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Research Article

Differential physiological responses to salt stress in rice landraces in Thailand

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Abstract

Soil salinity is a limitation factor for rice cultivation in the north-eastern Thailand. Understanding the mechanisms of salt stress tolerance is critical for maintaining or improving crop yield under salt stress. Over the past ten years, many landrace rice varieties have been examined for genetic diversity. To utilize the richness of natural resources and to explore novel genetic resources for developing a new variety, evaluating local rice varieties for salinity is still needed. This study investigated the physiological and biochemical responses of 8 landrace rice varieties in comparison to 'Pokkali', the standard salt-tolerant rice genotype, to clarify the major salt tolerance mechanisms in the landraces found in Thailand. After being exposed to 120 mM NaCl for 7 days, 'Pokkali' expressed physiological parameters in response to salt stress which are indicative of salt tolerance ability including low growth reduction, low Na^+/K^+ ratio, low chlorophyll degradation and low membrane integrity. In contrast, the landrace varieties displayed varying patterns of response. No landrace variety showed an outstanding ion exclusion mechanism as 'Pokkali', as evidenced by the fact that all landrace varieties showed approximately two times higher Na^+/K^+ ratios than 'Pokkali'. However, it was found that 'Surin' had a similar salt stress response to 'Pokkali' (low growth reduction, low chlorophyll degradation, low membrane integrity), except for the markedly increased proline and sugar accumulation, suggesting an osmotic adjustment mechanism. Therefore, this variety could be a potential genetic resource to be developed as a donor for the osmotic adjustment trait to improve salt-tolerant rice in the future.

Keywords: biodiversity; landrace rice; osmotic adjustment; proline; salt stress; salt tolerance

Introduction

Soil resources are one of the most important factors for crop cultivation. With an increase severity of climate change, soil salinization has become one of the major abiotic stress problems which severely affects crop

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production worldwide leading to the risk of food insecurity. Soils that accumulate salts (e.g., sodium chloride or sodium sulphate) in soil water greater than 4 dS m^{-1} is defined as saline soil (Atta *et al*., 2023). In Thailand, there are two types of salt-affected areas, inland saline soil and coastal saline soil. In the northeastern part of the country, the inland saline soil area covers approximately 18% of the region (Arunin and Pongwichian, 2015). This problem restricts agricultural crop cultivation area and deteriorates crop yield and quality.

Salt stress on plants results in several detrimental effects, including the retardation of plant growth and development, including inhibition of enzymatic activities, chlorophyll degradation and reduction in photosynthetic rate, or early senescence and death in some highly susceptible genotypes (Tabassum *et al*. 2021; Huang *et al*., 2020). Salt stress occurs due to the simultaneous occurrence of osmotic and ionic stresses, which can be considered as the primary effects. Additionally, oxidative stress can be considered as a secondary effect. The presence of salts in soil water causes ionic imbalance due to excess sodium ion accumulation and subsequently nutrient deficiency (Huang *et al*., 2020; Atta *et al*., 2023). Moreover, oxidative stress due to high level of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and singlet oxygen ($^1O_2$) which can interact with many crucial macromolecules and metabolites resulting in cellular damage (Singh *et al*., 2022).

Various mechanisms exist to protect plant cells against salt stress. Plants accumulate compatible solutes to counteract osmotic stress, develop ion compartmentation mechanisms from roots to shoots to cope with ion toxicity and generate enzymatic (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX) and non- enzymatic antioxidants (glutathione and ascorbate) to detoxify excess ROS (Akyol *et al*., 2022). The accumulation of compatible solutes or osmoprotectants (such as proline, glycine betaine and trehalose) in response to osmotic stress which can be induced by drought or salt stress is believed to contribute to resilient cellular osmotic adjustment (Zulfiqar *et al*., 2020; Singh *et al*., 2022). Among those compatible solutes, proline is widely reported for protecting plants from many abiotic stresses (Ghosh *et al*., 2022). For example, it was reported that higher proline and soluble sugar contents were found in salt-tolerant rice genotypes under salt stress conditions than the salt-sensitive ones, suggesting better osmoprotection against salt stress (Gerona *et al*., 2019). For regulation of ion homeostasis under salt stress, many genes and proteins regulated via SOS signaling pathway have been reported to function in controlling the level of cytoplasmic Na^+ ions by actively excluding Na^+ out of the cells or compartmentalizing them inside the vacuoles (Roy *et al*., 2014; Atta *et al*., 2023). Accumulation of excess Na^+ in the cytoplasm leads to oxidative stress, the condition of an imbalance between the rate of generation and elimination of ROS (Akyol *et al*., 2022). To control the level of ROS, it is known that plants increase high levels of enzymatic and non- enzymatic antioxidants to ameliorate oxidative stress. Higher levels of antioxidant enzyme activities such as SOD, CAT and POX have been reported in many plant species such as common vetch, rice and wheatgrass which are categorized as salt-tolerant genotypes compared with salt-sensitive ones (Sun *et al*., 2022; Srivastava and Sharma, 2022; Sheikh-Mohamadi *et al*., 2022).

Rice (*Oryza sativa* L.) is one of the main staple food crops for people in Asia including Thailand. Unfortunately, rice growing area in Thailand particularly, the northeastern part is limited due to natural saline soil problem leading to declining productivity. Genetic improvement to obtain new rice cultivars which are salt-tolerant is one of the solutions to increase rice productivity cultivated in saline soil areas. A challenge in breeding salt-tolerant rice is to find a potential donor parent which contained stress tolerant genes. It is known that the strong phenotype of plants for biotic and/or abiotic stress tolerance is a result of their genetic background, and the process of tolerance mechanisms is related to physiological responses and the complex gene regulations in specific conditions (Paudel *et al*., 2020). Luckily, high biodiversity of rice landrace varieties has been reported in the northeast region including the ability to resist biotic or abiotic stress (Pusadee *et al*., 2019; Kwanwah *et al*., 2020; Nounjan and Theerakulpisut, 2021; Khotasena *et al*., 2022). Although some landrace rice varieties have been categorized for biotic and/or abiotic tolerance, the characteristics of new varieties still need to be investigated, not only for keeping their data in gene bank collections but also for implying novel genetic resources for breeding programs. This study aimed to elucidate the physiological and biochemical responses of some landrace rice varieties under salt stress compared with 'Pokkali', the standard salt-tolerant rice genotype to clarify salt tolerance ability together with studying genetic diversity of those landrace rice varieties.

