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Morpho-physiological characterization of barnyard millet mutants for salt tolerance

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Abstract

The Indian Barnyard millet (*Echinochloa frumentacea*), is a climate-resilient crop noted for its wide adaptability, short growth cycle, high nutritional value and stress tolerance. The present study was conducted to assess the salt tolerance level of the barnyard millet mutants. Twenty-five barnyard millet mutants along with check (MDU1 and CO(KV)2) were subjected to varying concentrations of sodium bicarbonate (NaHCO$_3$) salt stress under controlled conditions. Germination percentage, root and shoot length, fresh and dry weight of seedlings were recorded, and stress tolerance indices were calculated. The analysis of variance revealed significant variation among mutants in response to salt stress. Highly tolerant mutants exhibited improved germination percentages and maintained favourable water relations under stress. The Relative Salt Injury Rate (RSIR) increased with higher salt levels, indicating increased sensitivity. Correlation analysis revealed a significant relationship between salt tolerance traits. Principal Component Analysis (PCA) helped to identify the main characteristics that caused variations among mutants. The significant contributors to this variation were Vigour Index (VI), Relative Growth Rate (RGR), Relative Water Content (RWC), and RSIR. Cluster analysis categorized mutants into four clusters, clearly distinguishing highly tolerant mutants from susceptible ones. Based on the findings promising salt-tolerant mutants, such as ACM21022, ACM21016, ACM21017, ACM21024, and ACM21014, were identified with the potential to contribute to future millet breeding programs.

Keywords: NaHCO$_3$, Salt tolerance, mutants, stress tolerance indices, correlation analysis, PCA, cluster analysis.

INTRODUCTION

As the global population grows rapidly, the challenge of ensuring an adequate food supply is exacerbated by climate change and resource depletion. To meet the rising demand, food production must increase by around 70 percent by 2050, necessitating innovative solutions (Food and Nations 2009){Food, 2009 #392}.The productivity of crop is hindered by various biotic and abiotic stresses. Soil salinity is a major threat to crop productivity, affecting 20% of cultivated and 33% of irrigated lands worldwide (Flowers 2004, Munns and Tester 2008).Mandal *et al.,* (2018) reported a rising trend in salt-affected areas, covering 1,128 million hectares globally, with South Asia alone experiencing impacts on 52 million hectares (Wicke *et al.,* 2011). India faces nearly 5% salt-affected soils across diverse regions. Soil salinization reduces cultivable land, harms productivity, biodiversity, and water quality. Crop yields drop significantly in saline conditions, causing global economic losses of $27.3 billion annually (Qadir *et al.,* 2014). India's increasing salt-affected soil threatens food security and economic progress.

Soil with high salt concentrations leads to water stress in plants by lowering osmotic potential, consequently affecting the overall soil water potential. On the other hand, sodicity negatively impacts crops by influencing soil physical and chemical properties(Letey 1985, Loveday and Bridge 1983).Sodic soils are characterized by having a high sodium (Na^+) content, an Exchangeable Sodium Percentage (ESP) above 15, and a high alkalinity with a pH greater than 8.5. The elevated sodium concentration, in comparison to other cations, causes soil aggregates to disperse, resulting in poor soil structure. This leads to increased runoff due to low permeability and waterlogging during rainy seasons, as well as surface crusting and high soil strength during dry spells. These conditions further hinder crop growth and productivity. Fortunately, millets present a promising solution. These resilient grains can thrive in harsh climatic conditions, making them a valuable resource for addressing food shortages. Their ability to be cultivated in harsh climatic conditions makes them especially important for vulnerable populations facing food insecurity. In essence, millets have the potential to play a vital role in providing sufficient and healthy food in the face of global challenges.

The Indian Barnyard millet, scientifically known as *Echinochloa frumentacea* (2n=36, 54), is a climate-resilient and nutritionally valuable crop, showcasing its wide adaptability, short growth cycle, stress tolerance, and exceptional nutritional attributes (Renganathan *et al.,* 2020, Saleh *et al.,* 2013). It was one among the early millets to be domesticated in India. Notably, it thrives in the mid hills of the Himalayan region in Uttarakhand, while also being a popular millet choice in Tamil Nadu. Barnyard millet demonstrates remarkable adaptability, making it suitable for cultivation in challenging environments such as dry farming areas and coastal regions (Sood *et al.,* 2015).

