PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol x(x): xx–xx
https://doi.org/10.14719/pst.2023

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RESEARCH ARTICLE

Morphological, biochemical and genetic variation of rice (*Oryza sativa* L.) genotypes to vegetative stage salinity stress

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OPEN ACCESS

ARTICLE HISTORY
Received: 12 August 2022
Accepted: 07 March 2023
Available online
Version 1.0 : 12 May 2023

Check for updates

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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Yah F N C, Shamsudin N A A, Razak M S F A, Rafii M Y, Bhuiyan M A R, Nordin M S, Salleh M S. Morphological, biochemical and genetic variation of rice (*Oryza sativa* L.) genotypes to vegetative stage salinity stress. Plant Science Today (Early Access). https://doi.org/10.14719/pst.2023

Abstract

Salinity is one of the most serious issues in rice cultivation and production. Salt stress significantly reduced seedling growth performance of rice. This research was conducted to study the effects of vegetative stage salinity stress on morphological, biochemical, molecular and genetic variation of 12 rice genotypes including 2 check varieties, MR297 (susceptible) and Pokkali (tolerant). The experiment was arranged in a split-plot design with 3 replications. Normal freshwater at 0 dS m^{-1} (L1), saline water at 6 dS m^{-1} (L2) and saline water at 12 dS m^{-1} (L3) were the main plot and rice genotypes were the sub-plot. In general, morphological and biochemical traits of all genotypes showed an overall reduction of about 47.41% in L3 as compared to L1 except for the tolerant check, Pokkali. The genetics and correlation analysis indicated that plant height, leaf size and standard evaluation system (SES) score might be used as a selection criterion in developing salt tolerant rice. The multivariate analysis revealed that a Malaysian landraces, Jarom Mas was clustered together with Pokkali as tolerant genotype. Screening using tightly linked Simple Sequence Repeat (SSR) markers (RM1287, RM10748, RM493) of salinity tolerant QTL, *Saltol* indicated that this QTL was absence in Jarom Mas. This finding might indicate the presence of other QTL associated with salinity tolerance in Jarom Mas. Further study on identifying the speculated QTL may be conducted to confirm this postulation.

Keywords

Chlorophyll, Proline, Rice, Salinity tolerant, *Saltol*

Introduction

Rice (*Oryza sativa* L.) is one of the world's most important crops that provides a staple sustenance for billions of people worldwide (1). Increasing demand for rice due to escalating world population necessitate higher production of rice. However, global rice productivity is affected by abiotic stresses. A study reported that abiotic stresses such as drought, high and low temperatures, salinity, submergence, and oxidative stress were responsible for more than 50% crop damages (2). Salinity is deemed as one of the most critical problems in rice cultivation. A total of 45 million hectares of land are currently affected by salt stress worldwide, with 20% of that area are arable land (3). Salt stress is projected to affect more than half of the world's arable land by 2050, implying that continuing salinization of limited

agricultural land would put pressure on food security (4). Moreover, rice plants are inherently sensitive to salt stress (8) specifically at the seedling (9) and reproductive growth stages (10). Salt stress interrupts the growth and development of rice (7), as well as the physiological and metabolic processes, mainly cellular osmotic and ionic homeostasis (10, 11). Salt stress also affecting gene expression, resulting in an increase in the production of osmoregulator and osmoprotectant (12) such as proline, which could be considered as a generic measure for salt stress alleviation (6).

In addition, yield improvement particularly genetic enhancement and development of salt tolerant rice varieties are crucial in overcoming the problem of salt stress. The selection and adoption of high-salt tolerance varieties have always been the preferred choice to improve productivity in salt-affected soils (4). The selection might be based on the phenotypic and/or genotypic attributes. The discovery of Quantitative Trait Loci (QTL) associated with salt tolerance has accelerating breeding for salinity tolerance in rice. A major salt tolerance QTL controlling Na^+/K^+ uptake ratio, widely known as *Saltol* was mapped on rice chromosome 1 (17). Molecular marker using Simple Sequence Repeat (SSR) microsatellite for *Saltol* have also been developed and used in marker-assisted selection (MAS) breeding of salt tolerant rice. The SSR marker was used due to high polymorphism in rice, highly reproducible, co-dominant and multi-allelic (15). Those tightly linked SSR marker to Saltol QTL may also be used for genotypic screening of rice germplasm for salt tolerant genotype. Nonetheless, the basis of plant breeding is genetic diversity. Hence, assessing genetic diversity and identifying superior genotypes are critical elements of any crop improvement programme (19). The present study thus was conducted to investigate the effects of vegetative stage salinity stress on the morphological, biochemical and genetic variation of selected germplasm in the International Islamic University Malaysia (IIUM) rice collection.

Materials and Methods

Plant material and experimental design

Twelve selected accessions from the IIUM rice collection were used (Table 1). All seeds were originally obtained from the Malaysian Rice Gene Bank, MARDI Seberang Perai, Pulau Pinang, Malaysia and was multiplied at the IIUM Kuantan Campus following previous study (20). The local rice cultivar MR297 was used as salinity susceptible check and Pokkali as salinity tolerant check. The experiment was arranged in a split-plot design with 3 replications whereby salinity stress as the main plot and the rice genotype as the sub-plot. The research was conducted in between February and March 2019 at the Glasshouse and Nursery Complex, Kulliyyah of Science, IIUM Kuantan Campus, Pahang, Malaysia.

