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Systematic assessment of salt tolerance based on morpho-physiological traits and genes related in inbred rice lines at the seedling stage

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Abstract. *Herawati R, Simarmata M, Masdar, Purwoko BS, Miswati. 2024. Systematic assessment of salt tolerance based on morpho-physiological traits and genes related in inbred rice lines at the seedling stage. Biodiversitas 25: 9-20.* Salinity stress is an abiotic constraint that limits rice productivity. Salinity-tolerant traits are very complex and involve many genes. Therefore, it is difficult to conclude how rice plants respond to salinity stress. This study aimed to investigate the genetic potential of 19 rice genotypes for salt tolerance as well as the quantitative impacts of varying salinity stress levels on a subset of the genotypes. Salinity tolerance is identified in two stages: assessing salinity stress in nutrient solutions using 0, 5,000 (EC 7.8 dS.m^{-1}), and 10,000 ppm (EC 15.62 dS.m^{-1}). Gene expression analysis detected genes controlling salinity stress tolerance using two pairs of specific primers: DST (Drought Salt Tolerance) and OsAPX (ascorbate peroxidase). The results showed that all lines could survive high salinity stress up to EC 7.8 dS.m^{-1}. Biplot is reflected in K-means grouping the 21 rice genotypes into three major groups, namely salt-sensitive (1 genotype, 4.75%), moderately salt-tolerant (2 genotypes, 9.5%), and salt-tolerant-very tolerant (18 genotypes, 85.7%). The DST and *OsAPX1* genes showed both genes were expressed under salinity stress, although some lines showed smears or even did not appear at all, namely in G3 and G7. This result is consistent with the PCA analysis, where genotypes G3 and G7 are categorized as moderately tolerant. This study reveals that screening at the seedling stage combined with marker-assisted selection can identify tolerant genotypes. Furthermore, conducting field trials on soil salinity with EC > 4 dS.m-1 is recommended to obtain salinity-tolerant lines as potential new varieties.

Keywords: Gene expression, inbred line, salt tolerance, seedling stage

INTRODUCTION

Utilization of marginal land, including coastal land, for rice cultivation is an effort to increase national rice production. The potential of Indonesia's coastal land is estimated at 1,060,000 ha, located on 106,000 km of coastline (Indonesia Statistics Center Agency 2014). Salinity-stressed soil is defined as soil with an electrical conductivity (EC = Electrical Conductivity) value reaching 4 desiSiemens/m (dS.m^{-1}) at 25ºC (Shrivastava and Kumar 2015). The high EC value is caused by the content of dissolved salts, especially NaCl, an element that dominates the soil water content. Soil salinity can inhibit plant growth and development. Rice has a significant sensitivity to salinity stress at different growth phases. According to Simoes et al. (2016), there was a more than 30% decrease in plant height and the number of productive tillers with a 4–8 dS.m^{-1} increase in soil EC. According to several studies, increased soil electrical conductivity (EC) has also been linked to a decrease in sugarcane growth components and physiological processes, including photosynthetic rate, stomatal conductance, and chlorophyll synthesis (Patade et al. 2011; Zhang et al. 2014).

The salinity-tolerant rice varieties can be assembled through a breeding program that combines tolerant and high-productivity traits from selected germplasm. Identification is needed to explore the genes of salinity stress resistance to improve superior traits in breeding rice lines. The first thing is to do preliminary screening in the laboratory to find rice lines that have resistance when grown on saline media in greenhouse conditions. Two main ways of screening rice for salinity tolerance are field-based mass screening and controlled environment screening using hydroponics or other artificial mediums (Ismail and Horie 2017). Due to changeable environmental factors (weather, soil heterogeneity, and quantity of salt deposition in the soil), mass screening in the field without replication in several years and locations is not trustworthy for identifying acceptable cultivars (Ismail and Horie 2017).

In general, research for salinity tolerance, especially at the seedling stage, is conducted in laboratories or greenhouses under controlled conditions using NaCl solutions or NaCl + CaCl mixtures (Huqe et al. 2021; Mohammadi et al. 2023). Screening germplasm or novel varieties for salinity tolerance using nutrient solutions in natural settings during early development stages is an easy and quick screening technique. However, further confirmation is needed to overcome existing weaknesses. Therefore, combining marker-assisted selection (MAS) techniques helps breeders perform selection more accurately.

One of the mechanisms of salinity stress in plants is controlled by the DREB2 gene (Huang et al. 2018; Herawati et al. 2021). Plant molecular responses to salinity

stress include changes in gene expression, enzymatic synthesis, etc. Plants exposed to salinity stress will activate the antioxidant defense system in plant cells, namely enzymatic and non-enzymatic components. Enzymatic components include catalase (CAT) and ascorbate peroxidase (APX). Non-enzymatic components include flavonoids, phenolics, glutathione, ascorbate, and carotenoids (Nahar et al. 2018; Sandhya et al. 2021). *OsAPX1* is a gene crucial in plant tissue signaling by encoding the enzyme ascorbate peroxidase (APX). APX is an enzyme expressed differently in abiotic stress that functions in metabolic pathways and antioxidant defense mechanisms vital as OsAPX1 is responsive to environmental changes, limiting the adverse effects of H_2O_2 (Monsur et al. 2022; Li et al. 2022). APX is a more efficient enzyme detoxifying H_2O_2 during stress (Das and Roychoudhury 2014). Inhibiting H_2O_2 accumulation, DST (Drought Salt Tolerance) binds directly to DBS (DST-binding sequence) elements in the promoters of genes involved in H_2O_2 homeostasis and promotes their transcription. In addition, stomatal closure is impacted by the restriction of H_2O_2 buildup, which eventually improves abiotic stress tolerance. (Zhang et al. 2014; Shen et al. 2021).

The screening method is an early and important step in selecting improved rice varieties and exploiting desirable genotypic variation in rice breeding programs for salinity tolerance. In addition, the evaluation of morpho-physiological rice genotype characteristics subjected to salinity stress was analyzed using Principle Component Analyses (PCA) to explore the relationship between morphological and physiological traits; breeders used this method to understand better the morpho-physiological changes of rice varieties at the seedling stage and produce high salinity-tolerant genotypes. This study aimed to investigate the genetic potential of 19 rice genotypes for salt tolerance as well as the quantitative impacts of varying salinity stress levels on a subset of the genotypes.

MATERIALS AND METHODS

The experiment was conducted in the greenhouse and Integrated Biotechnology Laboratory of the Crop Production Department, Faculty of Agriculture, Bengkulu University, from June to September 2023. A salinity stress tolerance assessment was conducted on 19 superior lines from previous breeding. As controls, Salumpikit and IR20 were used as resistant and susceptible check varieties, respectively (Table S1). Salinity tolerance is identified in two stages: assessing salinity stress in nutrient solutions and gene expression analysis to detect genes controlling salinity stress tolerance.

