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Article

# Analysis of Morphological, Physiological, and Biochemical Traits of Salt Stress Tolerance in Asian Rice Cultivars at Different Stages

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**Abstract:** Rice (*Oryza sativa* L.) is a primary energy food for Asian population. One of the most constraints in rice production is soil salinity because of the rice is very susceptible to salt. Meanwhile, many agricultural land in Asia are in the saline area. It is important to identify and develop salt-tolerant rice varieties that can adapt in Asian climate. By combining morphological, physiological, and biochemical assessments for screening the salt tolerant of 116 Asian rice cultivars was able to classify into tolerant, moderate, and sensitive rice cultivars under salinity stress condition, and also for understanding the salt tolerance mechanisms. The rice cultivars which belong to salt-tolerant including Pokkali from India, TCCP 266 from Philippines, IR 45427 also from Philippines, and Namyang 7 from Korea. Whereas, salt-sensitive rice varieties like IR29 from Philippines, IR58 also from Philippines, Daegudo from Korea, and Guweoldo also from Korea. The salt-tolerant varieties showed signs of tolerance, including lower percent reduction of percentage germination, root length, root fresh weight, shoot length, plant biomass, and chlorophyll content. In order to maintain the cellular osmotic balance under saline condition, the salt-tolerant ones exhibited less membrane damage, lower Na/K ratio, high proline and sugar accumulation, lower malondialdehyde (MDA) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Pokkali from India, TCCP 266 from Philippines, IR 45427 also from Philippines, and Namyang 7 from Korea are recommended as valuable germplasm resource for Asian rice breeding program in saline agricultural area.

**Keywords:** Asian rice; salt stress; morphology; physiology; biochemistry

## 1. Introduction

Rice (*Oryza sativa* L.) is one of the major food source for more than half of the global population. A 100 g of rice contains 78.2 g of carbohydrates, 6.8 g of protein, and provides 345.0 kcal [1]. Most of the global rice is produced and consumed in Asia [2–4]. In Asia, small farmers produced almost 90% of the rice which become their source of income [5]. One of the most constraints in rice production is soil salinity because of the rice is very susceptible to salt, especially at the seedling, early vegetative (3 leaf stage), and reproductive stages [6–11]. Based on the history, Mesopotamian civilization (now part of Iraq) collapsed due to the salinization in agricultural areas that cause crop failures [12]. Because of the human activities and natural phenomena, soil salinity is increasing. Soil salinity stress generally occur in the rice field area that have improper irrigation and drainage systems, and also in the coastal areas [13,14]. The effects of soil salinity stress also depend on the rice genotypes and the salt concentration. Indica rice is more salt tolerance compared with japonica rice subspecies [15]. Soil salinity is a serious problem in most of the rice-growing areas of Asian countries in the tropics and temperate regions. Most of the agricultural areas in the Asian regions belong to the saline soils [16,17]. Rice belongs to the most susceptible crop to soil salinity stress compared with barley and wheat [18]. Saline soils have Electrical Conductivity (ECe) more than 4 dSm<sup>-1</sup> (~40 mM NaCl) because of the present of salts including sodium chloride, bicarbonates, magnesium and calcium sulphates, and also

a number of inorganic ions, such as  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{CO}_3^{2-}$ ,  $\text{HCO}_3^-$ ,  $\text{SO}_4^{2-}$ , and  $\text{NO}_3^-$  [19–21]. Sodium chloride (NaCl) is the most abundant salt in the saline soils.

Saline soil condition makes roots difficult to uptake nutrients and water which induce osmotic and ionic stress in the rice plants [22]. Under salinity stress condition, large amounts of  $\text{Na}^+$  influx into the plant cells and improved the  $\text{Na}^+$  concentrations in the cytoplasm and vacuole which toxic to the metabolism mechanism and leading to the cell death due to the osmotic and ionic stresses [18,23]. Excessive salt concentration in the plant cells absolutely leads to reduce cell membrane stability, cell wall damage, cytoplasmic degradation, plasmolysis, endoplasmic reticulum damage, accumulation of malate, citrate, and inositol in the leaf, and increase proline concentration, which leading to decrease the grain yield [24]. Recent study found that Pokkali is the salt-tolerant rice cultivar and IR20 belongs to the salt-sensitive cultivar [25]. In the salt-tolerant cultivar maintains a higher  $\text{K}^+/\text{Na}^+$  ratio than salt-sensitive cultivar. Excess salts in the soil cause high osmotic pressure outside the roots which reduce the ability of root cells to take up water and nutrients from the soil. In order to adapt in the soil salinity stress, plants cells need to accumulate osmolytes, including proline, glycine, taurine, sugars, inositols, glycerol, sorbitol, etc. The salt-tolerant cultivar accumulates more proline than the salt-sensitive cultivar [26,27].

Salinity stress effects the morphological, physiological, and biochemical characteristics of the rice plants which varies with the growth stages, including reduction in plant height, productive tiller number, biomass, grain yield, filled grain per panicle, grain weight, grain quality, harvest index, photosynthetic activity, and increased  $\text{Na}^+$  and  $\text{Cl}^-$  uptake to the shoot [28–31]. Under salinity stress at 3.5  $\text{dSm}^{-1}$  or 50 mM NaCl, the grain yield of rice significantly reduced 90% [32,33]. Meanwhile, rice seedlings die at 10  $\text{dSm}^{-1}$  [34]. In order to cope the soil salinity stress, rice plants develop several mechanisms, such as antioxidants for reactive oxygen species (ROS) detoxification, ion homeostasis, biosynthesis and accumulation of osmolytes for osmo-protection, and programmed cell death [26,35].

