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RAPID SCREENING OF ALUMINUM TOLERANCE IN MAIZE USING HEMATOXYLIN STAINING

SKRINING CEPAT TOLERANSI ALUMINIUM PADA JAGUNG MENGGUNAKAN PEWARNAAN HEMATOKSILIN

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ABSTRACT

Developing aluminum (Al^{3+})-tolerant maize genotypes is an important objective in breeding programs for acidic soils. Rapid and reliable screening methods are required to identify tolerant genotypes at the seedling stage. This study evaluated the response of ten maize lines to Al^{3+} stress using a nutrient solution containing 10 ppm Al^{3+} combined with hematoxylin staining. The experiment was conducted from March to May 2025 at the Plant Breeding and Seed Science Laboratory, Faculty of Agriculture, Hasanuddin University, Makassar, using a completely randomized design (CRD) with three replications. Data were analyzed using analysis of variance, heritability, correlation, and cluster analysis. Hematoxylin staining classified the genotypes into four tolerance categories, consisting of 10% very tolerant, 30% tolerant, 60% comparatively tolerant, and no sensitive genotypes. Cluster analysis grouped the ten maize lines into three major clusters, indicating phenotypic variation in their responses to Al^{3+} stress. Among the evaluated lines, G1 exhibited the highest level of tolerance based on hematoxylin staining and cluster analysis. These findings indicate that hematoxylin staining provides a useful preliminary approach for distinguishing phenotypic responses among maize lines exposed to aluminum stress.

KATA KUNCI:
Keragaman genetik, morfologi akar, pemuliaan tanaman, seleksi dini, tanah masam.

ABSTRAK

Pengembangan galur jagung toleran terhadap cekaman aluminium (Al^{3+}) merupakan salah satu tujuan penting dalam program pemuliaan tanaman pada lahan masam. Metode skrining yang cepat dan andal diperlukan untuk mengidentifikasi genotipe toleran sejak fase bibit. Penelitian ini bertujuan mengevaluasi respons sepuluh galur jagung terhadap cekaman Al^{3+} menggunakan larutan nutrisi yang mengandung 10 ppm Al^{3+} dan pewarnaan hematoksilin. Penelitian dilaksanakan pada Maret–Mei 2025 di Laboratorium Pemuliaan Tanaman dan Ilmu Benih, Fakultas Pertanian, Universitas Hasanuddin, Makassar, menggunakan Rancangan Acak Lengkap dengan tiga ulangan. Data dianalisis menggunakan analisis ragam, heritabilitas, korelasi, dan analisis klaster. Hasil pewarnaan hematoksilin mengelompokkan genotipe menjadi empat kategori toleransi, yaitu 10% sangat toleran, 30% toleran, 60% agak toleran, dan tidak ditemukan genotipe peka. Analisis klaster mengelompokkan sepuluh galur ke dalam tiga klaster utama yang menunjukkan adanya variasi respons terhadap cekaman Al^{3+}. Di antara galur yang diuji, G1 menunjukkan tingkat toleransi tertinggi berdasarkan hasil pewarnaan hematoksilin dan analisis klaster. Temuan ini menunjukkan bahwa pewarnaan hematoksilin dapat digunakan sebagai pendekatan awal yang bermanfaat untuk membedakan respons fenotipik antar galur jagung yang terpapar cekaman aluminium.

1. INTRODUCTION

Maize (*Zea mays* L.) is one of the world's most important cereal crops, serving as a staple food, livestock feed, and industrial raw material. The increasing demand for maize driven by population growth and industrial development requires continuous improvement in productivity under diverse environmental conditions. However, maize production in tropical regions is frequently constrained by acidic soils, which occupy approximately 30–40% of the world's arable land. Under acidic conditions (pH <5.5), aluminum (Al^{3+}) becomes soluble and highly toxic to plants, representing one of the most limiting abiotic stresses for maize production. Aluminum toxicity inhibits root elongation, disrupts nutrient and water uptake, induces oxidative stress, and ultimately reduces crop productivity (Herawati *et al*., 2023). Plant adaptation to aluminum toxicity is a complex trait involving morphological, physiological, biochemical, and molecular mechanisms (Bhukya *et al*., 2024). Aluminum-tolerant genotypes generally maintain root growth through the exclusion of Al^{3+} from root tissues by releasing organic acids such as citrate and malate or through internal detoxification mechanisms (Chakraborty *et al*., 2024). Because these mechanisms are genetically controlled, breeding aluminum-tolerant maize cultivars has become one of the most effective strategies for improving productivity on acidic soils (Guo *et al*., 2024).