Materials and Methods

Plant materials and stress treatment

Eight rice genotypes were used for identifying salt stress tolerance compared to 'Pokkali'. Seeds of seven landrace rice varieties ('Surin', 'Kum', 'Lao Taek', 'Kon Jud', 'Sew Gliang', 'Maled Phai', 'Black Hmong') were kindly provided by the Department of Agronomy, Faculty of Agriculture, Khon Kaen University whereas seeds of 'Luempua', a well-known commercial landrace variety was purchased from a farmer in Phetchabun province. Seeds of standard salt-tolerant variety, 'Pokkali', were kindly provided by Rice Gene Discovery Unit, Kasetsart University, Kamphaeng Saen.

Rice plants were grown in hydroponic system in a greenhouse under natural sunlight conditions at the Faculty of Agriculture, Khon Kaen University, Thailand. Seeds were sterilized with 2% sodium hypochlorite for 10 min and washed repeatedly with distilled water. All rice cultivars were germinated in distilled water for 3 - 4 days, then were transferred to a container filled with 6 L of nutrient solution (Yoshida *et al.*, 1976). One germinated seed was planted in a hole of Styrofoam with nylon net bottoms. Every four days, the nutrient solution was replaced, and the pH was kept between 5.0 and 5.5 throughout the experiment. Salt treatment was started at 19 d after germination by adding salt (120 mM NaCl; EC 12 dS m^{-1}) into nutrient solution (salt stress). The solution EC was measured and adjusted daily in both control and salt treatment groups. Rice plants under both growth conditions were collected at 7 days after stress treatment. The collected leaf samples were stored at –80 °C and –20 °C for further analysis.

Determination of growth parameters, ion concentration and physiological parameters

Plants were weighed immediately after harvesting for fresh weight record. Then plants were dried in a hot-air oven at 70 °C until the dry weight was stabilized for recording dry weight. For Na and K ions, dried leaves were used to determine Na and K ions using an atomic absorption spectrometer (Corning, Model GBC932AAA, England) as described previously (Nounjan and Theerakulpisut, 2021). The relative water content (RWC) was determined at mid-day (11.30 am – 12.30 pm) according to the sampling and method of described by Nounjan *et al.* (2018). Electrolyte leakage (EL) and malondialdehyde (MDA) were determined following the method of Filek *et al.* (2012). Proline content was estimated according to Bates *et al.* (1973) using 0.05 g leaf samples. For total sugar, colorimetric analysis using anthrone and 3,5-dinitrosalicylic acid (DNS) reagents were applied (Dubey and Singh, 1999).

Determination of photosynthetic parameters

The method described by Arnon (1949) was used for estimating chlorophyll content by soaking 0.05 g leaf tissue in 10 mL 80% acetone overnight in the dark. The extract was centrifuged at 10000 × g for 5 min and the absorbance of the supernatant was read at 645 and 663 nm. Chlorophyll content was calculated using the formula: Total Chl (mg g^{-1} FW) = $[20.2(A_{645}) + 8.02(A_{663})] \times (V/1000W)$, where V = extract volume and W = leaf fresh weight. The maximum quantum efficiency of photosystem II (Fv/Fm) was measured in darkness after 7.00 pm using LI-6400-XT (LI-6400-XT, LI-COR, NE, USA).

Determination of antioxidant enzymes activity

Leaf tissue (0.5 g) was homogenized in 5 mL of grinding buffer (10 mM potassium phosphate buffer (pH 7.0) and 4% polyvinylpyrrolidone). The homogenate was centrifuged at 12,000 × g at 4 °C for 15 min. The supernatants were immediately used for estimation of enzyme activity. Superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) activities were estimated by following the methods described previously by Pamuta *et al*. (2021).

Genetic relationship among rice genotypes based on rice microsatellite markers

Eleven rice microsatellite (SSR) primers known to link with QTL related to salt tolerance, namely, RM411, RM16, RM257, RM5310, RM212, RM3412, RM3482, RM10696, RM10748, RM10843, and RM10852 were used to amplify genomic DNA from seedlings of the nine rice genotypes. The sequences of the primers are shown in Table 1. PCR amplification was performed in a 10 µl reaction mixture containing 50 ng template DNA, 0.2 µM of each forward and reverse primer, 0.2 µM of dNTP, 1.8 mM $MgCl_2$, 0.5-unit Taq polymerase and 1X PCR buffer. All amplifications were performed on a Blue-Ray biotech thermal cycler (Blue-Ray Biotech Corp., Taipei, Taiwan) under the following conditions: (1) pre-denaturing at 94 °C for 5 min; (2) 35 cycles of run, each followed by denaturing at 94 °C for 30 s, annealing at 55-60 °C for 30 s and extension at 72 °C for 2 min; (3) final extension at 72 °C for 5 min. The PCR amplification products were separated by electrophoresis in 4.5% denaturing polyacrylamide gel (PAGE) in a Sequi-Gen®GT nucleic acid electrophoresis system (BioRad Laboratories Inc., USA). DNA fragments were revealed using the silver staining procedure. The amplified bands were scored based on the presence or absence of bands, generating a binary data matrix of 1 and 0 for each marker system. The matrix was then analyzed using the NTSYS pc statistical package version 2.1. The genetic similarity based on Jaccard's similarity coefficients was calculated, and dendrogram displaying genetic relationships was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

Table 1. Sequences of SSR primers

Marker	Forward primer	Reverse primer
RM10696	5'CCTTCGACTCCATGAAACAAACG3'	5'TCTCTTTGCCCTAACCCTATGTCC3'
RM3412	5'AAAGCAGGTTTTCCTCCTCC3'	5'CCCATGTGCAATGTGTCTTC3'
RM212	5'CCACTTTCAGCTACTACCAG3'	5'CACCCATTTGTCTCTCATTATG3'
RM5310	5'TAGACAAAGCAACGGGTTCC3'	5'CGGAAGCAGGAGAATCGTAG3'
RM3482	5'TTGTTGTCAAGCTACGGTGG3'	5'CTGCTTCGTGATGTTGTTGG3'
RM10748	5'CATCGGTGACCACCTTCTCC3'	3'CCTGTCATCTATCTCCCTCAAGC5'
RM10843	5'CACCTCTTCTGCCTCCTATCATGC3'	3'GTTTCTTCGCGAAATCGTGTGG5'
RM10852	5'GAATTTCTAGGCCATGAGAGC3'	3'AACGGAGGGAGTATATGTTAGCC 5'
RM411	5'ACACCAACTCTTGCCTGCAT3'	5'TGAAGCAAAAACATGGCTAGG3'
RM16	5'CGCTAGGGCAGCATCTAAA3'	5'AACACAGCAGGTACGCGC3'
RM257	5'CAGTTCCGAGCAAGAGTACTC3'	5'GGATCGGACGTGGCATATG3'