Table 1. List of rice accessions

Genotype	Genotype name	Country of origin
V1	Apami	Malaysia
V2	Boewani	Suriname
V3	Basmati 370	India
V4	Cica 4	Colombia
V5	Dular	India
V6	Jarom Mas	Malaysia
V7	Kalarata 1-24	India
V8	Biris	Malaysia
V9	Haiboq	China
V10	MR297	Malaysia
V11	Pokkali	India
V12	MR253	Malaysia

Phenotypic evaluation and data collection

Planting procedures and salinity stress treatment

The planting procedures were based on a previous study (22, 23) with some modifications. All seeds (18 seeds per genotypes) were initially soaked in distilled water for 24 h prior to incubation for 48 h. The pre-germinated seeds were then sown in a 5 x 5" polybag with 500 g of topsoil (21). All genotypes were replicated thrice for each treatment in a block. The polybags were then placed in a container of about 10 cm height throughout the experiment. All containers were initially filled in with freshwater prior to salt stress treatment. About 2.0 g NPK (15:15:15) fertilizer has been applied in each polybag on the 14 days after sowing (DAS) (22). The salt stress treatment has been applied on 21 DAS by substituting freshwater with a diluted salt water at 6 dS m^{-1} for L2 and 12 dS m^{-1} for L3 respectively. The concentration of salt water was regularly checked and constantly refilled whenever necessary throughout the experiment.

Data collection

All morphological parameters such as plant height (cm), leaf size (cm), root length (cm), total dry weight (g) and number of leaves were recorded on 45 DAS (Table 2).

Table 2. Morphological parameters

S. No.	Parameter	Description
1.	Plant height	The height was measured from the basal to the shoot tip
2.	Leaf size	Multiplying leaf length with the leaf width
3.	Root length	The length was measured from the basal root to the root tips.
4.	Total dry weight	Dry weight of seedling after oven-dried at 70° C for 72 h
5.	Number of leaves	Number of leaves per seedling

Each measurement was taken twice to ensure its accuracy. Total dry weight was measured and recorded after oven-dried at 70 °C for 72 h (25). Chlorophyll content was determined following standard protocol (25) with some modifications. Fresh leaves were collected and immediately stored at -80 °C until further use. Next, about 0.1 g leaf was cut into small pieces of and grind with 2.0 mL 80% acetone. The homogenate was then centrifuged for 10 min at 9072 RCF. The supernatants were observed at specific wavelengths of 750.0 nm, 663.6 nm and 646.6 nm using the UV spectrophotometer (Perkin Elmer).

The chlorophyll *a*, chlorophyll *b*, and total chlorophyll (chl *a* +*b*) were then computed as follows:

Chl *a*: 13.71 ($A_{(663.6)} - A_{(750)}$) – 2.85($A_{(646.6)} - A_{(750)}$)

Chl *b*: 22.39 ($A_{(646.6)} - A_{(750)}$) – 5.42($A_{(663.6)} - A_{(750)}$)…….Eqn. (1)

Chl *a* +*b*: 19.54 ($A_{(646.6)} - A_{(750)}$) + 8.29($A_{(663.6)} - A_{(750)}$)

Where, A was the absorbance at 750 nm, 663.6 nm and 646.6 nm

Proline content was determined following methods described (26). About 0.1 g of fresh leaf was grinded and homogenized in 5.0 mL 3% aqueous sulfosalicylic acid. The extract was then mixed with 2.0 mL acid ninhydrin and 2.0 mL glacial acetic acid prior to boiling at 100 °C for 1 h. The resulting solutions were extracted and homogenized with toluene after cooling. The UV spectrophotometer was then used to measure the absorbance of toluene fraction at 520 nm. The proline calibration curve (Fig. 1) was also prepared based on the following formula:

$$\text{Proline (mmol g}^{-1}\text{ FW)} = \left[\frac{(\text{mg proline ml}^{-1} \text{ x ml toluene})}{\left(115.13 \text{ mg mmole}^{-1}/\text{g sample}/df\right)}\right] \quad \text{.....Eqn. 2}$$

Where; *df* was the dilution factor for sulfosalicylic acid used in the reaction, Proline MW; 115.13 g/mol

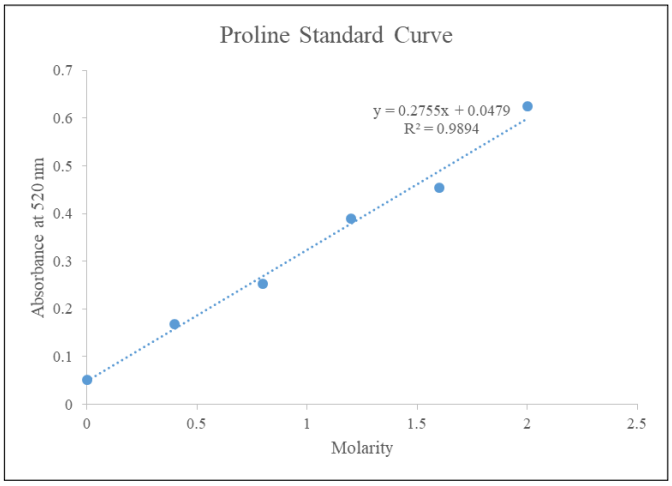

Fig. 1. The proline standard curve.

Molecular Analysis of Saltol QTL

Leaf sample of each genotype was collected on 21 DAS and dried using silica gel. The DNA was extracted following protocol established by the Malaysian Agricultural and Research Development Institutes (MARDI) (27). Frozen leaf samples were ground together with the extraction buffer using Tissue Lyser (Qiagen, Germany). Three tightly-linked SSR markers (RM1287, RM10748, RM493) of *Saltol* were used in the study (Table 3). The PCR reaction was conducted following protocol established by MARDI (27, 28). The PCR master mix was prepared by adding 10 uM of forward and reverse primer, 5 uM fluorescence labelled M13 (dye) primer, 2 uM dNTP and 1 uL Taq polymerase. The PCR profile was set with initial denaturation at 94 °C for 2 min, followed by 35 cycles of amplification at 94 °C for 30 s. Optimum annealing temperature of between 41-65 °C was determined prior to the experiment and set for 45 s. The final extension was set at 72 °C for 7 min respectively. PCR reaction was conducted using the GeneAmp PCR System 9700 and the products were multiplexed using fluorescent label M13 with PET, VIC FAM and NED fluorescent dyes. The ABI 3730 xl was then used to resolve the PCR products with GeneScan 500 LIZ as a standard ladder.

