	0.27	S	0.30	S
L911	0.14	HS	0.26	S	0.11	HS	0.17	HS
L913	0.34	S	0.31	S	0.32	S	0.32	S
L917	0.16	HS	0.11	HS	0.14	HS	0.14	HS
L1214	0.82	HT	0.78	T	0.83	HT	0.81	HT
L3233	0.74	T	0.82	HT	0.84	HT	0.80	HT
L3234	0.59	I	0.57	I	0.56	I	0.57	I
ZM421	0.49	I	0.76	T	0.72	T	0.66	T
ZM521	0.50	I	0.56	I	0.57	I	0.54	I
L5522	0.20	S	0.28	S	0.25	S	0.24	S
L5527	0.60	T	0.59	I	0.61	T	0.60	T

\* HS = highly susceptible, S = susceptible, I = Intermediate (Medium type), T = tolerant, HT= highly tolerant, ARL = Actual root length, RRL = relative root length, and RLR = Root length response.

## 4. Discussion

### 4.1. Correlation among Seedling Traits

Root length measurement is one of the primary criteria used for evaluation for aluminum tolerance in hydroponic conditions [36,37]. Other traits like ratio of root to shoot fresh weight [38], total dry weight of the roots, and total dry weight of the shoots have been used for identifying aluminum-tolerant genotypes. However, the inter-correlations among the traits may provide challenges for their effective utilization, as the desired trait could be positively correlated to an undesirable trait. The classification of ZM421 and ZM521 being tolerant to aluminum toxicity was unexpected as the two populations were developed for low and drought tolerance (<http://r4d.dfid.gov.uk/Project/60389/Default.aspx>, accessed 6 March 2015). This could be attributed the fact that selection for drought tolerance was sometimes done on soils with a pH below 4.5 [39,40], which could have resulted in co-selection for aluminum tolerance [34]. The observation made in this study is supported by the susceptibility of the variety, pool 16, which was developed for drought tolerance based on drought escape through early maturity. In addition, studies have also confirmed that selection for drought and low nitrogen tolerance in southern Africa has resulted in generating hybrids with wide adaptation [41]. Therefore, ZM421 and ZM521 populations should be exploited for the generation of inbred lines that are tolerant to aluminum toxicity.

Selection shapes the genetic make-up and thus targeted genetic improvement for aluminum tolerance will depend on the existence of genetic variation and identification of traits that are correlated to grain yield. Studies have shown that seminal root length and root dry weight are highly correlated to field grain yield under low and high nitrogen conditions, while total root length and root dry weight are strongly correlated to nutrient and water use efficiency in maize [42,43]. The relative

net root growth (RNRG) of maize inbred lines under hydroponic conditions is positively correlated to field performance of maize under aluminum toxicity, explaining between 24% and 35% [19]. All these research findings suggest that multiple routes can be used for stress tolerance breeding towards aluminum tolerance, and therefore each breeding program should find the optimal trait to use.

In this study, SRDM ratio was negatively correlated to all root traits (FRL, IRL, ARL, NOR, TDMm and RDM), indicating that there was a tendency for the plants to preferentially allocate resources to the roots as opposed to the shoots. However, the resources allocated to the roots were mostly used for root maintenance rather than root growth. This is supported by the significantly high negative correlations between SRL and NOR ( $r = -0.73$ ), and between SRL and RDM ( $r = -0.76$ ); the moderate negative correlation of SRL with TDM ( $r = -0.50$ ); and the weak negative correlation of SRL with SDM ( $r = -0.14$ ). Blair *et al.* (2009) observed a negative correlation between SRL and SRDM ratio in beans under aluminum toxicity. In this study, a strong positive correlation between SRL and SRDM ratio was observed, suggesting that increasing SRL will result in increasing the dry matter partitioned to the shoot rather than the roots in cereals.

Specific Root Length (SRL) is the index of root benefit to root cost, whereby root length is proportional to acquiring resources and root mass is proportional to construction and maintenance [27]. SRL integrates root length with root fineness, thereby representing the exploration and resource uptake of the plant; it has therefore been suggested as a useful trait in breeding [27,44]. Plants with high SRL build more root length for a given dry mass and are preferred as they have higher nutrient and water uptake [45]. Although high SRL is associated with high nutrient uptake, RGR, and water use efficiency, the trait has a short life span [46]. It is also important to note that high SRL is also associated with low tissue density or low diameter. Inbred lines L3233 and L1214 had high SRL and high RRL, indicating that more dry matter was allocated to root length, which could have resulted in increased uptake and thus sustained growth. The second highest inbred line for SRL was L3234, but it had intermediate RRL. This genotype (L3234), however, could have allocated more dry matter to root development such that growth was inhibited greatly, which resulted in medium aluminum tolerance. On the other hand, L911 had high SRL and low RRL, implying that more dry matter was allocated to root maintenance. In this regard, inbred lines L3233 and L1214 are potentially useful for aluminum tolerance breeding.

