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RESEARCH ARTICLE

Molecular Genetics

Assessing the impact of salinity stress on Na^+, K^+, and Ca^{2+} contents of 125 rice genotypes

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Abstract: The ionic balance of Na^+, K^+, and Ca^{2+} affects salinity tolerance in rice. This study examined the content of Na^+, K^+, and Ca^{2+} in seedling dry matter in 121 traditional rice accessions and four improved rice genatypes under salinity stress and control conditions. The experiment was conducted as a randomized complete block design with four replicates. Seedlings were raised for three days in *Yoshida* solution with an electrical conductivity (EC) of 6 dS/m and then exposed to a EC 12 dS/m salinity level for fourteen days. Salinity tolerance was assessed using a standard visual scoring system. Na^+, K^+, and Ca^{2+} contents were measured using a flame photometer. Salinity tolerance levels were inversely correlated with Na^+, Ca^{2+}, and Na:K ratio, and positively correlated with K^+. Inverse relationships were observed between K^+ and both Na^+ and Ca^{2+}, suggesting antagonism, while a positive correlation existed between Na^+ and Ca^{2+}. Analyzing shoot dry matter, Na^+, K^+, and Ca^{2+} contents in rice help to identify and select salinity-tolerant rice genotypes by prioritizing those with higher K^+ and lower Na^+ and Ca^{2+} levels. The order of Na^+, K^+, and Ca^{2+} in the dry matter of tolerant and moderately tolerant rice genotypes highlights that the ion balance is genotype dependent and not the sole determinant of salinity tolerance in rice. Further research is needed to determine the susceptibility of accessions to salinity stress, focusing on demonstrating decreased Na^+ and Ca^{2+} contents, and increased K^+ content across various stress scenarios and growth phases to understand their potential for salinity tolerance.

Keywords: Na^+, K^+, Ca^{2+} content, salinity tolerance, seedling stage, traditional rice accessions.

INTRODUCTION

Rice as the staple food for half of the world's population, ensures food security, livelihoods, and cultural heritage. It also offers potential solutions to global challenges such as hunger and climate change. Developing salinity tolerance in rice is essential for safeguarding global food security by enabling rice cultivation in saline-affected coastal areas and mitigating the impact of rising sea levels. Salinity stress has been shown to significantly reduce grain yield by 64.52%, primarily affecting seed set, panicle number, and grain quality, with a more pronounced impact in the reproductive stage (Zeng *et al*., 2023).

It is estimated that one billion hectares of land worldwide are salt affected accounting for 5% of the global land area. Furthermore, the increase in sea level by 12-22 cm in the 20th century and continuing rise by 0.34 cm annually affects rice production in Thailand, Vietnam, China, and Korea (Walthall *et al*., 2013; Hopmans *et al*., 2021). Introducing salinity-tolerant genotypes to these affected lands is a sustainable solution to fulfill the demand for rice production. However, the number of genetic variations associated with enhancing salt tolerance in the primary rice crop is limited, and our understanding of the underlying molecular mechanisms

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remains incomplete (Chen *et al.,* 2021; Alfatih *et al.,* 2023).

Sri Lanka has conserved several thousands of traditional rice accessions that are evolutionarily diverse and possess various beneficial traits (Shyamalee and Ranawake, 2024a & b). Selecting for salinity tolerant accessions directly broadens the gene pool for salinity tolerance in rice. Further, research endeavors should prioritize not only exploration of the wide array of genetic resources at our disposal, but also on identifying genetic traits related to salinity tolerance that may have been inadvertently overlooked during the domestication process. This initiative aims to reintroduce these valuable salinity tolerance attributes, especially those found in traditional accessions, into cultivated rice varieties. This knowledge can also be applied to other crops and help scientists to develop strategies for enhancing the resilience of various agricultural species to environmental stresses (Chen *et al.,* 2021).

