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RESEARCH PAPER

Assessment of genetic variability for aluminum tolerance in cowpea accessions screened in pots under field conditions

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Key Message: This study assessed genetic variability in ten cowpea accessions under aluminum stress. Key findings reveal significant differences among accessions for all traits. Based on aluminum tolerance indices, AC03, AC04, AC05, AC06, AC08, and AC09 were classified as highly tolerant, AC02 was deemed moderately tolerant and AC01, AC07, and AC10 were identified as highly susceptible.

Abstract

Aluminum toxicity is a major factor limiting crop productivity on acid soils, thus limiting food production. This study assessed the level of genetic diversity for aluminum tolerance in cowpea and the inter-character association of important traits for the effective selection of tolerant genotypes. Ten accessions of the crop were screened in pots filled with topsoil employing a 10 × 4 factorial experiment in a completely randomized design with three replicates. The four aluminum treatments imposed were 0, 50, 100, and 200 µM $AlCl_3$. The study found significant differences among accessions for all traits. Aluminum treatment affected all traits except seeds/plant and seed yield, with significant interaction effects for traits except emergence percentage and plant height. Heritability was high (≥ 60%) for all traits except pods/plant, which had moderate heritability (57.98%). Genetic advance was high (≥ 20%) for all traits except days to flowering (11.08%) and plant height (15.87%), showing moderate values. Based on aluminum tolerance indices, AC03, AC04, AC05, AC06, AC08, and AC09 were classified as highly tolerant, AC02 as moderately tolerant, while AC01, AC07, and AC10 were highly susceptible. Consequently, selection for the traits with high heritability and genetic advance would result in genetic gain and breeding progress for aluminum tolerance in cowpea for acidic soils in Nigeria and other tropical regions. © 2024 The Author(s)

Keywords: Acidity**,** Growth parameters**,** Heritability, Toxicity, Variability, Yield

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Introduction

Aluminum (Al) ranks first among metals and third among elements in abundance on Earth (Siecinska & Nosalewicz, 2016; Shetty et al., 2021). It is non-phytotoxic as long as the soil remains neutral or slightly acidic (pH values) because it exists in the insoluble oxides or aluminosilicate under this condition. However, the phytotoxic forms begin to manifest in soil solutions as soil acidity increases, eventually reaching levels that can negatively affect plant growth and development (Casierra-Posada et al., 2021; Wei et al., 2024). Excessive acidification of soils is a consequence of uninterrupted rigorous agriculture as well as the alteration of environmental conditions propelled by global climate change (Shetty et al., 2021). Al toxicity is a major factor limiting crop productivity on acid soils, thus limiting food production. When its concentration in soil surpasses 3 mg kg^{-1} of soil at a pH of 5.5, its toxicity is manifested (Casierra-Posada et al., 2021). Al inhibits root elongation in Al-sensitive plants due to its quick inhibition of cell division and cell expansion of root meristems (Phukunkamkaew et al., 2021) and can also obstruct the uptake of minerals and water (Wei et al., 2024). This can lead to serious drought stress and nutrient deficiency (Tang et al., 2002).

Presently, 40% of the world's arable lands are acidic in many subtropical and tropical areas (Tang et al., 2002; Phukunkamkaew et al., 2021), and more than 50% of the world's potentially arable lands (Siecinska et al., 2016; Asfawu et al., 2024). In Nigeria, up to 18% of the total land area is acidic (Ajayi, 2021). The largest amount of cowpea (*Vigna unguiculata* L. Walp) is produced in Nigeria, which stands at 36% of the world's total (FAO, 2020). Nigeria belongs to the tropical belt where agriculture is largely practiced on acidic soils as semi-subsistence farming (Akinrinde et al., 2006). This is a result of high population pressures that disallow enough fallow periods and push farmers into managing soil fertility to sustain productivity (Akinrinde et al., 2006). Agronomic strategies employed by farmers for the management of acid soils to sustain yield include the application of lime which enhances soil pH and phosphorus availability and as a consequence reduces aluminum toxicity and the use of organic matter which can produce various Al-organic acid complexes thereby reducing aluminum solubility, decrease the concentration available to plants, and consequently enhance the availability of phosphorus to plants. However, the impracticality of these soil improvement strategies in many regions lies in their high cost of deployment (Siecinska & Nosalewicz, 2016). Hence, as a cost-effective strategy, it is imperative to examine the source of aluminum tolerance present in the adapted crop species, such as cowpea in Nigeria and other tropical regions. Therefore, Al tolerance is a crucial crop improvement goal to increase crop productivity on acidic soils.

Cowpea is a major source of protein in Nigeria and other tropical and subtropical countries of the world (Akinrinde et al., 2005; Akinrinde et al., 2006). Although it has a higher tolerance to Al stress compared to other legumes, Al toxicity can be the major factor limiting its productivity in acid soils. Different mechanisms of Al tolerance in crops have been established, and studies have noted that the key to improving crop productivity in the tropics and subtropics is a function of access to acid-tolerant genotypes (Abdou Razakou et al., 2013). Wide genotypic variability to Al toxicity on acid soils has been reported in cowpea germplasm among researchers such as Iroh (2004); Akinrinde et al. (2005), leading to the selection of promising genotypes. Genotypic differences in the physiological, morphological, and yield traits of cowpea and a decrease in the protein content of seeds have been reported (Ezeh et al., 2007). Also, Ezeh et al. (2007) established the importance of genotype × Al effects on morphological and yield traits of cowpea.

Several studies on Al tolerance in cowpea exist based on several agronomic and yield traits. However, information regarding its level of genetic diversity and character association under aluminum stress employing the multivariate approach is limited. To develop Al-tolerant genotypes of cowpea in a plant breeding program, a better understanding of the presence and magnitude of the genetic diversity for Al tolerance in a gene pool is important. Heritability of traits and their genetic gains are critical to successful breeding programs since the strengths of such estimates provide the extent to which improvement can be made (Ajayi et al., 2014). Therefore, the present study was designed to assess the level of genetic diversity for aluminum tolerance in cowpea for the effective selection of Al-tolerant genotypes.