Assessment of salinity stress in nutrient solutions

Salinity stress assessment was conducted in the greenhouse of the Crop Production Department, Faculty of Agriculture, University of Bengkulu, using a modified Yoshida (1976) nutrient solution. The experiment was conducted from June to September 2023. NaCl, Yoshida solution, styrofoam, foam, distilled water, and labels are used. The experiment was arranged in a factorial Split Plot Design repeated three times, where the main plot was NaCl concentrations of 0, 5,000, and 10,000 ppm, equivalent to 0, 7.8 $dS.m^{-1}$ (moderate salinity), and 15.62 $dS.m^{-1}$ (high salinity), respectively. At the same time, the subplots were selected from 19 lines and two check varieties, tolerant and sensitive, respectively (Table 1).

Rice seeds were germinated in petri dishes. Two-week-old rice plants with three leaves were transferred to perforated styrofoam (1.5 cm thick) (90 holes 8cm x 8cm apart, 10 plants per genotype) and placed on 80cm x 50cm x 15cm pots containing Yoshida solution. The solution was adjusted to a pH of 9.0 and maintained during the stress treatment, which was carried out for 28 days. Observations were made on root length, shoot length, root dry weight, shoot dry weight, root shoot ratio, and leaf chlorophyll content (total chlorophyll, chlorophyll a, chlorophyll b, and carotene). Moreover, the salt injury observations were performed 28 days after transplanting. Salt injury is a necrotic symptom that begins at the tips of old leaves, resulting in the death of leaves and plants. Moreover, with modification, Gregorio et al. (1997) used as the standard score for visually evaluating salt injury in the rice seedling phase based (Table 1).

Expression of salinity-stress-tolerant genes

The study detected salinity-tolerant genes using two pairs of specific primers: *DST* (Drought Salt Tolerance) and *OsAPX* (ascorbate peroxidase). Primers were designed using Primer3Plus software and the NCBI gene bank database. The specifications for each primer are presented in Table 2.

Table 1. Modified standard evaluation score (SES) of visual salt injury at seedling stage

Score	Observation	Response
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips or few leaves whitish and rolled	Tolerant
5	Growth severely retarded; most leaves rolled; only a few are elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dry; some plants dying	Susceptible
9	Almost all plants are dead or dying	Highly susceptible

Table 2. Primer characteristics for detecting salinity stress tolerance genes

Target gene	Primer sequence (Forward/Reverse)	Size (bp)	Annealing temperature (ºC)
DST	F:CCTCATTGGGTCAGGAAGAA R: GGATCTCAGCCACCCACTTA	250-300	54ºC
OsAPX1	F: GTCTTCCTGATGCTACCAAG R:GCTCCGTGAAGTAAGAGTTG	350-400	58ºC

The leaf sample was taken in the first experiment in the salinity condition. Next, 0.1 g of rice leaves are homogenized with liquid nitrogen, and the total DNA is isolated according to the Wizard Genomic DNA Purification Kit protocol. The leaf powder is inserted in an Eppendorf tube of 2 mL, then 600 µl of Nuclei Lysis Solution is added, vortex for 1-3 seconds, then the sample is heated in a water bath at 65ºC for 15 minutes. After that, 3µl of RNase Solution is added and incubated at 37°C for 15 minutes. Then, 200 µl is added to the Protein Precipitation Solution and centrifuged again for 3 minutes at 13,000 rpm; then, centrifuged again for 1 minute at room temperature. The next step is to remove and dry the solution for 15 minutes. The latter adds 100µl of DNA Rehydration Solution, incubates at 65ºC for 1 hour, and incubates again for 1 night at 4ºC.

Total DNA was used as a gene amplification template consisting of a specific primary sequence of salinity sequencing genes using a Polymerase Chain Reaction (PCR) machine. DNA ladder of 100 bp was used as the marker; positive controls are Salumpikit DNA, while negative controls use IR20 genotypes as a sensory check. The PCR reaction was carried out in a volume of 10 µL consisting of 0.01 U/uL PhusionR DNA polymerase, 100 mM dNTPs, 0.4 mM forward primer, 0. 4 mM reverse primer, and 0.5 µL DNA template.

The PCR was performed at a pre-denaturation temperature of 94ºC for 5 minutes, followed by 35 cycles, i.e., 94ºC denaturation for 1 minute, then annealing for 2 minutes and an extension of 72ºC for two minutes, as well as an extension for the last step at 72ºC during 10 minutes. The amplification success was analyzed using DNA electrophoresis on a 1.5% TBE agarose gel (0.6 clamps to 1x TAE 40 ml buffer) and run at 100 V for 30 minutes. The results were painted with Biotium GelRed® Nucleic Acid Gel Stain 10,000X and visualized with an LED transilluminator.

DNA sequencing was performed on the four representative genotypes to prove the DST and OsAPX1 genes' similarity to other species. The sequence was done by BigDye® Terminator First Base Services (PT. Genetika Sains Indonesia). The sequencing results were edited with the Bioedit software version 7.2 and analyzed by ClustalX and Mega X version 10.2.6. The amino acids were analyzed using NCBI's Basic Local Alignment Search Tool X (BLASTX) (www.ncbi.nlm.nih.gov/BLAST/). In addition, the phylogenetic tree was constructed using the MEGA (Molecular Evolutionary Genetics Analysis) program version 6.0 with the Neighbor-Joining method 1000 times.

Data analysis

Data were analyzed with an ANOVA of a 5% significance level; if there were significant differences between treatments, the LSD (Least Significant difference) test was conducted at 1%. The Stress Tolerance Index (STI) uses the following formula (Fernandez 1993):

$$STI = \frac{(Yp)(Ys)}{(\bar{Y}p)^2}$$

Where: Yp represents the relative root length in a typical situation, while Ys represents a stressed-out situation. $\bar{Y}p$ stands for average relative root length in typical circumstances. STI ≤ 0.5 for sensitive genotypes, 0.5< STI ≤1.0 for tolerant medium genotypes, and ITC >1.0 for tolerant genotypes are the parameters used to assess the degree of tolerance to salinity stress.

The relative value of the STI of the morphophysiological character is used for primary component analysis (PCA) and cluster analysis to evaluate the different tolerance of the genotype inbred lines. The value-F is calculated according to the result of the PCA, which represents the different morphophysiological responses of genotypes to salinity. The Pearson correlation coefficient is used to determine the relationship between morphological-physiological characteristics. The assessment formula of each major component is obtained based on the composition assessment system matrix of the PCA. Determination of major component numbers based on PC eigenvalue > 1 (Jolliffe, 2002). The clustering is done using K-means to determine the genotype tolerant of salinity. The collected data were analyzed using R software version 4.1.2 (R Core Team 2021) with the FactoMineR package (Lê et al. 2008).

