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Morpho-physiological evaluation of rice for salt tolerance

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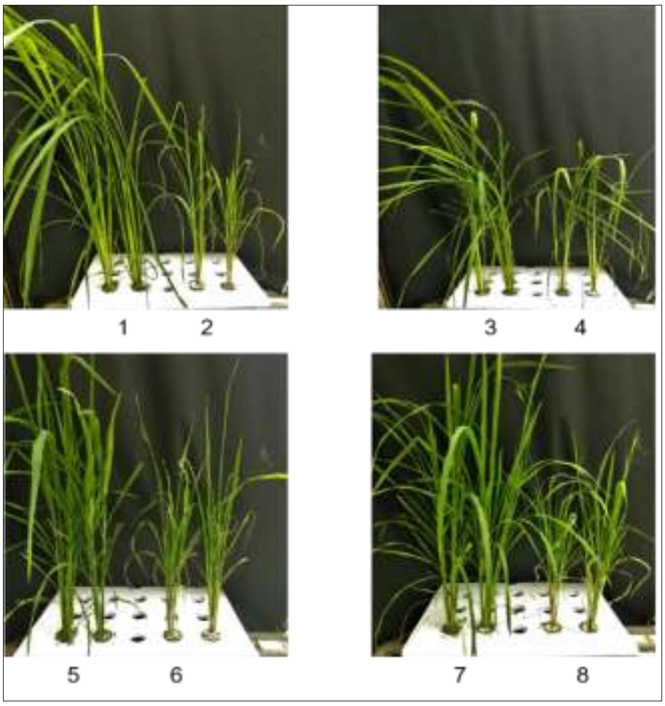
Abstract
Salinity is one of the major abiotic stressors limiting the growth and productivity of crops like rice. Salt stress is due to a higher proportion of sodium ions entering the plants. Excessive sodium ions in soil competes with potassium, the counter ion, affecting the intracellular Na/K ratio which is a vital indicator of plant's salt tolerant capacity. The Na^+ and K^+ concentrations in roots of salt tolerant genotypes (*Pokkali* and *Kuthiru*) and salt susceptible elite rice cultivars (ASD16 and IR64) were analysed during the late vegetative stage. A low Na/K ratio in roots of *Pokkali* and *Kuthiru* as compared to the salt susceptible genotypes clearly differentiated the salt tolerant and susceptible genotypes. Besides, other physiological parameters such as relative water content, chlorophyll stability index, root and shoot length, root and shoot dry weight also fell in favour of the salt tolerant genotypes. Therefore, understanding the morpho-physiological parameters can help in evaluating the salt stress tolerance ability of rice genotypes.

Keywords: Rice, salt stress, Na/K ratio, chlorophyll stability, Fv/Fm

Introduction
Rice, the major staple supplies the carbohydrate requirements of more than half of the world's population, particularly in the Asian continent (Sen *et al.*,2020) [17]. There are several biotic and abiotic factors which limits the productivity of rice. Salt stress turns out to be a major constraint severely affecting the growth and development particularly during the reproductive stage of rice (Singh *et al.*, 2021) [19]. Salinity is due to excess of chlorides and sulphates of calcium and magnesium within the soil as well as in irrigation water. Excessive influx of sodium ions in particular was the major cause of osmotic stress and ionic stresses leading to stunted growth, reduced leaf area and decreased tillering in rice. In addition, it leads to damages to cell membrane and chloroplast leading to a decreased photosynthetic efficiency (Hameed *et al*., 2021) [7].
Salt stress response function involves various adaptive mechanisms which include (i) osmotic regulation via accumulation of compatible solutes such as proline, glycine-betaine, (ii) ion exclusion through selective uptake of specific ions and minimizing the entry of unfavourable ions into the system, (iii) ion compartmentalization through sequestering salt ions particularly sodium into the vacuoles and thereby maintaining the cytosolic pH, (iv) antioxidant defence through scavenging reactive oxygen species (ROS) and minimizing the oxidative damage and (v) activation of stress responsive genes which includes several ion transporters and channels (Balasubramaniam *et al*., 2023; Brini and Masmoudi, 2012; Hasanuzzaman *et al*., 2021;Thompson *et al*., 2010;Chatterjee *et al*., 2022) [2, 3, 8, 23, 4]. Understanding these mechanisms is very crucial towards improving the salt tolerance ability in rice crop. In this study, we have explored a selected set of morpho-physiological parameters in ascertaining their role in salt tolerance using a contrasting set of rice genotypes for their response to salt stress. In addition, root Na/K also served as an important indicator in characterizing salt tolerance in rice.