The present study has been designed to delve into the variability exhibited among barnyard millet M_5 mutant lines concerning their tolerance to different level of salt concentrations.

MATERIALS AND METHODS

A total of 25 barnyard millet mutants along with two checks MDU1 and CO(KV)2 were selected for present study. The base materials were procured from the Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai. Karthikeyan (2022) conducted a mutation breeding programme in MDU1 barnyard millet using various dosages of Sodium azide and EMS. The best 25 mutants were then chosen based on yield and associated features. The details of the mutants were are in **Table1**. Factorial Completely Randomized Design (Factorial CRD) was adopted for assessing the salt stress tolerance of the barnyard millet M_5 mutant lines under *invitro* condition. The experimental factors encompassed the various mutants and concentrations of sodium bicarbonate ($NaHCO_3$). The roll towel method was utilized for germination experiments. Twenty - five seeds of each mutant were placed in a roll towel at 0mM, 15mM, 30mM, 45mM, 60mM and 75mM concentrations of $NaHCO_3$. To ensure robustness, three independent replicates were maintained for each unique combination of treatments. The roll towel containing the seeds were then placed within a controlled seed germination chamber. Seeds were considered to be germinated when both plumule and radicle were emerged (more than 2 mm). Physio – morphological parameters namely germination percentage, root length, shoot length, fresh weight and dry weight of the seedlings were recorded on the 14th day after germination. Based on the above parameters stress tolerance indices like relative germination rate (RGR), shoot length stress tolerance index, root length stress tolerance index, vigour Index (VI), and relative salt injury rate (RSIR) were calculated by using the following formulae.

$$RGR = \frac{Germination\ \%\ in\ treatment}{Germination\ \%\ in\ control}$$ (Li 2008)

$$RSL = \frac{Shoot\ length\ under\ stress}{mean\ shoot\ length\ of\ control}$$ (Krishnamurthy *et al.,* 2007)

$$RRL = \frac{Root\ length\ under\ stress}{mean\ root\ length\ of\ control}$$ (Krishnamurthy *et al.,* 2007)

$$RSIR = \frac{Germination\ \%\ of\ control - Germination\ \%\ in\ treatment}{Germination\ \%\ of\ control}$$ (Li 2008)

$$VI = (Shoot\ lenght + Root\ lenght)\ x\ Germination$$

(Abdul-Baki and Anderson 1973)

Statistical analysis: Utilizing R Studio software (Racine 2012), an analysis of variance (ANOVA) was carried out, employing the "*agricolae*" package (de Mendiburu and de Mendiburu 2019). To assess the correlation between traits, the '*corrplot*' package (Wei *et al.,* 2017) was employed. Principal Component Analysis (PCA) and Cluster analysis was performed using the '*FactoMineR*' and '*factoextra*' packages.

RESULTS AND DISCUSSION

The impact of salt on the morphological characteristics of different barnyard millet mutants was assessed through analysis of variance (ANOVA) after subjecting them to varying concentrations of $NaHCO_3$. The results revealed a highly significant difference in all observed traits for both treatments and mutants (**Table 2**). Moreover, interactions between mutants and treatment were also found to be significant, at a probability level of $p<0.05$. The mean square values attributed to salt treatment were highly significant across all examined traits at different concentrations.Arthi *et al.,* (2019) performed the same study and inferred similar significant differences among the genotypes.These findings underscore the notable influence of salt stress on the morphological traits of the studied mutants and emphasize the significance

Table 1. List of experimental materials derived from induced mutagenesis of MDU1 Barnyard Millet

Culture Name	Dosage	Mutagen
ACM21001	10mM	EMS
ACM21002	10mM	EMS
ACM21003	10mM	EMS
ACM21004	10mM	EMS
ACM21005	10mM	EMS
ACM21006	10mM	EMS
ACM21007	20mM	EMS
ACM21008	20mM	EMS
ACM21009	20mM	EMS
ACM21010	20mM	EMS
ACM21011	20mM	EMS
ACM21012	20mM	EMS
ACM21013	10mM	SA
ACM21014	10mM	SA
ACM21015	10mM	SA
ACM21016	10mM	SA
ACM21017	10mM	SA
ACM21018	10mM	SA
ACM21019	20mM	SA
ACM21020	20mM	SA
ACM21021	20mM	SA
ACM21022	20mM	SA
ACM21023	20mM	SA
ACM21024	20mM	SA
ACM21025	20mM	SA