The success of breeding programs largely depends on the availability of rapid, reliable, and inexpensive screening methods capable of distinguishing tolerant and susceptible genotypes at the early growth stage (Ofoe *et al*., 2023). Several screening approaches have been developed, including hydroponic culture, root elongation assays, nutrient solution experiments, physiological and biochemical measurements, molecular marker analysis, and hematoxylin staining. Hydroponic systems allow precise environmental control but require considerable time and laboratory facilities (Ur Rahman *et al*., 2024). Physiological and biochemical analyses provide detailed information regarding aluminum detoxification mechanisms but are relatively expensive and unsuitable for screening large breeding populations (Channapur *et al*., 2026). Likewise, molecular marker-assisted selection offers high accuracy but requires specialized equipment and validated genetic markers, limiting its routine application in many breeding programs (Liu *et al*., 2026). Among these methods, hematoxylin staining remains one of the simplest and most economical techniques for early screening of aluminum tolerance. Hematoxylin reacts with aluminum accumulated in root tissues, producing staining intensity proportional to aluminum accumulation (Huang & Ma, 2025). Consequently, tolerant genotypes generally exhibit lighter staining than susceptible ones because they accumulate less aluminum in root tissues (Li & Tian, 2023). Previous studies have demonstrated that hematoxylin staining is closely associated with root growth responses under aluminum stress and can effectively distinguish tolerant and susceptible maize genotypes (Le Poder *et al*., 2022).

Although hematoxylin staining has been widely used for several decades, its application still requires continuous evaluation, particularly when applied to newly developed breeding materials (Szőke *et al*., 2024). The relationship between staining intensity and aluminum tolerance may vary depending on the genetic background of the evaluated germplasm, environmental conditions, and scoring criteria used (Fang *et al*., 2024). Moreover, most previous studies primarily focused on visual staining responses or root elongation (S. Yang *et al*., 2022), whereas relatively few studies have integrated hematoxylin staining with seedling morphological traits and multivariate statistical approaches to evaluate the diversity of aluminum tolerance among tropical maize breeding lines.

This limitation represents an important research gap because breeding decisions require not only rapid visual screening but also supporting information regarding morphological performance and genetic relationships among candidate genotypes (He *et al*., 2024). Integrating hematoxylin staining with quantitative seedling traits and cluster analysis may improve the reliability of early selection (Oliveira *et al*., 2016; P *et al*., 2025a) and facilitate the identification of phenotypically distinct materials with potential aluminum tolerance suitable for hybrid development.

Therefore, the novelty of this study lies in the integration of hematoxylin staining, seedling morphological evaluation, heritability analysis, correlation analysis, and hierarchical cluster analysis (Dossa *et al*., 2023) to characterize phenotypic responses associated with aluminum tolerance of newly developed tropical maize lines. This integrated approach provides a more comprehensive evaluation than visual staining alone (Asfawu *et al*., 2024) and contributes additional information for early-stage selection in maize breeding programs targeting acidic soils.

Accordingly, this study aimed to evaluate the response of ten maize lines to aluminum stress using hematoxylin staining combined with seedling morphological traits and multivariate analysis (Mitreka *et al*., 2026) to identify promising genotypes for breeding programs on acidic soils. It was hypothesized that significant variation exists among maize lines in their response to aluminum toxicity (Vasconcellos *et al*., 2021) and that hematoxylin staining integrated with morphological characterization and cluster analysis can effectively discriminate aluminum-tolerant genotypes at the seedling stage.

2. MATERIALS AND METHOD

2.1 Place and Time

The experiment was conducted from March to June 2025 at the Plant Breeding and Seed Science Laboratory, Faculty of Agriculture, Hasanuddin University, Makassar, South Sulawesi, Indonesia (5°07′50″ S; 119°29′07″ E; 9 m above sea level). The experiment was carried out under controlled laboratory conditions at a temperature of 28 ± 2 °C, relative humidity of 70 ± 5 %, a 14 h light/10 h dark photoperiod**,** and a light intensity of approximately 350 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps.

2.2 Plant Materials and Chemicals

The study evaluated ten transgressive maize lines, namely G2×P2-1 (G1), G2×P2-2 (G2), G2×P5-1 (G3), G3×P1-1 (G4), G6×P1-1 (G5), G8×P4-1 (G6), G9×P2-1 (G7), G11×P4-1 (G8), G12×P1-2 (G9), and G24×P2-1 (G10). All chemicals used in this study were of analytical grade and purchased from Merck KGaA (Darmstadt, Germany)**.** Aluminum stress was imposed using aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$; ≥99 % purity)**.** Hematoxylin staining was performed using hematoxylin (≥95%) and potassium iodide (KI; ≥99%)**.** The pH of the nutrient solution was adjusted using 1 N HCl and 1 N NaOH**.** The nutrient solution consisted of 1.0 mM KCl, 1.5 mM NH_4NO_3, 1.0 mM $CaCl_2 \cdot 2H_2O$, 0.50 mM $Mg(NO_3)_2 \cdot 6H_2O$, 0.20 mM $MgSO_4 \cdot 7H_2O$, 45 µM KH_2PO_4, 77 µM Fe-EDTA, 33 µM H_3BO_3, 11.8 µM $MnCl_2 \cdot 4H_2O$, 3.06 µM $ZnSO_4 \cdot 7H_2O$, 0.80 µM $CuSO_4 \cdot 5H_2O$, and 1.07 µM $Na_2MoO_4 \cdot 2H_2O$**.**

2.3 Experimental Design

The experiment was arranged in a Completely Randomized Design (CRD) consisting of ten maize lines as treatments with three biological replications, resulting in 30 experimental units. Each experimental unit consisted of one plastic container containing 2 L of nutrient solution and ten uniform maize seedlings**,** resulting in a total of 300 seedlings used throughout the experiment. Five seedlings from each experimental unit were used for hematoxylin staining, while the remaining five seedlings were used for morphological and biomass evaluations. The mean value of the seedlings within each container was considered one experimental unit for statistical analysis.