Materials and Methods

Plant materials and experimental area

The ten (10) accessions involved had been previously screened under aluminum stress based on germination parameters (FAO, 2020) and are presented in Table 1. The location of the present study was the Plant Science and Biotechnology Experimental Field, Adekunle Ajasin University (Latitude 7.2^0 N, Longitude $5.44^'$ E, Altitude 423 m above sea level), Nigeria, between July and October, 2016. The accessions were supplied by the International Institute of Tropical Agriculture (IITA), Nigeria.

Table 1 Selected cowpea accessions and their previous aluminum tolerance statuses for germination and seedling parameters under laboratory conditions

Accession ID	Biological status	Tolerance for germination parameters	Code
TVu-199	Breeding material	Highly susceptible	AC01
TVu-207	Breeding material	Moderately susceptible	AC02
TVu-218	Breeding material	Moderately tolerant	AC03
TVu-235	Breeding material	Moderately tolerant	AC04
TVu-236	Breeding material	Moderately tolerant	AC05
TVu-241	Breeding material	Highly tolerant	AC06
IT98K-205-8	Unknown	Moderately susceptible	AC07
IT98K-555-1	Unknown	Moderately tolerant	AC08
TVu-4886	Landrace	Moderately susceptible	AC09
TVu-9256	Landrace	Highly tolerant	AC10

Experimental design and procedure

This experiment was performed following a modified procedure from Ezeh et al. (2007). Six hundred bottom-perforated plastic pots (5 L capacity) were each filled with topsoil collected near the experimental field (five pots per accession per treatment per replicate). The three perforations at the bottom of each pot were to enhance drainage during the experiment. The experimental field soil surface was covered with polythene before the arrangement of the plastic pots during the experiment.

A factorial experiment employing a 10 × 4 design was used. Subsequently, seeds were planted in individual pots, each containing 3.5 kg of topsoil. These pots underwent two rounds of treatment at one-week intervals, where 500 ml of $AlCl_3$ solutions at concentrations of 0, 50, 100, and 200 µM were applied. This experimental setup followed a completely randomized design (CRD) with three replicates. For each accession, three seeds were sown in each pot, with a total of five pots assigned to each treatment within each replicate. Ten (10) days after emergence, the seedlings were thinned to maintain only one plant per pot. This treatment application process continued weekly until the fifth week after planting (WAP).

Data collection

The emergence percentage was assessed 10 days after planting (DAP), while measurements for plant height and the number of leaves were recorded in the fifth week after planting (WAP). The number of days until the first flowering occurred was documented as plants began to flower. Furthermore, parameters including the number of pods per plant, the number of seeds per pod, seed yield per plant, as well as the number and length of primary roots, root dry, and fresh weight were determined at the point of maturity. To ascertain root parameters, the stems of the plants were severed from the roots, and soil was carefully removed from the roots through immersion in a large container of water. Following this, the main roots were counted, and the length of the tap root was measured. The root dry weight was established by subjecting them to oven drying at 80°C until reaching a constant weight, a process that spanned 24 hours.

Statistical analysis

The data underwent statistical analysis through analysis of variance (ANOVA) using the GLM (General Linear Model) procedure within SPSS software, version 20. Here, treatment was fixed while accession was random. Mean values were separated by LSD at $P \leq 0.05$. Accessions were ranked based on their level of tolerance to aluminum stress using the tolerance index (TI) calculated as $(X \times Y) / (\bar{X})^2$, where X was the mean performance of accession for a trait under the control treatment, and Y the average mean performance of accession for a trait under aluminum treatments, and $\bar{X}$ was the grand mean of all accessions for that trait under the control treatment. Accessions with mean TI greater than the grand mean of TI were deemed more tolerant, while the ones with lower values were more susceptible.

Estimates of genetic parameters were performed according to Ojo & Ayuba (2016) with modifications as follows:
Error variance (V_E) = σ_E^2 = Mean square error (MS_E). Genotype × treatment variance (V_{GT}) = $\sigma_{GT}^2 = (MS_{GT} - MS_E)/r$.
Genotypic variance (V_G) = σ_G^2 = $(MS_G - MS_E)/rT$. Phenotypic variance (V_P) $= V_G + V_{GT}/T + V_E/rT$. Genotypic coefficient of variation (GCV) $= \frac{\sqrt{V_G}}{\bar{X}} \times 100$. Phenotypic coefficient of variation (PCV) $= \frac{\sqrt{V_P}}{\bar{X}} \times 100$. Broad-sense heritability (H^2) = $V_G/(V_G + (V_{GT}/T) + (V_E/rT))$.
Genetic advance (GA) $= \frac{VG}{\sqrt{VP}} \times k$; k = 2.06 (selection differential).
Genetic advance as percent of the mean (GAM) = $\frac{GA}{\bar{X}} \times 100$; where T, $\bar{X}$, and r are the number of treatments, grand mean of trait, and replicates, respectively. Genetic parameters were categorized following the criteria outlined in Ajayi et al. (2014) as follows: GCV and PCV were classified as low (0–10%), moderate (10–20%), and high (above 20%). Broad-sense heritability was categorized as low (0–30%), moderate (30–60%), and high (above 60%). Similarly, genetic advance as a percentage of the mean (GAM) was classified as low (0–10%), moderate (10–20%), and high (above 20%).

Results

Analysis of variance (ANOVA) for measured traits under aluminum treatment

ANOVA revealed a highly significant effect of accession for all measured traits. Aluminum treatment was highly significant for all traits except for the number of seeds per plant and seed yield per plant. Furthermore, the accession × treatment effect was highly significant for all traits except for emergence percentage, plant height, and the number of days to first flowering. The coefficient of variation among measured traits varied from 8.42% in the number of days to first flowering to 38.88% in seed yield per plant (Table 2).