Table 2. Analysis of variance for morpho- physiological traits

Traits	Mutants	Treatments	Mutants:Treatments	Residuals
GP	148^{**}	3464^{**}	47^{**}	14
SL	6.97^{**}	130.38^{**}	1^{*}	0.75
RL	6.7^{**}	439.8^{**}	1.7^{**}	0.7
RGR	0.013^{**}	3.426^{**}	0.005^{**}	0.002
RWC	369^{**}	15653^{**}	67^{**}	0
RSL	0.0569^{**}	1.0972^{**}	0.0066^{**}	0.0052
RRL	0.0368^{**}	2.6627^{**}	0.0074^{**}	0.0032
RSIR	0.017^{**}	3.307^{**}	0.005^{**}	0.001
VI	14625^{**}	293387^{**}	36663^{**}	18291

**Significance at $p < 0.01$; * Significance at $p < 0.05$. GP, germination percentage; SL, shoot length; RL, root length; RGR- Relative growth rate; RWC, Relative water content; RSL, Relative shoot length; RRL, Relative root length; RSIR, Relative Salt Injury Rate; VI, Vigour index

of mutant-specific responses to varying levels of salt treatment. Seed germination profoundly impacts on crop establishment in the plant life cycle (Hatfield and Prueger 2015). Salt stress induces reduction in germination rate, exhibiting mutant-specific variability. Among the barnyard millet mutants tested ACM21018, ACM21022 and ACM21024, exhibited improved germination percentages (>45%) even at highest $NaHCO_3$ stress (75Mm). Also, three mutants exhibited lower germination percentage (<20%) due to increased salt

Table 3. Effect of salt stress on morpho physiological parameters governing salt tolerance induced by $NaHCO_3$ under *invitro* condition