Experimental design, statistical analysis, and principal component analysis

The experiment was set up in a randomized complete block design with 4 replications for each treatment (control and salt stress), and each replicate contains twelve seedlings of each rice genotype. The data was analyzed with SPSS ver. 20 for statistical testing using One-way ANOVA. The significant difference among means was determined using the Duncan's multiple range test (DMRT) where $p \leq 0.05$ was considered significantly different. The mean data was used to analyze Pearson's correlation, which revealed the relation among variables. The raw data was standardized using Z-scores before unsupervised analysis. Principal component analysis (PCA) and Hierarchical cluster analysis (HCA) were conducted using the standardized data. PCA results were expressed as loading plot and score plot that were constructed from PC1 and PC2. The HCA heatmap displayed the response patterns of variables, and different rice genotypes were clustered depending on the patterns. Pearson's correlation, PCA, and HCA were analyzed and visualized using R programming for Window.

Results

Growth parameters, ion accumulation and water balance

As shown in Figure 1A and 1B, the growth performance of seedlings of the nine rice cultivars showed a decreasing trend under salt stress conditions (120 mM NaCl) in terms of fresh weight (FW) and dry weight (DW), in comparison with the control. Under salinity, the average means FW ranged from 0.88 g to 2.09 g, the lowest FW was found in 'Kum' whereas the highest FW was noted in 'Pokkali'. However, FW of 'Black Hmong' was reduced the most (from 3.36 g in the control to 1.54 g in salt-stressed plants which accounted for 54% reduction). Notably, FW reduction percentage of 'Surin' (24%) was the lowest and less than that of the standard salt-tolerant, 'Pokkali' (35%) (Figure 1A).

Dry weight of plants under salt stress ranged from 0.13 g to 0.34 g, in 'Kum' and 'Pokkali', respectively (Figure 2). The reduction percentage varied from 21% ('Surin') to 50% ('Black Hmong'). It is intriguing that the lowest reduction in DW was recorded in 'Surin' (21%) which was lower than that of 'Pokkali' (27%) (Figure 1B). 'Kon Jud', 'Sew Gliang' and 'Black Hmong' were among the varieties that suffered almost 50% DW reduction.

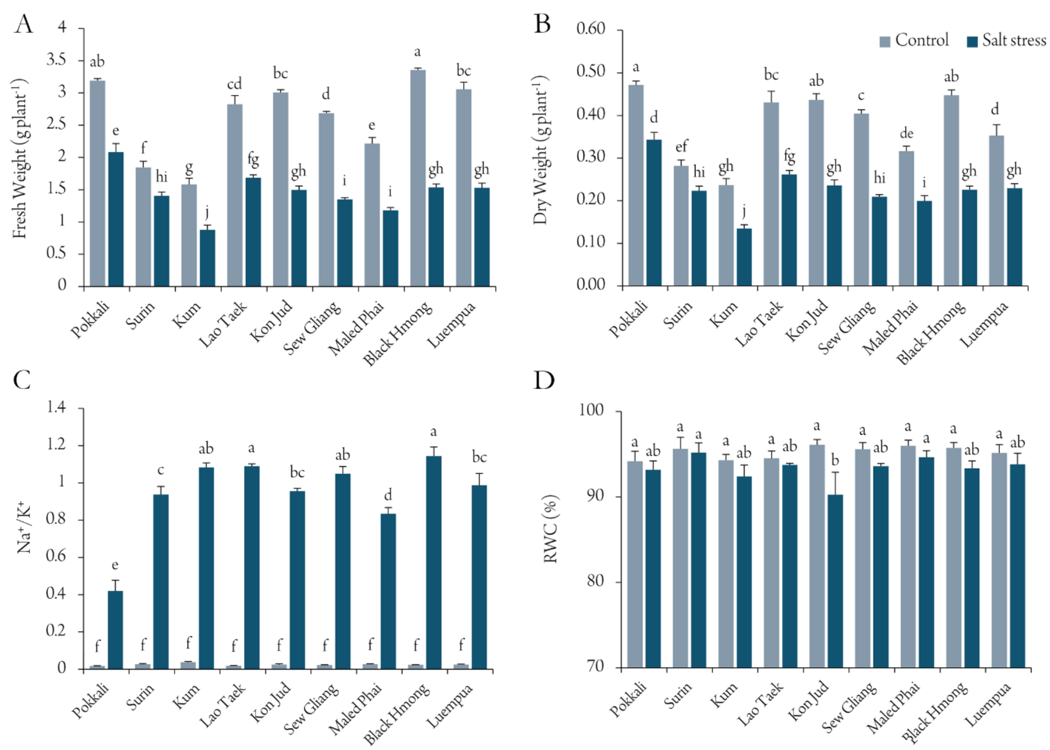

Figure 1. Fresh weight (FW), dry weight (DW), ratio between Na^+/K^+, and relative water content (RWC) of rice plants under control (light bars) and salt stress (black bars) for 7 days

Values are means of four replications ± SE. Different letters indicated the means are significantly different (p ≤0.05).

Rice seedlings after 7 days of stress in 120 mM NaCl expressed a dramatic increase in Na^+/K^+ ratio compared with the control group (Figure 1C). The Na^+/K^+ ratios of salt-stressed plants were 25 to 58 times higher than those of the controls. All landrace varieties showed a significantly higher Na^+/K^+ ratio than 'Pokkali' which increased from 0.02 to 0.42 (accounted for 25 times increase compared to untreated plants). Among the landraces, 'Maled Phai' had the lowest value of Na^+/K^+ ratio (0.83) followed by 'Surin' (0.94) while

'Black Hmong' was the highest (1.17). The Na^+/K^+ ratios of the landraces under salt stress were 1.98 to 2.71 times higher than that of 'Pokkali'.

For relative water content (RWC) in seedling leaves (Figure 1D) which was determined at mid-day (11.30 am – 12.30 pm). Most varieties tended to have a good ability to maintain RWC, except for 'Kon Jud' which showed a significant decrease compared to other landrace varieties (decreased from 96.10% in control to 90.26% in salt stressed plants).