Exceeding the critical threshold for soil salinity (3.0 dS/m) has adverse effects on various panicle and grain related characteristics in rice (Mumtaz *et al.,* 2018). A soil electrical conductivity (EC) of 6.0 dS/m led to a 50% reduction in rice grain yield, with most rice lands in the region falling into the moderately saline (EC 4-8 dS/m) and highly saline (EC 8-16 dS/m) categories, posing a threat to crop health (Mondal *et al.,* 2001; Djaman *et al.,* 2020). The high concentrations of solutes damage internal membranes, causing failure in osmotic adjustment in the cytoplasm with Na^+ and Cl^- accumulation and storing reactive oxygen species (Kumar *et al.,* 2013). Failure in osmotic adjustment in plants reduces root growth, accelerates leaf drying, and decreases dry matter production, including yield (Fischer, 1996). Different plant parts tolerate osmotic potential differently (Farooq *et al.,* 2021). Furthermore, Na^+ concentration is different within the same plant (Rivelli *et al.,* 2002; de Lacerda *et al.,* 2003).

Salinity creates a series of complex interactions in plant metabolism, defense mechanism and nutrient requirements (Grattan & Grieve, 1999). Na^+ is considered a toxic element for most plants, including rice, when present in excess concentrations in the soil (Gepstein *et al.,* 2005). Additionally, elevated salinity caused by Na^+, reduces transpiration and photosynthesis by closing the stomata (Khan *et al.,* 2023). Stomatal closure increases the leaf temperature, which affects shoot elongation (Rajendran *et al.,* 2009). The toxic concentration of Na^+ is reached before the Cl^- toxicity concentration (Khan *et al*., 2023). Hence, examining Na^+ levels in plants under salinity stress is justifiable when salinity stress is induced using NaCl.

The defense against Na^+ damage in the cell is achieved by minimizing Na^+ entry into the root through the Na^+ transport pathway, maximizing efflux, minimizing xylem loading, accelerating phloem transportation, partitioning Na^+ into different parts of the plants, and ultimately expelling Na^+ out of the leaves (Tester & Davenport, 2003). Accumulation of compatible solutes including proteins is commonly reported in salt-tolerant plants (Tester & Davenport, 2003; *Gao et al*., 2023). Some rice varieties have developed mechanisms to tolerate high levels of accumulated Na^+. These mechanisms involve the selective uptake and compartmentalization of Na^+ in vacuoles thereby reducing its toxic effects in the cytoplasm (Porcel *et al.,* 2016).

A sufficient supply of K^+ can reduce the uptake and accumulation of Na^+ in plant tissues (Gregorio, 1997). Absorption of K^+ while excluding Na^+ to maintain a low Na: K ratio is a strategy for salinity tolerance (Koyama *et al.,* 2001). A high level of K^+ can help counteract the toxic effects of Na: K (Shahzad *et al.,* 2022). Adequate levels of K^+ in plants maintain the correct ion balance and osmotic potential, essential for maintaining cell turgor and overall plant health, especially under saline conditions. Therefore, K^+ content in salinity-sensitive rice genotypes is lower than in salt-tolerant varieties (Asch *et al.,* 2000).

Calcium ions (Ca^{2+}) increases the Ca: Na ratio, ameliorating salinity (Aslam *et al.,* 2003). Plant membrane bound Ca^{2+} is important for cell wall structure, ion transportation and ion exchange (Rengel, 1992; Marschner, 1995). Ca^{2+} competes with Na^+ for binding sites on cell membranes, reducing the influx of Na^+ into plant cells (Rengel, 1992). The opening and closing of stomata in rice plants, which affect water loss and gas exchange, are regulated by Ca^{2+} (Wang *et al.,* 2014). Supplementing Ca^{2+} to salt-stressed plants increases chlorophyll content, reduces H_2O_2 accumulation, and protects against oxidative damage, while also helping to maintain water balance and reduce membrane injury through enhanced antioxidant enzyme activity. Ca^{2+} supplementation is an effective remediation technique for saline affected rice fields (Tahjib-Ul-Arif *et al.,* 2018). Na^+ content and Na-Ca selectivity are useful criteria for evaluating salinity tolerance in rice (Zeng *et al.,* 2003).