Statistical and genetic analysis

Data collected was analyzed using the analysis of variance (ANOVA) at $p \leq 0.05$ followed by Duncan's new multiple range test (DNMRT) using the Statistical Analysis System (SAS) software. Pearson's correlation analysis was also conducted using SAS software while the multivariate analysis was performed using R-studio software. GeneMapper version 5 was used to score the raw data generated by ABI 3730 xl. Peak Scanner was later used to determine the allele size. Quantitative genetic parameters such as genotypic variance, phenotypic variance, phenotypic coefficient of variance (PCV), genotypic coefficient of variance (GCV), broad-sense heritability and expected genetic advance (GA) were calculated using formula described by previous study (29). Variance components were estimated using PROC VARCOMP using SAS software and the genetic parameters were computed based on the expected mean square (EMS) formula.

Results and Discussion

Effects of salinity stress on morphological and biochemical traits

In general, there was a trend of reduction in seedling growth performance mainly plant height, leaf size, root length, total dry weight and number of leaves (Fig. 2). This observation was corroborated with previous studies which reported significant reduction in seedling growth performance of cereals such as wheat and rice (31, 32). In the present study, the highest plant height reduction of about 39.56% was recorded in Basmati 370, followed by the susceptible check, MR297 (31.93%). In contrast, tolerant check (Pokkali) recorded the lowest plant height reduction of only 16.3% (Fig. 2A). Interestingly, Jarom Mas, a Malaysian landraces demonstrated statistically similar plant height under L2 and L3, a similar trend showed by Pokkali (Fig. 2A). This result suggested that Jarom Mas might withstand salinity stress just like Pokkali (Fig. 3). It was described that plant metabolism particularly rate of cell elongation and multiplication would be affected by

Table 3. Tightly linked SSR marker to Saltol QTL

Marker	Annealing temp (° C)	Expected size (bp)	Forward primer sequence	Reverse primer sequence
RM1287	43.1	162	GGAAGCATCATGCAATAGCC	GGCCGTAGTTTTGCTACTGC
RM10748	41.6	95	CATCGGTGACCACCTTCTCC	CCTGTCATCTATCTCCCTCAAGC
RM493	44.8	211	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG

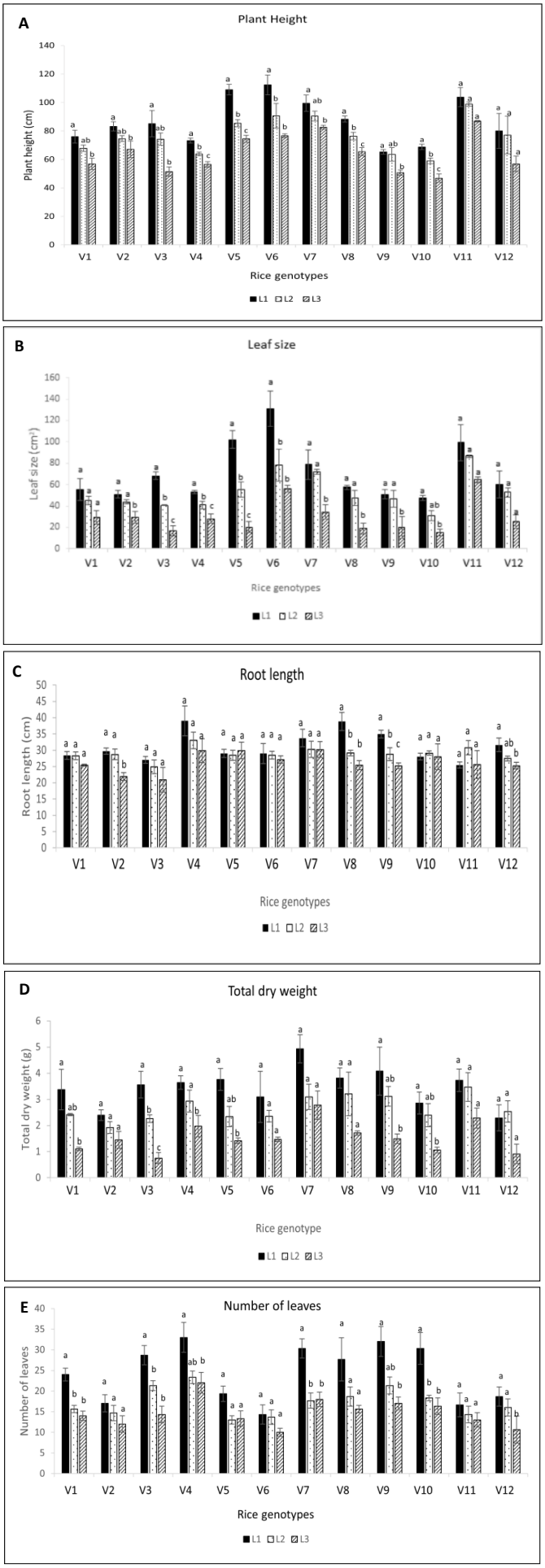

Fig. 2. Effects of salinity stress on: **A**) plant height (cm); **B**) leaf size (cm); **C**) root length (cm); **D**) total dry weight (g); and **E**) number of leaves. The alphabetical letters indicated a significant difference at ($p \leq 0.05$).