Photosynthetic parameters

Total chlorophyll content of all cultivars was varied (ranging from 3.27 to 5.45 mg g^{-1} FW) (Figure 2A). High total chlorophyll was observed in 'Surin', 'Pokkali' and 'Kum' whereas low total chlorophyll was found in 'Luempua' and 'Kon Jud'. Total chlorophyll content of 'Lao Taek', 'Maled Phai', 'Sew Gliang' and 'Black Hmong' was in between those two groups. Under salinity stress, the chlorophyll content of all varieties was significantly reduced compared to the control group, except for 'Surin'. Notably, the highest chlorophyll content under stress was recorded in 'Surin' followed by 'Pokkali', 'Lao Taek', 'Maled Phai', 'Sew Gliang', 'Kum', 'Black Hmong', 'Kon Jud' and 'Luempua' (ranging from 4.89 to 2.41 mg g^{-1} FW).

For Fv/Fm, there were no significant differences in this value (0.79 to 0.77) among rice varieties in the non-stress condition, except for 'Black Hmong' which showed slightly lower Fv/Fm (0.76) than other varieties (Figure 2B). Under salt treatment, some rice varieties could maintain Fv/Fm ('Pokkali' and 'Kum'), some rice varieties showed a slight decrease in this value ('Surin', 'Sew Gliang', 'Black Hmong' and 'Luempua') and some rice varieties showed a dramatic reduction ('Lao Taek', 'Kon Jud' and 'Maled Phai') compared to those of control group. Among these, 'Kon Jud' also suffered the greatest reduction in Fv/Fm.

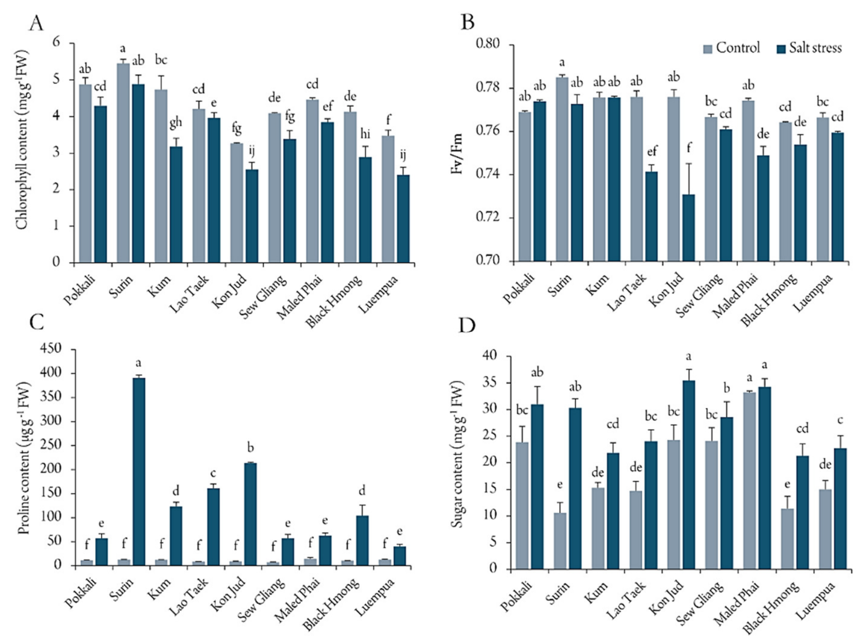

Figure 2. Chlorophyll content, maximum quantum efficiency of PSII photochemistry (Fv/Fm), proline content, and total sugar of rice plants under control (light bars) and salt stress (black bars) for 7 days.
Values are means of four replications ± SE. Different letters indicated the means are significantly different (p ≤0.05).

Proline and sugar content

A prominent effect of salt stress on proline content was observed and its content was significantly increased in all cultivars compared with the control (Figure 2C). 'Surin' showed the highest proline accumulation in the salt stress conditions (391.12 µg g^{-1} FW; 98% increase), followed by 'Kon Jud' (213.69 µg g^{-1} FW) and 'Lao Taek' (161.36 µg g^{-1} FW) while the lowest accumulation was seen in 'Luempua' (40.15 µg g^{-1} FW) followed by 'Pokkali' (57.41 µg g^{-1} FW). Under salt stress, 'Surin' accumulated 6.8 times higher proline content than 'Pokkali'. The similar trend was observed for sugar content. Leaf sugar content under non-stress conditions varied considerably from 10.64 ('Surin') to 33.21 ('Maled Phai') mg g^{-1} FW. When plants were grown under salt stress conditions, sugar content increased in all rice varieties except for 'Maled Phai'. Sugar increased remarkably in 'Surin', 'Black Hmong', 'Lao Taek', 'Luempua' and 'Kon Jud' which increased by 65%, 47%, 39%, 34% and 31%, respectively compared to those of control groups. However, the highest amount of sugar was observed in 'Kon Jud' (35.44 µg g^{-1} FW) and 'Maled Phai' (34.29 µg g^{-1} FW). In contrast, 'Kum', 'Black Hmong' and 'Luempua' had the lowest sugar content under the stress condition compared to other varieties (Figure 2D).

Lipid peroxidation and membrane damage

There were no significant differences in leaf malondialdehyde (MDA) content in all rice varieties under control conditions. Conversely, under saline condition, MDA content dramatically increased in 'Kon Jud', 'Luempua', 'Sew Gliang' and 'Black Hmong' (approximately 36-50% compared to those of control plants). The highest MDA content was found in 'Luempua' followed by 'Kon Jud' and 'Sew Gliang'. For 'Pokkali' and 'Maled Phai', salt stress did not alter the leaf MDA content, and 'Surin' showed a slight non-significant increase (Figure 3A). Similarly, electrolyte leakage (EL) percentages in all rice varieties were not different under non-stress conditions (Figure 3B). Salt stress treatments seriously affected the EL parameter. The EL dramatically increased when the landrace varieties were exposed to salt stress (increased by 76-97% when compared to those of control) while EL of 'Pokkali' was unaffected. 'Kon Jud' had the highest EL value (86%) followed by 'Black Hmong' (80%) and 'Lao Taek' (66%), respectively. On the other hand, 'Pokkali' had the lowest EL percentage which was only 9% increase. Among local varieties 'Surin' showed the lowest EL under salt stress.