RESULTS AND DISCUSSION

Assessment of salinity stress in nutrient solutions

The scoring for salinity tolerance in 19 rice inbred lines and 2 check varieties ranged from 3.0–6.33 at 5,000 ppm treatment (equivalent to 7.8 $dS.m^{-1}$) and 7.67–9.0 at 10,000 ppm NaCl treatment (equivalent to 15.62 $dS.m^{-1}$) as shown in Table 3. Almost all lines showed tolerance at 5,000 ppm treatment with a score of 3.0-3.67, which is consistent with the tolerant check variety Salumpikit's score of 3.67. In comparison, the susceptible check variety IR20 scored 6.33, indicating this variety is close to sensitive. Almost all lines tested at 10,000 ppm NaCl showed sensitivity ranging from sensitive to highly sensitive, indicating almost all leaves were browning and dying; salinity stress

significantly reduces plant growth, especially at 10,000 ppm stress, equivalent to 15.62 $dS.m^{-1}$ (high salinity), causing physiological damage, drying leaves, and even causing plant death (Table 3). In this study, salinity stress at 5,000 ppm is still at the tolerance limit, which is indicated by the survival growth of rice lines; hence, further assessment can be done until the end of observation.

Analysis of variance showed significant differences in salinity treatments for all morpho-physiological variables ($P \leq 0.001$ and $P < 0.05$), except for the shoot dry weight character (Table 4). The genotypes tested showed significant differences ($P \leq 0.001$) in all characteristics except root length and dry weight. There was an interaction between salinity stress and genotype on shoot length and physiological traits (total chlorophyll, chlorophyll a, chlorophyll b, and carotene) (Table 4). These interactions affect genotype and salinity is important to determine tolerant varieties under salinity stress conditions.

Effect of salinity stress on morpho-physiological characters

Boxplot analysis showed that there were significant differences in the reduction of morphology (root length, shoot length, root dry weight, shoot dry weight, and root shoot ratio) and physiological (chlorophyll a, chlorophyll b, total chlorophyll, and carotene) characters (Figure 1). A significant decrease was found in the 10,000 ppm treatment on root length (Figure 1a), shoot length (Figure 1b), root dry weight (Figure 1c), root shoot ratio (Figure 1e), and all physiological characteristics (Figure 1f-j), but did not show a significant decrease in shoot dry weight characters (Figure 1d). The finding in this study is that there is no difference between the control and 5,000 ppm stress on the root length and shoot length characteristics (Figure 1a-b). However, it shows a significant difference in other characteristics. This shows that the plants are still metabolized normally and grow well.

Pearson's correlations

The relationship between morpho-physiological characters was analyzed using Pearson correlation (Figure 2). Almost all characters had positive correlations, but there were significant and positive correlations in the characteristics of RSR/RDW ($r = 0.8$, $p<0.001$), Ch a/Ch b ($r = 0.87$, $p<0.001$), T Ch/Ch a ($r = 0.96$, $p<0.001$), T_Ch/Ch b ($r = 0.97$, $p<0.001$), and RL/SL ($r = 0.62$, $p<0.001$). Therefore, the relationship between characters was analyzed, as illustrated in Figure 3. The relationship between characters had a strong and positive correlation, as observed from the regression coefficient. The response of root dry weight to root shoot ratio has a regression equation $y = 0.29 + 21x$ ($R^2 = 0.65$, $p<0.000$) (Figure 3a), the response of root length to shoot length has a regression equation $y = 0.23 + 0.35x$ ($R^2 = 0.39$, $p<0.000$) (Figure 3b); and the response of total chlorophyll to chlorophyll a and chlorophyll b has regression equations $y = 0.12 + 0.48x$ and $y = 0.12 + 0.52x$ ($R^2 = 0.9$, $p<0.000$), respectively (Figure 3c–d). The red line shows the fitting curves, while the 95% confidence is the blue shading.

Table 3. Assessment of SES scores in inbred rice line following 14 days of 5,000 and 10,000 ppm NaCl exposure

Genotype	5000 ppm	Leaf color	10000 ppm	Leaf color	Genotype	5000 ppm	Leaf color	10000 ppm	Leaf color
G1	3.00	Green	7.67	Light brown	G12	3.67	Green	8.33	Brown
G2	3.33	Light green	8.33	Brown	G13	3.67	Green	8.33	Brown
G3	3.67	Green	7.67	Light brown	G14	3.67	Green	8.33	Brown
G4	3.67	Green	8.33	Brown	G15	3.67	Green	7.67	Light brown
G5	3.67	Green	8.33	Brown	G16	3.00	Green	8.33	Brown
G6	3.67	Green	8.33	Brown	G17	3.00	Green	7.67	Light brown
G7	3.67	Green	9.00	Brown	G18	3.00	Green	7.67	Light brown
G8	3.67	Green	9.00	Brown	G19	3.67	Green	7.67	Light brown
G9	3.67	Green	7.67	Light brown	Ir20	6.33	Light brown	9.00	Brown
G10	3.67	Green	7.67	Light brown	Sapumpikit	3.67	Green	7.67	Light brown
G11	3.67	Green	8.33	Brown					

Note: The modified standard evaluation score (SES) by Gregorio et al. (1997): (1) highly tolerant; (3) tolerant; (5) moderately tolerant; (7) sensitive; (9) highly sensitive

Table 4. Analysis of variance across the 19 inbred lines rice, treatments, and their interaction for the morpho-physiological traits measured at the seedling stage, 28 days after sowing; root length (RL), shoot length (SL), root dry weight (RDW), shoot dry weight (SDW), root shoot ratio (RSR), chlorophyll a (Ch_a), chlorophyll (Ch_b), total chlorophyll (T_Ch), carotene (Car)

Source	RL	SL	RDW	SDW	RSR	Ch_a	Ch_b	T_Ch	Car
Salinity (S)	***	***	*	ns	*	***	***	***	***
Genotype (G)	ns	***	ns	***	*	***	***	***	***
S x G	ns	***	ns	ns	ns	***	***	***	***

Note: Significant levels ***,*, and ns mean P-value < 0.001, 0.05, and non-significant, respectively