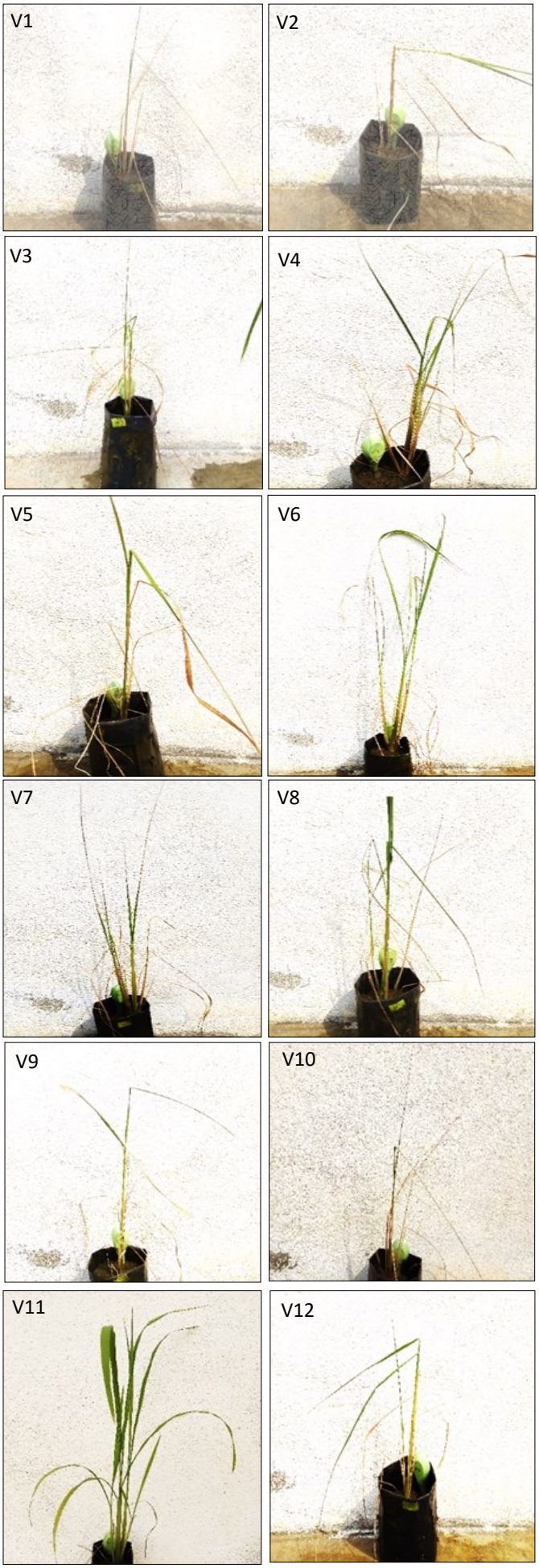

Fig. 3. Rice seedling (**V1-V12**) at 12 dS m^{-1} (L3) on 45 DAS.

salinity stress (34). Similar trend was also observed in the leaf size, in particular Basmati 370 (reduction of about 75.22%) and MR297 (reduction of about 68.01%) as shown in Fig. 2B. However, there was no significant reduction in the root length of all genotypes except for Haiboq, Biris and MR253 (Fig. 2C). Previous study also reported that detrimental effects of salinity stress was higher in the shoot as compared to the root (35). A similar result was also previously reported (36). In the case of total dry weight, the highest reduction of about 78.98 % was recorded in Basmati 370 (Fig. 2D). It was previously reported that total dry weight of rice seedlings was significantly affected by salinity stress (10). The decreased in total dry weight could be due to reduction in the rate of photosynthesis per unit leaf area, which limits the supply of carbohydrates for shoot growth (37). Moreover, Haiboq and MR297 recorded the highest reduction in the number of leaves of about 46.88 % and 46.16 % (Fig. 2E), as compared to other genotypes. The number of leaves of Pokkali (tolerant check) and Jarom Mas, however, were unaffected. Similar observation was also reported earlier (38). A reduction in the number of leaves could be due to an increased in sodium chloride accumulation in the cell wall and cytoplasm (12), and also could be attributed to the absence of leaf primordia formation in rice at higher salt concentration (39).

In addition, a similar decreasing trend as salinity level increases was also observed in the chlorophyll content (Fig. 4). Chlorosis on leaves caused by a high rate of chlorophyll degradation could be considered as a normal response of rice to salt stress (40). The reduction in chlorophyll '*a*' (Chl *a*), however, was found to be greater than the reduction in chlorophyll '*b*' (Chl *b*) as shown in Fig. 4A and 4B. A similar outcome was also previously reported which demonstrated that Chl a was more affected by salt stress compared to Chl b (40). A reduction in chlorophyll content could be due to the inhibitory effect of accumulating salt ions in chlorophyll production (41). Interestingly, tolerant check, Pokkali, recorded the highest total chlorophyll content at 34.103 mg/L under L3, followed by Jarom Mas at 19.332 mg/L (Fig. 4C), a possible indicator of tolerant ability to salt stress. Proline content in Pokkali was not significantly affected by salt stress treatment (Fig. 4D), indicating that it was not under stress condition. Proline may function as signaling compound as well as molecular chaperone (23). As a signaling compound, proline will be accumulated as a sign of stress under high salt condition (42). Similar trend was observed in susceptible check, MR297, which recorded significantly high proline content under L3 as compared to L1 (Fig. 4D). Proline may also play a role in stress tolerance mechanism (43, 44). Under salinity stress, proline will be accumulated and function as osmoprotectant in the regulation of osmotic balance and protection of subcellular structures (45). This might explain higher proline content in Jarom Mas under L3 (Fig. 4D).

Genetic variation of rice genotypes in response to salinity stress

Standard Evaluation System (SES) is the most popular indicator used to evaluate the salt-stress symptom of rice.