Mutants	GP		RL		SL		RGR		RSL		RRL		RSIR		RWC		VI	
	0mM	75mM	0mM	75mM	0mM	75mM	0mM	75mM	0mM	75mM	0mM	75mM	0mM	75mM	0mM	75mM	0mM	75mM
ACM21001	99.68	16.33	12.98	5.48	8.79	5.89	1.00	0.16	1.00	0.70	1.00	0.43	0.00	0.84	94.09	37.91	2393.94	185.67
ACM21002	99.40	29.00	13.56	4.38	10.46	6.57	1.00	0.29	1.00	0.65	1.00	0.33	0.00	0.69	94.62	41.99	2423.17	317.55
ACM21003	99.85	23.68	11.13	5.98	11.03	3.25	1.00	0.24	1.00	0.29	1.00	0.49	0.00	0.73	95.87	31.09	2362.53	218.57
ACM21004	99.57	19.60	10.29	5.12	10.28	3.42	1.00	0.20	1.00	0.33	1.00	0.45	0.00	0.80	94.83	22.06	2148.11	167.38
ACM21005	99.82	39.64	13.05	5.40	10.80	5.46	1.00	0.40	1.00	0.50	1.00	0.42	0.00	0.57	93.92	44.21	2414.72	430.49
ACM21006	99.29	31.65	11.13	5.80	11.13	3.73	1.00	0.32	1.00	0.33	1.00	0.52	0.00	0.64	92.82	20.85	2377.45	301.62
ACM21007	99.61	32.82	10.02	5.84	10.37	5.24	1.00	0.33	1.00	0.51	1.00	0.58	0.00	0.64	94.11	44.67	2330.86	363.65
ACM21008	99.90	37.14	14.27	5.58	11.30	5.40	1.00	0.37	1.00	0.49	1.00	0.39	0.00	0.58	91.80	47.91	2554.81	407.80
ACM21009	99.49	29.15	14.54	6.30	13.02	6.16	1.00	0.29	1.00	0.44	1.00	0.42	0.00	0.69	95.32	40.89	2742.61	363.21
ACM21010	99.53	11.40	12.99	6.49	9.82	5.53	1.00	0.11	1.00	0.56	1.00	0.48	0.00	0.89	90.72	31.25	2631.82	136.96
ACM21011	99.77	29.12	11.74	5.35	8.69	7.00	1.00	0.29	1.00	0.82	1.00	0.45	0.00	0.69	90.15	55.00	2038.93	359.63
ACM21012	99.57	35.60	12.06	6.31	9.56	6.40	1.00	0.36	1.00	0.66	1.00	0.51	0.00	0.57	91.84	49.05	2652.24	452.48
ACM21013	99.82	35.69	10.07	5.85	8.79	6.88	1.00	0.36	1.00	0.79	1.00	0.58	0.00	0.61	92.15	54.52	1982.22	454.33
ACM21014	99.94	39.82	13.61	5.04	10.45	7.04	1.00	0.40	1.00	0.67	1.00	0.37	0.00	0.57	94.14	54.84	2537.50	481.14
ACM21015	99.84	31.89	16.01	3.64	10.90	6.20	1.00	0.32	1.00	0.58	1.00	0.23	0.00	0.66	87.25	39.02	2687.81	313.80
ACM21016	99.89	39.89	14.57	5.84	12.84	6.72	1.00	0.40	1.00	0.55	1.00	0.42	0.00	0.56	95.67	58.04	2445.09	**501.02**
ACM21017	99.68	39.94	13.01	5.31	12.63	7.22	1.00	0.40	1.00	0.57	1.00	0.48	0.00	0.62	93.83	**68.93**	2402.41	**500.45**
ACM21018	99.05	**46.58**	13.42	6.49	11.28	5.24	1.00	0.41	1.00	0.57	1.00	0.48	0.00	**0.53**	91.49	**59.16**	2357.33	476.00
ACM21019	99.34	37.02	10.25	4.48	9.16	5.51	1.00	0.37	1.00	0.59	1.00	0.43	0.00	0.61	87.63	48.21	2103.91	369.80
ACM21020	99.96	39.47	14.29	5.63	11.56	6.01	1.00	0.39	1.00	0.57	1.00	0.38	0.00	0.57	90.04	41.36	2643.14	459.43
ACM21021	99.43	32.85	12.73	4.02	11.21	6.54	1.00	0.33	1.00	0.59	1.00	0.32	0.00	0.66	93.80	48.85	2379.34	346.90
ACM21022	99.85	**49.08**	13.20	5.80	12.16	7.98	1.00	0.49	1.00	0.68	1.00	0.44	0.00	**0.51**	92.50	**64.24**	2465.14	**676.32**
ACM21023	99.63	28.85	12.04	5.69	10.81	6.73	1.00	0.29	1.00	0.63	1.00	0.48	0.00	0.68	91.62	49.33	2277.05	358.32
ACM21024	99.86	**47.92**	12.64	5.25	10.68	6.57	1.00	0.41	1.00	0.61	1.00	0.48	0.00	**0.52**	95.22	**62.95**	2002.63	483.54
ACM21025	99.67	27.59	11.73	3.78	8.79	7.22	1.00	0.28	1.00	0.83	1.00	0.32	0.00	0.68	85.47	44.50	2045.14	303.49
MDU 1	99.10	31.59	11.31	5.61	9.30	6.97	1.00	0.32	1.00	0.75	1.00	0.50	0.00	0.63	92.89	57.77	1998.52	397.61
CO (kv) 2	98.50	28.38	10.79	5.79	8.19	6.26	1.00	0.28	1.00	0.78	1.00	0.54	0.00	0.69	93.98	52.98	1912.84	342.08
MAX	99.96	49.08	16.01	6.49	13.02	7.98	1.00	0.49	1.00	0.83	1.00	0.58	0.00	0.88	95.87	68.93	2742.61	676.32
MIN	98.5	11.40	10.02	3.64	8.19	3.25	1.00	0.11	1.00	0.29	1.00	0.23	0.00	0.50	85.47	20.85	1912.84	136.96
MEAN	99.59	32.54	12.50	5.42	10.52	6.04	1.00	0.33	1.00	0.59	1.00	0.44	0.00	0.65	92.51	47.10	2344.86	376.64
SD	0.33	8.27	1.56	0.77	1.32	1.15	0.00	0.08	0.00	0.14	0.00	0.08	0.00	0.08	2.64	11.96	240.75	117.67