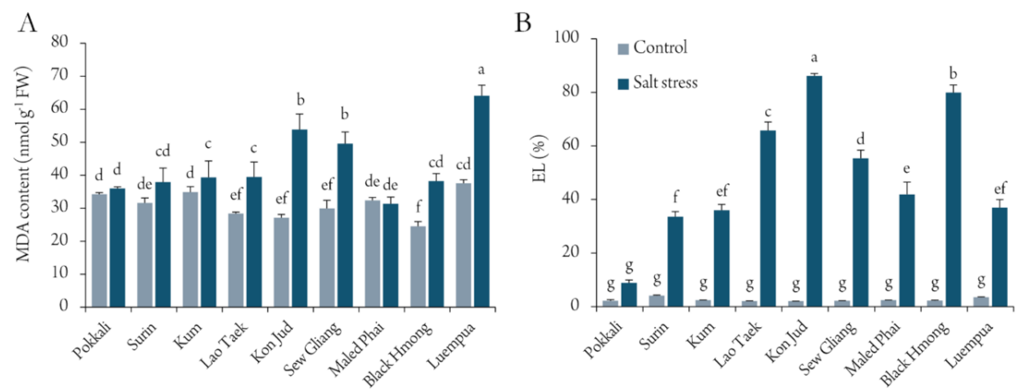

Figure 3. Lipid peroxidation (malondialdehyde; MDA) and electrolyte leakage (EL) of rice plants under control (light bars) and salt stress (black bars) for 7 days.

Values are means of four replications ± SE. Different letters indicated the means are significantly different (p ≤0.05).

Antioxidant enzymes activities

The fluctuation trends of SOD, CAT, and POX activities were detected in all rice cultivars under both normal and salt-stress conditions. Under salt treatment, 'Pokkali', 'Lao Taek', and 'Maled Phai' had a small reduction in SOD compared to the control group (approximately 14-29% decrease). The salt-stressed 'Surin', 'Black Hmong', and 'Luempua' plants exhibited a substantial decrease in SOD activity (42-56%), while the salt-stressed 'Kon Jud' plants showed greater SOD activity relative to the control plants (26%). There were no notable alterations in the activity of SOD in 'Kum' and 'Sew Gliang' under both conditions (Figure 4A). For CAT, the activity of CAT in controlled 'Luempua' showed the highest level whereas the lowest level was found in 'Surin'. The level of this enzyme in other landrace rice varieties was in between that of 'Luempua' and 'Surin'.

When exposed to salt stress, different trends were observed: increase ('Surin', 'Kum' and 'Maled Phai'), no detectable change ('Pokkali' and 'Lao Taek') decrease ('Kon Jud', 'Sew Gliang', 'Black Hmong' and 'Luempua') when compared to those of control plants. However, CAT activity did not show a significant difference between the control and salt treatment groups (Figure 4B). For POX, no significant differences in POX activity were found in all varieties. However, when NaCl was applied, Kon Jud produced the highest POX activity (0.62 μmol min^{-1} mg^{-1} protein) followed by 'Kum' (0.48 μmol min^{-1} mg^{-1} protein) and 'Black Hmong' (0.39 μmol min^{-1} mg^{-1} protein). Slight increases and no changes in POX activity were found in other rice varieties (Figure 4C).

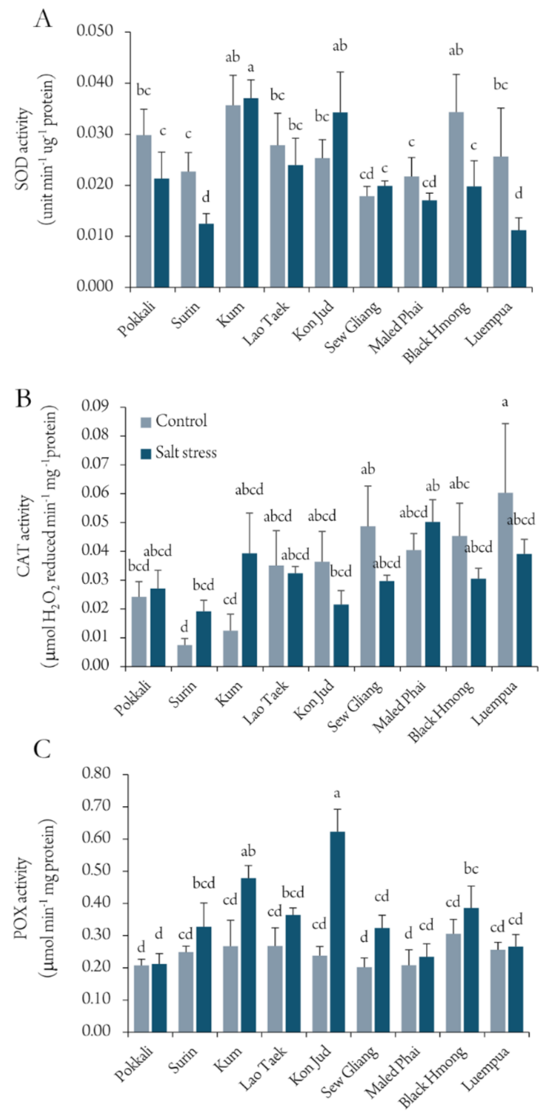

Figure 4. Activity of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) of rice plants under control (light bars) and salt stress (black bars) for 7 days.

Values are means of four replications ± SE. Different letters indicated the means are significantly different (p ≤0.05).

Correlation among physiological traits and clustering analysis based on physiological parameters

The percentage of changes in 13 physiological parameters (FW, DW, Na^+/K^+, RWC, chlorophyll, Fv/Fm, proline, sugar, MDA, EL, activity of SOD, CAT and POX) between the salt stress and control condition were used to determine the correlations among parameters and the results are displayed in Figure 5. The changes in growth were negatively correlated with changes in Na^+/K^+, MDA, and EL. The correlations between the percent changes in FW and percent changes in Na^+/K^+, MDA, and EL were -0.563, -0.660, and -0.677, respectively, and they are all highly significant ($p < 0.01$). Similarly, the correlations between the percent changes in DW and percent changes in Na^+/K^+, MDA, and EL were -0.605, -0.496, and -0.773 which are all highly significant ($p < 0.01$). Therefore, the increase in Na^+/K^+, MDA, and EL was associated with the decrease in growth. Not surprisingly, changes in Na^+/K^+ had strong positive correlations with MDA ($r = 0.774$) and EL ($r = 0.723$). Changes in RWC, proline and sugar tended to have positive correlations with changes in FW and DW, but they are not statistically significant.