Role of Na^+, K^+, and Ca^{2+} in salinity stress

Na^+, K^+, and Ca^{2+} are involved in complex physiological and biochemical processes under salinity stress (Luan *et al.,* 2009; Machado & Serralheiro, 2017). The exclusion of Na^+ and absorption of K^+ to maintain low Na^+ levels in the solute reduces Ca^{2+} availability and transport to growing parts of the plant (Grattan & Grieve, 1999). Conversely, low-K^+ or high-Na^+ levels induce Ca^{2+} activity that accelerates signaling pathways for salinity tolerance (Luan *et al*., 2009). Reduced Ca^{2+} absorption is essential for salinity tolerance (Läuchli, 1999; Zhu, 2002) and maintaining low cytoplasmic Ca^{2+} activity is also a strategy for salinity tolerance (Läuchli, 1999). Ca^{2+} induces K^+ uptake more effectively than Na^+ (Hasegawa *et al.,* 2000). Salinity-tolerant genotypes should be able to withstand high Na^+ content in the soil and uptake a higher amount of K^+ than Na^+ (Khan *et al.,* 2023). Different genotypes within a species may exhibit distinct regulatory mechanisms for maintaining Na^+, K^+, and Ca^{2+} ion balance when subjected to salinity-induced stress conditions. Therefore, a systematic assessment of germplasm to uncover these regulatory pathways is crucial in the context of breeding programs.

Parameters to assess salinity tolerance in rice

A series of studies used different parameters to evaluate salinity tolerance (Zeng *et al.,* 2002). Parameters such as shoot and root length, shoot and root fresh weight or dry weight (Lang *et al.,* 2000; Mahmood *et al.,*2009; Ranawake & Nakamura, 2012; Shyamalee & Ranawake, 2024b), physiological characteristics (Blumwald *et al.,* 2000; Chunthaburee *et al.,* 2015) reproductive stage traits (Counce & Wells, 1990), seedling survival rate (Zeng *et al.,* 2002; Lang *et al.,* 2000), and plant vigour (Yeo *et al.,* 1990) have been used to assess and compare salinity tolerance in rice.

The visible impact of salinity stress on plant growth and health is evaluated by using the visual scoring system (Gregorio *et al.,* 1997). Further, Na^+, K^+, and Ca^{2+} content have already been confirmed as criteria for salinity tolerance in rice (Gregorio & Senadhira,1993; Ahmad *et al.,* 2007; Khan & Panda, 2008; Luan *et al.,* 2009; Machado & Serralheiro, 2017). This study aims to uncover genotype-specific responses to salinity and the underlying mechanisms governing Na^+, K^+, and Ca^{2+} under NaCl-induced salinity stress. The objectives were to analyze Na^+, K^+, and Ca^{2+} accumulation in the seedling dry matter of rice genotypes under salinity stress and to determine their correlation with salinity tolerance levels. The study included 121 traditional rice accessions with different levels of salinity-tolerance and four improved rice genotypes at the seedling stage (unpublished data). The protocol established by Gregorio *et al.* (1997) was used to assess the level of salinity tolerance in rice genotypes. Analyzing Na^+, K^+, and Ca^{2+} contents in various rice genotypes under salinity stress are a valuable approach for understanding genotype specific tolerance mechanisms, and guiding breeding efforts to develop more resilient and productive rice varieties for saline environments.

MATERIALS AND METHODS

One hundred and twenty one (121) traditional rice accessions and four improved rice genotypes were collected from the Plant Genetic Resources Center, Gannoruwa, Peradeniya, Sri Lanka (PGRC, 1999) (*Supplementary Table 1*). The study followed a randomized complete block design, with four replicates, with 10 seedlings in each. Rice seeds were germinated in distilled water for 3 ds and then transferred to *Yoshida* solution (Gregorio *et al*., 1997) with an electrical conductivity (EC) of 6 dS/m. The EC of the *Yoshida* solution was adjusted using a calibration curve constructed by dissolving known amounts of NaCl in distilled water. Germinated rice seeds were placed in slits made in styrofoam sheets, which were floated on the *Yoshida* solution in plastic cups. After initial 3-day period in *Yoshida* solution of EC 6 dS/m, the salinity level increased to 12 dS/m and the solution replaced in every 2 ds. The seedlings were then grown in this solution for 14 consecutive days. Preliminary studies were conducted to optimize the experimental conditions using several salinity-susceptible and tolerant rice genotypes.