Table 2 Mean square values of accession, treatment, and accession × treatment interaction of cowpea under aluminum stress

Source of variation	Accession	Treatment	Accession × treatment	Error	CV (%)
DF	9	3	27	80	
EM	2203.33**	5345.56**	310.99^{ns}	546.67	33.48
PH	16.05**	2.63**	1.87^{ns}	2.92	13.53
NL	22.74**	5.45**	2.04**	1.41	16.89
DFF	128.98**	26.18**	12.78^{ns}	19.93	8.42
PDP	78.18**	6.01**	36.38**	6.10	24.09
SPP	54.63**	3.85**	11.08**	4.44	26.08
SDPL	8590.25**	461.07^{ns}	4594.47**	1005.75	36.71
SDYPL	118.72**	6.95^{ns}	42.8**	19.03	38.88
NRT	71.58**	3.03**	17.34**	2.49	13.05
RTL	113.9**	62.01**	34.41**	3.93	13.23
DWR	0.55**	0.33**	0.12**	0.03	25.85

***: Significant at $P \leq 0.05$; ns: Not significant;* DF: Degree of freedom; CV: Coefficient of variation. EM: Emergence percentage; PH: Plant height; NL: Number of leaves per plant; DFF: Number of days to first flowering; PDP: Number of pods per plant; SPP: Number of seeds per pod; SDPL: Number of seeds per plant; SDYPL: Seed yield per plant; NRT: Number of roots per plant; RTL: Root length per plant; DWR: Dry weight of roots.

Effects of accession × aluminum treatment interactions on growth and yield traits

Table 3 presents the effects of accession × aluminum treatment interactions on growth traits of cowpea accessions under aluminum stress. The emergence percentage was significantly reduced by aluminum stress (especially at 200 µM) in all accessions between the ranges of 33% to 60% over the control in AC03, AC06, AC07, and AC09, where the reduction was insignificant. The number of leaves per plant in AC03, AC06, AC08, and AC10 was enhanced significantly by aluminum stress over the control. The number of roots per plant was significantly increased over the control mainly by 50 and 100 µM aluminum in most accessions, while root length and dry root weight were significantly inhibited in AC03, AC05, and AC06 mainly by 100 µM aluminum. However, as presented in Table 4, the number of days to first flowering was significantly enhanced in AC05 and AC06 by 50 µM, which also significantly inhibited the number of pods per plant in accessions AC06, AC07, and AC10, ranging between 48% and 66% reduction over the control. Aluminum treatment of 50 and 100 µM significantly increased seeds per pod, especially in AC04, AC06, and AC07, however, they did not cause a significant increase in the number of seeds per plant and seed yield per plant among the accessions.

Genetic variation, heritability, and genetic advance of measured traits

Across all the measured traits, it was consistently observed that the phenotypic coefficient of variation (PCV) exceeded the genotypic coefficient of variation (GCV). PCV and GCV exhibited a range from moderate to high values for most traits, except for plant height (8.27% PCV, 8.85% GCV) and days to first flowering (5.59% PCV, 6.02% GCV), where they remained low, staying below or equal to 10%. Broad-sense heritability was notably high for all traits except the number of pods per plant, which was at 57.98%. The heritability values spanned from 62.28% for the number of seeds per plant to an impressive 91.28% for the number of leaves per plant. Nevertheless, when considering genetic advance as a percentage of the mean, it appeared to be moderate for plant height and days to first flowering. In contrast, for all other traits, the genetic advance was substantial (Table 5).

Aluminum tolerance indices and ranking of cowpea accessions

Table 6 presents the results on aluminum tolerance indices of growth and yield traits of cowpea with their ranks in the bracket. Accessions AC03, AC04, AC05, and AC06 had above-average tolerance for most traits measured. The rank sum (RS) and mean ranks ($\bar{R}$) were highest in AC07 (78.00, 7.09), while the lowest (42.00, 3.82) were observed in AC03. Based on the mean ranks, accessions with a mean rank lesser than the grand mean (GM) of ranks (5.21) were categorized as highly tolerant accessions; these were AC03, AC04, AC05, AC09, AC06, and AC08. The moderately tolerant accession (AC02) had a value a bit higher than the GM. The highly susceptible accessions were the ones with higher mean ranks compared to the GM, and these included accessions AC01, AC10, and AC07.

Table 3 Effects of accession × aluminum interaction on the growth traits of cowpea accessions under aluminum stress

Emergence percentage (%)										
Treatment	**AC01**	**AC02**	**AC03**	**AC04**	**AC05**	**AC06**	**AC07**	**AC08**	**AC09**	**AC10**
Control	100.00^{b}	93.33^{b}	80.00^{a}	100.00^{b}	100.00^{b}	86.67^{a}	53.33^{a}	100.00^{b}	80.00^{a}	100.00^{b}
50 µM	66.67^{a}	53.33^{ab}	73.33^{a}	80.00^{ab}	80.00^{ab}	80.00^{a}	33.33^{a}	100.00^{b}	66.67^{a}	40.00^{a}
100 µM	73.33^{ab}	40.00^{a}	66.67^{a}	80.00^{ab}	66.67^{a}	80.00^{a}	26.67^{a}	80.00^{ab}	60.00^{a}	53.33^{a}
200 µM	66.67^{ab}	60.00^{ab}	66.67^{a}	66.67^{a}	66.67^{a}	66.67^{a}	40.00^{a}	53.33^{a}	60.00^{a}	53.33^{a}
±SE (13.49)										
LSD (30.65)										
Plant height (cm)										
Control	15.44^{a}	15.23^{b}	11.92^{a}	13.84^{b}	13.63^{a}	11.77^{a}	11.37^{a}	11.09^{a}	13.60^{a}	12.70^{b}
50 µM	15.24^{a}	12.58^{a}	12.35^{a}	13.29^{ab}	14.15^{a}	11.60^{a}	11.38^{a}	12.71^{a}	12.99^{a}	9.60^{a}
100 µM	15.09^{a}	12.88^{a}	11.73^{a}	11.37^{a}	13.27^{a}	11.71^{a}	11.60^{a}	11.65^{a}	12.88^{a}	11.73^{b}
200 µM	14.32^{a}	13.07^{a}	12.53^{a}	12.57^{ab}	12.80^{a}	11.68^{a}	11.43^{a}	11.60^{a}	12.98^{a}	12.94^{b}
±SE (0.99)										
LSD (2.08)										
Number of leaves per plant										
Control	7.85^{a}	8.23^{a}	3.78^{a}	5.53^{a}	5.32^{a}	4.11^{a}	6.08^{a}	6.93^{a}	9.75^{a}	6.89^{ab}
50 µM	6.90^{a}	7.55^{a}	6.27^{b}	6.43^{a}	5.97^{a}	6.33^{b}	6.50^{a}	9.73^{b}	8.75^{a}	5.33^{a}
100 µM	8.22^{a}	8.44^{a}	6.87^{b}	5.01^{a}	6.28^{a}	6.05^{b}	6.78^{a}	9.72^{b}	8.58^{a}	6.67^{ab}
200 µM	8.15^{a}	8.24^{a}	6.96^{b}	5.53^{a}	5.72^{a}	5.44^{ab}	7.06^{a}	9.17^{b}	9.67^{a}	8.22^{b}
±SE (0.69)										