GP, germination percentage; SL, shoot length; RL, root length; RGR- Relative growth rate; RWC, Relative water content; RSL, Relative shoot length; RRL, Relative root length; RSIR, Relative Salt Injury Rate; VI, Vigour index concentration **(Table 3**), likely resulted from diminished osmotic potential, ion accumulation, and nutrient uptake (Afzali *et al.,* 2011). These factors collectively disrupt the seeds' physiological and biochemical functions, impeding aerobic respiration. RSIR increases with increased salt levels(Williams *et al.,* 2019). Three mutants displayed a low RSIR (0.5) at 75mM, while others exhibited higher RSIR (0.6-1.0) (**Table 3**), signifying salt sensitivity (Sreenivasulu *et al.,* 2000), attributed to membrane damage and reduced water uptake (Prasanthi Kumari *et al.,* 2013). Morphological traits decline with increasing salt concentrations, affecting root and shoot growth (**Table 3**) due to salt's disruption of cell division and differentiation (Tahjib-Ul-Arif *et al.,* 2018).

Water loss within the plant contributes to a reduction in Relative Water Content (RWC), which serves as a dependable and widely used indicator for evaluating salt tolerance (Sánchez-Rodríguez *et al.,* 2010). RWC demonstrates variations across mutants and salt levels (**Table 3**). Three mutants (ACM21017, ACM21022, ACM21024) maintain higher RWC (>60%) at 75mM, indicative of its tolerance nature. Conversely, six mutants exhibit lower RWC (<40%), indicating sensitivity and inefficient water uptake (Islam *et al.,* 2011). Decreased RWC implies reduced turgor pressure and constrained cell expansion (Katerji *et al.,* 1997). Tolerant plants sustain favourable water relations and RWC (Khanna-Chopra and Selote 2007).

Vigour index decreased significantly with the increasing salt level as shown in the **table 3**. Reduction in vigour index of 99% was observed in 75 mM concentration. Salt stress affects the metabolisms in plants which ultimately lead to reduction in growth and productivity of plants (Shafi *et al.,* 2009). Mutants ACM21022, ACM21016 and ACM21017 have highest vigour index of more than **500** compared to other mutants.

Correlation: Association between salt tolerance related traits was estimated through simple correlation analysis. Two of the major salt tolerance traits, RGR and VI ($r = 0.95^{**}$, $p < 0.01$) exhibited a highly significant and positive association among each other. These results

ACM21010 **ACM21022**

MDU1 **CO(KV)2**

Fig. 1. Seedling growth in different concentrations of $NaHCO_3$ (0mM, 15mM, 30mM, 45mM, 60mM and 75mM)

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were in accordance with Subramanian *et al.,* (2020) for RGR and VI. Under salt stress, VI exhibited a highly significant and positive correlation with RWC (r = 0.80**, p< 0.01),while RGR also showed a significant correlation with RWC (r = 0.68**, p< 0.01) (**Fig. 2**). Similarly, SL also highly correlated with RWC (r = 0.77**, p< 0.01).RSIR showed significant negative correlation with all the traits recorded. RSIR exhibited high negative association with RGR (r = -0.95**, p< 0.01) and VI (r = -0.86**, p< 0.01). The present study revealed that the salt tolerance indices, RGR and VI, showed high positive association with each other and with RWC indicating that the mutants with high RWC contributes positively towards salt tolerance. The study also shown that another key trait, RSIR has shown high negative correlation with important traits *viz*., RGR and VI. This has shown that the mutants with low RSIR values can perform better under high salt concentration (Rehman *et al.,* 2000). Hence, the current study provides insights for the direct and indirect selection of contributing traits toward salt tolerance.