2.4 Aluminum Treatment

The aluminum stock solution was prepared by dissolving $AlCl_3 \cdot 6H_2O$ in distilled water and subsequently adding it to the nutrient solution to obtain a final concentration of 10 mg Al L^{-1}. The concentration of 10 ppm Al^{3+} was selected because it has been widely used in aluminum tolerance screening studies in maize and has been demonstrated to effectively inhibit root growth while producing distinct differences in hematoxylin staining intensity without causing complete seedling mortality (Zhao *et al*., 2022). The pH of the nutrient solution was maintained at 4.0 ± 0.1 (Zishiri *et al*., 2022) using 1 N HCl or 1 N NaOH, and was monitored twice daily using a calibrated digital pH meter. The solution volume was maintained at 2 L by daily addition of distilled water, whereas the nutrient solution was completely renewed every three days. Throughout the experiment, the nutrient solution was continuously aerated using an aquarium air pump to maintain adequate dissolved oxygen levels.

2.5 Seed Germination and Hematoxylin Staining

Seeds were surface-sterilized with 1% sodium hypochlorite (NaOCl) for 5 min, followed by three rinses with sterile distilled water. Seeds were germinated on moist filter paper placed in 9 cm Petri dishes for seven days. The seedlings were subsequently transferred into the aluminum-containing nutrient solution. After three days of aluminum treatment, five seedlings were randomly selected for hematoxylin staining. Seedlings were immersed in 0.2% hematoxylin solution containing 0.02% potassium iodide (KI) for 20 min under gentle shaking (20 rpm), followed by rinsing with distilled water for 15 min (Ding *et al*., 2022). Root staining intensity was visually scored as follows: 1 = very tolerant (no staining), 2 = tolerant (light staining), 3 = comparatively tolerant (moderate staining), and 4 = susceptible (dark staining).

2.6 Measurements

The observed variables included root length (RL), shoot length (SL), number of root branches (NRB), fresh root weight (FRW), fresh shoot weight (FSW), dry root weight (DRW), dry shoot weight (DSW), and hematoxylin staining score. Fresh biomass was measured using an analytical balance with a precision of 0.001 g**.** The samples were subsequently oven-dried at 70 °C for 72 h until constant weight before determining dry biomass.

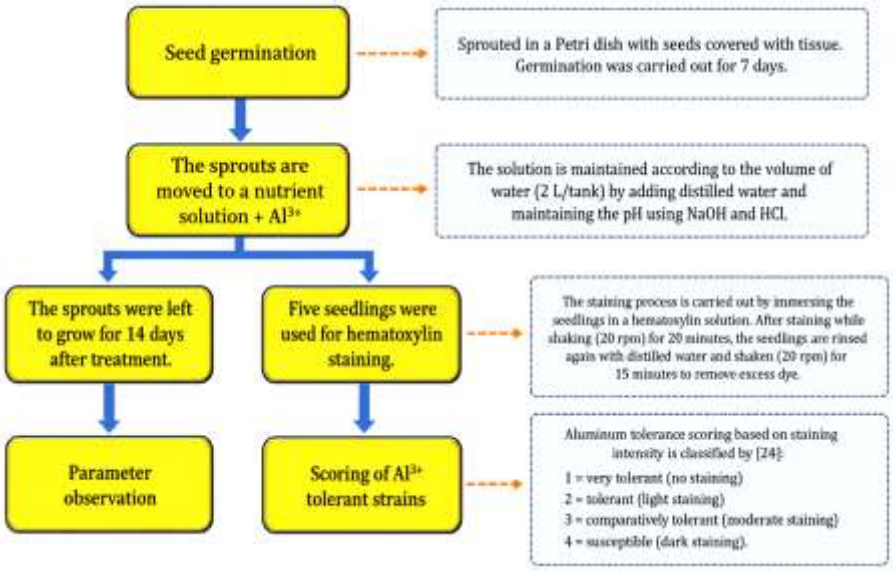


Figure 1. Experimental workflow used for aluminum tolerance screening in maize.

2.7 Statistical Analysis

Prior to statistical analysis, the residuals were tested for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene's test. Quantitative traits were analyzed using analysis of variance (ANOVA) based on the Completely Randomized Design (CRD) model:

$$Yij=\mu+Gi+\varepsilon ij \quad (1)$$

Note: YijYij = observation of the *i*-th maize line in the *j*-th replication; μμ = overall mean; GiGi = fixed effect of genotype; εijεij = experimental error.