Materials and Methods
Two salt tolerant genotypes i.e., *Pokkali*, an internationally known salt tolerant land race widely cultivated in the coastal tracts of Kerala and *Kuthiru*, a less explored land race cultivated in Kaipad tracts of Kerala. The two salt susceptible cultivars include ASD16 an elite

rice cultivar popular in Tamil Nadu and IR64 an international acclaimed mega variety. All four genotypes were hydroponically raised using Yoshida medium (pH 4.5; Yoshida *et al*., 1976) [24] in greenhouse maintained at 30 °C (± 3 °C), 85% relative humidity and 12h light and dark cycles for 60 days. The hydroponic system consisted of 2-inch net cups filled with perlite as anchorage material and a 12-litreplastic tray container. Six similar trays were maintained, three each for treatment and control, respectively and each of the genotypes were individually in net cups. Fresh nutrient solution was changed in five days interval, the salt stress was imposed on 60 days old plants by adding 150 mM NaCl to the Yoshida nutrient solution for a two weeks period (Fig.1). Observations were recorded from fully expanded 3rd leaf uniformly across the three biological replicates at the end of two weeks of stress phase.



1. *Pokkali* under control condition; 2- *Pokkali* under salt stress; 3- *Kuthiru* under control condition, 4- *Kuthiru* under salt stress; 5- ASD16 under control condition, 6-ASD16 under salt stress; 7- IR64 under control condition; 8- IR64 under salt stress

Fig 1: Comparison of rice genotypes between unstressed (control) and salt stressed conditions

Estimation of relative water content (RWC)

Fresh weight ~ 0.5g of leaf discs from the 3rd leaf of all three biological replicates from every genotype was subjected to RWC analysis. Inter-venal area was excluded during the preparation of leaf discs. The leaf samples were then incubated at 20 °C for hours using double distilled water and blotted on a tissue paper to remove excess water from the leaf samples. The turgid weight was then taken as described by Sairam *et al*., (2002) [16]. The leaf samples were kept on a brown paper cover and dried in hot air oven at 70 °C for two days. The dry weight of the leaf samples was finally recorded. The relative water content (%) is estimated as below.

$$\text{Relative water content (\%)} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

Analysis of chlorophyll stability index (CSI)

Chlorophyll stability index was assessed based on the protocol described by Kaloyereas, (1958) [11]. Approximately, 1g of leaf tissue was sampled and placed in a test tube containing 10ml of double distilled water. Two replicates of the same were prepared, one set was kept at room temperature for one hour and the other set was kept in water bath at a temperature of 55 °C for one hour. After an hour, decanted the water, ground the leaf tissue using 80% acetone, centrifuged at 10000rpm for 10 minutes and finally made up to 10ml using 80% acetone. The chlorophyll stability index (CSI) was calculated using the following formula.

$$\text{Chlorophyll stability index (\%)} = \frac{\text{OD at 652 nm of treated sample}}{\text{OD at 652 nm of treated sample}} \times 100$$

Wherein,
Treated sample = one gram of leaf sample kept at 55 °C in water bath for one hour
Control sample = one gram of leaf sample kept at room temperature for one hour

Measurement of mean root length and shoot length

Root and shoot lengths of both control and stressed plants were recorded in centimetres. Root length was measured from the collar region to the root tip whereas shoot length was taken from the collar region to uppermost leaf tip.

$$\Delta RL = \frac{\text{Mean root length in (stress)} - \text{Mean root length (control)}}{\text{Mean root length (control)}} \times 100$$

$$\Delta SL = \frac{\text{Mean shoot length (stress)} - \text{Mean shoot length (control)}}{\text{Mean shoot length (control)}} \times 100$$

Measurement of root and shoot dry weight

For the measurement of root and shoot dry weight, the root and shoot portions were separated from the stressed and control plants and washed thoroughly. Care was taken to minimize the loss of tissue while washing. Samples were finally dried in a hot air oven at 60 °C for five days and finally weight was recorded.