Visual scoring of salinity tolerance

Plants were evaluated according to visual symptoms described in the following standard evaluation system for salinity tolerance (Gregorio *et al*.,1997):

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

Evaluation of Na^+, K^+ and Ca^{2+} content

The seedlings collected after exposing to salinity stress were used to assess the Na^+, K^+ and Ca^{2+} content. The seedlings were quickly rinsed in distilled water to remove external materials and then dried in an oven at 65 ^oC for 5 ds. Hundred milligrams (100 mg) of dried leaf tissues were measured in three replicates for each rice accession. Leaf samples were digested according to the method described by O'Halloran *et al*., (1997). The samples were tightly covered with lids, and all vials were packed tightly in a box to keep them on the shaker. The vials were thoroughly shaken on a shaker to suspend all plant materials in the dilute acid for 2 ds at room temperature while agitating occasionally. After 2 days, the samples were filtered to remove all solid materials. Standard solutions were prepared using KCl, NaCl, and $CaCl_2$ as references. A calibration curve was constructed by plotting the absorbance against the concentration of the certified reference solutions. The Na^+, K^+ and Ca^{2+} content in the samples were subsequently determined using this calibration curve, with three replicates, using a flame photometer (Sherwood, 360).

Data analysis

The content of Na^+, K^+, Ca^{2+}, and the Na^+ :K^+ ratio of traditional rice accessions under salinity stress and control conditions were analyzed using ANOVA and mean separation was performed using Duncan's multiple range test. Correlation analysis was conducted using SPSS (SPSS, Inc, 2011). Rice accessions were ranked based on their Na^+, K^+, and Ca^{2+} content in the dry matter to compare their ion levels.

RESULTS AND DISCUSSION

Among the 125 rice genotypes visually scored, *Randhunipagal2866* was found to be salinity tolerant while *Suduheenati3397, Bathkiriel3550, Madathawalu3718, Matholuwa3214, Moddaikaruppan3388, Polayal3639, Lumbini3613, Madaelgalle3508, Mudaliwi3672*, and *Sinnakaruppan3391* were moderately salinity tolerant at the seedling stage. All other rice accessions were either susceptible or highly susceptible (*unpublished data*).

Na^+ reduction and salinity tolerance

Na^+ accumulation was more significant in stressed plants than in plants under controlled cenditions for each rice genotype (Table 1). Since a high concentration of Na^+ can damage shoots and leaves under salinity stress (Lin *et al.,* 2004), reducing Na^+ within the plant is important to develop salinity tolerance. Out of the 125 rice genotypes, 51 (40.15%) rice accessions, with the highest Na^+ accumulation, were found to be salinity susceptible

Table 1: Na^+, K^+, and Ca^{2+} contents (ppm) in shoot dry matter of tolerant and moderately tolerant rice genotypes

TA/MTA	Accession	Na^+ (C)	Na^+ (T)	Rank	K^+ (C)	K^+ (T)	Rank	Ca^{2+} (C)	Ca^{2+} (T)	Rank	Na^+/K^+ (C)	Na^+/K^+ (T)
T	*Randhunipagal2866*	17.2	52.15^{c}	12	68.2	45^{bc}	110	5	8.75^{d}	125	0.25	1.16^{bcd}
MT	*Suduheenati3397*	26	47.6^{c}	6	201.6	44.5^{bc}	109	5	8.75^{d}	124	0.27	0.61^{d}
MT	*Bathkiriel3550*	25	50.75^{c}	9	145	68.9^{a}	121	5	11^{cd}	119	0.17	0.74^{cd}
MT	*Madathawalu3718*	17.6	51.1^{c}	11	130	49.8^{abc}	116	7.5	7.5^{d}	126	0.14	1.03^{bcd}
MT	*Matholuwa3214*	22	52.2^{c}	13	112	37.2^{cd}	104	5	15^{abc}	99	0.2	1.4^{abc}
MT	*Moddaikaruppan3388*	12.8	55.3^{bc}	18	108	46.25^{bc}	114	7.5	11^{cd}	120	0.12	1.2^{bcd}
MT	*Polayal3639*	16	56.15^{bc}	22	99	59.4^{ab}	119	5	11^{cd}	117	0.26	1.05^{bcd}
MT	*Lumbini3613*	16	76.3^{ab}	38	112	49.95^{abc}	117	10	17.5^{ab}	75	0.14	1.53^{abc}
MT	*Madaelgalle3508*	15	79.95^{ab}	48	126	38.1^{cd}	106	7.5	^{13bc}	110	0.12	2.1^{ab}
MT	*Mudaliwi3672*	16.8	82.8^{ab}	51	115.2	38.2^{cd}	107	2.5	13^{bc}	108	0.15	2.17^{ab}
MT	*Sinnakaruppan3391*	25.2	97^{a}	75	129	36.7^{d}	101	10	21^{a}	44	0.2	2.64^{a}