Soil salinity tolerance is a quantitative traits which controlled by multiple genes and highly affected by the environmental conditions [36–40]. The screening of rice for salinity tolerance is also complex. Thus, salinity screening under laboratory conditions is more controllable, rapid and accurate than field screening. Under the field screening, environmental conditions such as dynamic climate factors and soil heterogeneity might influence the accuracy of the salinity effects on the rice plants [41]. The potential indicators for salt tolerance screening for morphological, physiological, and biochemical characteristics are germination percentage, germination time, seedling vigor index, root length, shoot length, plant biomass, cell-membrane stability,  $\text{Na}^+/\text{K}^+$  ratio, proline content, malondialdehyde (MDA) content, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) content, sugar content, ethylene content, and chlorophyll content [42,43]. International Rice Research Institute (IRRI) has been released the standard evaluation score for visual salinity injury from 1 to 9 [6]. Salt tolerant is indicated by the lower score (1) and the salt sensitive is denoted by the higher score (9) based on the leaf symptoms, tiller number, and the growth characteristics under salinity conditions. Identification of quantitative trait loci (QTL) and cloning of genes correlated to salt tolerance in rice accelerate developing salt-tolerance rice varieties (Qin et al., 2020). Meanwhile, not many genes associated with the salt tolerance have been isolated and applied in rice breeding programs.

Many QTLs associated with the salinity tolerance have been identified by using mapping populations derived from crosses between salt-tolerant rice varieties and salt-sensitive varieties. The most popular marker in QTL mapping is single nucleotide polymorphism (SNP) [44,45]. A recombinant inbred line (RIL) population that derived from a cross between salt-tolerant Pokkali and salt-sensitive IR29 has been used to identify a major QTL Saltol on chromosome 1 which involved in regulating  $\text{Na}^+/\text{K}^+$  homeostasis [46]. Another QTLs that have been identified are qSKC-1, qSNC-7, qSE3, and qST1 which plays an important role in salt tolerance at the different growth stages [26]. Genome-wide association studies (GWAS) accelerates the breeding of salt-tolerant rice varieties because of the availability of rice reference genome and next-generation sequencing (NGS) techniques. GWAS identified more accurate genomic locations associated with the salinity tolerance. A total of twenty-one QTLs and two candidate genes correlated to salinity tolerance were identified by using GWAS with 664 rice varieties [47]. GWAS was also conducted to identify salt-tolerant loci

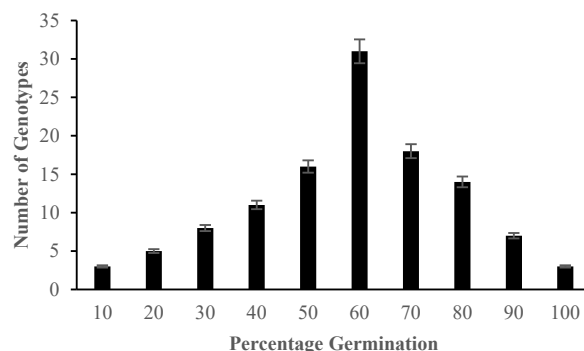
during reproductive stage [48,49]. A number of genes associated with the grain yield under salinity stress condition were also identified by using GWAS with 708 rice genotypes [50].

Analysis of genomic, transcriptomic, proteomics, and metabolomics also important in identifying genes correlated to salinity tolerance in rice. Under salinity stress, a series of changes in the rice plants, including the gene expression, protein content, and metabolite concentrations [51,52]. A number of potential genes associated with the salinity tolerance can be identified by comparing the transcriptome, proteome, and metabolome characteristics of salt-tolerant rice varieties with salt-sensitive varieties under salinity stress versus normal conditions. The most important goal in relieving the soil salinity problem is to identify and develop the rice varieties with high tolerance to salinity stress. Rice genotypes with highly salinity tolerant can be identified by using effective screening methods and provide donor alleles for salt tolerance to develop high salinity tolerant varieties through rice breeding program. By understanding the mechanism of salinity tolerance in the rice plants based on the morphological, physiological, biochemical, and genetics effects, the development of rice varieties with high salinity tolerant by genetic engineering techniques can be accelerated [28,53]. Breeding for rice salt tolerance is a major goal for rice breeders in agricultural areas with saline soil condition to ensure the food sustainability. The objectives of this research is to screen the salt tolerance of Asian rice cultivars based on the morphological, physiological, and biochemical characteristics.

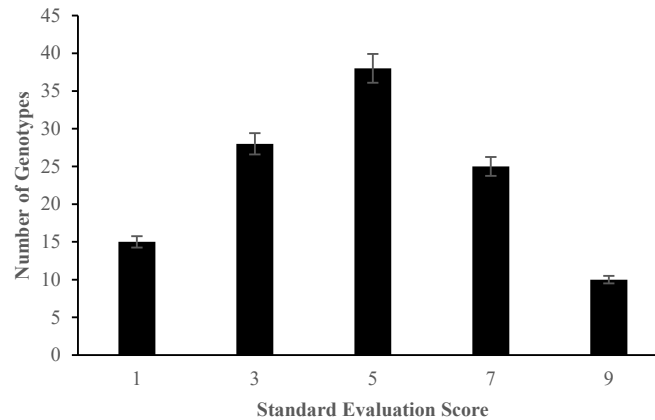
## 2. Results

### 2.1. Morphological Responses of Asian Rice Cultivars to Salt Stress at Seedling and Early Vegetative Stages

All of the Asian rice varieties showed normal growth in the control condition. Percentage germination of Asian rice cultivars under salinity condition of 200 mM NaCl showed reduction compared with the normal condition because of the salt caused retardation in plumule and radicle length. Under salinity stress conditions, the Asian rice cultivars had a wide range of percentage germination (Figure 1) and visual symptoms of salt injury at the seedling stage ranging from score 1 (salt tolerant) to score 9 (salt susceptible) (Figure 2). Based on the SES of visual salt injury at the seedling stage, 31 rice cultivars were identified as salt tolerant, 45 cultivars were classified as moderately salt tolerant, and 40 cultivars belong to salt susceptible. The most salt tolerant were Pokkali from India, TCCP 266 from Philippines, IR 45427 also from Philippines, and Namyang 7 from Korea. Meanwhile, the most salt susceptible rice varieties were IR29 from Philippines, IR58 also from Philippines, Daegudo from Korea, and Guweoldo also from Korea.