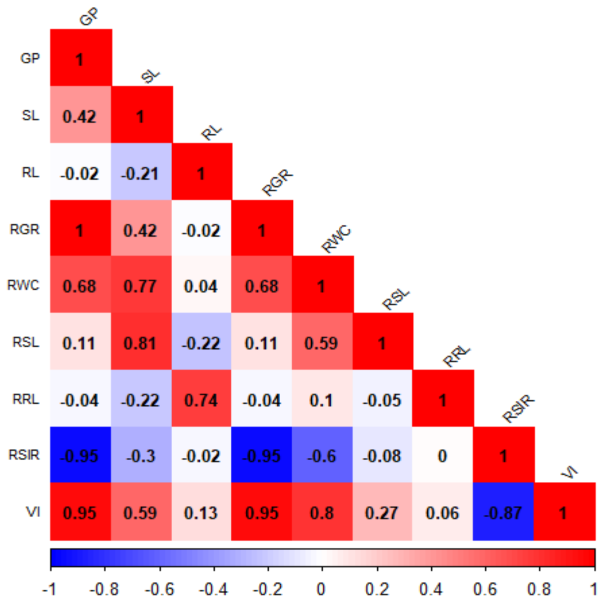

Fig. 2. Correlogram showing association between traits associated with salt tolerance GP, germination percentage; SL, shoot length; RL, root length; RGR- Relative growth rate; RWC, Relative water content; RSL, Relative shoot length; RRL, Relative root length; RSIR, Relative Salt Injury Rate; VI, Vigour index

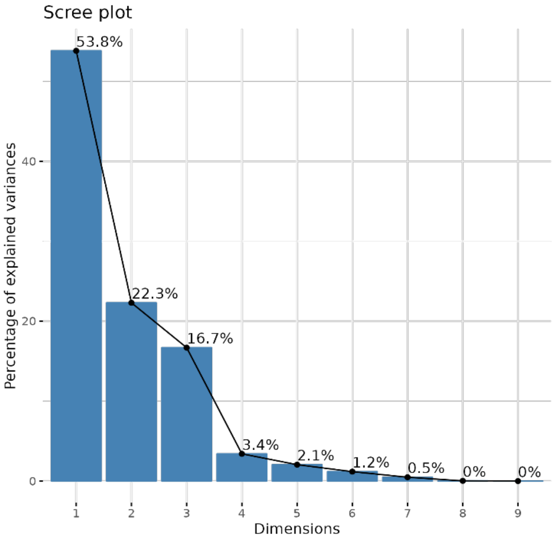

Fig. 3. Scree plot showing the degree of variation contributed by the two principal components towards overall variation

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Principal component analysis (PCA): Principal component analysis measures the contribution of each component to total variance (Sinha and Mishra 2013). The first three principal components (PCs) with eigenvalues greater than 1 collectively explained 92.82% of the total variation. PC1, with an eigenvalue of 4.84, accounted for 53.82% of the variation, followed by PC2 (22.30%) and PC3 (16.69%) as in Table 4. A scree plot visualized the variance contributions of each principal component (**Fig. 3**). In PC1, traits VI, RGR, RSIR, and RWC exhibited high loadings of 0.94, 0.87, 0.76, and 0.75, respectively, indicating their substantial contribution to this component and thus to most of the total variation. In PC2, RRL and RSL had high contributions (0.52 and 0.36). PC3's variation was primarily due to RSL (0.37) and RRL (0.36). Plotting individual salt tolerant traits against PC1 and PC2 revealed that VI and RGR were crucial contributors to overall variation, followed by RSIR, RWC, and SL.

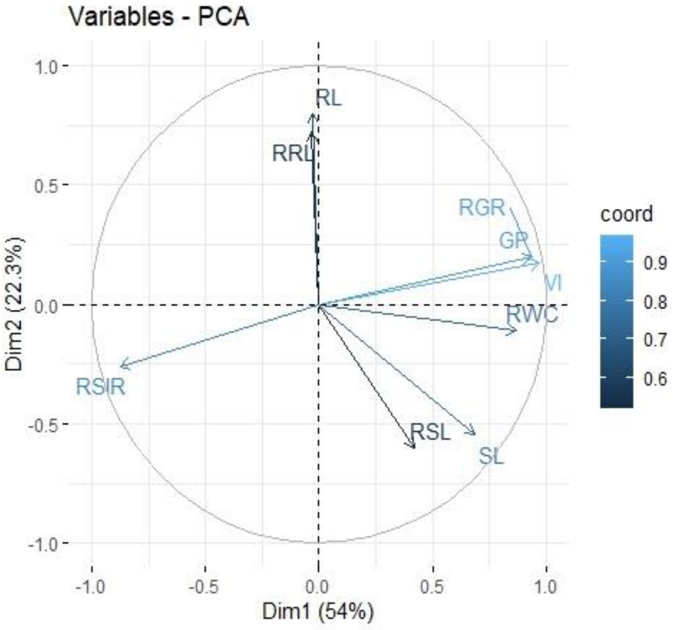

Fig. 4. PCA analysis plotting variation on two principal components contributed by individual traits associated with Salt tolerance