Analysis of Na and K contents

After measuring the dry weight of roots, the tissues were used for estimating the sodium and potassium contents. The root tissues from each of the three biological replicates were powdered individually using liquid nitrogen and 0.2 g of the powdered sample was used in the further analysis. The root samples were transferred into 100 ml conical flask and digested using 10 ml of triple acid which included nitric acid: sulphuric acid: perchloric acid in 9:2:1 ratio and left overnight. For the complete digestion, the flasks were kept on a hot sand bath until the solution turned clear. The digested sample was filtered through Whatman no.1 filter paper and the volume were finally made to 100 ml using a volumetric flask (Overman and Davis,1947) [13]. Five standards ranging from 20 to 100 ppm were prepared using NaCl and KCl for sodium and potassium respectively and the values were expressed in ppm.

Statistical analysis: To evaluate the significant differences in each trait between the genotypes under salt stress and control conditions student ‘t’ test was performed. Significance was analysed at p value of 0.05% level and the mean comparison was done using Duncan’s multiple range test using AgriWASPstat 2.0 software.

Results

Effect of salt stress on physiological parameters

The relative water content was significantly decreased in all the four genotypes under 150mM NaCl stress. Salt tolerant genotypes *viz*., *Pokkali* and *Kuthiru* showed a moderate reduction of about 9.09% and 11.06%with respect to their controls. However, salt susceptible genotypes *viz*., ASD16 and IR64 showed a significant reduction of 16.97% and 22.73%, respectively, compared to their unstressed counterparts (Fig. 2).

Each bar represents an average relative water content from three biological replicates. Values are mean ±SE (n=3); superscript ‘a’ is the best treatment and ‘d’ is the poorest treatment.

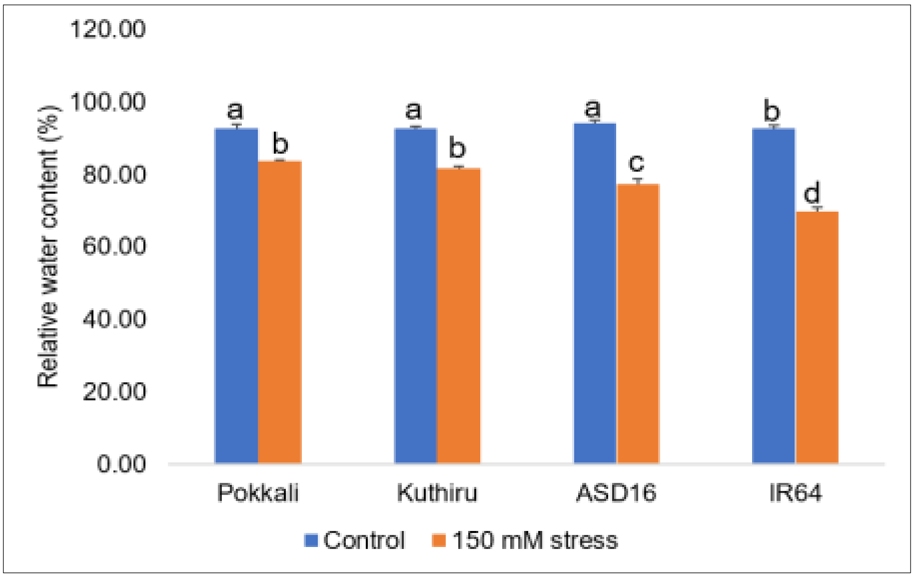


Fig 2: Relative water content measured under control and 150 mM NaCl stress

All the four genotypes showed a significant reduction in chlorophyll stability under 150 mM NaCl stress. Increase in the Na^{+} levels upon salt stress had severely affected the stability of the chlorophyll pigments in IR64 with a reduction of 10% whereas, ASD16 had showed a reduction of ~ 3% which is almost close to salt tolerant genotypes. Salt tolerant genotypes *viz*., *Pokkali* had a decrease of 2.3% whereas *Kuthiru* had 2.6% reduction in their chlorophyll stability index as compared to their respective controls (Fig. 3).