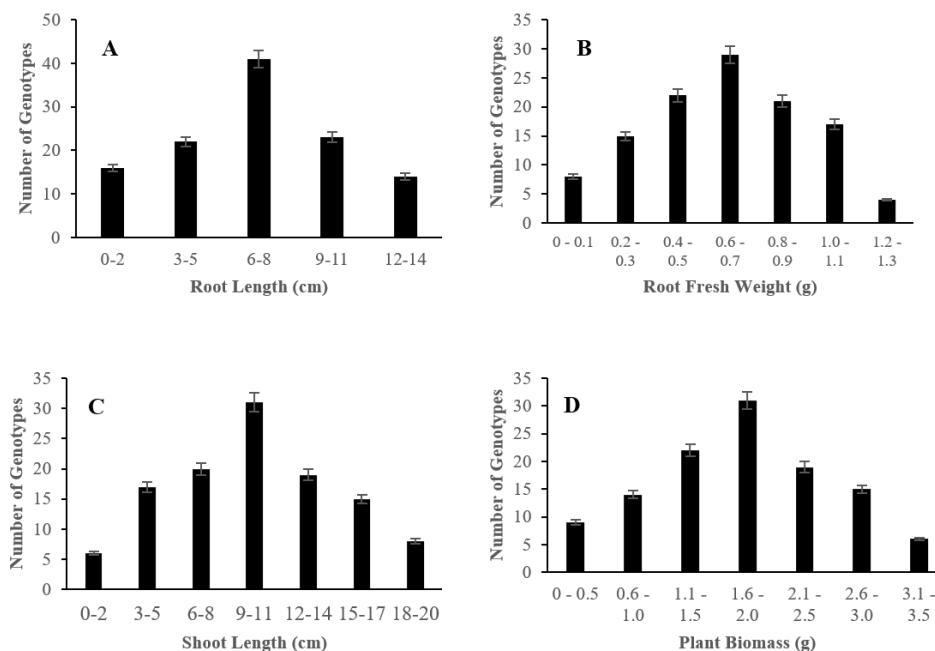


**Figure 1.** Percentage germination of 116 Asian rice cultivars under salinity stress condition 200 mM NaCl.



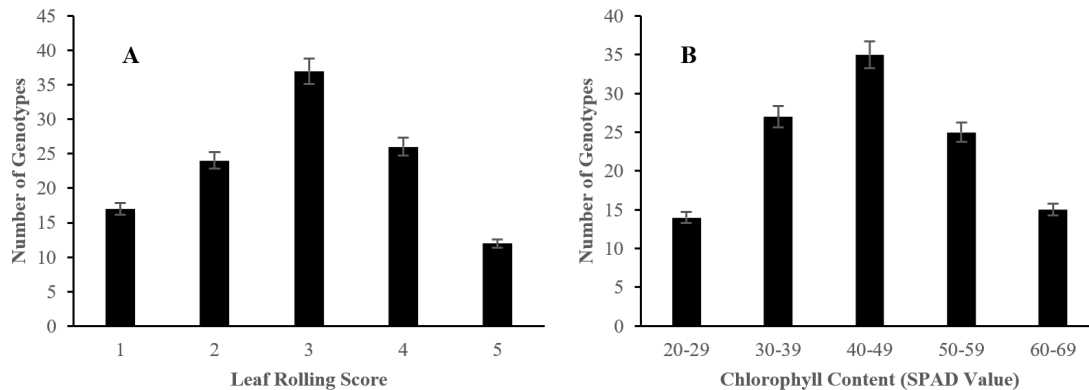
**Figure 2.** Standard evaluation score (SES) of visual salt injury at seedling stage of 116 Asian rice cultivars.

Another morphological responses to salinity stress condition, including root length, root fresh weight, shoot length, and plant biomass, showed variation among the Asian rice cultivars (Figure 3). In all of the rice cultivars, root length, root fresh weight, shoot length, and plant biomass were reduced under salinity stress condition, and the reduction was high as 59%, 51%, 61%, and 55% respectively. Pokkali from India, TCCP 266 from Philippines, IR 45427 also from Philippines, and Namyang 7 from Korea showed the lowest reduction for root length, root fresh weight, shoot length, and plant biomass. Meanwhile, IR29 from Philippines, IR58 also from Philippines, Daegudo from Korea, and Guweoldo also from Korea had the highest reduction for root length, root fresh weight, shoot length, and plant biomass. All of the Asian rice cultivars displayed root length reduction ranged from 33% to 59%; the range reduction for root fresh weight from 35% to 51%; the shoot length had range reduction from 37% to 61%; and the reduction ranged for plant biomass from 31 to 55%.



**Figure 3.** Effect of salinity stress condition on root length (a), root fresh weight (b), shoot length (c), and plant biomass (d) of 116 Asian rice cultivars.

Rice plants developed leaf symptoms, such as leaf rolling, yellowing, and necrotic lesions when rice is grown under salinity stress condition. Leaf rolling leads to minimize the water loss by respiration caused by water deficit under salinity stress condition. Salinity stress at the early vegetative stage of rice plants with 3 leaves; leaf rolling, yellowing, necrotic lesions, drying of leaves and other senescence symptoms were observed in all Asian rice cultivars with a wide range of variation (Figure 4). The damage of leaves under salinity stress condition related to the accumulation of  $\text{Na}^+$  from root to shoot.