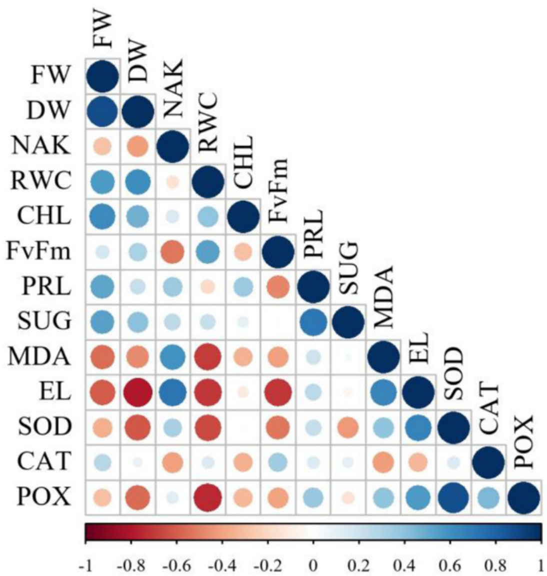

Figure 5. Correlation among changes in 13 physiological parameters under salt stress (CAT, catalase activity; CHL, total chlorophyll; DW, dry weight; EL, electrolyte leakage; FW, fresh weight; Fv/Fm, maximum quantum efficiency of PSII; MDA, malondialdehyde; NAK, Na^+/K^+; POX, peroxidase activity; PRL, proline; RWC, relative water content; SOD, superoxide dismutase activity; SUG, total sugar)

To group the salt tolerance of genotypes, hierarchical cluster analysis was performed based on the pattern and intensity of changes in 13 physiological parameters in response to salt stress. As shown in Figure 6, 'Pokkali', 'Maled Phai', and 'Kum' are grouped together in Cluster I. 'Lao Take', 'Sew Gliang', 'Black Hmong' and 'Luempua' formed Cluster II. The levels of changes in RWC, chlorophyll, proline and CAT activity are similar in 'Pokkali' and 'Maled Phai', hence these varieties are closely linked. The patterns of physiological changes in 'Surin' and 'Kon Jud' are distinct and separated from the remaining varieties. It is noted that the discriminating characters that differentiated 'Surin' from the remaining varieties included the lowest reduction in FW and DW, and outstanding increases in proline and sugar. The physiological responses that make 'Kon Jud' distinct from others are the reduction in RWC and Fv/Fm, and elevation of MDA, EL and POX activity.

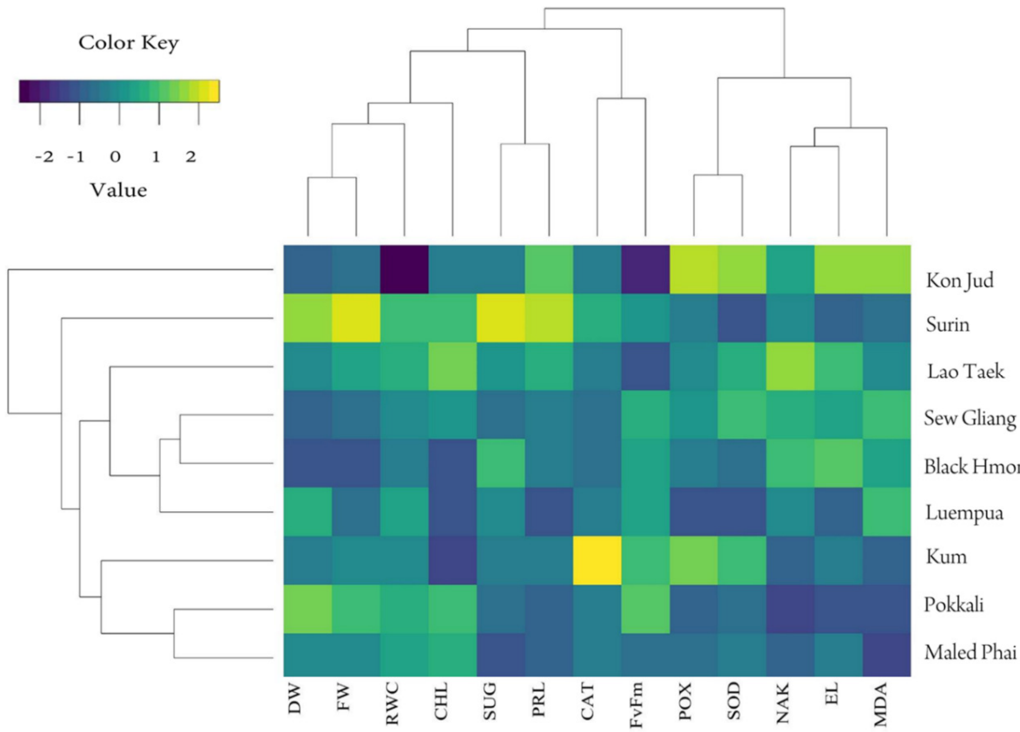

Figure 6. Cluster dendrogram of 9 rice genotypes based on similarity of changes in 13 physiological parameters under salt stress. (CAT, catalase activity; CHL, total chlorophyll; DW, dry weight; EL, electrolyte leakage; FW, fresh weight; Fv/Fm, maximum quantum efficiency of PSII; MDA, malondialdehyde; NAK, Na^+/K^+; POX, peroxidase activity; PRL, proline; RWC, relative water content; SOD, superoxide dismutase activity; SUG, total sugar)

Genetic relationship among rice genotypes based on rice microsatellites

All the eleven SSR markers amplified polymorphic bands in the nine rice genotypes. A dendrogram constructed based on genetic similarity using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated differentiation of the nine rice genotypes by eleven markers (RM411, RM16, RM257, RM5310, RM212, RM3412, RM3482, RM10696, RM10748, RM10843, and RM1085) as shown in Figure 7. The UPGMA cluster analysis led to the grouping of the nine genotypes in two major clusters (Cluster-I and -II). Cluster I contain 'Pokkali', 'Kum', 'Lao Taek' and 'Kon Jud' in the subcluster Ia, while 'Surin' was separated in Ib. In Cluster II, 'Sew Gliang', 'Black Hmong' and 'Luempua' are genetically similar and grouped in subcluster IIa while 'Maled Phai' was separated into subcluster IIb. Among all genotypes, 'Black Hmong' and 'Luempua' are most genetically similar based on the diversity of DNA sequences amplified from SSR markers linked to salt-tolerant genes.