TA: Tolerant accessions, MTA: Moderately tolerant accessions, (C): Control seedling, (T): Seedlings under salinity stress, Rank: Rank of the rice cultivar among 125 rice genotypes when arranged according to the increasing order of Na^+, K^+, Ca^{2+} contents.

The same letters in the same column were not significantly different in the DMRT test at 5% probability level

or highly susceptible. *Madaelgalle*3508, *Mudaliwi3672,* and *Sinnakaruppan3391* were among the rice genotypes that accumulated a relatively higher amount of Na^+ in the shoot dry matter while still being moderately salinity tolerant (Table 1). Further, among the eleven salinity tolerant and moderately salinity tolerant rice accessions, seven rice accessions (*Suduheenati*3397*, Bathkiriel*3550*, Madathawalu*3214*, Randhunipagal*2866*, Matholuwa*3214*, Moddaikaruppan*3388, and *Polayal*3639) were included among the lowest twenty-two rice accessions with Na^+ accumulation. Furthermore, when the rice cultivars were arranged according to increasing order of Na^+ accumulation under salinity stress, 85.8% of the studied rice accessions with high values were consistently found to be highly susceptible or susceptible to salinity stress.

Among the tolerant and moderately tolerant rice accessions, (referred to as TMT) *Madaelgalle*3508, *Mudaliwi*3672 and *Sinnakaruppan3391* were moderately tolerant with high Na^+ content in the shoot dry matter (Figure 1). In these accessions, salinity tolerance likely developed in the plant cells by tolerating a higher Na^+ level at the initial stages of growth (Cheeseman, 1988; Yeo *et al.,* 1990). Additionally, these three rice accessions may have adopted other salt tolerant mechanisms such as maintaining low Cl^- or Zn^+, proline accumulation, or high activity of antioxidant enzymes (Dionisio-Sese & Tobita, 1998; Läuchli, 1999; Zhu, 2002) rather than maintaining a low Na^+ content. *Pokkali* is a salinity tolerant rice accession (Gregorio & Senadhira,1993; Waziri *et al*., 2016), but the four accessions used in the present study recorded Na^+ content of 91.85-160 ppm and were not salinity tolerant (*data not shown*).