Table 4. Principal components of traits associated with Salt tolerance

Parameters	PC1	PC2	PC3
Eigen Value	4.84	2.01	1.5
Variance %	53.82	22.3	16.69
Cumulative %	53.82	76.13	92.82
GP%	0.88	0.04	0.08
SL(cm)	0.47	0.30	0.16
RL(cm)	0.00	0.64	0.24
RGR	0.87	0.04	0.08
RWC	0.75	0.01	0.12
RSL	0.18	0.36	0.37
RRL	0.00	0.52	0.36
RSIR	0.76	0.07	0.09
VI	0.94	0.03	0.00

PC, principal component; GP, germination percentage; SL, shoot length; RL, root length; RGR- Relative growth rate; RWC, Relative water content; RSL, Relative shoot length; RRL, Relative root length; RSIR, Relative Salt Injury Rate; VI, Vigour index.

Table 5. Cluster mean for morpho- physiological traits for salt tolerance

Traits	Cluster I (n= 5)	Cluster II (n=5)	Cluster III (n=12)	Cluster IV (n=5)
GP%	20.53	30.09	34.66	41.93
SL (cm)	4.36	6.71	6.02	7.11
RL (cm)	5.77	4.23	5.75	5.45
RGR	0.21	0.30	0.35	0.42
RWC	28.63	45.87	49.17	61.80
RSL	0.44	0.69	0.61	0.62
RRL	0.47	0.33	0.47	0.44
RSIR	0.76	0.68	0.62	0.58
VI	202.04	328.27	406.27	528.49

Values in parenthesis represent the number of mutants present in each cluster. GP, germination percentage; SL, shoot length; RL, root length; RGR-Relative growth rate; RWC, Relative water content; RSL, Relative shoot length; RRL, Relative root length; RSIR, Relative Salt Injury Rate; VI, Vigour index.

A biplot analysis displayed the dispersion and diversity among variables (traits) and mutants(**Fig. 5**). Four discrete quadrants in the biplot separated tolerant mutants from others. Highly tolerant mutants were positioned in the third and fourth quadrants, aligned with key tolerance traits. Moderately tolerant mutants were clustered near the origin, some overlapping with tolerant ones. Moderately susceptible and susceptible mutants were located away from the origin, indicating their sensitivity.

The biplot analysis indicated that the key traits VI, RGR, GP, and RWC prominently influenced the performance of highly tolerant mutants under salt stress. Conversely, RSIR negatively impacted susceptible and moderately susceptible mutants. Therefore, the PCA biplot effectively differentiated between tolerant and susceptible mutants and highlighted the traits contributing to variation under salt stress.Furthermore, in the current study, PCA analysis was effectively employed to identify the primary attributes that contribute to stress tolerance, in line with previous findings(Lapuimakuni S *et al.,* 2018, Vaezi *et al.,* 2020).

Cluster analysis: The hierarchical cluster analysis using Ward.D2 Euclidean distancescategorized the 25 mutants based on salt tolerance traits, resulting in four distinct clusters (**Fig. 6**). Cluster III (n=12) was found to be the