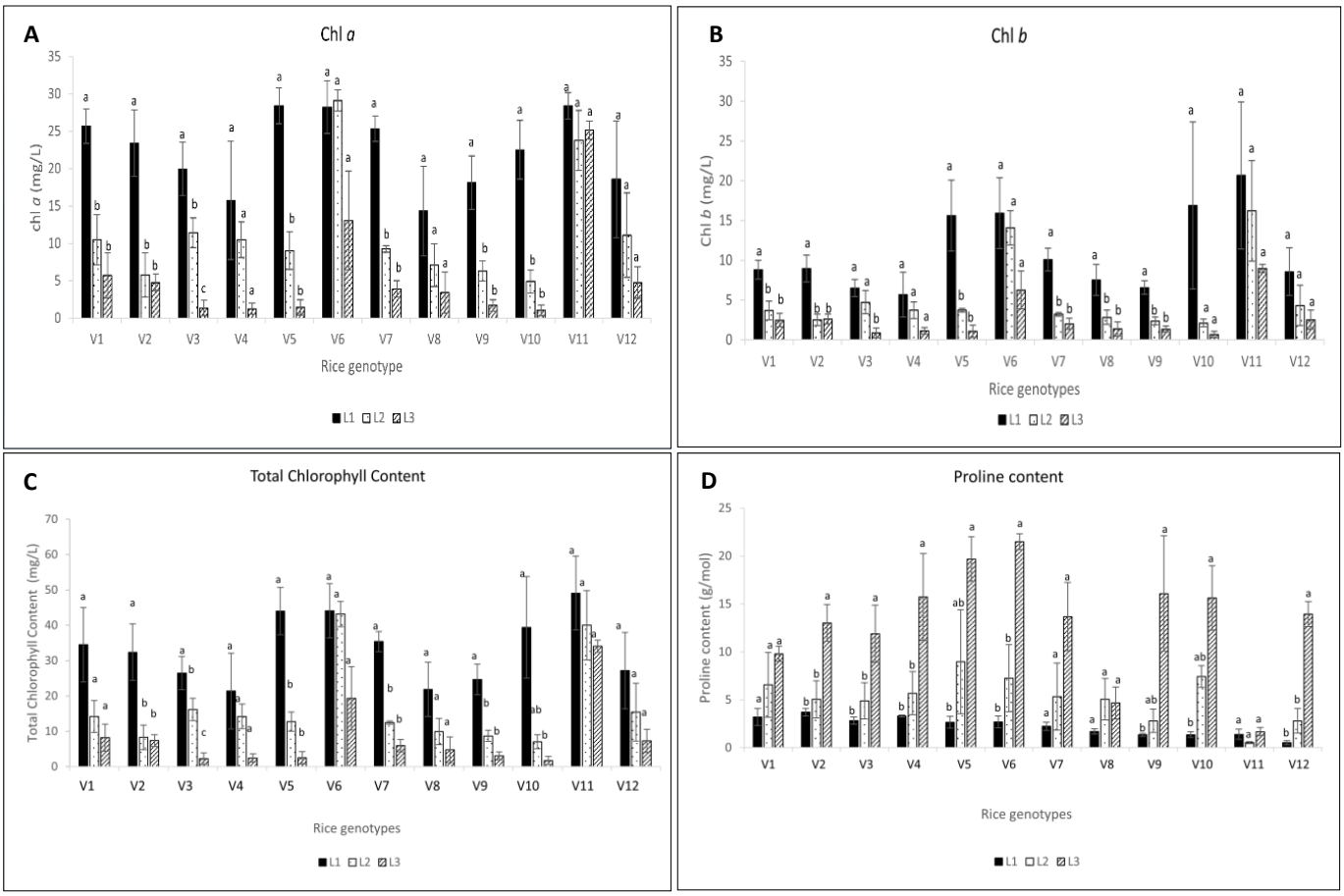

Fig. 4. The biochemical traits under different levels of salinity: **A**) chlorophyll *a* (mg/L); **B**) chlorophyll *b* (mg/L); **C**) total chlorophyll (*a*+*b*) (mg/L); and **D**) proline content (g/mol). The alphabetical letters indicated a significant difference at ($p \leq 0.05$).

The range of SES is between '1' to '9' with a score '1' indicates highly tolerant while score '9' indicates highly susceptible. Previous study reported that scores range between '3' and '5' indicated a moderate tolerant ability to salinity stress (48). In the present study, Pokkali (V11) recorded SES score '1' under L2 and score '2' under L3 (Table 4), indicating high degree of tolerant to salinity. Under L3, Jarom Mas (V6) could be considered as moderately tolerant genotype with SES score of '4'. The rest of genotypes, however, been classified as susceptible with SES score of between '6' and '7' (Table 4).

Table 4. The SES score under L2 (6 dS m^{-1}) and L3 (12 dS m^{-1}) salt stress treatment.

Accession	L2	L3
V1	3	7
V2	3	5
V3	5	7
V4	4	7
V5	5	7
V6	3	4
V7	4	7
V8	4	7
V9	5	7
V10	7	7
V11	1	2
V12	3	6

As shown in Table 5, the mean square analysis of the studied traits indicated extremely significant at p≤0.001 for all traits except for the root length. Similar result was also reported earlier (49). The mean comparison analysis under L3 (Table 6) indicated that Pokkali recorded significantly higher plant height at 86.867 cm followed by Kalarata 1-24 (82.567 cm) and Jarom Mas (76.6 cm). The leaf size of Jarom Mas and Pokkali was also significantly larger as compared to other genotypes. Moreover, Pokkali and Jarom Mas were also recorded significantly higher chlorophyll content as compared to other genotypes (Table 7). According to previous report, tolerant genotype may retain high chlorophyll content under salinity stress condition with Pokkali being commonly used as tolerant donor in rice breeding (16).

Table 5. Mean squares of morphological and biochemical traits under L3 salt stress treatment

Traits	Genotypes (df – 11)	Block (df – 2)	Error (df – 22)
PH	527.925***	3.211 ^{ns}	33.511***
LS	716.569***	230.483^{ns}	85.097***
RL	26.380^{ns}	96.644**	13.450*
TDW	1.029***	0.815**	0.187***
NOL	33.846***	52.111***	7.323***
CHLA	148.952***	11.247 ^{ns}	18.744***
CHLB	19.001***	0.404 ^{ns}	3.002***
CHLAB	272.977***	15.914 ^{ns}	36.2498***
PROLINE	95.852**	10.196 ^{ns}	27.569*

Notes: *Significant at p≤ 0.05, **highly significant at p ≤0.01, *** extremely significant at p ≤0.001, **PH**: Plant height (cm), **LS**: Leaf's size (cm), **RL**: Root length (cm), **TDW**: Total dry weight (g), **NOL**: Number of leaves, **CHLAB**: Total chlorophyll *a+b* (mg/L).