Whenever significant differences among maize lines were detected ($P < 0.05$), mean comparisons were performed using Tukey's Honest Significant Difference (HSD) test at the 5% significance level. Broad-sense heritability (H^2) was estimated from the genotypic and phenotypic variance components obtained from ANOVA, whereas relationships among measured traits were evaluated using Pearson's correlation analysis. Prior to hierarchical cluster analysis, all variables were standardized to zero mean and unit variance to eliminate differences in measurement scales. Cluster analysis was performed using the squared Euclidean distance and the average-linkage method because this approach provides a balanced compromise between within-cluster compactness and robustness to extreme observations. The number of clusters was determined by visual inspection of the dendrogram at the selected rescaled-distance threshold. All statistical analyses were conducted using R software version 4.4.1 (R Core Team, Vienna, Austria) through RStudio version 2024.04.2.

3. RESULTS AND DISCUSSION

3.1 Morphological and Biomass Responses of Maize Genotypes under Aluminum Stress

Overall, all graphs demonstrated significant variation in growth performance among genotypes across the evaluated parameters, including root length, shoot length, number of root branches, fresh root weight, fresh shoot weight, dry root weight, and dry shoot weight. Genotypes with a more developed root system generally exhibited greater shoot growth and biomass accumulation. The different letters displayed on each graph indicate significant differences among genotypes based on the post-hoc multiple comparison test. These findings suggest that genetic factors play a crucial role in determining plant growth performance, indicating that these growth-related traits can serve as reliable selection criteria for identifying genotypes with superior vigor and greater adaptive potential. Genotype G1 consistently exhibited superior root length and vigorous biomass accumulation, whereas G2 and G10 showed relatively lower performance.

3.2 Distribution of Aluminum Tolerance Based on Hematoxylin Staining

Table 1 summarizes the distribution of maize lines according to aluminum tolerance categories, whereas Figure 3 illustrates the percentage of each category. The tolerance of the genotypes was classified into four categories: very tolerant, tolerant, comparatively tolerant, and sensitive. Based on the classification results displayed, 10% of the genotypes were categorized as very tolerant, while 30% were included in the tolerant group. The majority of the genotypes (60%) were classified as comparatively tolerant, while there were no genotypes categorized as sensitive to aluminum. The results showed that the majority of genotypes had moderate ability to survive in environments with high aluminum levels, but only a small number truly display optimal resilience. The absence of sensitive genotypes was a positive indication that all tested genetic material had adaptive potential to acidic soil conditions (Sopandie, 2025). This information is very relevant in the efforts of plant breeding for marginal lands, as genotypes with the categories of very tolerant and tolerant can be used as genetic donors in the development of superior varieties resistant to aluminum stress.