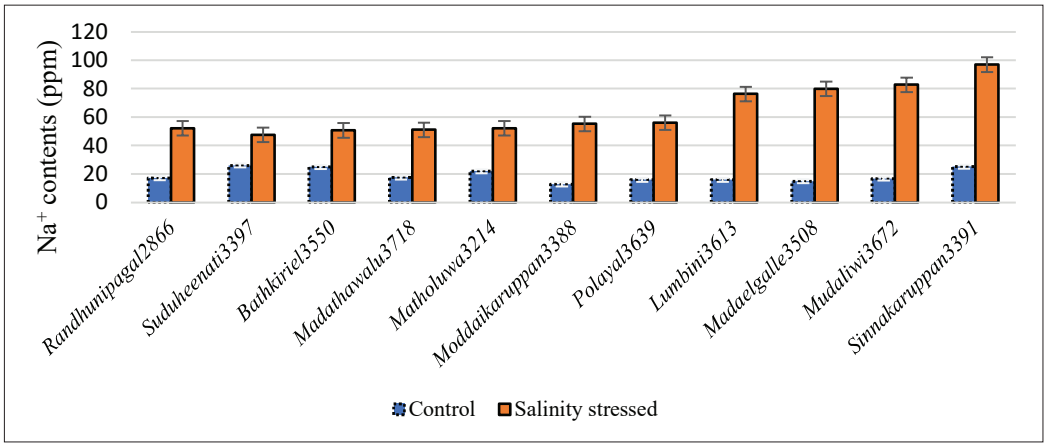

Figure 1: Differential Na^+ content in seedling dry matter of salinity tolerant (*Randhunipagal2866*) and moderately tolerant (all other accessions) rice genotypes under control and salinity-stressed conditions.

K^+ accumulation and salinity tolerance

K^+ content of stressed seedlings was lower than those of control seedlings in all the studied rice accessions except for *Godawee3919*. Among the eleven salinity-TMT rice accessions all were among the top twenty-six rice accessions with the highest K^+ content (Table 1). Previous studies (Gregorio & Senadhira,1993; Chaum *et al.,* 2009; Ahmad *et al.,* 2007; Khan & Panda, 2008; Lokeshkumar *et al.,* 2023) have reported lower K^+ content under salinity stress in salinity-tolerant rice accessions. However, *Randhunipagal2866* and *Sinnakruppan3391* showed insignificant differences in K^+ content between the control and salinity-stressed plants (Figure 2). Some highly susceptible rice accessions (26) also had the highest K^+ content. While *Godawee3919, Godaheenati3724,* and *Massamba2349* (*Supplementary Table 1*) ranked among the top ten rice accessions with the highest K^+ content, they were not among the salinity TMT accessions. Further, these three accessions were not found among the twenty rice genotypes with the lowest Ca^{2+} content. This suggests that relying solely on a genotype-specific pattern of K^+ accumulation in rice seedling dry matter under salinity stress is insufficient to confer salinity tolerance.

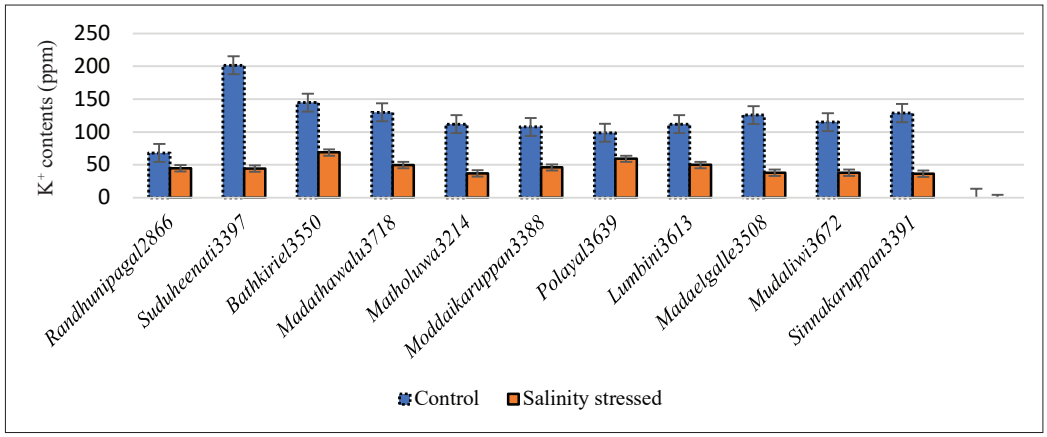

Figure 2: Differential K^+ content in seedling dry matter of salinity tolerant (*Randhunipagal2866*) and moderately tolerant (all other accessions) rice genotypes under control and salinity-stressed conditions.