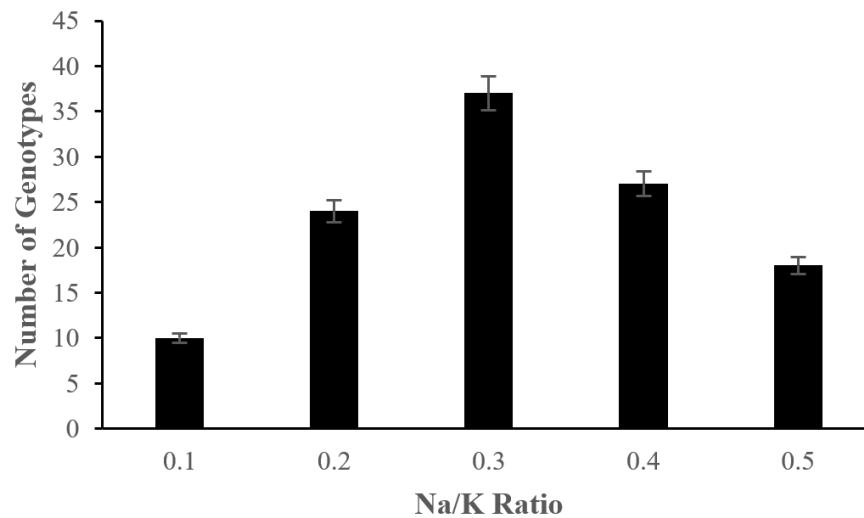
**Figure 4.** Leaf symptoms of leaf rolling (a) and chlorophyll content (b) from 116 Asian rice cultivars under salinity stress condition.

Salinity stress condition reduced the total chlorophyll content significantly in the Asian rice cultivars which belong to the salt-susceptible cultivars. The decrease of chlorophyll content in 116 Asian rice cultivars showed a wide range variation (Figure 4). Reduction of chlorophyll content influences the low photosynthetic activity. This result was in line with the other crops, including peas [56], wheat [57], rapeseed [58], and safflower [59].

## 2.2. The Effect of Salinity Stress on Physiological and Biochemical Characteristics of Asian Rice Cultivars at Seedling and Early Vegetative Stages

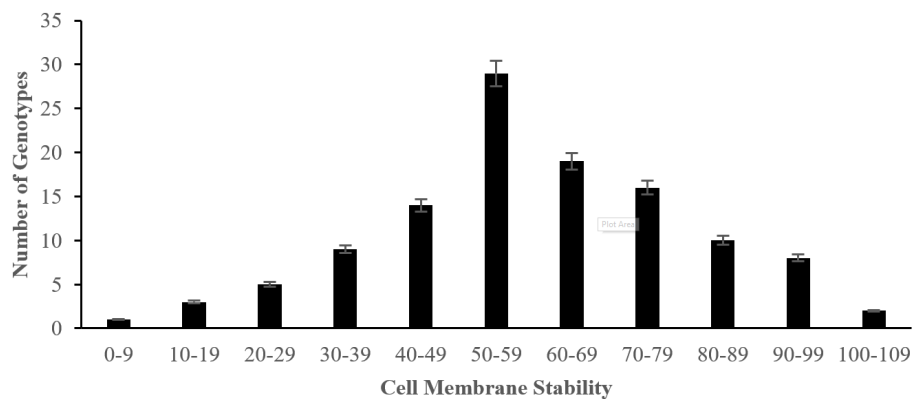
Under salinity stress condition, sodium (Na) content in all of the Asian rice cultivars were increased significantly. The highest accumulation of sodium was found in the salt-susceptible rice varieties such as IR29 from Philippines, IR58 also from Philippines, Daegudo from Korea, and Guweoldo also from Korea followed by salt-moderate tolerant including Nipponbare from Japan, Padi Tarab from Malaysia, and Ciharang from Indonesia. Meanwhile, the lowest sodium concentration was observed in the shoots of salt-tolerant varieties but had higher potassium (K) accumulation than the sensitive ones, such as in Pokkali from India, TCCP 266 from Philippines, IR 45427 also from Philippines, and Namyang 7 from Korea. The Na/K ratio in all 116 Asian rice varieties exhibited a wide variation (Figure 5).





**Figure 5.** Na/K ratio of 116 Asian rice varieties under salinity stress condition.

Cell membrane stability of the rice plants under salinity stress tolerant was influenced by the osmotic adjustment. With the increasing of  $\text{Na}^+$  concentration in the cells, water potential inside of the cells is reducing and effect the cell membrane stability. Salt-sensitive rice varieties like IR29 from Philippines, IR58 also from Philippines, Daegudo from Korea, and Guweoldo also from Korea accumulated high concentration of  $\text{Na}^+$  which experience toxicity and cell damage (Figure 6).



**Figure 6.** Effect of salinity stress condition on cell membrane stability of 116 Asian rice varieties.

In order to maintain the cellular osmotic balance under saline condition, the rice plant cells accumulate compatible solutes or metabolites such as proline, malondialdehyde (MDA), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and sugar content. All of the 116 Asian rice cultivars showed fluctuated compatible solutes under salinity stress condition (Figure 7). In the salt-tolerant rice varieties such as Pokkali from India, TCCP 266 from Philippines, IR 45427 also from Philippines, and Namyang 7 from Korea accumulated higher amount of proline and sugar than the salt-sensitive varieties. Meanwhile, in the salt-tolerant varieties accumulated lower concentration of MDA and  $\text{H}_2\text{O}_2$  compared to the sensitive ones.