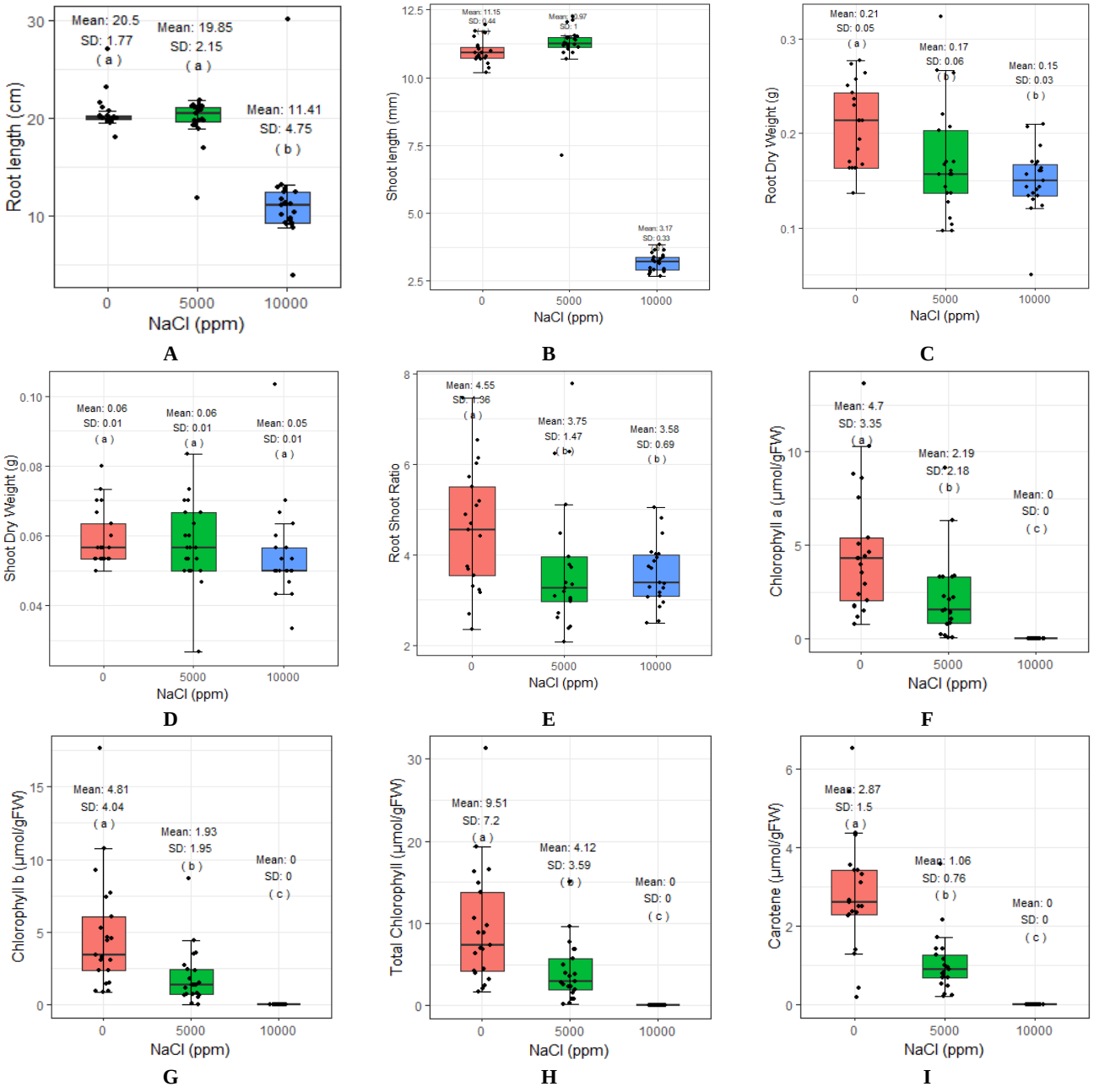

Figure 1: Box plots for morpho-physiological features and growth that display natural variation and the impact of varying salinity treatments on the average of (A) shoot length (cm); (B) root length (cm); (C) root dry weight (g); (D) shoot dry weight (g); and (E) root shoot ratio; µmol.gFW-1 for chlorophyll a (F), µmol.gFW^{-1} for chlorophyll b (G), µmol.gFW^{-1} for total chlorophyll (H), and µmol.gFW^{-1} for carotene (I). The horizontal line in the box shows the median value, SD=standard deviation, and the genotypes below the first quartile or above the third quartile are outliers in specific attributes. Significant differences ($P < 0.05$) are shown by different letters.

Salinity tolerance index (STI)

The varying analysis results on each character will make it difficult for researchers to select genotypes. Therefore, a selection index for each character will help breeders conduct further representative analysis. The salinity tolerance index was defined as the observations under salinity divided by the mean values of the controls. The 5,000 ppm stress treatment analyzed the salinity tolerance index because almost all genotypes showed survival growth. STI values showed varying results on all morpho-physiological characters (Table 5). STI on morphological characters for all genotypes ranged from 0.22 to 1.18, while in susceptible check varieties, IR20 was between 0.22 and 0.58, and in tolerant check varieties, Salumpikit was between 0.83 and 1.02. In addition to physiological characteristics, STI ranged from 0.2–0.9, indicating that salinity stress directly affects plant physiological processes.

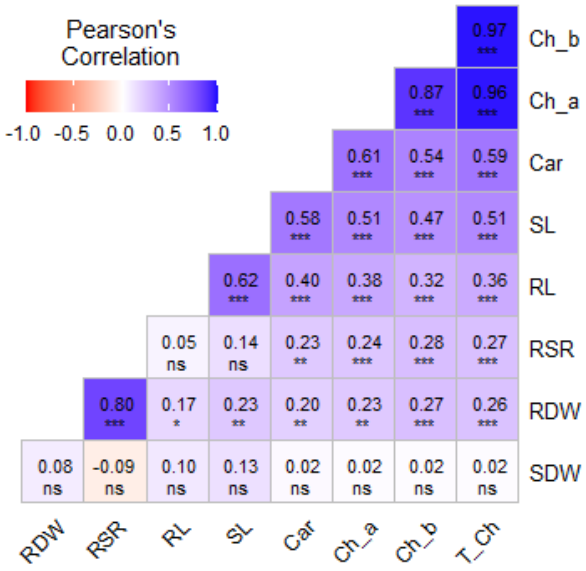

Figure 2. Pearson correlations among morpho-phisiological traits of inbred rice lines; root length (RL), shoot length (SL), root dry weight (RDW), shoot dry weight (SDW), root shoot ratio (RSR), chlorophyll a (Ch_a), chlorophyll (Ch_b), total chlorophyll (T_Ch), carotene (Car) Significant level ***,**, *, and ns means P-value < 0.001, 0.01, T 0.05, and not significant, respectively.