LSD (1.61)										
Root length (cm)										
Control	10.95^{a}	9.35^{ab}	16.42^{b}	22.96^{c}	23.63^{c}	23.27^{c}	19.50^{b}	10.00^{a}	15.90^{b}	14.65^{b}
50 µM	12.81^{a}	11.08^{bc}	22.43^{c}	12.89^{a}	18.32^{b}	21.47^{bc}	16.44^{a}	11.14^{ab}	10.32^{a}	20.04^{c}
100 µM	13.50^{a}	13.36^{c}	13.42^{a}	18.40^{b}	15.48^{a}	11.43^{a}	16.90^{ab}	13.40^{bc}	11.86^{a}	10.19^{a}
200 µM	11.67^{a}	8.13^{a}	13.67^{a}	11.56^{a}	19.43^{b}	20.41^{b}	14.79^{a}	14.67^{c}	11.79^{a}	11.62^{a}
±SE (1.15)										
LSD (2.67)										
Dry weight of roots (g)										
Control	0.49^{a}	0.40^{a}	1.07^{c}	0.83^{a}	1.47^{b}	1.06^{b}	0.40^{a}	0.54^{a}	0.67^{a}	0.44^{a}
50 µM	0.58^{a}	0.42^{a}	0.77^{b}	0.79^{a}	0.77^{a}	0.90^{ab}	0.36^{a}	0.50^{a}	0.52^{a}	0.44^{a}
100 µM	0.50^{a}	0.46^{a}	0.47^{a}	0.79^{a}	0.55^{a}	0.82^{a}	0.40^{a}	0.58^{a}	0.52^{a}	0.42^{a}
200 µM	0.45^{a}	0.45^{a}	1.03^{c}	0.70^{a}	1.30^{b}	1.00^{ab}	1.25^{b}	0.59^{a}	0.45^{a}	0.48^{a}
±SE (0.10)										
LSD (0.23)										
Number of main roots per plant										
Control	9.89^{b}	7.67^{b}	14.59^{bc}	13.07^{a}	11.47^{a}	11.42^{a}	12.83^{bc}	12.08^{a}	13.98^{c}	13.35^{c}
50 µM	9.75^{b}	10.50^{c}	12.00^{a}	17.32^{b}	13.40^{ab}	14.67^{b}	10.75^{ab}	11.80^{a}	10.62^{b}	7.27^{a}
100 µM	6.50^{a}	7.11^{b}	16.53^{c}	13.84^{a}	18.61^{b}	13.00^{ab}	10.67^{a}	16.75^{b}	13.07^{c}	9.33^{a}
200 µM	13.00^{c}	4.75^{a}	14.36^{b}	15.33^{b}	16.25^{c}	12.79^{ab}	12.94^{c}	11.83^{a}	7.73^{a}	10.63^{b}
±SE (0.91)										
LSD (2.10)										

Table 4 Effects of accession × aluminum interaction on the yield traits of cowpea accessions under aluminum stress

Number of days to first flowering										
Treatment	**AC01**	**AC02**	**AC03**	**AC04**	**AC05**	**AC06**	**AC07**	**AC08**	**AC09**	**AC10**
Control	52.98^{a}	49.68^{a}	54.96^{a}	56.20^{a}	59.20^{ab}	61.50^{b}	54.06^{a}	51.00^{a}	49.83^{a}	51.67^{a}
50 µM	48.39^{a}	50.11^{a}	50.33^{a}	54.13^{a}	55.65^{a}	52.57^{a}	53.67^{a}	49.40^{a}	53.56^{a}	51.67^{a}
100 µM	48.63^{a}	49.67^{a}	50.40^{a}	57.64^{a}	58.56^{ab}	55.91^{ab}	54.22^{a}	50.44^{a}	51.08^{a}	49.28^{a}
200 µM	49.75^{a}	50.47^{a}	54.79^{a}	53.58^{a}	62.53^{b}	57.00^{ab}	57.39^{a}	50.67^{a}	48.50^{a}	48.64^{a}
±SE (2.58)										
LSD (5.80)										
Number of pods per plant										
Control	13.33^{b}	13.33^{a}	11.67^{ab}	10.00^{a}	5.67^{a}	10.33^{bc}	9.33^{b}	14.67^{b}	14.00^{a}	10.67^{b}
50 µM	13.33^{b}	10.67^{a}	15.00^{b}	15.67^{b}	4.67^{a}	5.33^{a}	4.33^{a}	13.00^{ab}	17.33^{a}	3.67^{a}
100 µM	3.00^{a}	12.33^{a}	12.00^{ab}	12.33^{ab}	18.33^{c}	7.67^{ab}	6.00^{ab}	10.00^{a}	16.67^{a}	8.33^{b}
200 µM	12.33^{b}	10.33^{a}	10.00^{a}	9.67^{a}	10.67^{b}	12.67^{c}	8.00^{b}	10.33^{a}	16.33^{a}	10.67^{b}
±SE (1.43)										
LSD (3.39)										
Number of seeds per pod										
Control	5.30^{a}	5.80^{a}	11.46^{a}	7.47^{a}	11.74^{b}	8.60^{b}	8.26^{a}	5.89^{a}	4.66^{a}	7.27^{a}
50 µM	5.79^{a}	6.71^{a}	9.79^{a}	12.55^{c}	6.51^{a}	11.88^{c}	11.47^{b}	4.94^{a}	5.97^{a}	9.50^{a}
100 µM	5.82^{a}	5.89^{a}	11.82^{a}	8.92^{ab}	11.71^{b}	5.25^{a}	12.03^{b}	4.50^{a}	6.59^{a}	9.12^{a}
200 µM	4.74^{a}	7.33^{a}	11.53^{a}	11.68^{bc}	7.32^{a}	11.55^{c}	6.43^{a}	6.03^{a}	6.46^{a}	7.10^{a}
±SE (1.22)										
LSD (2.84)										
Number of seeds per plant										
Control	70.80^{a}	80.69^{a}	136.25^{a}	74.98^{a}	65.86^{a}	88.46^{ab}	77.06^{a}	86.45^{a}	65.82^{a}	77.50^{a}
50 µM	77.81^{a}	72.23^{a}	145.04^{a}	195.33^{a}	30.05^{a}	64.48^{a}	51.33^{a}	62.18^{a}	105.53^{a}	36.33^{a}
100 µM	17.31^{a}	73.68^{a}	141.02^{a}	111.69^{a}	220.93^{a}	40.17^{a}	72.46^{a}	45.03^{a}	112.01^{a}	76.21^{a}
200 µM	60.22^{a}	73.73^{a}	115.54^{a}	113.72^{a}	79.90^{a}	146.95^{a}	51.41^{a}	61.99^{a}	101.08^{a}	76.10^{a}
±SE(18.31)										
LSD (ns)										
Seed yield per plant (g)										
Control	10.46^{a}	10.46^{a}	12.66^{a}	10.54^{a}	9.28^{a}	12.96^{a}	9.34^{a}	12.36^{a}	8.66^{a}	10.71^{a}