<b>SL</b>	-	0.68	0.83	0.75	1															
<b>PB</b>	0.91*	0.73	0.87*	0.91*	0.92*	1														
<b>PH</b>	-	0.67	0.88*	0.68	0.79	0.92*	1													
<b>LR</b>	0.93*	0.53	0.51	0.42	0.39	0.41	0.37	1												
<b>CC</b>	-	0.56	-0.73	0.83	0.74	0.86	0.79	-0.59	1											
<b>CMS</b>	0.92*	0.51	0.81	0.69	0.51	0.62	0.84	0.68	0.36	1										
<b>Na/K</b>	-	-	-0.61	-0.47	-0.68	-0.57	-	0.55	-0.27	-0.35	1									
<b>Proline</b>	0.85*	0.47	-0.58	-0.53	-0.53	-0.41	0.62	0.61	-0.22	-0.29	-0.47	1								
<b>MDA</b>	-	-	-0.51	-0.42	-0.41	-0.47	-	0.69	-0.22	-0.41	0.97*	-0.39	1							
<b>H<sub>2</sub>O<sub>2</sub></b>	0.89*	0.67	-0.62	-0.39	-0.36	-0.58	0.68	0.54	-0.27	-0.32	0.96*	0.42	0.85	1						
<b>Sugar</b>	0.95*	-	-0.64	-0.54	-0.31	-0.64	-	0.62	-3.48	-0.37	-0.51	0.38	0.41	0.25	1					
	-	0.61					0.79													
	0.87*	-					-													
	-0.14	0.73					0.61													
	0.92*	-					-													
	-	0.64					0.57													
	0.90*																			
	0.57																			
	0.91*																			
	-																			
	0.91*																			

\* Significant at the  $p < 0.05$ . SS: salinity score, PG: percentage germination, RL: root length, RFW: root fresh weight, SL: shoot length, PB: plant biomass, PH: plant height, LR: leaf rolling, CC: chlorophyll content, CMS: cell membrane stability, Na/K: Na<sup>+</sup>/K<sup>+</sup> ratio, Proline: proline content, MDA: malondialdehyde content, H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide content, Sugar: sugar content.

### 3. Discussion

Seedlings grown in salinity stress condition of 116 Asian rice cultivars exhibited different visual symptoms of salt injury. Salinity condition decreased percentage germination of Asian rice cultivars due to the osmotic and ionic stress cause insufficient water absorption which lead to the toxic effects on the seed embryo. Based on the interaction between salinity condition and rice cultivars, among the Asian rice cultivars there is a wide difference in response to salinity stress condition. Salt-tolerant seedlings were distinguished from the salt-sensitive seedlings under salinity stress condition. Our results are consistent with the previous studies which mentioned that different genotypes of barley, cabbage, and *Suaeda* species showed different responses to salinity condition with regard to percentage germination [60–63]. Salinity condition decreased the radicle and plumule growth. The retardation of the radicle and plumule length because of the reduction in turgor cells.

Visual salt injury begins with the reduction of effective leaf area. Salt-sensitive rice cultivars had high score for leaf rolling and leads to the decrease of photosynthetic activity. The distribution for root length, root fresh weight, shoot length, and plant biomass across 116 Asian rice cultivars under salinity stress condition showed a wide fluctuations. Root length and shoot length were shorter in salinity condition, compared to the normal condition. Root fresh weight and plant biomass of salt-susceptible cultivars showed higher percent reduction than salt-tolerant cultivars. Lower percent reduction of root length, root fresh weight, shoot length, and plant biomass were recorded in Pokkali from India followed by TCCP 266 from Philippines, IR 45427 also from Philippines, and Namyang 7 from Korea. On the other hand, higher percent reduction of root length, root fresh weight, shoot length, and plant biomass were exhibited by IR29 from Philippines, IR58 also from Philippines, Daegudo from Korea, and Guweoldo also from Korea. Salt-tolerant rice cultivars displayed less growth reduction than salt-sensitive cultivars under salinity stress condition.

Cell membrane stability of 116 Asian rice cultivars were affected by the salinity stress condition. In the salt-sensitive rice cultivars, the cell membrane structure was damaged by the salt which increases the membrane permeability, and destroy the plasma membrane; as a result, the plant growth reduced [64]. In order to maintain the Na/K balance in the shoot, the plant absorb more K and exclude the toxic Na [39,65]. In this study, salt-tolerant rice cultivars showed the ability to absorb more K than Na in order to maintain the Na/K balance in the shoot. According to Ponnampuruma

[66], K concentration in the shoot has a positive correlation with salinity tolerance because of the K is important in stomatal functions. Gregorio and Senadhira [39] also reported that salt-tolerant rice cultivars had higher K concentration and lower Na content in the shoot. This Na/K balances and maintains of low Na/K ratio are part of the salt tolerance mechanisms and could be promising criterion for salt-tolerant selection [15,67].

Variation of chlorophyll content in 116 Asian rice varieties can be used as a potential salinity stress indicator because chlorophyll content reduced in the salt-sensitive rice varieties under salinity stress condition. The salt-sensitive rice varieties, including IR29 from Philippines, IR58 also from Philippines, Daegudo from Korea, and Guweoldo also from Korea showed lower chlorophyll content compared to the salt-tolerant varieties. Salinity stress condition causes oxidative stress to the chloroplast that reduced the size and number of chloroplast in the leaves by inhibiting the synthesis of chloroplast [69–71]. Chlorophyllase enzyme activity which degrade the chlorophyll increases under salinity stress condition that lead the decrease of photosynthetic activity [16].