Assessment of salt tolerant genotypes using principal component analysis (PCA)

The Eigenvalue, percentage of diversity, and loading plot of the main components on morpho-physiological characters of 19 inbred rice lines are presented in Table 6. Determining the number of main components based on eigenvalue PC > 1 indicates that PC1 and PC2 can be used to form a biplot. PCA analysis was conducted to identify the main components of morphological-physiological characters of rice genotypes that best describe the response to salinity stress and to identify salinity-tolerant genotypes. The two components, PC1 and PC2, accounted for 50.66% and 17.39%, respectively, so the cumulative percentage variation of PC1 and PC2 was 68.05% among rice genotypes (Figure 4a). Furthermore, it allows the visualization of complex data on morpho-physiological parameters of 19 inbred rice lines separated by PC1 and PC2.

The genotype that shows the highest value against the presumption of STI based on the morphophysiological parameters of the genotypes for PC1 and PC2, located in the upper-left and lower-liberal corner of the biplot, is considered to be tolerant to highly tolerant of salinity. The genotype is considered quite tolerant, with the mean value for PC2 and PC1 at the top right of the graph.

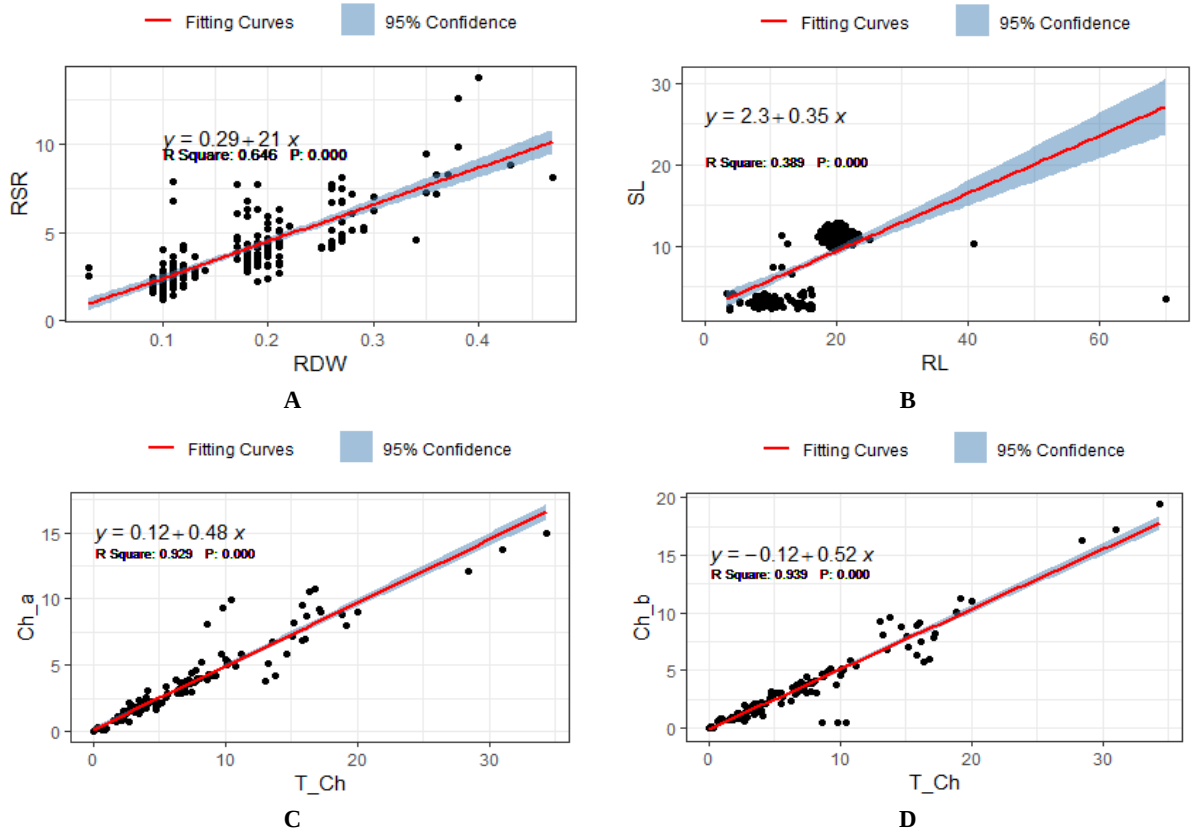

Figure 3. Strong correlation between characters. A. root dry weight response to root shoot ratio; B. root length response to shoot length; C. total chlorophyll response to chlorophyll a; D. total chlorophyll response to chlorophyll b). The red line shows fitting curves, while 95% confidence is the blue shading.

By contrast, the genotype shows low values in the morpho-physiological character of PC1, and PC2 is included in the lower-right part of the chart and is considered sensitive to salinities. Because PC1 and PC2 collectively explain more than half (68.05%) of the variations and make more important contributions to separating genotypes into different categories. K-means classified 21 padi genotypes into three major groups, i.e., including salt-sensitive (1 genotype, 4.75%), sufficiently salt-tolerant (2 Genotypes, 9.5%), salt-highly tolerant (18 Genotyps, 85.7%) (Figure 4b).

PCR analysis using specific primers showed consistency with the biplot clustering results. The amplification results of DST (Drought Salt Tolerance) and OsAPX (ascorbate peroxidase) genes in 19 upland rice lines and check varieties are presented in Figure 4.C. All lines expressing the OsAPX gene appeared to produce a single band at a sequence size of 400 bp, except for the sensitive check variety IR20. However, the expression of the DST gene was not amplified in genotypes G3 and G7, indicating that the genotype is moderately salinity-tolerant (Figure 4.C).

Table 5. The salinity tolerance index of inbred rice lines for morpho-physiological traits in 19 inbred rice lines exposed to 5,000 ppm NaCl shows different salinity tolerance responses.

Genotype	STI*								
	RL**	SL	RDW	SDR	RSR	Cha	Chb	ChT	Car
G1	0.99	0.90	1.19	0.77	0.51	0.93	0.77	0.85	0.63
G2	1.04	0.98	0.93	0.90	0.43	0.67	0.72	0.99	0.76
G3	0.96	1.03	0.94	0.89	1.03	0.29	0.38	0.49	0.23
G4	0.92	1.07	1.07	0.95	0.94	0.86	0.82	0.82	0.82
G5	1.04	1.06	0.87	1.18	1.11	0.82	0.86	0.84	0.76
G6	0.98	1.22	0.92	0.68	0.49	0.61	0.93	0.77	0.84
G7	0.96	1.17	0.80	0.80	0.85	0.35	0.57	0.44	0.62
G8	0.95	1.06	1.18	1.08	1.18	0.79	0.87	0.78	0.82
G9	1.07	1.03	0.83	1.50	0.33	0.76	0.81	0.93	0.92
G10	1.09	1.16	0.72	0.82	0.52	0.96	0.72	0.91	0.81
G11	1.07	0.98	1.11	1.00	0.76	0.94	0.84	0.84	0.88
G12	1.05	1.00	1.09	0.87	0.55	0.66	0.67	0.88	0.89
G13	1.08	0.98	1.18	1.01	0.95	0.75	0.63	0.89	0.85
G14	0.98	1.06	0.99	0.99	1.14	0.92	0.73	0.82	0.79
G15	1.02	0.98	0.93	0.90	0.94	0.65	0.84	0.83	0.88
G16	1.05	1.01	1.06	0.79	0.95	0.97	0.83	0.86	0.94
G17	1.10	0.99	1.03	1.05	1.03	0.61	0.77	0.75	0.71
G18	0.96	1.00	0.98	0.92	0.46	0.89	0.89	0.84	0.85
G19	0.75	0.99	1.12	0.97	0.57	0.81	0.83	0.72	0.83
IR20	0.49	0.42	0.43	0.36	0.45	0.58	0.36	0.46	0.22
Salumpikit	1.02	0.99	0.96	1.02	0.92	0.89	0.97	0.83	0.87