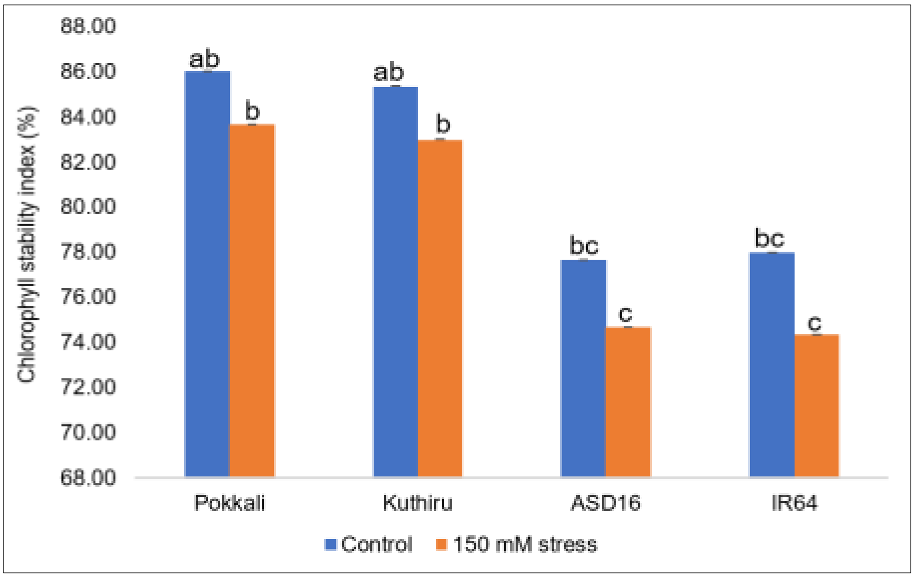


Fig 3: Chlorophyll stability index measured under control and 150 mM NaCl stress

Each bar represents an average chlorophyll stability index from three biological replicates. Values are mean ±SE (n=3); superscript ‘a’ is the best treatment and ‘c’ is the poorest treatment.

The mean root and shoot lengths showed a positive value under salt stress in *Pokkali* and *Kuthiru*. Whereas, in salt susceptible genotypes such as ASD16and IR64, the mean of root and shoot length exhibited negative trend. *Pokkali* showed a ΔRL of 20.45 followed by *Kuthiru* with a ΔRL of 13.63 whereas, ASD16with a ΔRL of -10.29 and IR64 with -22.44. With regard to shoot length, *Pokkali* had a ΔSL of 3.94 and *Kuthiru* at 1.11. However, ASD16 and IR64 showed a negative ΔSL of -0.64 and -9.94, respectively. (Table 1).

Table 1: Mean root length and mean shoot length under control and 150 mM NaCl stress

	MSL (cm) control	MSL (cm) 150 mM stress	MRL (cm) control	MRL (cm) 150mMstress	ΔSL	ΔRL
Pokkali	118.3± 0.88^{b}	123±1.15^{a}	14.66±1.52^{d}	17.66±0.881^{c}	3.94	20.45
Kuthiru	119±1.45^{ab}	121±1.15^{ab}	14.66±0.577^{d}	16.66±0.333^{c}	1.11	13.63
ASD16	104.66±0.57^{cd}	103.33±1.45^{d}	22.66±0.577^{a}	20.33±0.333^{b}	-0.641	-10.29
IR64	114±1.73^{c}	102.66±0.88^{d}	16.33±0.577^{cd}	12.66±0.333^{e}	-9.94	-22.44

Each table value is an average of the observations from three biological replicates. Values are mean ±SE (n=3); superscript ‘a’ is the best treatment and ‘e’ is the poorest treatment.

With respect to shoot dry weight, *Pokkali* and *Kuthiru* showed a slight reduction in their shoot dry weight whereas there was a considerable decrease in ASD16 and IR64 (Fig.4). Under salt stress, the root dry weight of all the four genotypes was found to be reduced significantly (Fig. 5).