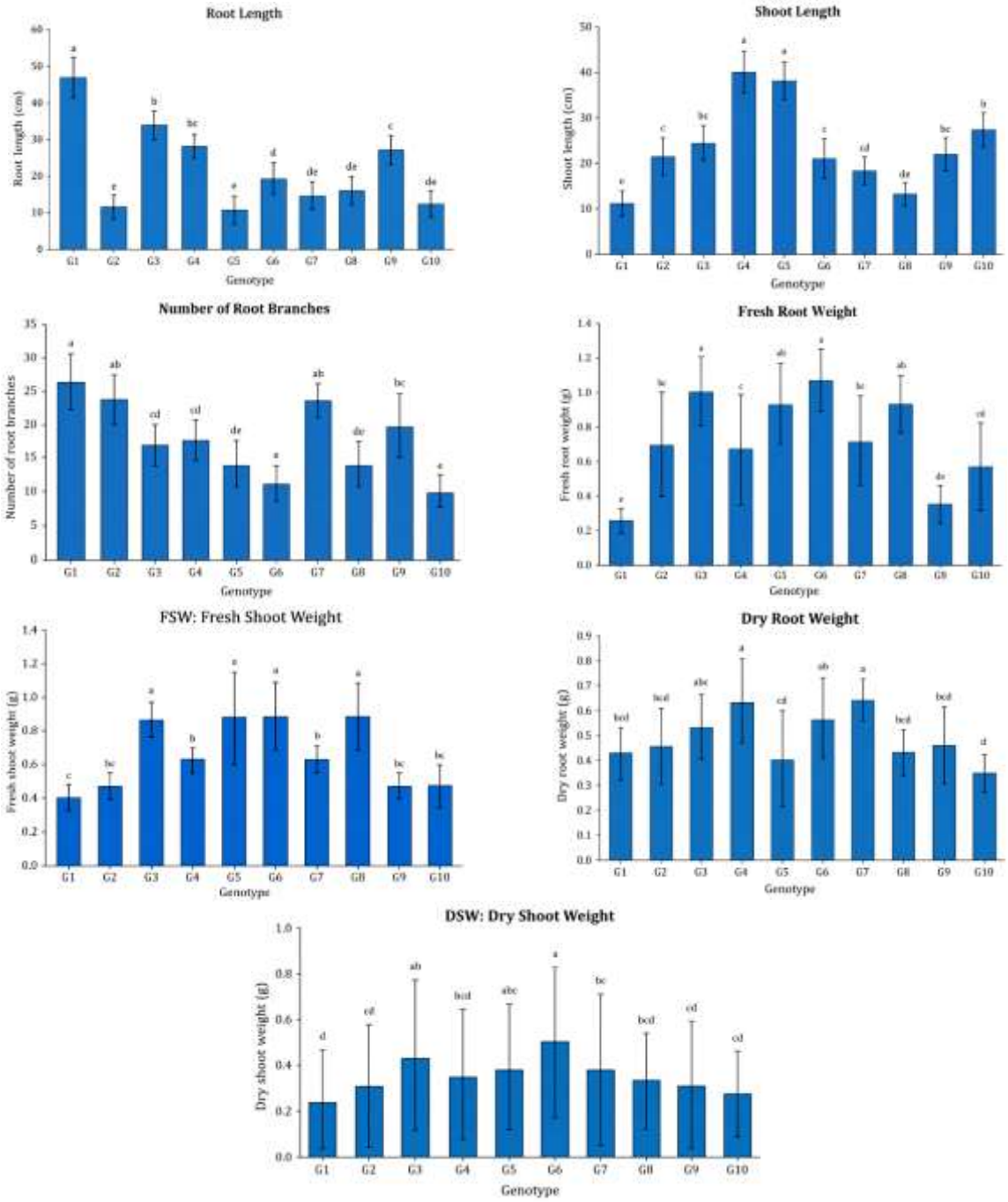


Figure 2. Performance of ten maize genotypes for each observed trait under aluminum stress conditions. Data are presented as mean ± standard deviation (SD) based on three replications. Different lowercase letters above the bars indicate statistically significant differences among genotypes according to the Tukey's Honest Significant Difference (HSD) test at the 5% significance level.

Hematoxylin staining is widely recognized as a rapid and reliable phenotypic screening method for evaluating aluminum (Al) tolerance because hematoxylin forms a stable blue-purple complex with Al ions accumulated in root tissues (Simpkins, 2020). Consequently, roots with higher Al accumulation exhibit darker staining, whereas roots excluding or detoxifying Al display weaker staining. This staining response has been extensively used as an indirect indicator of aluminum

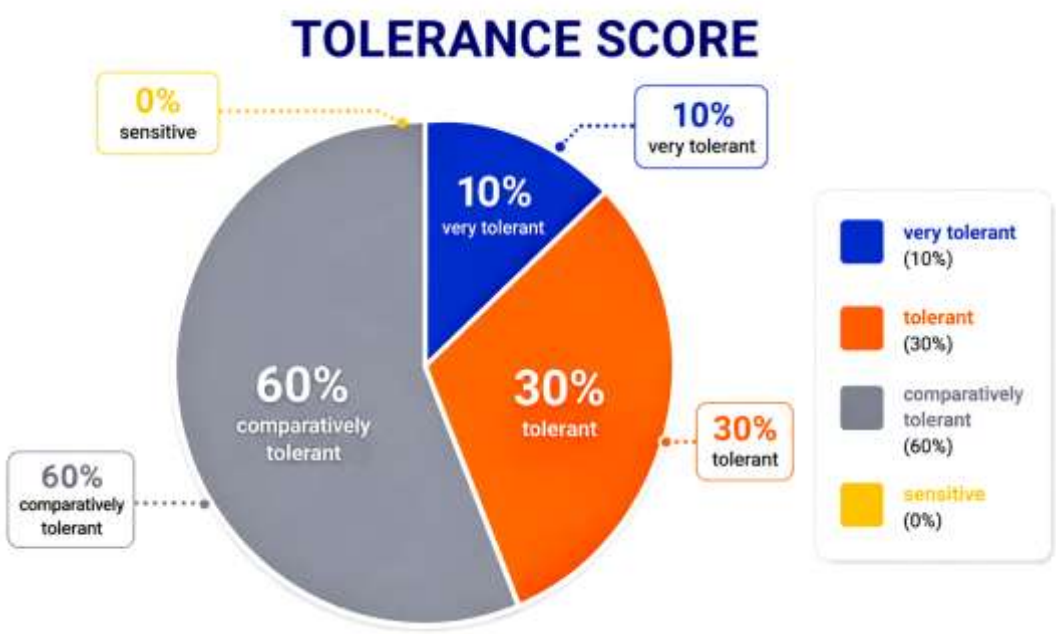
accumulation in cereals, particularly during the seedling stage, where Al toxicity primarily affects root tissues (Kochian *et al*., 2015; Ma, 2007; Ryan *et al*., 2011).