Na^+/K^+ ratio and salinity tolerance

The Na^+/K^+ ratio was higher in all rice genotypes under salinity stress than in control seedlings (Table 1). Among the 125 rice accessions, 88% of salinity susceptible and highly susceptible rice accessions had the highest Na^+/K^+ ratios. All eleven salinity-TMT rice accessions were among the thirty-seven rice accessions with the lowest Na^+/K^+ ratios. A reduced Na^+/K^+ ratio confers salinity tolerance in rice and other crops (Chaum *et al.,* 2009; Lokeshkumar *et al.,* 2023). Maintaining a low Na^+/K^+ ratio in plants is a mechanism for salinity tolerance (Gregorio, 1997; Asch *et al.,* 2000; Munns, 2005). This has been experimentally proven by demonstrating reduced K^+ absorption with the addition of Na^+ into the medium (Ahmad *et al.,* 2007). A study reported a negative association between Na^+/K^+ ratio and salinity tolerance, and a positive association between K^+ and Ca^{2+} contents and salinity tolerance, using a plant stimulant to enhance salinity tolerance (Lokeshkumar *et al.,* 2023).

Assalam *et al.* (2003) reported similar results using 1.0 mM Ca^{2+} and higher concentrations of Ca^{2+} in a tissue culture medium. Similar results have also been reported by Goudarzi and Pakniyat (2008). The rice cultivars *Nona Bokra, Pokkali, SR26B* and *Lunishree,* with reduced Na^+/K^+ ratios, have performed better under salinity stress than the known salinity susceptible rice genotypes, *IR28, IR29, M1-48*, and *Begunbuchi* (Gregorio & Senadhira,1993; Ahmad *et al.,* 2007; Khan & Panda, 2008). Salt tolerance in seedlings increased after application of a plant stimulant to reduce the Na^+/K^+ balance (Mekawy *et al*., 2024).

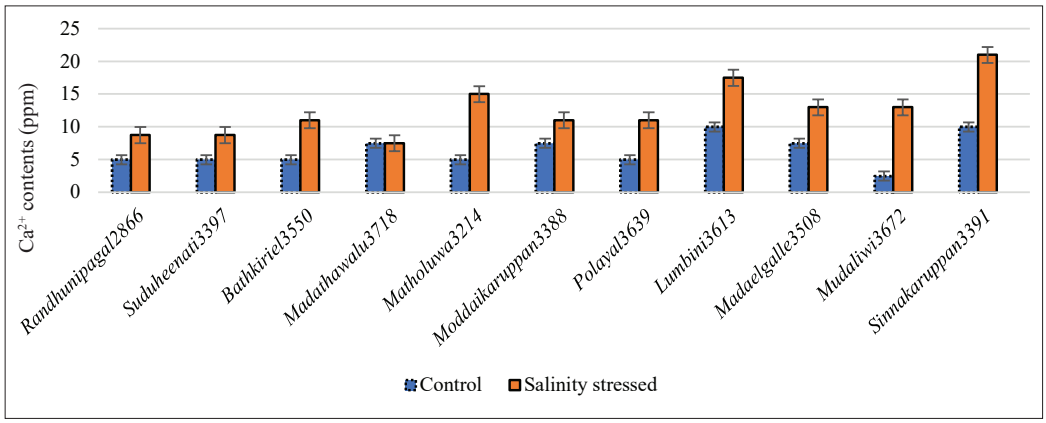

Figure 3: Differential Ca^{2+} content in seedling dry matter of salinity tolerant (*Randhunipagal2866*) and moderately tolerant (all other accessions) rice genotypes under control and salinity-stressed conditions.

Ca^{2+} uptake and salinity tolerance

The Ca^{2+} content increased in all rice genotypes with salinity stress compared to their respective control seedlings (Figure 3). Some rice varieties have developed selective ion transport mechanisms that allow them to preferentially take up Ca^{2+} over Na^+, even under saline conditions. These varieties are better equipped to maintain a healthier sodium-calcium balance and are more salt-tolerant (Song *et al.,* 2006). A similar effect has been reported by Cao *et al*. (2024) and Mahmood *et al*. (2024). Additionally, Orcan and Orcan (2024) demonstrated that the application of $CaCl_2$ resulted in reduced proline content, indicating decreased salinity stress.