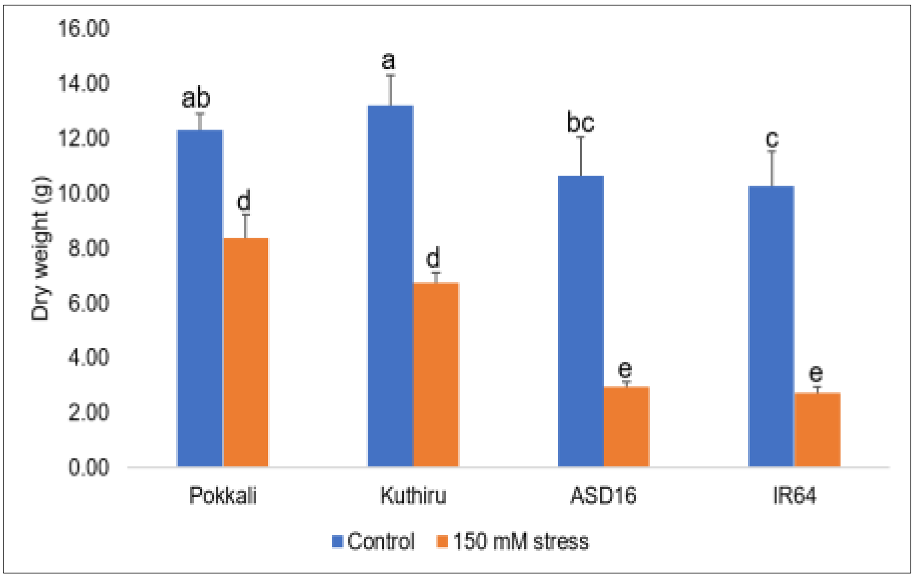


Fig 4: Shoot dry weight measured under control and 150 mM NaCl stress

Each bar represents average shoot dry weight from three biological replicates. Values are mean ±SE (n=3); superscript ‘a’ is the best treatment and ‘e’ is the poorest treatment.

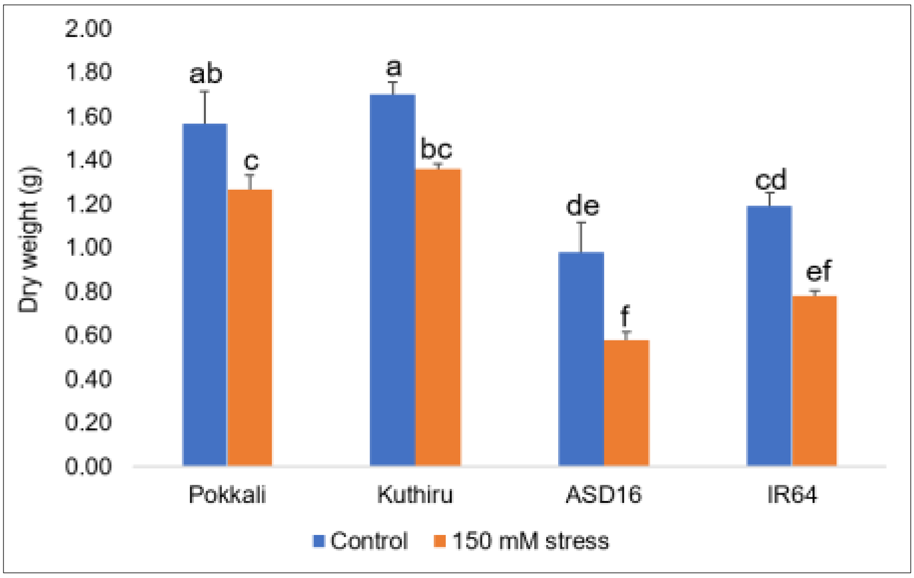


Fig 5: Root dry weight measured under control and 150 mM NaCl stress

Each bar represents an average root dry weight from three biological replicates. Values are mean ±SE (n=3); superscript 'a' is the best treatment and 'f' is the poorest treatment.

Role of Na/K ratio on salt tolerance

A significant difference in Na/K ratio in roots was observed in salt tolerant and susceptible genotypes under salt stress. Salt tolerant genotypes had Na/K values toward the lower side in roots of *Pokkali*at 0.38, followed by *Kuthiru* at 0.52. High Na/K ratio was observed in among ASD16and IR64 at 0.88 and 1.81, respectively (Fig. 6).