The relatively lighter staining observed in several maize lines in the present study suggests lower Al accumulation in root tissues and therefore a relatively greater tolerance to Al stress. Conversely, darker staining indicates greater Al accumulation, which is generally associated with increased sensitivity to aluminum toxicity. Nevertheless, hematoxylin staining reflects only the phenotypic consequence of Al accumulation and should not be interpreted as direct evidence of the underlying physiological or molecular mechanisms. Confirmation of aluminum tolerance requires complementary physiological, biochemical, or molecular analyses (P *et al*., 2025b).

Aluminum toxicity primarily inhibits root elongation by rapidly restricting cell division and cell expansion within the root apex. Aluminum ions bind strongly to negatively charged components of the cell wall and plasma membrane, reducing cell wall extensibility and disrupting nutrient and water uptake. Consequently, root elongation is inhibited within a few hours after Al exposure (Y. Yang *et al*., 2024), whereas reductions in shoot growth generally occur as secondary effects resulting from impaired root function (Kochian *et al*., 2015; Ryan *et al*., 2011). Therefore, root-related traits are commonly regarded as reliable indicators for evaluating aluminum tolerance during early plant development (Mariano & Keltjens, 2004).

Table 1. Distribution of Maize Lines Based on Aluminum Tolerance Categories

Tolerance category	Staining score	Number of genotypes	Percentage
Very tolerant	1	1	10
Tolerant	2	3	30
Comparatively tolerant	3	6	60
Susceptible	4	0	0



Note: The pie chart shows the distribution of genotype percentage based on the level of tolerance to aluminum stress, categorized into four groups: very tolerant (10%), tolerant (30%), comparatively tolerant (60%), and sensitive (0%).

Figure 3. Percentage Distribution of Aluminum Tolerance Categories

Table 2. Squared Euclidean Distance Matrix among Maize Lines under Aluminum Stress

Case	Squared Euclidean Distance									
	1:G1	**2:G2**	**3:G3**	**4:G4**	**5:G5**	**6:G6**	**7:G7**	**8:G8**	**9:G9**	**10:G10**
1:G1	0.000	13.649	27.085	24.881	38.450	41.092	20.383	24.744	6.503	21.606
2:G2	13.649	0.000	13.716	11.648	13.328	17.960	4.276	8.982	3.804	8.651
3:G3	27.085	13.716	0.000	7.975	8.417	3.550	9.129	7.380	13.547	19.561
4:G4	24.881	11.648	7.975	0.000	11.231	13.485	8.248	16.492	9.385	16.017
5:G5	38.450	13.328	8.417	11.231	0.000	8.597	15.672	7.768	16.484	10.196
6:G6	41.092	17.960	3.550	13.485	8.597	0.000	11.513	7.482	20.295	21.129
7:G7	20.383	4.276	9.129	8.248	15.672	11.513	0.000	10.534	7.981	18.510
8:G8	24.744	8.982	7.380	16.492	7.768	7.482	10.534	0.000	11.544	9.892
9:G9	6.503	3.804	13.547	9.385	16.484	20.295	7.981	11.544	0.000	7.229
10:G10	21.606	8.651	19.561	16.017	10.196	21.129	18.510	9.892	7.229	0.000

3.3 Euclidean Distance Matrix

The Euclidean distance matrix was used to quantify the phenotypic similarity among maize lines based on all measured traits following exposure to aluminum (Al^{3+}) stress. Smaller Euclidean distance values indicate greater phenotypic similarity between genotypes, whereas larger values indicate greater phenotypic dissimilarity. The analysis revealed that the squared Euclidean distances among the evaluated maize lines ranged from 3.550 to 41.092 (Table 2). The smallest distance was observed between G3 and G6 (3.550), followed by G2 and G9 (3.804) and G2 and G7 (4.276). These relatively small distances indicate that the corresponding genotype pairs exhibited similar phenotypic responses to aluminum stress based on the measured traits. In contrast, the largest squared Euclidean distance was recorded between G1 and G6 (41.092), followed by G1 and G5 (38.450) and G1 and G3 (27.085). These relatively large distances indicate greater phenotypic dissimilarity between the respective genotype pairs under the same aluminum stress conditions.

Overall, the variation in squared Euclidean distance values indicates the presence of phenotypic diversity among the evaluated maize lines (Wang *et al*., 2024). These similarity relationships provide the basis for the hierarchical cluster analysis presented in the subsequent section. However, squared Euclidean distance values describe only the degree of phenotypic similarity derived from the measured traits and should not be interpreted as evidence of the underlying physiological, genetic, or molecular mechanisms responsible for aluminum tolerance.