#### 4.2. Phenotypic and Genotypic Coefficients of Variation, Heritability, and Genetic Advance as Percent of the Mean

The genetic improvement for aluminum toxicity will depend on the amount of genetic variability and heritability of the trait. Since the genetic variation might be heritable or non-heritable, estimating the heritability of the trait is important. The observed phenotypic variability is partitioned into heritable and non-heritable components with the phenotypic and genotypic coefficient of variation, heritability, and genetic advance [47]. The broad sense heritability ( $H^2_{bs}$ ) provides information on the relative magnitude of genetic and environmental variation [48]. Therefore,  $H^2_{bs}$  is of little help to breeders if the genetic gain (GG) of the trait is not known as it provides the breeder with the expected genetic progress made during breeding. In the present study, most of the traits had high heritability coupled with high genetic gain, suggesting a preponderance of additive gene effects [49,50]. Therefore, the

traits with high  $H^2_{bs}$  and GG can be used for selecting for aluminum tolerance [51,52]. Based on these propositions, all traits are potentially useful as selection traits except for RRL. Furthermore, all the traits in this study had almost equal GCV and PCV, which is acceptable, although breeders desire higher GCV than PCV [49].

#### 4.3. Principal Component Analysis and Cluster Analysis

Many root traits and their responses to aluminum toxicity have been used in breeding and selection of tolerant genotypes in cereals [53,54]. Principal component analysis can be used to identify the most influential traits, so first two PCs were used to identify influential traits. All important traits (loadings  $\geq 0.30$ ) in PC1 and PC2 were considered influential. The inter-relationships between the traits were considered and six traits out of 13 were identified as potential for selection. These traits had high heritability and genetic gain; however, the order of importance based on genetic gain are NRG, SLR, SLRratio, FRL, SRL, and RRL.

The dendrogram based on the seedling traits allowed for the categorization of the 14 genotypes into three main clusters. Clear distinctions were discerned between inbreds L3233 and L5522, the heterotic patterns of southern Africa. The three populations were also clearly separated, with ZM421 and ZM521 in one cluster and pool16 in another cluster. The population pool 16 represents early breeding for drought tolerance through escape. On the other hand, ZM421 and ZM521 represent recent developments in breeding for multiple stress tolerance.

#### 4.4. Implications on Plant Breeding in Zambia

Substantial variation in maize inbred line response to aluminum concentration was observed as evidenced by the highly significant genotype by environment interaction. This indicates that the comprehensive maize hybrid breeding program that was used in the early years [52] had resulted in co-selection for aluminum tolerance, especially when the breeding focused on developing drought-tolerant genotypes in the late 1980s [53]. Studies have shown that breeding for drought tolerant results in aluminum tolerance [54]. Therefore, co-selection for aluminum tolerance could have occurred, just as Masole and Gumbo (1980) reported good genetic gain for drought tolerance and earliness during breeding. Despite this, the traits that confer tolerance to aluminum toxicity were not highly concentrated in the inbred lines as selection for aluminum tolerance was not done. Now that there are many hybrids on the market, breeding for specific adaptation or ecological regions is the major focus of the Zambian breeding program. The observed variation for aluminum tolerance in maize germplasm can be enhanced for breeding. Based on the overall classification of maize inbred lines, the mode of gene action can be studied by making crosses among the different tolerance classes. Furthermore, populations can be formed by inter-mating the highly tolerant, tolerant, and intermediate inbred lines. The resulting populations can then be improved for yield performance under Al conditions. These populations would later be used for extracting inbred lines. In addition, exotic inbred lines that are tolerant to low pH or Al concentration can be systematically introgressed in these inbreds.

## 5. Conclusions

The observed genetic variation of maize inbred lines in response to aluminum concentration can be exploited for developing aluminum tolerant hybrids. The high heritability and genetic advance over mean observed in most of the root traits indicates that additive gene action is at play. Therefore, selection for these traits would result in genetic gain and breeding progress. NRG, SLR, and RLR with high heritability and genetic gain are traits that should be used for selection. NRG had the highest genetic gain followed by SLR, supporting the superiority of root traits in evaluating for aluminum tolerance. The root tolerance index that incorporates many traits should be used to identify tolerant genotypes. The two populations found to be tolerant to aluminum toxicity, ZM421 and ZM521, should be used in breeding works.

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## Author Contributions

All Authors contributed equally to the work.

## Conflicts of Interest

The authors declare no conflict of interest.

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