Moreover, as shown in Table 8, phenotypic variances (VP) of all traits were higher compared to genotypic variances (VG). Similarly, the genotypic coefficient of variation (GCV) of all traits was also slightly lower compared to the phenotypic coefficient of variation (PCV). This indicated that all traits were influenced by the environment. Previous study showed that PCV and GCV value greater than 20 could be regarded as high, whereas values less than 10 were considered as low (50). The highest PCV was recorded in chlorophyll *a* (CHLA) at 145.087, followed by the total chlorophyll (CHLAB), chlorophyl *b* (CHLB) and leaf size (LS) at 135.047, 114.895 and 57.597 respectively (Table 8). The lowest PCV was recorded in root length (RL) and plant height (PH) with the value of 19.95 and 20.23 respectively. The GCV of CHLA, CHLAB, CHLB and LS was also recorded high values at 121.249, 111.789, 91.906 and 48.603 respectively. Small differences between PCV and GCV on traits PH, NOL, RL and LS indicating high genetic influence on those traits. In contrast, a greater difference between PCV and GCV indicated high environmental influences as reflected in traits TDW, CHLA, CHLB, CHLAB and PROLINE. According to one report, selection made based on that traits that having greater differences between PCV and GCV would not be effective due to high environmental variance (51).

In addition, the broad-sense heritability (BSH) was in between 24.2% and 83.1% (Table 8). The highest BSH was recorded in PH at 83.1% followed by LS, CHAB and TDW at 71.2%, 68.5% and 60.0% respectively. Higher heritability value indicated that the trait could be inherited by the next generation thus selection made based on these traits would be effective (52). The lowest heritability was recorded in the RL and PROLINE at 24.3% and 45.2% (Table 8). However, BSH need to be paired with genetic advance (GA) and genetic advance as % of mean (GAM) to reflect the effect of additive gene in the studied trait (53). In the present study, GA was ranged between 0.84 (TDW) and 25.22 (LS) while the GAM was in between 190.2% (CHAB) and 8.035% (RL). According to an earlier report, GAM could be categorized into low (0-10%), moderate (10-20%) and high (≥20%) respectively (53). A trait that has high BSH and GAM values would indicate that the trait is being regulated by additive gene and selection made using the trait will be effective (54). As shown in Table 8, LS and PH recorded high BSH and GAM values suggesting that selection for salinity tolerant genotype could be made using these 2 traits.

Correlation and multivariate analysis

The Pearson correlation analysis indicated that LS recorded positive correlation with PH at r = 0.689 (p ≤0.0001), TDW at r = 0.331 (p <0.05), and CHAB at r = 0.761 (p<0.0001), as shown in Table 9. However, there was no significant correlation between LS and RL, NOL and

Table 6. Mean comparison of morphological traits under L3 salt stress treatment

Genotype	PH	LS	RL	TDW	NOL
V1	56.867^{ef} ±3.9	29.3933^{bc} ±6.44	25.400^{a} ±0.3	1.103^{de} ±0.06	14.0^{bcde} ±1.15
V2	67.1^{cde} ±5.88	29.7^{bc} ±5.583	21.933^{a} ±1.19	1.448^{cde} ±0.32	12.0^{cde} ±2
V3	51.433^{f} ±3.24	16.897^{bc} ±4.78	20.900^{a} ±3.81	0.748^{e} ±0.21	14.33^{bcde} ±2.03
V4	56.467^{ef} ±2.05	28.04^{bc} ±4.43	29.833^{a} ±3.67	1.975^{bc} ±0.41	22.0^{a} ±2.52
V5	74.5^{bcd} ±2.47	20.2667^{bc} ±4.96	29.900^{a} ±2.51	1.413^{cde} ±0.11	13.33^{bcde} ±1.86
V6	76.6^{abc} ±1.43	56.1233^{a} ±3.01	27.100^{a} ±1.15	1.466^{cde} ±0.088	10.0^{e} ±1
V7	82.567^{ab} ±1.31	34.2167^{b} ±6.7	30.200^{a} ±2.43	2.775^{a} ±0.54	18.0^{a} ±1.73
V8	65.433^{de} ±2.79	18.8867^{bc} ±5.313	25.400^{a} ±1.42	1.711^{bcd} ±0.08	15.66^{bcd} ±0.88
V9	50.67^{f} ±1.92	19.8967^{bc} ±10.29	25.200^{a} ±0.889	1.489^{cde} ±0.18	17.0^{bc} ±1.53
V10	46.833^{f} ±2.91	15.12^{c} ±3.25	27.967^{a} ±4.012	1.059^{de} ±0.11	16.33^{bc} ±2.03
V11	86.867^{a} ±0.54	64.45^{a} ±3.083	25.567^{a} ±4.283	2.282^{ab} ±0.38	13.0^{bcde} ±1.73
V12	56.867^{ef} ±5.43	25.21^{bc} ±6.396	25.233^{a} ±1.073	0.910^{de} ±0.37	10.67^{de} ±3.28

Notes: **PH**: Plant height, **LS**: Leaf size, **RL**: Root length, **TDW**: Total dry weight, **NOL**: Number of leaves. Means followed by different letters are statistically different at ($p \leq 0.05$) among genotypes. Values after ± represent standard error of the mean.