Salt-tolerant rice varieties accumulated high proline under salinity stress condition [72,73]. In this study, salt-tolerant varieties such as Pokkali from India followed by TCCP 266 from Philippines, IR 45427 also from Philippines, and Namyang 7 from Korea also showed higher proline concentration compared to the salt-sensitive varieties. These results were consistent with Ghosh et al. [74] who found that salt-tolerant rice cultivars like Nonabokra and Pokkali under salinity stress condition displayed the high proline concentration in seedling stage. Under salinity stress condition, proline plays an important role in protection the protein against denaturation by regulate the redox potential and acts as source of nitrogen and carbon for post salt stress recovery in the rice plants [75–77].

Under salinity stress condition, malondialdehyde (MDA) is increasing in the salt-sensitive rice cultivars such as IR29 from Philippines, IR58 also from Philippines, Daegudo from Korea, and Guweoldo also from Korea. MDA represents the biological membrane damage because MDA is the primary product of decomposition process from unsaturated fatty acids due to oxidative stress under 200 mM NaCl [76,78–84]. The amount of hydrogen peroxide ( $H_2O_2$ ) showed a wide variation in 116 Asian rice cultivars under salinity stress condition. Salt-tolerant rice varieties like Pokkali from India followed by TCCP 266 from Philippines, IR 45427 also from Philippines, and Namyang 7 from Korea exhibited lower  $H_2O_2$  than in the salt-sensitive ones. Sugar content of salt-tolerant rice varieties showed significantly increase under salinity stress condition. These results consistent with the previous study by Chang et al. [85] that found many sugars like raffinose, glucose, fructose, sucrose, galactose, mannose, ribose, xylose, melibiose, galactitol, mannitol, rhamnase, ribose, and erythritol were increasing in the leaves of the salt-tolerant rice cultivars such as Fatmawati and Dendang under salinity stress condition. The accumulation of sugars under salinity stress condition is to escape from the osmotic stress [86–90].

At the seedling and early vegetative stages, salt-tolerant rice varieties showed higher morphological characteristics such as percentage germination, root length, root fresh weight, shoot length, plant biomass, plant height, and chlorophyll content; and also higher physiological and biochemical traits like cell membrane stability, proline, and sugar content. Meanwhile, salt-sensitive ones had high leaf rolling, Na/K ratio,  $H_2O_2$ , and MDA content. These results were supported by the previous studies such as Peng et al. [91], Zhang et al. [92], Dwiningsih et al., [93], and Bhowmik et al. [10]. Many studies indicated that all of the growth stages in rice plants under salinity stress condition showed different responses to salt [94]. The most sensitive stage to salt is during fertilization and flowering, followed by early vegetative stage, germination, and maturity [95–97]. The tolerance responses of rice cultivars to the salinity stress condition may become the foundation of breeding tolerant rice cultivars in the saline agricultural area.

## 4. Materials and Methods

### 4.1. Plant Materials

A total of 116 rice genotypes originally from Asian countries (Table 1) were screened for salinity tolerance at the seedling stage and early vegetative stage (3 leaf stage). Seeds were sterilized with

10% (v/v) NaClO for 15 minutes and washed with distilled water. Pokkali was used as the salt-tolerant standard check and IR29 was used as salt-sensitive check [54].

**Table 1.** Rice varieties for salt stress tolerance screening.

#	Rice Varieties	Country	Sub-population
1	Karang Serang	Indonesia	TRJ
2	Rojolele	Indonesia	TRJ
3	Cempo Ireng	Indonesia	TRJ
4	Ciherang	Indonesia	IND
5	Mayang Khang	Indonesia	IND
6	Sipirasikkam	Indonesia	TRJ
7	Mitak	Indonesia	TRJ
8	Dara	Indonesia	AUS
9	B805D-Mr-16-8-3	Indonesia	IND
10	Tia Bura	Indonesia	TRJ
11	C 5560	Thailand	TEJ/TRJ
12	Nam Dawk Mai	Thailand	IND
13	Bkn 6987-68-14	Thailand	IND
14	Td 70	Thailand	IND
15	Cntr80076-44-1-1-1	Thailand	IND
16	Nahng Sawn	Thailand	IND
17	Quinimpol	Philippines	TRJ
18	TCCP 266	Philippines	IND
19	IR 4482-5-3-9-5	Philippines	IND
20	IR 45427	Philippines	IND
21	IR 9660-48-1-1-2	Philippines	IND
22	IR 238	Philippines	IND
23	IR 2061-214-2-3	Philippines	IND
24	IR 2462	Philippines	IND
25	IR 58614-B-B-8-2	Philippines	IND
26	Pakkali	Philippines	ARO
27	IR64	Philippines	IND
28	IR58	Philippines	IND
29	IR29	Philippines	IND
30	Taichu Mochi 59	Taiwan	TRJ
31	Ai Chueh Ta Pai Ku	Taiwan	IND
32	Ragasu	Taiwan	TEJ/TRJ
33	Tobura	Taiwan	TEJ/TRJ
34	Kao Chio Lin Chou	Taiwan	IND
35	Taino 38	Taiwan	IND/AUS
36	Nanton No. 131	Taiwan	TRJ/(admix)