50 µM	10.97^a	11.70^a	20.34^a	22.98^a	5.73^a	8.89^a	6.74^a	8.64^a	12.78^a	4.95^a
100 µM	2.32^a	11.39^a	21.30^a	14.00^a	15.90^a	5.96^a	10.25^a	5.60^a	14.83^a	7.84^a
200 µM	8.26^a	11.53^a	15.60^a	13.60^a	10.87^a	17.78^a	7.11^a	9.12^a	13.81^a	10.55^a
±SE (2.52)										
LSD (ns)										

Means followed by the same superscripts within a column are not significantly different at $P \leq 0.05$ using the Least Significant Difference (LSD). SE: Standard error.

Table 5 Estimates of genetic parameters for growth and yield traits of cowpea accessions under aluminum stress

Trait	GM	σ^2_G	σ^2_P	σ^2_E	σ^2_{GT}	GCV (%)	PCV (%)	H^2 (%)	GAM (%)
EM	69.83	138.06	163.98	546.67	-78.56	16.83	18.33	84.19	31.79
PH	12.63	1.09	1.25	2.92	-0.35	8.27	8.85	87.20	15.87
NL	7.03	1.78	1.95	1.41	0.21	18.98	19.86	91.28	37.52
DFF	52.99	9.09	10.16	19.93	-2.38	5.69	6.02	89.47	11.08
PDP	10.85	6.01	7.21	6.10	10.09	22.62	24.77	57.98	29.53
SPP	8.08	4.18	5.10	4.44	2.21	25.30	27.94	81.96	47.15
SDPL	86.38	632.04	1014.91	1005.75	119.24	29.10	36.88	62.28	47.31
SDYPL	11.22	8.31	11.88	19.03	7.91	25.72	30.75	69.95	44.26
NRT	12.09	5.76	7.21	2.49	4.95	19.85	22.21	79.89	36.48
RTL	14.98	9.16	12.02	3.93	10.16	20.20	23.14	76.14	36.30
DWR	0.67	0.04	0.05	0.03	0.03	29.85	33.37	80.00	55.90

GM: Grand mean; σ^2_G: Genotypic variance; σ^2_P: Phenotypic variance; σ^2_E: Error variance; σ^2_{GT}: Genotype × treatment variance; GCV: Genotypic coefficient of variation; PCV: Phenotypic coefficient of variation; H^2: broad sense heritability; GAM: Genetic advance as a percent of the mean. EM: Emergence percentage; PH: Plant height; NL: Number of leaves per plant; DFF: Number of days to first flowering; PDP: Number of pods per plant; SPP: Number of seeds per pod; SDPL: Number of seeds per plant; SDYPL: Seed yield per plant; NRT: Number of roots per plant; RTL: Root length per plant; DWR: Dry weight of roots.

Table 6 Aluminum tolerance indices and (ranks) based on growth and yield traits of cowpea accessions under aluminum stress

Accession	EMI	PHI	NLI	DFFI	PDPI	SPPI	SDPLI	SDYPLI
AC01	0.86 (4)	1.35 (1)	1.47 (4)	0.89 (4)	0.99 (5)	0.49 (8)	0.54 (9)	0.65 (9)
AC02	0.59 (9)	1.15 (2)	1.60 (2)	0.85 (1)	1.16 (3)	0.66 (5)	0.87 (6)	1.05 (4)
AC03	0.69 (6)	0.85 (6)	0.61 (9)	0.97 (5)	1.13 (4)	2.17 (1)	2.69 (1)	2.09 (1)
AC04	0.95 (2)	1.01 (5)	0.75 (8)	1.06 (7)	0.98 (6)	1.41 (3)	1.55 (2)	1.54 (2)
AC05	0.89 (3)	1.07 (3)	0.77 (7)	1.19 (9)	0.49 (9)	1.71 (2)	1.07 (4)	0.87 (6)
AC06	0.82 (5)	0.80 (7)	0.59 (10)	1.16 (8)	0.69 (7)	1.41 (3)	1.09 (3)	1.22 (3)
AC07	0.22 (10)	0.76 (9)	0.99 (6)	1.02 (6)	0.45 (10)	1.41 (3)	0.66 (8)	0.65 (9)
AC08	0.97 (1)	0.78 (8)	1.59 (3)	0.87 (2)	1.28 (2)	0.52 (6)	0.72 (7)	0.83 (7)
AC09	0.62 (7)	1.03 (4)	2.12 (1)	0.87 (2)	1.84 (1)	0.51 (7)	1.03 (5)	1.04 (5)
AC10	0.61 (8)	0.85 (6)	1.12 (5)	0.88 (3)	0.63 (8)	1.07 (4)	0.72 (7)	0.72 (8)
Grand mean	0.72	0.97	1.16	0.98	0.97	1.13	1.09	1.07