Note: *Salinity Tolerance Index was defined as the observations under salinity divided by the control means; criteria for determining the level of tolerance to drought stress are STI ≤ 0.5 for sensitive genotypes (S); 0.5< STI ≤1.0 for medium tolerant (MT) genotypes; and STI >1.0 for tolerant genotypes (T): ** root length (RL), shoot length (SL), root dry weight (RDW), shoot dry weight (SDW), root shoot ratio (RSR), chlorophyll a (Ch_a), chlorophyll (Ch_b), total chlorophyll (T_Ch), carotene (Car).

Table 6. Eigenvalues, percentage of variance, and the component loadings of morpho-physiological traits inbred lines exposed to 5,000 ppm NaCl

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Eigenvalue	4.560	1.565	0.912	0.599	0.526	0.424	0.197	0.134	0.082
% of variance	50.665	17.394	10.131	6.651	5.846	4.711	2.190	1.494	0.916
Component loading for each character*									
RL	-0.363	0.276	-0.318	0.061	-0.275	0.505	0.147	-0.335	-0.472
SL	-0.304	0.388	-0.380	-0.461	0.222	0.010	-0.501	0.139	0.282
RDW	-0.309	0.191	0.418	-0.243	-0.670	-0.420	-0.087	-0.032	-0.027
SDR	-0.316	0.220	-0.151	0.768	0.130	-0.428	-0.195	0.018	-0.035
RSR	-0.076	0.575	0.614	0.115	0.294	0.338	0.182	0.100	0.171
Cha	-0.297	-0.456	0.398	0.066	0.133	0.300	-0.610	0.003	-0.248
Chb	-0.391	-0.175	0.102	-0.239	0.454	-0.293	0.296	-0.607	0.044
ChT	-0.391	-0.307	-0.089	0.198	-0.275	0.297	0.180	0.038	0.713
Cr	-0.426	-0.158	-0.050	-0.146	0.156	-0.088	0.395	0.698	-0.307

Note: *Root length (RL), shoot length (SL), root dry weight (RDW), shoot dry weight (SDW), root shoot ratio (RSR), chlorophyll a (Ch_a), chlorophyll (Ch_b), total chlorophyll (T_Ch), carotene (Car).

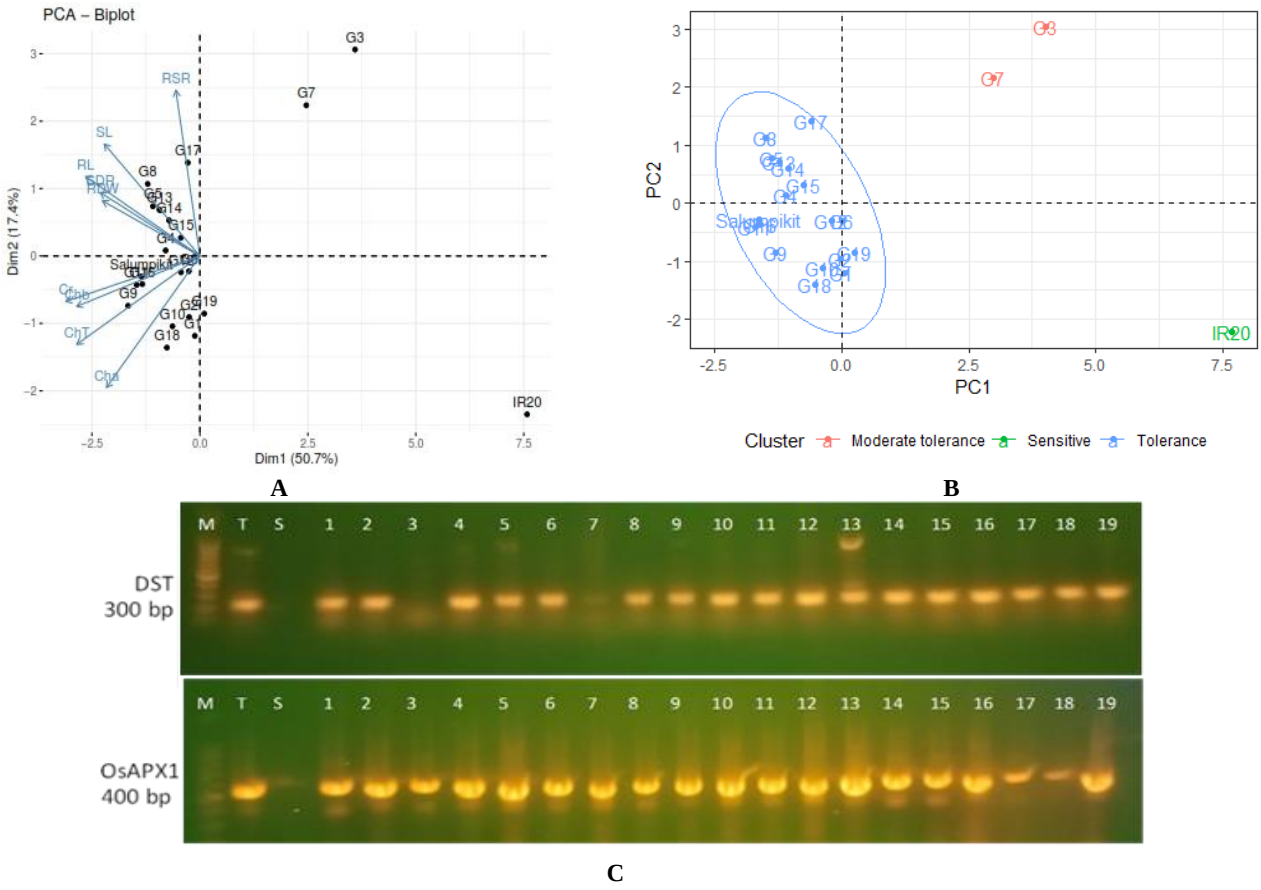

Figure 4: Biplot-PCA of all traits and salinity tolerance from 19 genotypes and two check varieties at the seedling stage. A. Principal component analysis (PCA) for the first two principal components (PC) scores, PCA1 vs. PCA2, describing the classification of 19 rice genotypes and Salumpikit, IR20 as check tolerance and susceptible respectively into morpho-physiological traits exposed to 5,000 ppm NaCl; B. K-means showed clustering genotype base on stress tolerance index ; C. DST and OsAPX1 gene expression in 19 upland rice lines with Salumpikit (T) and IR20 (S) as positive and negative control respectively (M= DNA ladder of 100 kb)