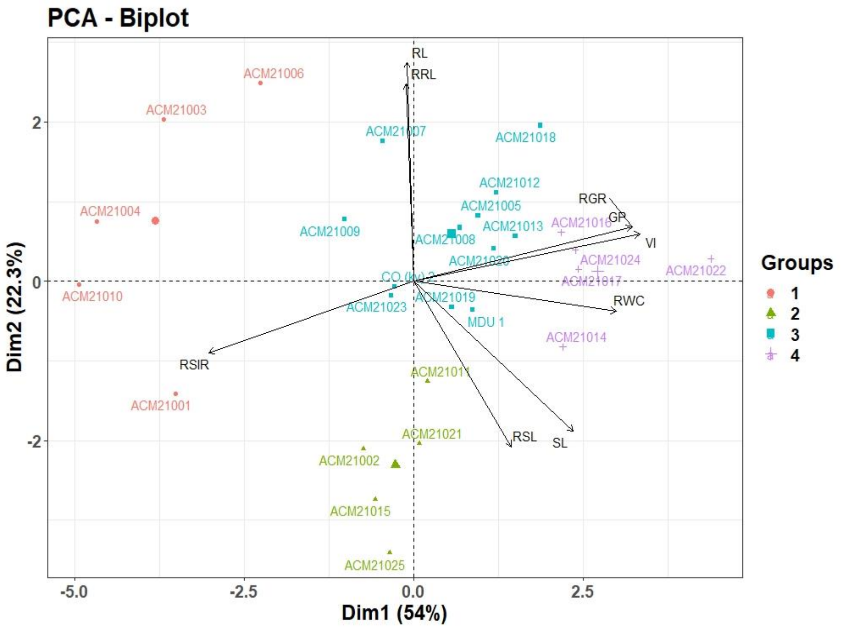

Fig. 5. Principal component analysis biplot plotting all the 25Mutants along with two checks based on the variation contributed towards two principal components

Fig. 6. Hierarchical cluster analysis based on the morpho- physiological traits. Euclidean distance and Ward's algorithm were used to evaluate the salt tolerance of 25 mutant lines along with the check. The red line indicates highly sensitive group; green line, sensitive group; purple line, moderately tolerant and blue line indicates tolerant group.

largest, while the remaining three clusters comprised five mutants each. Cluster IV included five highly tolerant mutants (ACM21022, ACM21016, ACM21017, ACM21024, ACM21014) with the highest mean values for salt tolerance traits: VI (528.49), RGR (0.42), RWC (61.80), and the lowest mean of RSIR (0.58). Cluster III housed twelve moderately tolerant mutants with the second-highest mean values for VI (406.27), RGR (0.35), RWC (49.17), and second lowest of RSIR (0.62). Cluster II consisted of five moderately susceptible mutants, with mean values for VI (328.27), RGR (0.30), RWC (45.87), and RSIR (0.68). Cluster I encompassed five susceptible mutants with the lowest mean values for salt tolerance traits: VI (202.04), RGR (0.21), RWC (28.63), and the highest RSIR (0.76). The range of mean values for each salt tolerance trait among tolerant (cluster IV) and susceptible clusters (Cluster I) were as follows: VI (202.04-528.49), RGR (0.21-0.42), RWC (28.63-61.80), RSIR (0.58-0.76).

The average inter-cluster distance was highest between Clusters I and IV (9.37), while it was lowest between Clusters I and II (6.69). The average intra-cluster distance was highest in cluster I (5.31) and it was lowest in Cluster IV (2.89) (**Table 6**). This indicated significantly higher variation among mutants in Clusters I (susceptible) and IV (highly tolerant), and comparatively lower variation between Clusters I (susceptible) and II (moderately susceptible). The intra-cluster distance confirms strong association among the five tolerant mutants in Cluster IV. This study revealed a distinct clustering pattern, effectively segregating tolerant and susceptible mutants. Mean values across clusters displayed significant trait variations. Cluster IV, containing highly tolerant mutants, exhibited the highest mean values for positively associated traits (VI, RGR, RWC), and the lowest for the negatively associated trait RSIR. These findings align with previous studies in wheat, rice, and barley genotypes under salt and drought stress (Ahmad *et al.,* 2008, Singh *et al.,* 2015, Zeng 2005)

This study has identified highly salt-tolerant barnyard millet mutant lines namely ACM21022, ACM21016, ACM21017, ACM21024, and ACM21014.These mutants

Table 6. Average Intra and Inter Cluster Distances of cluster groups

	1	2	3	4
1	**5.31**	6.69	7.36	9.37
2		**4.25**	5.84	6.39
3			**3.85**	5.47
4				**2.89**

Values in bold represents the intra cluster distance demonstrated superior performance in terms of VI, RGR, RWC, and lower RSIR under salt stress condition. Their favourable position in the Principal Component Analysis (PCA) biplot further affirms their adaptability. Additionally, the cluster analysis grouped these promising mutants into a distinct cluster (Cluster IV), emphasizing their collective potential. These findings hold substantial implications for future breeding programs aimed at developing salt-tolerant millet varieties in barnyard millet, which can play a pivotal role in addressing the challenges posed by climate change and global food security.

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