EMI: Emergence percentage index; PHI: Plant height index; NLI: Number of leaves per plant index; DFFI: Number of days to first flowering index; PDPI: Number of pods per plant index; SPPI: Number of seeds per pod index; SDPLI: Number of seeds per plant index; SDYPLI: Seed yield per plant index; NRTI: Number of roots per plant index

Table 6 continue

Accession	RTLI	DWRI	*RS*	$\bar{R}$	σR
AC01	0.49 (8)	0.46 (8)	70	6.36	2.58
AC02	0.37 (10)	0.33 (10)	61	5.55	3.45
AC03	0.98 (5)	1.51 (3)	42	3.82	2.68
AC04	1.18 (3)	1.17 (4)	44	4.00	2.19
AC05	1.51 (1)	2.37 (1)	48	4.36	2.94
AC06	1.49 (2)	1.79 (2)	55	5.00	2.68
AC07	1.13 (4)	0.49 (7)	78	7.09	2.34
AC08	0.47 (9)	0.56 (6)	55	5.00	2.72
AC09	0.65 (7)	0.62 (5)	49	4.45	2.25
AC10	0.74 (6)	0.37 (9)	71	6.45	1.86
Grand mean	0.90	0.97	57.30	5.21	2.57

RS: Rank sum; R: Mean ranks; σR: Standard deviation of ranks. RTLI: Root length per plant index; DWRI: Dry weight of roots index

Discussion

The significant effect of accession revealed in this study for all measured traits indicates a sufficient level of genetic variation among the accessions under aluminum stress, as reported by Ojo & Ayuba (2016). This finding highlights the potential for genetic improvement (Asfawu et al., 2024). The significant effect of aluminum treatment on most traits suggested that the treatment was effective on the measured traits except for seeds per plant and seed yield per plant. The highly significant effect of the accession × treatment interaction on measured traits indicated that the response of accessions varied under different treatments (Akinrinde & Neumann, 2006; Villagarcia et al., 2001), apart from emergence percentage and the number of days to first flowering. These results are consistent with the responses of these accessions regarding germination and seedling traits under aluminum stress in laboratory conditions (Ajayi, 2021). In this context, identifying accessions with high stability and superior yield across different levels of aluminum stress would be crucial for maximizing yield and beneficial for cowpea breeding programs focused on aluminum tolerance. Aluminum treatment affected all measured traits among the accessions, showing both inhibitory and stimulatory effects, with responses to aluminum stress being dependent on the accession, as reported by Ezeh et al. (2007); Kushwaha et al. (2017) in cowpea.

In the current investigation, elevated levels of aluminum stress were found to negatively affect the emergence rate of plants. Notably, this inhibitory effect became more pronounced as the concentration of aluminum increased, aligning with the findings of Alamgir & Akhter (2009). The height of cowpea plants, as reported by Ezeh et al. (2007); Kushwaha et al. (2017) displayed variability among varieties. In contrast, Akinrinde and Neumann (2006) disputed this finding concerning plant height and other yield-related characteristics, except for the number of pods per plant. Furthermore, shoot and root growth were reported to be dependent on the cultivar in wheat (Alamgir & Akhter, 2009) and rice (Phukunkamkaew et al., 2021), with low concentrations being stimulatory and high concentrations being inhibitory. Poozesh et al. (2007) noted that one of the initial effects of aluminum toxicity is its negative influence on plant growth during the seedling stage. Akinrinde et al. (2006) observed that cowpea plants exposed to aluminum treatment displayed a significant increase in height compared to untreated plants. However, Ezeh et al. (2007) reported no significant difference in height for aluminum-treated cowpea. The stimulatory effect of aluminum toxicity on the growth of sugar maple at the seedling stage was noted by Schier & McQuattie (2002), whereas Massot et al. (1992); Yan and Tinker (2006); Singh et al. (2022) reported inhibitory effects on the shoot growth of beans, plant height of soybeans, and shoot length of rice seedlings under aluminum stress, respectively. In the present study, the number of leaves was higher for most treated plants across accessions, indicating a stimulatory effect of aluminum stress. AC03 was among the most tolerant accessions, exhibiting improved plant height and number of leaves under aluminum stress. However, this finding contradicts the reports of Yan and Tinker (2006) regarding rice genotypes grown under aluminum stress.

Stimulatory and inhibitory effects were observed among accessions for the number of roots, root length, and dry root weight. Regarding root length, a predominant inhibitory effect was noted in most accessions, aligning with the findings of Tang et al. (2002); Akinrinde et al. (2006) who reported significant inhibition of root elongation among wheat and cowpea genotypes treated with 20 and 30 µM of aluminum, respectively. Conversely, Kushwaha et al. (2017) reported a stimulatory effect on cowpea root length at higher aluminum concentrations. Additionally, the dry root weight under aluminum stress, as reported by Massot et al. (1992); Kushwaha et al. (2017) support the findings of the present study. Aluminum stress has been shown to negatively impact root growth and development (Poozesh et al., 2007), with studies revealing that soybean root lengths were adversely affected by aluminum stress (Singh et al., 2022). Numerous investigations have demonstrated that the primary reaction to aluminum stress occurs in the roots (Yan & Tinker, 2006; Yan et al., 2007), with aluminum-susceptible genotypes displaying stunted root development, distortions, and discoloration (Hede et al., 2002; Casierra-Posada et al., 2021). A significant decline in root traits has also been documented in rice under heightened aluminum stress, attributed to the inhibition of indole acetic acid (IAA) synthesis and the accumulation of abscisic acid (ABA), leading to cell senescence (Phukunkamkaew et al., 2021). However, aluminum-tolerant wheat exhibited longer roots compared to susceptible lines, which was linked to increased levels of Al-induced soluble sugars in their root cells, essential for promoting root elongation and improving moisture uptake under aluminum stress (Giannakoula & Moustakas, 2010). Additionally, root elongation among tolerant maize lines was associated with higher proline and carbohydrate content, which helps maintain osmotic potential in the roots to sustain moisture uptake (Giannakoula & Moustakas, 2010). Thus, root parameters are critical indicators for screening aluminum tolerance in crop species (Richard et al., 2015; Phukunkamkaew et al., 2021). Consequently, accession AC03, which displays elevated values for specific root traits under aluminum stress, is a promising candidate for developing aluminum-tolerant genotypes in breeding programs targeting aluminum tolerance. Consistent with Ezeh et al. (2007); Kushwaha et al. (2017) in cowpea, the study also observed both inhibitory and stimulatory effects on various accessions regarding yield-related traits. Except for AC08 and AC10, most accessions generally showed increased seed yield, seeds per pod, and seeds per plant under aluminum stress conditions. However, the number of pods per plant declined in most accessions when subjected to aluminum stress.