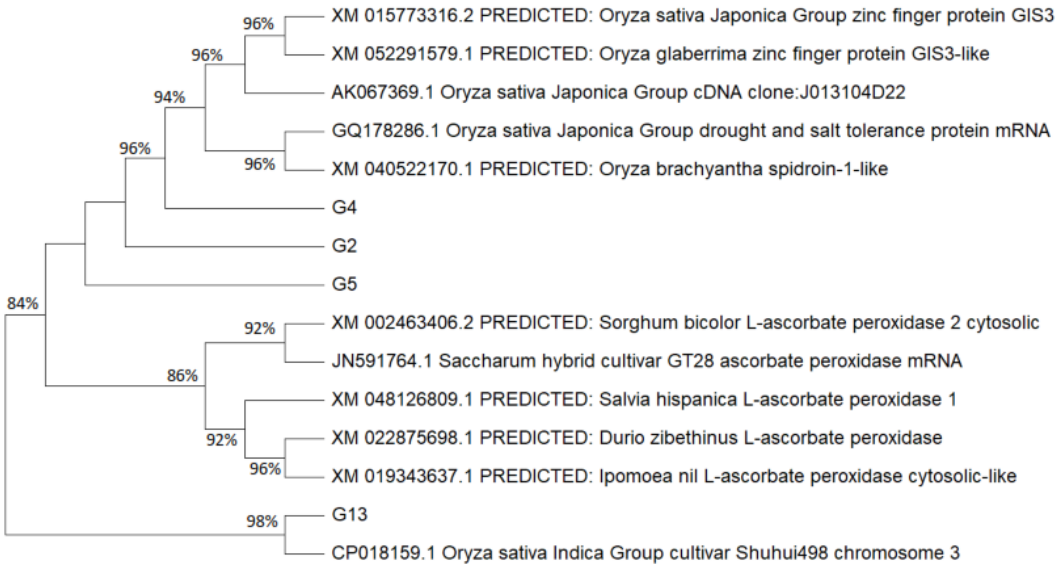

Figure 5: Phylogenetic analysis of the nucleotide sequences of the DST (drought salt tolerance) and OsAPX (ascorbate peroxidase) genes resulted in two distinct groups with homologs of 86-98%

The sequencing performed on four genotypes considered representative of salinity-tolerant in this study is presented in Figure 5. Phylogenetic construction of nucleotide sequences of DST (Drought Salt Tolerance) and OsAPX (ascorbate peroxidase) genes with homologous regions in different accession numbers resulted in two distinct groups, namely the Oryza sativa group and other plant groups that encode drought and salt tolerance (DST) and L-ascorbate peroxidase cytosolic (OsAPX) with 86–98% similarity.

Discussion

The modified standard evaluation system was used to determine the threshold limit of genotype tolerance to salinity stress at the early stages of plant growth during the seedling phase. Scoring revealed that all tested lines were only able to survive the 5000 ppm salinity stress equivalent to 7.8 $dS.m^{-1}$. In comparison, at the 10,000 stress equivalent to 15.62 $dS.m^{-1}$, all plants experienced severe injury in the first two weeks of treatment until the end of observation showed all plants had died. Saline soil has an EC of saturated paste extract >4 $dS.m^{-1}$, equivalent to 40 mmol of salt per liter. Growth and yield of cultivated plants are generally reduced at soil ECs of 4 $dS.m^{-1}$ or more; even sensitive plants can be adversely affected at ECs of 3 $dS.m^{-1}$ (Bañón et al. 2022; Melo et al. 2023).

Salinity at 3.89 dS/m reduced plant height, pod number, 1000-seed weight, and harvest index in sensitive genotypes, while in tolerant genotypes, it occurred at DHL 7.82 $dS.m^{-1}$ (Hossain et al. 2008). Previous researchers have widely reported the critical limit of mung beans to salinity stress. Based on a 10% yield reduction, they are 1.0 $dS.m^{-1}$ (Evans 2006), 1.8 $dS.m^{-1}$ (Yadav et al. 2011), and 1.5–3.3 $dS.m^{-1}$ (Cardon et al. 2012). Mung bean tolerance to salinity is related to physiological processes in the plant. Previous studies revealed that the level of tolerance of mung bean to salinity is related to membrane permeability and cell osmotic pressure (Nawaz et al. 2021; Al Murad et al. 2023), its ability to accumulate K (Hao et al. 2021; Joshi et al. 2022), its ability to inhibit Na translocation from root to shoot (Hu et al. 2022), its ability to accumulate water in leaves, proline and glycine betaine, and inhibit chlorophyll degradation (Shafi et al. 2019; Sofy et al. 2020;). Tolerance in mung beans is also related to enzymatic reactions in the cell. Increasing salt concentration from 50 to 200 mM NaCl increased catalase activity 2.4 times and peroxidase 2.8 times (antioxidative enzymes) (Al Murad et al. 2023).

Boxplot analysis showed significant differences in the reduction of morphological (root length, shoot length, root dry weight, shoot dry weight, and root shoot ratio) and physiological (chlorophyll a, chlorophyll b, total chlorophyll, and carotene) characters (Figure 1). Salinity stress causes osmotic stress, where the water potential increases, reducing water uptake, which leads to a decrease in relative leaf water content (Elhakem 2020; Zhang et al. 2023), which in turn causes cell dehydration (Katuwal et al. 2020). The water potential at field capacity was -0.033 MPa.

When the osmotic pressure in the rhizosphere exceeds the osmotic pressure in the root cells, it will inhibit the absorption of water and nutrients, causing the plant to be exposed to wilting and death due to a lack of water. It can be seen that there is a significant decrease in the treatment of 10,000 ppm in the characteristics of root length, shoot length, root dry weight, root shoot ratio, and all physiological characteristics, but not a significant decrease in the characteristic of shoot dry weight. Interestingly, this study shows no difference between the control and 5,000 ppm stress on root length and shoot length characteristics, although it shows a significant difference for other characteristics. Previous studies found significant decreases in the physio-biochemical attributes (such as total chlorophyll, carotenoids, K^+/Na^+ ratios, and catalase activity), growth traits (such as shoot length and dry weight), yield traits (such as the number of pods $plant^{-1}$ and seed yield ha^{-1}), and seed quality (such as protein and oil %) of soybean in actual saline soil (EC = 7.46 dS m^{-1}) (Taha et al. 2020). The critical limit of soybean-based on yield reduction is 5 $dS.m^{-1}$ (Yadav et al. 2011). In general, the critical phase of salinity stress for most plant species is during germination and seedling growth (Shiade and Boelt 2020; Tlahig et al. 2021; Tarchoun et al. 2022), so these phases are often used for salinity-tolerant selection (Shiade and Boelt 2020; Tlahig et al. 2021; Tarchoun et al. 2022).