Table 7. Mean comparison of biochemical traits under L3 salt stress treatment

Rice genotype	CHLA	CHLB	CHLAB	PROLINE
V1	5.706^{c} ±3.02	2.482^{b} ±0.83	8.188^{c} ±3.83	9.782^{bcd} ±0.79
V2	4.770^{c} ±1.08	2.614^{b} ±0.61	7.384^{c} ±1.68	13.02^{abc} ±1.91
V3	1.363^{c} ±1.05	0.873^{b} ±0.63	2.236^{c} ±1.69	11.89^{abc} ±2.98
V4	1.255^{c} ±0.79	1.151^{b} ±0.41	2.406^{c} ±1.17	15.72^{ab} ±4.54
V5	1.429^{c} ±1.005	1.072^{b} ±0.77	2.501^{c} ±1.77	19.69^{ab} ±2.32
V6	13.080^{b} ±6.6	6.252^{a} ±2.38	19.33^{b} ±8.97	21.49^{a} ± 0.83
V7	3.907^{c} ±1.13	2.015^{b} ±0.68	5.922^{c} ±1.79	13.68^{abc} ±3.56
V8	3.400^{c} ±2.75	1.364^{b} ±0.89	4.764^{c} ±3.64	4.66^{cd} ±1.65
V9	1.685^{c} ±0.76	1.356^{b} ±0.4	3.041^{c} ±1.12	16.09^{ab} ±6.012
V10	1.071^{c} ±0.72	0.670^{b} ±0.42	1.741^{c} ±1.15	15.62^{ab} ±3.36
V11	25.143^{a} ±1.22	8.961^{a} ±0.52	34.10^{a} ±1.72	1.669^{d} ±0.43
V12	4.742^{c} ±2.12	2.536^{b} ±1.19	7.278^{c} ±3.32	13.94^{abc} ± 1.29

Notes: **CHLA**: Chlorophyll *a* (mg/L), **CHLB**: Chlorophyll *b* (mg/L), **CHLAB**: Total chlorophyll *a*+*b* (mg/L), and **PRO**: Proline (g/mol). Means followed by different letters are statistically different at $p \leq 0.05$ among genotypes. Values after ± represent standard error of the mean.

Table 8. Genetic variance of morphological and biochemical traits of rice genotypes under L3 treatment

TRAITS	MEAN	VG	V E	VP	PCV	GCV	BSH	GA	GAM
PH	64.350	164.805	33.511	198.315	21.884	19.950	0.831	24.108	37.464
LS	29.850	210.490	85.097	295.588	57.597	48.604	0.712	25.221	84.491
RL	26.219	4.310	13.450	17.760	16.073	7.918	0.243	2.107	8.035
TDW	1.532	0.281	0.187	0.467	44.643	34.595	0.601	0.846	55.227
NOL	14.694	8.841	7.323	16.164	27.360	20.235	0.547	4.530	30.827
CHLA	5.434	43.403	18.744	62.147	145.087	121.249	0.698	11.342	208.733
CHLB	2.513	5.333	3.002	8.335	114.895	91.906	0.640	3.805	151.446
CHLAB	7.946	78.909	36.250	115.159	135.047	111.789	0.685	15.148	190.626
PROLINE	13.105	22.761	27.569	50.330	54.136	36.406	0.452	6.609	50.434

Genotypic variance, **VE**: Variance of error, **VP**: Phenotypic variance, **PCV**: Phenotypic coefficient of variance, **GCV**: Genotypic coefficient of variance, **BSH**: Broad sense heritability, **GA**: Genetic Advance, **GAM**: Genetic Advance as Percentage of Mean (%).

PROLINE. The SES score, however, was negatively correlated with PH (r = -0.565, p< 0.0001), LS (r = -0.765, p <0.0001) and CHLAB (r = -0.723, p< 0.0001). A similar correlation between these traits was also reported (36, 45). The multivariate analysis was then conducted to classify rice genotypes into a group based on their tolerant and susceptibility to salinity stress. The SES score, PH and LS were used in the multivariate analysis due to high

Table 9. Pearson correlation coefficients of the studied traits

Traits	LS	RL	PH	TDW	NOL	CHLA	CHLB	CHLAB	PROL	SES
RL	-0.041	1.00								
PH	0.689***	0.131	1.00							
TDW	0.331*	0.454*	0.568**	1.00						
NOL	-0.187	-0.334*	-0.155	0.442*	1.00					
CHLA	0.747***	-0.002	0.559**	0.312	-0.253	1.00				
CHLB	0.785***	-0.024	0.575**	0.288	-0.276	0.980***	1.00			
CHLAB	0.761***	-0.008	0.565**	0.307	-0.260	0.998***	0.989***	1.00		
PROL	-0.262	0.186	-0.208	-0.190	0.0005	-0.334*	-0.243	-0.311	1.00	
SES	-0.765***	0.059	-0.565**	-0.296	0.153	-0.720***	-0.723***	-0.723***	0.175	1.00

Notes: *Significant at p< 0.05, **highly significant at p <0.01, *** extremely significant at p <0.001, **PH**: Plant height, **LS**: Leaves size, **RL**: Root length, **TDW**: Total dry weight, **NOL**: Number of leaves, **CHLA**: Chlorophyll *a*, **CHLB**: Chlorophyll *b* **CHLAB**: Total chlorophyll *a+b*

heritability, genetic advance and correlation values. Multivariate analysis revealed that rice genotypes involved in the present study could be classified into 3 different groups with Group I (Highly susceptible), Group II (Susceptible) and Group III (Tolerant), as shown in Fig. 5. In total, 6 genotypes were classified as highly susceptible (Group I) mainly Apami (V1), Basmati 370 (V3), Cica 4 (V4), Haiboq (V9), MR297 (V10) and MR253 (V12). Four genotypes mainly Boewani (V2), Dular (V5), Kalarata 1-24 (V7) and Biris (V8) as susceptible (Group II) while the remaining 2 genotypes, Jarom Mas (V6) and Pokkali (V11) as tolerant (Group III) respectively. The present study, hence identified a Malaysian landrace, Jarom Mas as a new tolerant genotype to salinity stress. Pokkali on the other hand is widely used as a tolerant check for salinity stress (35).