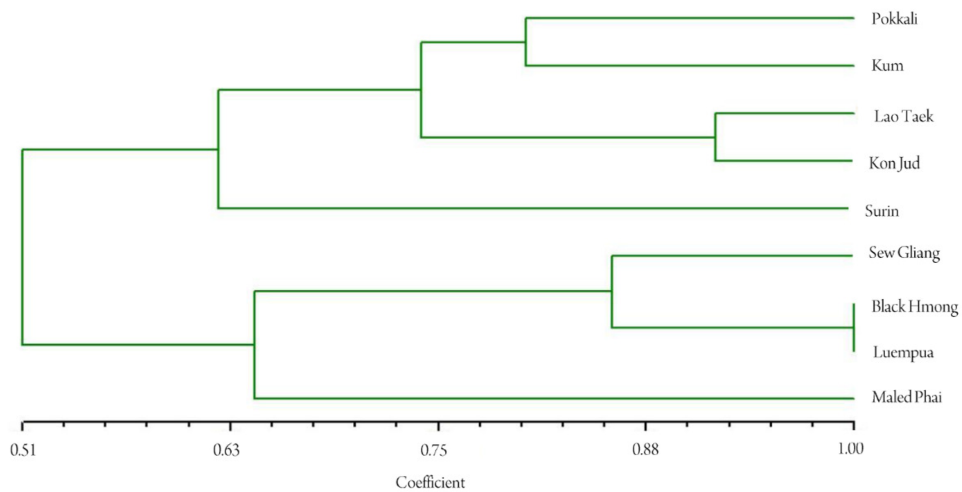

Figure 7. Dendrogram showing genetic relationship among of rice genotypes based on similarity of PCR-amplified products from 11 SSR markers

Discussion

The effects of salt stress on growth and physiological responses were investigated in eight different Thai landrace rice varieties in comparison with 'Pokkali', the standard salt-tolerant rice genotype. Under salt stress, the growth performance of seedlings in nine rice cultivars showed a decreasing trend under the salt stress condition (120 mM NaCl) based on the reduction in FW and DW in comparison to the controls. The results showed that 'Surin' and 'Pokkali' suffered a lower reduction (20-27%) in FW and DW compared to other rice varieties indicating these varieties suffered a less negative impact from salt stress. To survive under salt stress, plants have generated many mechanisms to protect plant cells from salt stress such as regulating ion homeostasis, activating the osmotic stress pathway, alteration of photosynthetic process and induction of antioxidant enzymes (Atta *et al*., 2023). Thus, salt tolerant plants often showed relatively better growth and had fewer symptoms than susceptible genotypes. As 'Pokkali' is well-known for its salt tolerance ability, 'Surin' which had the same trend for FW and DW reduction under salt stress might be categorized as a salt tolerant rice. On the other hand, landrace rice varieties which recorded approximately 50% growth reduction compared to untreated plants i.e., 'Black Hmong', 'Sew Gliang' and 'Kon Jud' tended to be more sensitive to salinity stress (Figure 1A and B). Numerous reports stated that salt stress induced more deteriorative phenotypic appearance in salt-sensitive plants than salt-tolerant plants (Chakraborty *et al*., 2020; Sun *et al*. 2022; Sogir *et al*., 2023).

Ion toxicity especially from excess Na^+ and Cl^- ions disrupts plant growth and development. Keeping balance between Na^+ and K^+ is necessary to maintain normal cellular metabolic functions. A high K^+/Na^+ (or low Na^+/K^+) ratio is an important indicator to indicate salt tolerance in plants (Atta *et al*., 2023). In this present study, all landrace rice varieties showed more than two times higher Na^+/K^+ ratio than 'Pokkali'. Nevertheless, among landrace rice varieties 'Maled Phai' had the significantly lower Na^+/K^+ ratio than others (Figure 1C). This variety could have better ability to maintain Na^+ and K^+ ratio than other landrace rice varieties suggesting good Na^+/K^+ homeostasis regulation. Na^+ detoxification and ion homeostasis are the most prominent salt tolerance mechanisms in plants, and salt tolerance and salt exclusion are strongly correlated (Gerona *et al*., 2019; Huang *et al*., 2020; Rasel *et al*., 2020).

Salt stress conditions restrict the water uptake of plants, resulting in a reduction in RWC of all cultivars in seedling leaves. Thus, the RWC of salt-sensitive genotypes under salt stress tends to be lower than that of salt-tolerant ones. Ma *et al*. (2018) reported that the RWC was an important parameter to determine plant growth and physiological adversity in various growth conditions, healthy tolerant plants with low salt injury were found to maintain high RWC. Interestingly, there were no significant differences in RWC of landrace rice varieties in both non-stress and stress conditions except for 'Kon Jud' of which RWC declined significantly (Figure 1D), indicating that 'Kon Jud' most suffered from osmotic stress, which was induced by salt stress.

Degradation in chlorophyll content is typically reported when plants were subjected to salt stress, and it was found that a greater reduction was observed in salt-sensitive genotypes than in salt-tolerant ones (Mahlooji *et al*., 2018; Tabassum *et al*. 2021; Rahiniza *et al*., 2023). Other photosynthetic parameters such as Fv/Fm are also used for rapid screening of salt tolerance plants. In this study, a greater reduction in chlorophyll content was observed in 'Kum', 'Black Hmong', 'Luempua', and 'Kon Jud' (Figure 2A), indicating that these varieties might be sensitive to salt stress. 'Kon Jud', in particular, showed the lowest chlorophyll content and Fv/Fm under salt stress (Figure 2A and 2B). In contrast, 'Surin' and 'Pokkali' showed less reduction in chlorophyll content and Fv/Fm, implying less damage from salt stress. Since 'Pokkali', a salt-tolerant genotype, exhibited less chlorophyll decrease under salt stress, it can be inferred that 'Surin', which had a similar trend, could also be classified as salt-tolerant. Gerona *et al*. (2019) pointed out that high ability to retain photosynthetic ability was an essential mechanism of salt-tolerant genotypes. Furthermore, prior research conducted by our group on the identification of mechanisms for salt tolerance in rice revealed that chlorophyll retention and high photosynthetic performance contributed to salt tolerance (Nounjan *et al*., 2018; Nounjan *et al*., 2020; Pamuta *et al*., 2020). The maintenance of chlorophyll content during salt stress is a prerequisite for maintaining photosynthetic efficiency leading to plant growth promotion (Zahra *et al*., 2022). Less chlorophyll degradation in 'Surin' and 'Pokkali' could be associated with less growth reduction in these two varieties (Figure 1A and 1B).