Considering all parameters, the tolerance indices effectively categorized accessions into distinct tolerance classes. Specifically, AC03, AC04, AC05, AC06, AC08, and

AC09 emerged as the most tolerant accessions, demonstrating above-average values across all drought tolerance indices. AC02 showed a moderate level of tolerance, while AC01, AC10, and AC07 were identified as highly susceptible accessions. This ranking differs from the results obtained for the accessions regarding germination and seedling traits under laboratory conditions (Ajayi, 2021). However, accession AC03 was consistently tolerant, while AC01 was consistently susceptible under both screening methods. Significant differences were observed among accessions for each of the tolerance indices, with each accession responding uniquely to the tolerance indices of various traits (Massot et al., 1992; Yan & Tinker, 2006; Alamgir & Akhter, 2009). Several aluminum tolerance indices have proven effective in distinguishing different genotypes of crop species, with one of the most effective being the aluminum tolerance index based on root parameters (Hede et al., 2002; Lisitsyn & Amunova, 2015).

Previous studies have provided insights into aluminum tolerance in cowpea and various other crop species. Nevertheless, there is a notable scarcity of information concerning the genetic variability among cowpea genotypes when subjected to aluminum stress conditions. The presence and extent of genetic diversity within a crop's gene pool are crucial factors for the success of any breeding program. Furthermore, heritability assessments of traits and the determination of genetic advance play a pivotal role in the selection process, as they indicate the potential for improvement. In the present study, the combination of high GCV and PCV for traits such as the number of pods per plant, seeds per pod, seeds per plant, seed yield per plant, number of roots, root length, and dry weight of roots indicates that the accessions exhibit a broad genetic base for these characteristics under aluminum stress. Meanwhile, the moderate PCV and GCV exhibited by emergence percentage and number of leaves per plant indicate the presence of moderate variability within the studied genetic stock for aluminum tolerance. However, the low PCV and GCV observed in plant height and days to first flowering suggest that these traits may be less responsive to improvement through selection. These results are consistent with Ojo & Ayuba (2016) regarding yield, plant height, and root parameters as reported by Ojo et al. (2016). The small disparities between GCV and PCV for most traits signify a strong genetic influence on these traits. Moderate heritability was observed in pods per plant, while other traits exhibited high heritability along with moderate to high GAM, as demonstrated in the study by Singh et al. (2022). This implies the prevalence of additive gene effects and the effective transmission of these traits to offspring. Consequently, selecting these traits is likely to be an effective strategy for enhancing aluminum stress tolerance in cowpea, particularly in tropical regions. These findings are consistent with previous research by Ojo & Ayuba (2016) in soybeans.

Conclusion

The potential for enhancing aluminum tolerance depends on the presence of adequate genetic variation among accessions screened under aluminum stress conditions. The current study has confirmed the existence of ample variability and heritability in growth, root, and yield parameters among cowpea accessions screened in pots under aluminum stress. The genetic diversity observed among these accessions can be harnessed for the development of tolerant lines through hybridization. The moderate to high heritability and GAM observed for all traits in the pot experiment provide a valuable basis for selection. As a result, focusing on these traits in the selection process is likely to lead to genetic improvements and breeding advancements for aluminum tolerance in cowpea. The utilization of tolerance indices, based on multiple quantitative traits, proves effective in identifying aluminum-tolerant accessions. In this context, accessions AC03, AC04, AC05, AC09, AC06, and AC08 were categorized as highly tolerant, while AC02 exhibited moderate tolerance. Accessions AC01, AC10, and AC07 were identified as highly susceptible. Among these accessions, AC03 and AC04, ranking highest among the highly tolerant ones, can be selected as parental candidates for hybridization. Additionally, they are recommended for cultivation in acidic soils, not only in Nigeria but also in other tropical regions. Accessions AC03, AC05, and AC09 can play a valuable role in improving the susceptible accessions through hybridization programs aimed at enhancing aluminum tolerance.

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References

Abdou Razakou, I. B. Y., Mensah, B., Addam, K. S., & Akromah, R. (2013). Using morpho-physiological parameters to evaluate cowpea varieties for drought tolerance. *International Journal of Agricultural Science Research*, *2*(5), 153–162.

Ajayi, A. (2021). Genotypic differences in aluminum tolerance of cowpea accessions utilizing germination parameters. *International Journal of Life Sciences and Biotechnology*, *4*(2), 254–273. https://doi.org/10.38001/ijlsb.871871.

Ajayi, A. T., Adekola, M. O., Taiwo, B. H., & Azuh, V. O. (2014). Character expression and differences in yield potential of ten genotypes of cowpea (*Vigna unguiculata* L. Walp). *International Journal of Agricultural Sciences*, *4*(3), 63–71.

Akinrinde, E. A., Iroh, L., Obigbesan, G. O., Hilger, T., Romheld, V., & Neumann, G. (2005). Response of cowpea varieties to phosphorus supply on an acidic alumi-haplic-acrisol from Brazil. *Nigerian Journal of Soil Science*, *16*, 115-120.

Akinrinde, E. A., & Neumann, G. (2006). Evaluation of differences in tolerance to aluminum toxicity among some tropical cowpea (*Vigna unguiculata*) genotypes. *Pakistan Journal of Biological Sciences*, *9*(5), 954–960.

Akinrinde, E. A., Iroh, L., Obigbesan, G. O., Hilger, T., Romheld, V., & Neumann, G. (2006). Differential expression of aluminum tolerance mechanisms in cowpea genotypes under phosphorus limitation. *Journal of Applied Sciences*, *6*(4), 854–859. https://doi.org/10.3923/jas.2006.854.859.