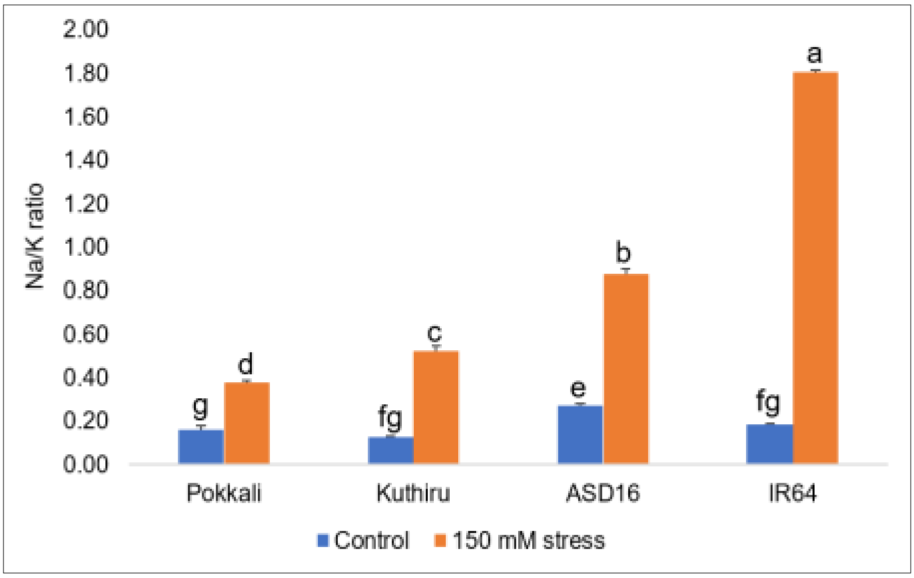


Fig 6: Na/K ratio in roots under control and 150 mM NaCl stress

Each bar represents an average Na/K ratio from three biological replicates. Values are mean ±SE (n=3); superscript 'a' is the best treatment and 'g' is the poorest treatment.

With respect to potassium contents under salt stress, salt tolerant genotypes *viz.*, *Pokkali* and *Kuthiru* did not show any significant difference in their root K^+ concentrations whereas, salt susceptible genotypes ASD16 and IR64 showed a significant decrease in their K^+ content (Fig. 7).

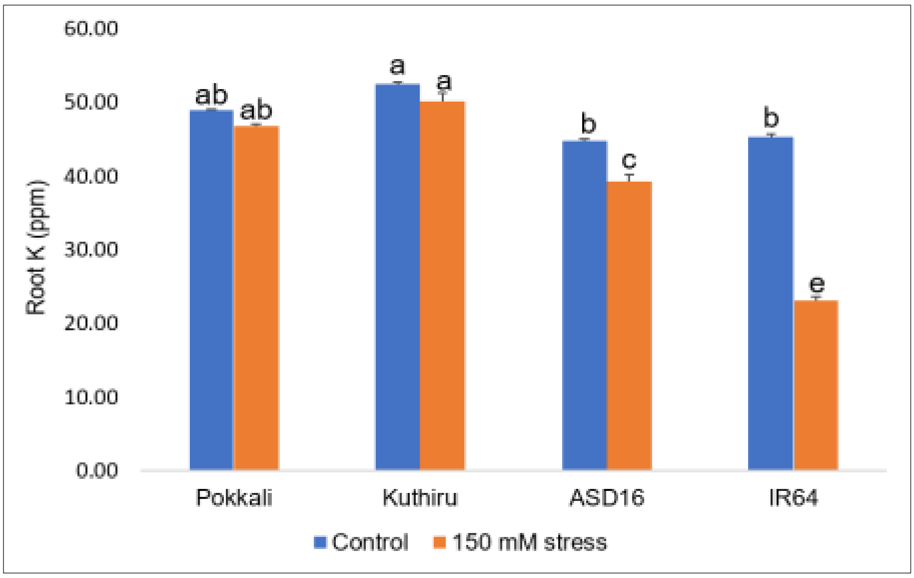


Fig 7: Potassium content in roots under control and 150 mM NaCl stress

Each bar represents an average K^+ content in roots from three biological replicates. Values are mean ±SE (n=3); superscript 'a' is the best treatment and 'e' is the poorest treatment.