Lack of water due to salinity stress causes disturbances in the photosynthesis process. Plants' photosynthetic rate is very low under salinity stress (Kwon et al. 2019). The effect of salinity stress on chlorophyll degradation is presented in Figure 1. Salinity stress at a concentration of 5,000 ppm is a tolerance limit that can still be used for chlorophyll analysis, namely the degradation of chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids. Pigment degradation is an appropriate indicator for observing plant responses to high osmotic conditions such as drought and salinity stress (Nxele et al. 2017).

Carotenoids play an important role in photosynthesis, especially in energy capture. Carotenoids strongly absorb much green light in sunlight, which chlorophyll cannot do. Then, the carotenoids transfer the excited energy to chlorophyll, leading to efficient photosynthesis. In addition, carotenoids also protect chlorophyll formation (Zulfiqar et al. 2021; Simkin et al. 2022). Therefore, plants will survive if they can maintain the content of total chlorophyll and total carotenoids under salinity stress. This study showed a wide range of values for each character, making it difficult for researchers to select the genotypes evaluated. Therefore, a selection index for each character will help breeders conduct a more representative analysis.

Principal component analysis (PCA) has previously been used to categorize salinity tolerance in canola (Brassica napus L., Naheed et al. 2021) and corn (Zea mays L., Wijewardana et al. 2016). Multidimensional analysis allows us to identify the variables that best describe plant tolerance to the variable response. Moreover, PCA can provide indications and explanations of the important component properties contributing to salinity tolerance (Negrão et al. 2017). In the current study, PCA analysis revealed that the root length and shoot length, which are grouped together, suggest that the two are more strongly correlated than the physiological character. The Salinity

Tolerance Index (STI) has previously been used to identify genotypes and parameter substances capable of resisting salinity (Ravelombola et al. 2017).

The results on PCA classification on genotypes are generally consistent with those obtained from calculating STI values for two extreme groups (high salt tolerance and sensitive salt). Medium categories (low and moderate salt toleration) differ slightly from some genotypes. The results of this study also revealed that all genotypes tested were grouped under high salt stress (7.8 $dS.m^{-1}$), resulting in genotypes that survived and even tolerated high salinity stress. This was expected because the genotypes tested were IR148 derivatives of the cross IR 79971-B-369-B-B (Herawati et al. 2021). The progeny population has been shown to have the QTL 12.1 marker, which can maintain yield under severe drought stress in the reproductive phase before flowering. The mechanism of drought stress is similar to salinity stress in plants, one of which is controlled by the DREB2 gene (Huang et al. 2018; Herawati et al. 2021).

The visual symptom showed significant differences between the resistant control (Salumpikit) and the susceptible check (IR20). Significant differences were found in all variables where the susceptible check had suppressed growth. Almost all lines had a good growth response, equivalent to the tolerant check varieties. Some lines, such as G13, G16, and G17, even have a higher growth response than the tolerant check. Increasing Na concentration in the soil reduces plant tissues' K++ and Ca content (Khan et al. 2021; Sarwar et al. 2022). Low Ca^{2+} uptake by plants disrupts cell membrane activity and integrity and promotes Na^{2+} accumulation in plant tissues. The potassium (K) concentration decrease in plant tissues may be caused by the antagonism of Na and K absorption in the roots, inhibiting K absorption and K transport in the xylem. Potassium plays an important role as a catalyst for various enzymes. Reduced K leads to decreased activity of enzymes such as nitrate reductase, which converts NO_3^- into NH3 (a protein constituent) (Singh et al. 2016; Ashraf et al. 2018). Mg^{2+} concentration decreases under saline conditions (Galkanda-Arachchige et al. 2021; Lim et al. 2022). Salinity reduces N and P content in all tissues (Sadak et al. 2020; Naveed et al. 2020). The decrease in N uptake is due to the interaction between Na^+ with $NH4^+$ and Cl with NO (Liu et al. 2020; Prodjinoto et al. 2021).

Analysis of gene expression using *DST* and *OsAPX1* in inbred lines under salinity stress showed that these two genes were expressed, although some lines showed smears or did not appear. The DNA fragment size of the *OsAPX1* gene in both was between 300 bp and 400 bp. The *OsAPX1* gene is a gene that encodes antioxidant enzymes, namely catalase and ascorbate peroxidase. The gene will be expressed more if rice is exposed to abiotic stress, one of which is salinity stress. *OsAPX1* gene expression will increase under abiotic stress compared to normal conditions (Figure 6-7). Similarly, it was reported that *OsAPX1* gene expression in rice under abiotic stress showed a higher expression level than normal conditions (Rossatto et al. 2017; Kim et al. 2018). The phylogenetic tree based on nucleotide sequences revealed a very close relationship with Oryza sativa, Saccahrum sp, Sorghum bicolor, and other genus groups that encode DST and OsAPX1 genes under salinity stress with homologs of 97-98%.

OsAPX1 as a gene is important in signaling in plant tissues by encoding the enzyme ascorbate peroxidase (APX). APX is an enzyme expressed under abiotic stress that functions in metabolic pathways and antioxidant defense mechanisms and plays an important role in drought stress in rice (Nahar et al. 2016). APX is a more efficient enzyme detoxifying H_2O_2 during stress (Das and Roychoudhury 2014). DST binds directly to DBS elements in the promoters of genes related to H_2O_2 homeostasis and activates their transcription, thereby inhibiting H_2O_2 accumulation. The inhibition of H_2O_2 accumulation affects stomatal closure and, ultimately, increases abiotic stress tolerance (Shen et al. 2021; Zangani et al. 2023). DST contributes to stomatal movement by regulating genes involved in ROS homeostasis. The DST mutation regulates 24 peroxidation precursors accumulating H_2O_2 in guard cells and triggers stomatal closure, increasing drought and salinity stress tolerance. Previous studies found that Arabidopsis mutants lacking cytosolic and chloroplastic ascorbate peroxidases responsible for removing H2O2 were more tolerant to salinity stress (Kameoka et al. 2021). The study showed that the estimation of salinity tolerance using STI, PCA, and molecular markers is more comprehensive at the seedling phase.

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