It is believed that osmoregulation or osmotic adjustment is one of the major salt tolerance mechanisms in plants (Zelm *et al*., 2020). A process of osmotic adjustment is associated with an accumulation of compatible solutes. Among the organic osmolytes, proline was reported to be an efficient compatible solute which alleviated negative effects from salt stress and enhanced plant growth (Hasanuzzaman and Fujita, 2022). In this present study, high accumulation of proline and sugar contents were found in all rice varieties in response to salt stress (Figure 2C and 2D). Among these, 'Surin' had the highest percent increase in proline and sugar accumulation, suggesting high osmotic adjustment ability in this variety which could lead to an efficient water uptake, hence stable RWC (Figure 1D). The major salt tolerance mechanism in plants involves not only ion exclusion but also osmotic, and tissue tolerance (Roy *et al*., 2014). Tissue tolerance refers to the capacity of cells or tissues to tolerate high internal Na^+ and Cl^- concentrations (Prusty *et al*., 2018). Although 'Surin' expressed high Na^+/K^+ ratio (Figure 1C), its growth performance under salt stress was comparable to, if not better, than 'Pokkali' (Figure 1A and 1B). Thus, this could be assumed that the processes contributed to salt stress tolerance in this variety could be the tissue tolerance mechanism. In addition, Chakraborty *et al*. (2020) suggested that tissue tolerance was a trait with a high potential to be used to improve salt-tolerant rice. On the other hand, compatible solutes accumulation in 'Pokkali' was not outstanding. These findings indicated that tissue tolerance or osmotic adjustment were not the major mechanism contributing to salt tolerance in this cultivar.

Salinity stress has been reported to induce oxidative stress in many plants. Salt stress causes an imbalance of oxygen metabolism within plant cells and leads to oxidation of fatty acids in cell membranes leading to a loss of membrane integrity (Guo *et al*. 2019). Thus, membrane damage can be reflected in elevated MDA and EL levels. Generally, when rice plants were exposed to salt stress, high MDA and EL were noted. Among the investigated rice varieties, 'Kon Jud' seemed to suffer the most from oxidative stress, as evidenced by the fact that MDA content was recorded as the second highest and expressed the highest EL percentage (Figure 3A and 3B). This may be associated with a low boost in SOD activity and a low level of CAT, though high activity of POX was found under salt treatment (Figure 4). In contrast, 'Pokkali' had less damage from oxidative stress due to no remarkable changes in MDA content, and a non-significant increase in EL (Figure 3A and 3B).

Interestingly, there was no significant change in SOD, CAT, and POX activities in 'Pokkali'. This might be because this cultivar had a good ion exclusion mechanism that eliminated excessive Na^+ ion accumulation in the tissues which is the major cause of oxidative stress (Figure 1C). Several previous studies reported that salt-tolerant plants tend to have low levels of MDA and EL, which indicated they could be resistant to the negative effects of salt stress (Prusty *et al*., 2018; Pamuta *et al*., 2021). For 'Surin', it exhibited less increase in MDA levels under salt stress conditions and displaying the lowest EL percentage compared to other landrace varieties (Figure 3A and 3B). This indicated that 'Surin' could possess the capability to preserve membrane integrity, though no remarkable increase in antioxidant enzyme activities was observed (Figure 4). In this case, a marked increase in proline, which was predominantly built up in this variety during salt stress (Figure 2C), might act as a non-enzymatic antioxidant molecule (Srivastava and Sharma, 2022), leading to low level of lipid peroxidation (Figure 3A) and the preservation of membrane integrity (Figure 3B).

Salt stress affected growth and physiological responses in both salt tolerant and salt sensitive cultivars. Clustering of rice varieties based on SSR markers linked to salt-tolerant genes (Figure 7) was partially similar to the patterns of physiological responses among varieties (Figure 6). The genetically similar 'Pokkali' and 'Kum' expressed similar levels of change in MDA, Fv/Fm, sugar and RWC, although these parameters did not translate into maintenance of growth performance in 'Kum'. High percentage growth reduction in 'Kum' is most probably associated with the much higher Na^+/K^+ (Pamuta *et al*., 2021). On the other hand, 'Sew Gliang', 'Black Hmong' and 'Luempua', showed similar degrees of responses in many physiological traits including Fv/Fm, Na^+/K^+, MDA, FW, RWC and CAT activity resulting in similar reduction in FW and DW, and hence they were grouped together (Figure 6). Interestingly, these three varieties were closely related based on SSR markers (Figure 7). Therefore, evaluation of varietal relatedness based on molecular markers linked to salt-tolerance genes can be indicative of salt tolerance levels and may be applied for screening large collection of rice germplasm.

Conclusions

Evaluating landrace rice varieties for salinity stress tolerance based on growth and physiological parameters found that salt stress affected physiological attributes of different rice genotypes to varying degrees. Based on percentage growth reduction 'Surin' achieved similar levels of salt tolerance to 'Pokkali', while the remaining landrace varieties suffered much greater growth reduction. Salt tolerance mechanism of the standard salt tolerant rice, 'Pokkali' resulted from lower accumulation of Na^+ and higher accumulation of K^+ indicating superior ion exclusion mechanisms. In contrast, the salt tolerance of the landrace rice variety 'Surin' was presumably associated with the osmotic adjustment ability indicated by the outstanding accumulation of the osmolytes, proline and sugar. The ability of 'Surin' to maintain growth despite high Na^+/K^+ indicated that it may possess good tissue tolerance characters which need to be elucidated in the future. This variety could be a potential genetic resource to be developed as a donor for improving salt-tolerant rice in the future.

Authors' Contributions

Conceptualization: NN, JS and PT; Data curation: NN and DP; Funding acquisition: PT; Investigation; NN and DP; Methodology: NN, DP and JS; Project administration: PT; Resources: JS; Supervision: PT; Validation: JS and PT; Writing - original draft: NN; Writing - review and editing: NN, DP, JS and PT. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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