3.4 Cluster Analysis

Hierarchical cluster analysis using the Average Linkage method was performed to classify the maize lines according to their phenotypic similarity based on all measured traits following aluminum (Al^{3+}) treatment. The clustering results are presented as a dendrogram (Figure 4), illustrating the similarity relationships among the evaluated genotypes based on Euclidean distance. The dendrogram separated the ten maize lines into three major clusters**.** Cluster I consisted of G2, G4, G7, G9, and G10, whereas Cluster II comprised G3, G5, G6, and G8. In contrast, G1 formed an independent cluster and was separated from the remaining genotypes at a relatively greater Euclidean distance. The clustering pattern indicates that genotypes grouped within the same cluster exhibited relatively similar phenotypic responses based on the measured traits, whereas genotypes connected by longer branches showed greater phenotypic dissimilarity.

The distinct separation of G1 indicates that its overall phenotypic response differed from that of the remaining maize lines under aluminum stress. However, hierarchical clustering describes only the similarity relationships among the evaluated genotypes and should not be interpreted as evidence of physiological, genetic, or molecular mechanisms underlying aluminum tolerance (Miftahudin *et al*., 2007)**.**

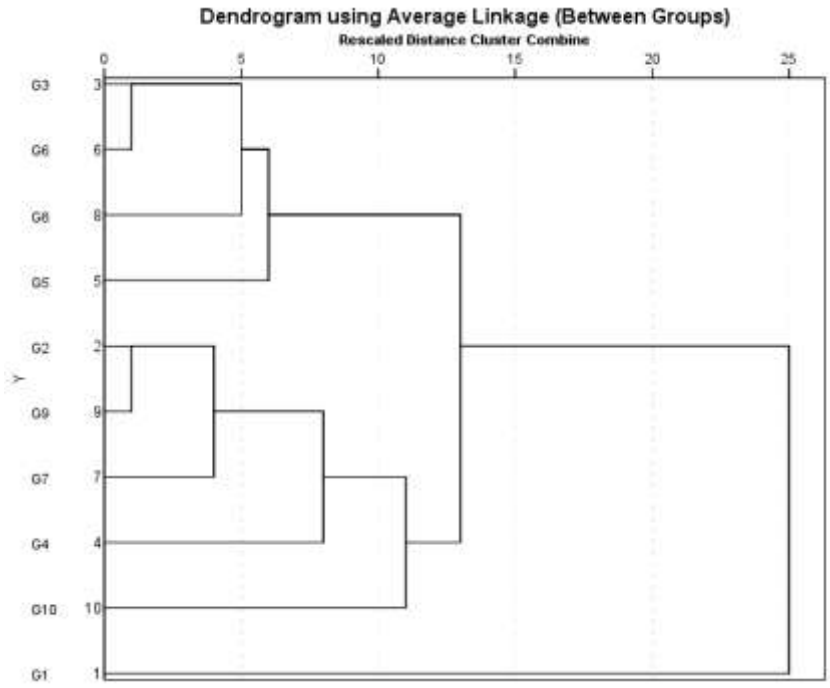


Figure 4. Hierarchical Cluster Dendrogram of Maize Lines under Aluminum Stress

Therefore, the clustering results provide an overview of phenotypic diversity among the evaluated maize lines and may serve as supporting information for genotype selection in maize breeding programs targeting aluminum-stress environments (Bernardeli *et al*., 2025).

3.5 Analysis of Variance and Heritability

Analysis of variance revealed that all measured growth traits differed significantly ($P < 0.01$) among the evaluated maize lines under aluminum stress (Table 3). These results indicate substantial phenotypic variation among the tested genotypes, suggesting that the measured traits are appropriate for evaluating aluminum tolerance (Giaveno & Miranda Filho, 2000).

Broad-sense heritability estimates indicated that most traits exhibited moderate to high heritability. Root length showed the highest heritability ($H^2 = 0.91$), followed by shoot length (0.86), fresh root weight (0.76), number of root branches (0.73), and fresh shoot weight (0.72). In contrast, root dry weight and shoot dry weight exhibited relatively lower heritability values (0.48 for both traits).

The relatively high heritability observed for several growth traits indicates that phenotypic variation among the evaluated maize lines was predominantly associated with genetic differences rather than environmental variation under the experimental conditions. From a breeding perspective, traits exhibiting relatively high heritability together with biological relevance to aluminum stress may serve as useful auxiliary criteria during early-stage screening (Salamah *et al*., 2017). However, heritability alone is insufficient to justify these traits as direct selection criteria because aluminum tolerance is a complex quantitative trait controlled by multiple physiological mechanisms (Setyowidianto *et al*., 2017). Selection decisions should therefore integrate multiple phenotypic traits (Habiba *et al*., 2022) and ideally be supported by estimates of genetic advance and subsequent field validation (Dong *et al*., 2024).

Table 3. Analysis of Variance (ANOVA) and Broad-Sense Heritability Estimates

character	Treatment	VG	VP	CV (%)	H2 (%)
Root Length	30.66**	129.11	142.17	16.0	0.91
Shoot Length	18.80**	78.55	91.79	15.0	0.86
Number of Root Branches	9.18**	29.87	40.84	19.0	0.73
fresh root weight	10.47**	0.06	0.09	20.0	0.76
Fresh Shoot Weight	8.67**	0.03	0.05	18.0	0.72
Dry Root Weight	3.78**	0.01	0.01	18.0	0.48
Dry Shoot Weight	3.73**	0.00	0.01	19.0	0.48

Notes: **: significant at α = 1%, VG: Variance of genotypes, VP: Variance of phenotypes, CV: Coefficient of variation, H: Heritability.