Discussion

In this investigation, different morpho-physiological parameters such as relative water content, chlorophyll stability index, dry weight of root as well as shoot, root and shoot length and Na-K contents were analysed in four rice genotypes under salt stress and control conditions. Salt tolerant rice landraces (*Pokkali* and *Kuthiru*) and salt susceptible rice cultivars (ASD16 and IR64) were subjected to 150 mM NaCl stress from the 60^{th} day of sowing for a two weeks period. *Pokkali* is a universal salt tolerant landrace with excellent salt tolerant capabilities and *Kuthiru* is one another salt tolerant land race from the state of Kerala. On the contrary, ASD16 and IR64 were known salt susceptible but elite rice cultivars. Excessive accumulation of salts near the rice root system affects the water conductance thereby leads to a reduction in the relative water content (Suriya-Arunroj *et al*., 2004; Polash *et al*., 2018) [21, 14]. Thus, estimation of relative water content can be an ideal parameter towards understanding the salt tolerant response in rice.

A significant reduction in the relative water content was found in all the four rice genotypes under 150 mM NaCl stress. However, salt susceptible genotypes such as ASD16 and IR64 showed a larger variation as compared to salt tolerant genotypes *Pokkali* and *Kuthiru*. Further, the chlorophyll stability index (CSI) was known as an important parameter in determining the salt tolerant capabilities in plants (Mohan *et al*., 2000; Singh *et al*.,2013) [12, 20]. Higher CSI ensures the intactness of the chlorophyll pigment under salt stress (Babu *et al*.,2009) [1]. In this study, CSI of all four genotypes showed a significant decline after two weeks of salt stress. Interestingly, *Pokkali* and *Kuthiru* had only a slight reduction in their CSI values which indicates their ability to withstand salt stress on the other hand, ASD16 and IR64 had a very low CSI values indirectly indicating their susceptibility to salt stress.

In addition to the above physiological parameters, morphological parameters such as root and shoot length as well as dry weight under control and salt stressed conditions were analysed. Salt stress negatively impacted the root and shoot length, root and shoot dry weight, number of tillers per plant, pollen viability, seed germination as well as plant yield (Shahi *et al*., 2015; Reddy *et al*., 2017) [18, 15]. In this study, *Pokkali* showed a higher root and shoot length after salt stress followed by *Kuthiru* whereas, ASD16 and IR64 revealed a significant decrease in their root as well as shoot lengths. This increase in root and shoot lengths in *Pokkali* and *Kuthiru* clearly indicates their salt tolerant capabilities. With regard to the dry weight of shoot as well as root, a significant decline was found in all selected genotypes. Even though, a reduction was found among the salt tolerant genotypes, the difference was found to be more prominent only among salt susceptible genotypes such as ASD16 and IR64.

Till date, researchers were mainly focusing on the Na^+ exclusion mechanism for imparting salinity tolerance to plants. Regulation of potassium levels and in turn lowering the Na/K ratio was given only secondary importance. This results in the disruption of plant metabolism since more than 50 enzymes were activated by the cellular potassium (Horst Marschner,1995) [9]. Due to similar ionic radius and hydration energy, Na^+ competes with K^+ uptake at sites *viz*., shaker type channels such as non-selective cation channels (NSCC) and HKT transporters (Tester and Davenport, 2003; Garciadeblas *et al*. 2003) [22, 5]. Therefore, exclusion of Na^+ and increased uptake of K^+ plays a major role in determining the salt tolerance capabilities of a plant (Golldack *et al*. 2003; Keisham *et al*. 2018) [6, 11]. Here, we demonstrate that salt tolerant genotypes will be capable of maintaining a low Na/K ratio under salt stress whereas, salt susceptible genotypes to have an elevated Na/K ratio. Interestingly, *Pokkali* and *Kuthiru* could well maintain their root K^+ levels even after salt stress as compared to ASD16 and IR64, thereby affecting the Na/K homeostasis only in susceptible genotypes.

Conclusion

We report that salt stress essentially influences the morpho-physiological parameters *viz*., Na/K ratio, relative water content, chlorophyll stability index, root and shoot length, root and shoot dry weight. Further potassium levels within the plant can also serves an efficient indicator of stress tolerance. Therefore, these parameters will be helpful in understanding the salt tolerant nature of rice genotypes.

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Conflict of Interest: The authors disclose no conflict of interest.

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