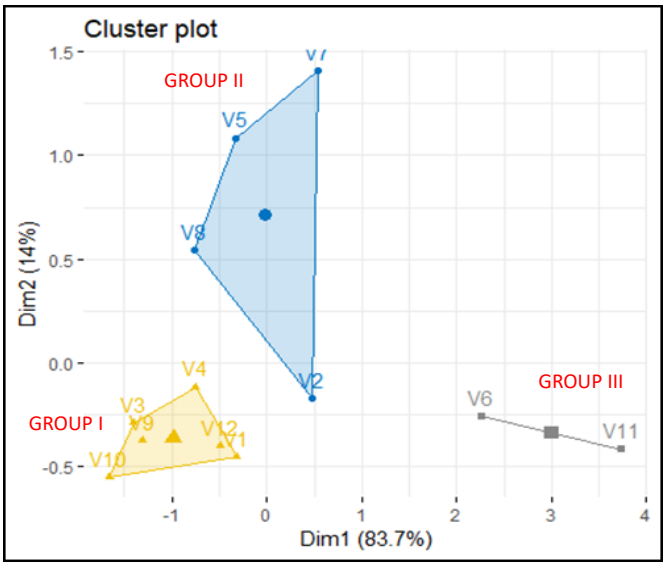

Fig. 5. Multivariate analysis of rice genotypes under salt stress.

Genotyping and Allele scoring

The scoring profile for each SSR marker (RM1287, RM10748, RM493) of *Saltol* was shown in Table 10. The allele size of each genotype was determined based on the peak on electropherogram obtained from the analysis using GeneMapper and Peak Scanner. All 3 SSR markers were highly polymorphic as there were more than 1 allele at one specific locus. In fact, SSR marker is widely used for fingerprinting and diversity studies of rice cultivars and their wild relatives due to high polymorphism rate (47). In the present study, there were 9 different alleles recorded in RM1287 and RM493 while RM10748 recorded only 5 different alleles (Table 10). Previous study indicated that

Table 10. Scoring profile of tightly-linked SSR marker to Saltol QTL

SSR Marker	RM1287			RM10748			RM493		
Genotype	Size of PCR product (bp)	Allele	Group	Size range (bp)	Allele	group	Size of PCR product (bp)	Allele	Group
Apami	193	CC	3	101	BB	2	255	BB	2
Boewani	182	DD	4	110	DD	4	227	CC	3
Basmati 370	197	EE	5	101	BB	2	227	CC	3
Cica 4	173	FF	6	103	CC	3	240	DD	4
Dular	200	BB	2	101	BB	2	252	EE	5
Jarom Mas	194	GG	7	101	BB	2	234	FF	6
Kalarata 1-24	173	FF	6	165	EE	5	247	GG	7
Biris	184	HH	8	110	DD	4	286	HH	8
Haiboq	173	FF	6	101	BB	2	261	JJ	9
MR297	179	JJ	9	101	BB	2	255	BB	2
Pokkali	204	AA	1	97	AA	1	249	AA	1
MR253	179	JJ	9	101	BB	2	255	BB	2

RM1287 and RM493 were a good flanking marker of *Saltol* with RM1287 flanking to the upper part of Saltol region and RM493 flanking to the lower part of *Saltol* region (centromeric to *Saltol*) in Chromosome 1 (46). The RM10748, on the other hand was a tightly-linked peak marker to *Saltol* (46). The allelic size of RM1287, RM10748 and RM493 in the reference genotype, Pokkali was confirmed and validated within the range of expected size of PCR product at 204 bp, 97 bp and 249 bp respectively. The susceptible check, MR297 recorded similar allele size as MR253 for all markers at 179 bp (RM1287), 101 bp (RM10748) and 255 bp (RM493). Meanwhile, a newly identified tolerant genotype in the present study, Jarom Mas recorded smaller allele size for RM1287 (194 bp) and RM493 (234 bp) but a bigger allele size for RM10748 (101 bp) as compared to Pokkali. Hence, this result suggested that *Saltol* was not presence in Jarom Mas. Further study might be conducted to identify potentially novel QTL associated with tolerant ability to salinity stress in Jarom Mas.

Conclusion

In general, morphological and biochemical traits of all genotypes showed an overall reduction of about 47.41% in L3 as compared to L1 except for the tolerant check, Pokkali. The multivariate analysis revealed that a Malaysian landraces, Jarom Mas was clustered together with Pokkali as tolerant genotype in Group III. Screening using tightly linked SSR markers of *Saltol*, however, indicated that *Saltol* was not presence in Jarom Mas. Hence, this finding might suggest the presence of other QTL associated with salinity tolerance in Jarom Mas. Further study on identifying the speculated QTL may be conducted to confirm this postulation.

Acknowledgements

This study was supported by the Fundamental Research Grant Scheme (FRGS) Project (FRGS/1/2019/WAB01/UIAM/01/1) of the Ministry of Higher Education Malaysia (MOHE) and the International Islamic University Malaysia (IIUM) Research Management Grant (RMCG20-012-0012). We are grateful to the Kulliyyah of Science, IIUM Kuantan and Centre for Marker Discovery and Validation (CMDV), Malaysian Agricultural and Research Development Institute (MARDI) for providing research facilities. We also acknowledged assistance from Ms. Site Noorzuraini Abd Rahman of the Genebank and Seed Centre, MARDI for providing rice germplasm for this study.

Authors contributions

FNCY conduct the experiment. NAAS and MSFAR involved in the biochemical and molecular genetic analysis. FNCY and MSS performed statistical analysis and draft the manuscript. MARB and NAAS reviewed and revised the manuscript. MSN, MYR and MSS conceptualized, coordinate and monitor the study. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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