37	Hsin Hsing Pai Ku	Taiwan	IND
38	Tainung 45	Taiwan	IND
39	Ao Chiu 2 Hao	China	IND
40	Chun 118-33	China	IND
41	Kin Shan Zim	China	IND
42	Pan Ju	China	IND
43	Kechengnuo No. 4	China	IND
44	4484	China	IND
45	4595	China	IND
46	You-I B	China	IND
47	Chunjiangzao No. 1	China	TEJ
48	Shimizu Mochi	Japan	TEJ
49	Norin 11	Japan	TEJ
50	Tamanishiki	Japan	TEJ
51	Niwahutaw Mochi	Japan	TEJ
52	Somewake	Japan	TEJ
53	A 5	Japan	TEJ
54	C.B. Ii	Japan	AUS
55	Fujisaka 5	Japan	IND
56	Nipponbare	Japan	TEJ
57	Khao Phoi	Laos	TEJ/TRJ
58	Khao Luang	Laos	TRJ/(admix)
59	Padi Pohon Batu	Malaysia	TRJ
60	Acheh	Malaysia	IND
61	Mahsuri	Malaysia	IND
62	Padi Tarab Arab	Malaysia	TRJ
63	Nc 1/536	Pakistan	AUS
64	Red	Pakistan	AUS/(Admix)
65	Santhi 990	Pakistan	IND/AUS
66	Daudzai Field Mix	Pakistan	AUS
67	Jp 5	Pakistan	IND/AUS
68	Won Son Zo No. 11	Korea	IND
69	Daegudo	Korea	TEJ
70	Guweoldo	Korea	TEJ
71	Namyang 7	Korea	TEJ
72	Yong Chal Byo	Korea_South	TEJ
73	Thang 10	Vietnam	IND
74	Cm1_ Haipong	Vietnam	IND
75	Nang Bang Bentre	Vietnam	AUS
76	Lua Chua Chan	Vietnam	TRJ

77	Soc Nau	Vietnam	IND
78	Heo Trang	Vietnam	IND
79	Pd 46	Sri Lanka	IND
80	Bakiella 1	Sri Lanka	IND
81	Gallawa	Sri Lanka	AUS
82	Ittikulama	Sri Lanka	AUS
83	Karayal	Sri Lanka	AUS
84	Amane	Sri Lanka	IND
85	Thavalu	Sri Lanka	AUS
86	Patnai 6	Myanmar	AUS
87	Buphopa	Myanmar	TEJ/TRJ
88	Kaukkyi Ani	Myanmar	TRJ
89	A100943-R	Myanmar	AUS
90	Nsgc 5953	Myanmar	IND
91	A 36-3	Myanmar	IND
92	Jumli Dhan	Nepal	TEJ/TRJ
93	N-2703	Nepal	AUS
94	Bhim Dhan	Nepal	TEJ/TRJ
95	Juppa	Nepal	IND
96	Tauli	Nepal	AUS
97	Darmali	Nepal	TEJ/ARO
98	Dhan	Nepal	IND
99	Tchampa	Iran	AUS
100	Phudugey	Bhutan	AUS
101	Jyanak	Bhutan	TEJ/TRJ
102	Wir 3039	Tajikistan	TEJ
103	Ak Tokhum	Azerbaijan	ARO
104	Gasym Hany	Azerbaijan	ARO
105	Celiaj	Azerbaijan	TEJ
106	Shimla Early	Iraq	IND/AUS
107	A 152	Bangladesh	IND/TRJ
108	Dnj 179	Bangladesh	AUS
109	Dj 24	Bangladesh	AUS
110	Dj 102	Bangladesh	AUS
111	Dnj 121	Bangladesh	AUS
112	Aswina 330	Bangladesh	AUS
113	Tranoep Beykher	Cambodia	IND
114	Srav Prapay	Cambodia	IND
115	Simpor	Brunei	TRJ
116	Pokkali	India	IND

\* IND = indica; TEJ = temperate japonica; TRJ = Tropical japonica; ARO = aromatic; AUS = aus.

#### 4.2. Salinity Screening at Seedling and Early Vegetative Stage

Concentration of salt (NaCl) used in this experiment was 200 mM. Ten seeds of each rice genotype were germinated in the petri dishes containing blotting paper and were treated with 200 mM NaCl and kept in the incubator at 30°C. For the control condition, seeds were germinated with distilled water. After 12 days of salt treatment, germinated seedlings were transferred to a hydroponic system containing Yoshida's medium [55] with 200 mM NaCl. Rice plants were grown in a growth-chamber at temperature 28°C/24°C for day/night with 65% humidity, and light intensity 500  $\mu\text{Em}^{-2}\text{s}^{-1}$  until early vegetative stage (3 leaf stage). Morphological, physiological, and biochemical responses, including root length, root fresh weight, shoot length, plant biomass, leaf rolling, chlorophyll content,  $\text{Na}^+/\text{K}^+$  ratio, cell-membrane stability, proline content, malondialdehyde (MDA) content, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) content, and sugar content were measured at early vegetative stage (3 leaf stage).

#### 4.3. Measurement of Morphological, Physiological, and Biochemical Traits

##### 4.3.1. Determination of Percentage Germination and Standard Evaluation Score for Salt Injury

The indicator for germinated seeds when the radicle had protruded through the seed coat, hypocotyl was extended, and cotyledon was unfolded. Percentage germination and visual symptoms of salt injury at the seedling stage was evaluated based on the standard evaluating score at 12 days of salt treatment (Table 2) [6].

**Table 2.** Standard evaluation score (SES) of visual salt injury at seedling stage [6].

Score	Observation	Tolerance
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	Moderately tolerant
7	elongating	Susceptible
9	Complete cessation of growth; most leaves dry; some plants dying	Highly susceptible
	Almost all plants dead or dying	

##### 4.3.2. Measurement of Root Length, Root Fresh Weight, Shoot Length, and Plant Biomass

Root length was measured from maximum length of the root for each rice plant at early vegetative stage (3 leaf stage). Whole weight of the roots of each rice plant was determined as the root fresh weight. Shoot length was measured from the ground surface to the tallest leaf tip with a ruler. The roots and shoot of each rice plant were weighted fresh and determined as plant biomass.

##### 4.3.3. Determination of Leaf Rolling

The leaf rolling score on the three leaves of each plant was identified at early vegetative stage (3 leaf stage) based on the standard evaluation system for rice [6]. The range in score is from 1 to 5, 1 indicating unrolled leaves and fully turgid, 2 indicating leaves are folded (Deep-V-shaped), 3 indicating leaves are fully cupped (U-shaped), 4 indicating leaves margins touching (O-shaped), and 5 indicating completely rolled leaves.