Table 4. Pearson Correlation Coefficients among Growth Traits.

	RL	SL	NRB	FRW	FSW	DRW	DSW
RL	1.00	-0.16ns	0.47**	-0.39*	-0.25 ns	0.07 ns	-0.08 ns
SL		1.00	-0.23 ns	0.15 ns	0.15 ns	0.10 ns	0.09 ns
NRB			1.00	-0.39*	-0.45*	0.20 ns	-0.06 ns
FRW				1.00	0.78**	0.20 ns	0.34 ns
FSW					1.00	0.08 ns	0.17 ns
DRW						1.00	**0.62****
DSW							1.00

Notes: ns: not significant, *: significant effect, **: highly significant effect, RL: root length, CL: Shoot Length, NRB: number of root branches, FRW: fresh root weight, FSW: fresh shoot weight, DRW: dry root weight, DSW: dry shoot weight.

3.6 Pearson Correlation Analysis

Pearson correlation analysis was performed to evaluate the relationships among maize growth traits under aluminum stress (Table 4). Most trait combinations exhibited weak to moderate correlations, whereas several trait pairs showed statistically significant relationships.

A highly significant positive correlation ($P < 0.01$) was observed between fresh root weight and fresh shoot weight ($r = 0.78$), as well as between root dry weight and shoot dry weight ($r = 0.62$). These positive associations indicate that increased root biomass tended to be accompanied by increased shoot biomass under the experimental conditions. In contrast, the number of root branches showed significant negative correlations with fresh root weight ($r = -0.39$) and fresh shoot weight ($r = -0.45$), indicating that an increase in root branching was not necessarily associated with greater biomass accumulation under aluminum stress. The remaining trait combinations were not significantly correlated ($P > 0.05$), suggesting relatively weak linear relationships among those variables within the evaluated population. Overall, biomass-related traits exhibited the strongest (Mishra *et al*., 2024) and most consistent associations among the measured characters. Pearson correlation analysis describes the linear association between variables but does not imply causation (Luz *et al*., 2024). Therefore, these relationships should be interpreted together with the results of variance analysis, heritability estimates, and other phenotypic evaluations when assessing genotype responses to aluminum stress.

The correlation analysis provides additional biological insights into the relationships among growth traits under aluminum stress. The significant positive correlation between fresh root weight and fresh shoot weight indicates that maize lines capable of maintaining root biomass also tended to maintain shoot growth (Zhang *et al*., 2024). Since roots represent the primary target of aluminum toxicity (Badu-Apraku *et al*., 2013), better root development likely contributes to improved water and nutrient acquisition (Akinwale *et al*., 2014), thereby supporting shoot biomass accumulation. Similar relationships have also been reported in previous studies evaluating

aluminum tolerance in maize and other cereal crops (Sang *et al*., 2022). In contrast, the absence of significant correlations between root length and shoot length suggests that these traits responded independently under the experimental conditions. Root elongation is directly affected by Al toxicity (Giannakoula *et al*., 2008), whereas shoot elongation is influenced by multiple physiological processes (Sihaloho *et al*., 2015) and generally responds later than root growth. Consequently, shoot length alone may not adequately reflect the degree of aluminum tolerance during early seedling development.

Overall, the present study demonstrates that hematoxylin staining provides an effective preliminary screening approach for distinguishing phenotypic variation among maize lines exposed to aluminum stress. Nevertheless, further evaluation under acidic soil conditions together with physiological, biochemical, and molecular investigations is required to verify the stability of the observed responses and to elucidate the mechanisms responsible for aluminum tolerance

4. CONCLUSIONS

This study successfully evaluated the responses of ten maize lines to aluminum stress using hematoxylin staining under nutrient solution supplemented with Al^{3+} (10 ppm) as an early screening approach for aluminum tolerance. Most lines were classified as comparatively tolerant (60%), followed by tolerant (30%) and very tolerant (10%), while no susceptible lines were identified. Cluster analysis grouped the tested lines into three major clusters, with line G1 clearly separated from the others, indicating a distinct phenotypic response under the experimental conditions. The relatively high heritability estimates observed for several growth-related traits, particularly root length and shoot length, indicate that variation in these traits was predominantly influenced by genetic factors under the experimental conditions. However, high heritability alone is insufficient to justify these traits as indirect selection criteria for aluminum tolerance. Their effectiveness as selection indicators should be further verified through estimates of genetic advance, selection response, and validation under field conditions**.** Therefore, the present findings provide preliminary information for identifying promising parental materials and serve as a foundation for further studies aimed at developing aluminum-tolerant maize varieties.

5. ACKNOWLEDGMENTS

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