Alamgir, A. N. M., & Akhter, S. (2009). Effects of aluminum on seed germination and seedling growth of wheat (*Triticum aestivum* L.). *Bangladesh Journal of Botany*, *38*(1), 1–6.

Asfawu, F., Nayagam, G., Fikiru, E., & Azmach, G. (2024). Breeding maize (*Zea mays* L.) for aluminum tolerance through heterosis and combining ability. *International Journal of Agronomy*, *2024*. https://doi.org/10.1155/2024/9950925.

Casierra-Posada, F., Arias-Salinas, J. J., & Rodríguez-Quiroz, J. F. (2021). Excess aluminum tolerance of the common water hyacinth (*Eichhornia crassipes*) under greenhouse conditions. *Chilean Journal of Agricultural Research*, *81*(4), 597–606. https://doi.org/10.4067/S0718-58392021000400597.

Ezeh, K. N., Omogoye, A. M., & Akinrinde, E. A. (2007). Aluminum influence on the performance of some cowpea (*Vigna unguiculata*) varieties on a Nigerian alfisol. *World Journal of Agricultural Sciences*, *3*(4), 517–522.

FAO. (2020). FAOSTAT data (Version 8-29-2020). http://faostat.fao.org

Giannakoula, A., & Moustakas, M. (2010). Aluminum stress induces up-regulation of an efficient antioxidant system in the Al-tolerant maize line but not in the Al-sensitive line. *Environmental and Experimental Botany*, *67*(3), 487–494.

Hede, A. R., Skovmand, B., Ribaut, J., & Stolen, O. (2002). Evaluation of aluminum tolerance in a spring rye collection by hydroponic screening. *Plant Breeding*, *121*(3), 241–248.

Iroh, L. (2004). *Plant growth, nutritional status, and final yield in cowpea genotypes grown on an acid mineral soil with different levels of phosphorus supply* (Master's thesis). Universität Hohenheim.

Kushwaha, J. K., Pandey, A. K., Dubey, R. K., Singh, V., Mailappa, A.S., & Singh, S. (2017). Screening of cowpea [*Vigna unguiculata* (L.) Walp] for aluminium tolerance in relation to growth, yield and related traits. *Legume Research*, *40*(3), 434–438. https://doi.org/10.18805/lr.v0i0.7016.

Lisitsyn, E. M., & Amunova, O. S. (2015). Genetic variability of spring common wheat varieties in aluminum tolerance. *Russian Journal of Genetics: Applied Research*, *5*(1), 48–54.

Massot, N., Poschenrieder, C., & Barceló, J. (1992). Differential response of three beans (*Phaseolus vulgaris*) cultivars to aluminum. *Acta Botanica Neerlandica*, *41*(3), 293–298.

Ojo, G. O. S., & Ayuba, S. A. (2016). Genetic variation and correlation among seedling and mature plant traits of soybean evaluated in acid sand culture and on acid/neutral soil fields of Nigeria. *Journal of Agricultural Science*, *8*(5), 86–94. https://doi.org/10.5539/jas.v8n5p86.

Phukunkamkaew, S., Tisarum, R., Pipatsitee, P., Samphumphuang, T., Maksup, S., & Cha-um, S. (2021). Morpho-physiological responses of indica rice (*Oryza sativa* sub. indica) to aluminum toxicity at the seedling stage. *Environmental Science and Pollution Research*, *28*(23), 29321–29331. https://doi.org/10.1007/s11356-021-12590-1.

Poozesh, V., Cruz, P., Choler, P., & Bertoni, G. (2007). Relationship between aluminum resistance of grasses and their adaptation to an infertile habitat. *Annals of Botany*, *99*(5), 947–954.

Richard, C., Munyinda, K., Kinkese, T., & Osiru, D. S. (2015). Genotypic variation in seedling tolerance to aluminum toxicity in historical maize inbred lines of Zambia. *Agronomy*, *5*, 200–219.

Schier, G. A., & McQuattie, C. J. (2002). Stimulatory effects of aluminum on the growth of sugar maple seedlings. *Journal of Plant Nutrition*, *25*(12), 2583–2589.

Shetty, R., Vidya, C. S. N., Prakash, N. B., Lux, A., & Vaculík, M. (2021). Aluminum toxicity in plants and its possible mitigation in acid soils by biochar: A review. *Science of the Total Environment*, *765*, 142744. https://doi.org/10.1016/j.scitotenv.2020.142744.

Siecinska, J., & Nosalewicz, A. (2016). Aluminum toxicity to plants is influenced by properties of the root growth environment affected by other co-stressors: A review. *Reviews of Environmental Contamination and Toxicology*, *243*, 1-26. https://doi.org/10.1007/398_2016_15.

Singh, V. K., Chander, S., Sheoran, R. K., Anu-Sheoran, O. P., & Garcia-Oliveira, A. L. (2022). Genetic variability for aluminum tolerance in sunflower (*Helianthus annuus* L.). *Czech Journal of Genetics and Plant Breeding*, *4*, 201–209.

Tang, Y., Garvin, D. F., Kochian, L. V., Sorrells, M. E., & Carver, B. F. (2002). Physiological genetics of aluminum tolerance in the wheat cultivar Atlas 66. *Crop Science*, *42*(5), 1541–1546. https://doi.org/10.2135/cropsci2002.1541.

Villagarcia, M. R., Carter, T. E., Rufty, T. W., Niewoehner, A. S., Jennette, M. W., & Arrellano, C. (2001). Genotypic rankings for aluminum tolerance of soybean roots grown in hydroponics and sand culture. *Crop Science*, *41*(5), 1499–1507.

Wei, Y., Han, R., & Yu, Y. (2024). GmMYB183, a R2R3-MYB transcription factor in tamba black soybean (*Glycine max*. cv. Tamba), conferred aluminum tolerance in Arabidopsis and soybean. *Biomolecules*, *14*(6). https://doi.org/10.3390/biom14060724.

Yan, W., & Tinker, N. A. (2006). Biplot analysis of multi-environment trial data: Principles and applications. *Canadian Journal of Plant Science*, *86*(3), 623–645.

Yan, W., Kang, M. S., Ma, B., Woods, S., & Cornelius, P. L. (2007). GGE biplot vs. AMMI analysis of genotype-by-environment data. *Crop Science*, *47*(2), 643–653.