##### 4.3.4. Analysis of Chlorophyll Content

Chlorophyll content of the fully expanded leaves on the top of each plant was measured by using Soil and Plant Analyzer Development (SPAD)-502 Plus Chlorophyll Meter (Spectrum Technologies, USA). Leaf was inserted into the sample slot of the SPAD in such a way to avoid the midrib and five reading was measured for each leaf.

#### 4.3.5. Measurement of Na/K Ratio

The ratio of sodium (Na) and potassium (K) concentration in the root and shoot were measured for each rice genotype grown under salinity condition of 200 mM at early vegetative stage (3 leaf stage). Each rice plant was rinsed with the distilled water and then dried at 65°C for 2 days. The dried tissue sample for each rice genotype was ground by mortar and pestle. A total of 100 mg of ground sample were digested in 3 ml hydrogen peroxide and 5 ml of nitric acid for 3 hours at 152–155°C. Then, the digested sample was diluted to a final volume of 12.5 ml. The concentration of Na and K were quantified by using a flame photometer (ANA-135, Tokyokoden, Tokyo, Japan). The estimated concentration of Na and K were calculated based on the standard curve.

#### 4.3.6. Measurement of Cell Membrane Stability

Leaf cell membrane stability (CMS) of each rice genotype under salinity condition was determined by using this equation:

$$\text{CMS (\%)} = 1 - (E1/E2) \times 100$$

Leaf samples were washed with distilled water and then, placed in 10 ml of deionized water at 10°C for 18 hours. Next, the samples were heated at 52°C for 1 hour in a water bath. In order to diffuse the electrolytes from leaf tissue to aqueous media, the samples were incubated at 10°C for 24 hours. The samples were shaken and initial conductance (E1) was measured for each sample. All of the samples were then autoclaved at 121°C and 0.10 MPa for 15 minutes in order to kill the plant tissue and release the electrolytes. The samples were placed in incubator at 25°C for cooling down, then the samples were shaken, and the final conductance (E2) was measured.

#### 4.3.7. Measurement of Proline Content

A total of 0.5 g fresh leaf sample of each rice genotype was diluted in 10 ml of 3% aqueous sulfosalicylic acid and centrifuged for 1 minute at 3000 rpm. The 2 ml of supernatant was reacted with 2 ml of glacial acetic acid and 2 ml of ninhydrin acid at 100°C for 1 hour. Exactly 2 ml of Toluene was used to extract the chromophore. Absorbance of the chromophore was measured at 520 nm by using Genesys 10-s UV/Vis Spectrophotometer (Thermo Spectronic, Waltham, MA, USA) and Toluene as the blank. A standard curve for proline content was quantified by using purified proline (Sigma Aldrich, Melbourne, VIC, Australia). Proline content of each rice genotype was calculated by using the following formula.

$$((\mu\text{g proline/mL} \times \text{mL toluene})/115.5 \mu\text{g}/\mu\text{mole}) \times (\text{g sample}/5) = \mu\text{moles proline gram FW}^{-1}$$

#### 4.3.8. Measurement of Malondialdehyde (MDA) Content

Exactly 0.5 g of rice leaf from each rice genotype was cut and homogenized with 1.5 ml of a 0.5% (w/v) thiobarbituric acid solution that consisting of 20% (w/v) trichloroacetic acid and 1.5 ml of distilled water. The solution was heated for 25 minutes at 95°C, and then the reaction was stopped by placing the samples on ice. Next, the solution was centrifuged, absorbance of the supernatant was measured at 532 and 600 nm. The extinction coefficient of  $\text{mM}^{-1} \text{cm}^{-1}$  was used to calculate the MDA content and expressed as nanomoles per gram (nmol/g) of fresh weight.

#### 4.3.9. Measurement of Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) Content

About 0.1 g of leaf from each rice genotype was diluted in 3 ml of 5% (w/v) trichloroacetic acid and incubated for 3 hours at 4°C. Next, 1 ml of FOX reagent was added to the 0.2 ml of supernatant of the sample, then mixed and incubated for 15 minutes at 25°C. Absorbance of the solution was read at 560 nm. The H<sub>2</sub>O<sub>2</sub> content was expressed as micromoles per gram ( $\mu\text{mol/g}$ ) of fresh weight.



#### 4.3.10. Measurement of Sugar Content

Leaf sample from each rice genotype was ground and 1.0 g of ground sample was added to the 1 ml of distilled water. Then, 1 ml Anthrone reagent was added to the suspension and incubated for 8 minutes at 25°C. Absorbance of the solution was read at 630 nm. The content of soluble sugar in each rice genotype was calculated by using a standard graph and expressed in milligram per gram (mg/g) of fresh weight.

#### 4.4. Statistical Analysis

The experiment of salinity screening was conducted in a randomized completely block design (RCBD) with five replications. The salinity treatment and control conditions were compared by Least Significant Difference (LSD) test at 0.05 probability level.

### 5. Conclusions

Combining morphological, physiological, and biochemical assessments for screening the salt tolerant of 116 Asian rice cultivars was able to classify into tolerant, moderate, and sensitive rice cultivars under salinity stress condition, and also for understanding the salt tolerance mechanisms. The rice cultivars which belong to salt-tolerant including Pokkali from India followed by TCCP 266 from Philippines, IR 45427 also from Philippines, and Namyang 7 from Korea. Whereas, salt-sensitive rice varieties like IR29 from Philippines, IR58 also from Philippines, Daegudo from Korea, and Guweoldo also from Korea. The identified salt-tolerant rice varieties potentially provide a valuable germplasm resource in the breeding programs for developing salt-tolerant rice.

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