Although the lowest Na^+/Ca^{2+} containing rice accessions were not salinity TMT (*Supplementary Table 1*) in the present study, eight salinity TMT accessions, namely *Mudaliwi3672, Madaelgalle3508, Polaya3639l, Bathkiriel3550, Moddaikaruppan3388, Suduheenati3397, Randhunipagal2866,* and *Madathawalu3718,* were among the eighteen rice accessions that recorded the lowest Ca^{2+} accumulation with salinity stress (Table 1).

Na^+, K^+, and Ca^{2+}

Twenty rice genotypes that recorded the lowest Na^+, Na^+/K^+, and Ca^{2+} contents as well as the highest K^+ content in the 125 rice genotypes studied were closely monitored for salinity tolerance. Interestingly, the group included the salinity-tolerant *Randhunipagal2866*, as well as moderately tolerant accessions *Madathawalu3718*, *suduheenati339*, and *Bathkiriel3550* (Table 1). The Na^+, K^+, and Ca^{2+} contents in the dry matter of these salinity TMT rice accessions were plotted in 2-D and 3-D graphs to clearly display the relative positions of among the recorded maximum and minimum Na^+, K^+, and Ca^{2+} contents in the studied accessions. (*Supplementary Figures 1A and 1B*). Maintaining a low Ca^{2+} activity in the cytoplasm is one of the strategies for salinity tolerance (Läuchli, 1999). Spraying water under NaCl-induced salinity stress has been shown to increase the uptake of Ca^{2+} by rice plants, thereby enhancing their salinity tolerance (Song *et al.* 2006). Under $NaHCO_3$ stress, rapeseed plants experienced a reduction in Ca^{2+} absorption in their stems and leaves, leading to an imbalance of K^+ and Na^+ across different tissues (Cao *et al.*, 2024).

Three other moderately tolerant accessions, *Polayal3639, Moddaikaruppan3388, and Mudaliwi3672*, were not among the twenty genotypes with the lowest Na^+, but they were among the twenty lowest Ca^{2+}, and the highest K^+ containing accessions. The other moderately tolerant *Madaelgalle3508* accession was among the lowest Ca^{2+} containing twenty rice accessions, but not included in any other groups. This suggests that the salinity-mitigating impact of Ca^{2+} is sufficient to confer moderate salinity tolerance in the *Madaelgalle3508* accession, diminishing the harmful effects of NaCl. Moderately tolerant *Sinnakaruppan3391* recorded the highest Ca^{2+} accumulation under salinity stress (Table 1), and was not included in the group of twenty rice accessions with the lowest Na^+ or the highest K^+ content. It is unlikely that the prominent strategy for enhancing the salinity tolerance of *Sinnakaruppan3391* involves the regulation of Na^+, K^+, Na^+/K^+, and Ca^{2+} balance when compared to other potential strategies. Only *Madaelgalle3508* and *Moddaikaruppan3388* were included in the group of twenty rice accessions that recorded the lowest Na^+/K^+ ratio. None of the TMT rice accessions were included in the lowest Na^+/K^+ containing twenty rice accessions though it has been reported that the reduction of Na^+/K^+ or Na^+/Ca^{2+} ratio within the saline solution resulted in a decrease in the concentrations of Na^+ and Cl^- in the shoot (Muhammed *et al.,* 1987).

Not all favorable modes of action of Na^+, K^+, and Ca^{2+} exist in each salinity TMT rice genotype. The interaction between Na^+ and Ca^{2+} uptake is influenced by the genetic makeup of the genotype and specific environmental conditions. Certain rice genotypes may have different strategies for coping with salinity stress, which can impact the balance between Na^+ and Ca^{2+} uptake.

Validation of salinity tolerance in rice accessions at the seedling stage according to correlations of shoot Na^+, K^+, and Ca^{2+} content

Salinity tolerance was positively correlated with K^+ content and negatively correlated with Na^+, Na^+/K^+, and Ca^{2+} content. Among Na^+, K^+, and Ca^{2+}, a positive correlation was recorded only between Na^+ and Ca^{2+} (Table 2). Wright *et al.* (1995) reported that Ca^{2+} in the plant increases Na^+ uptake. *Madaelgalle3508, Mudaliwi3672,* and *Sinnakaruppan3391* were moderately tolerant, with the highest recorded Na^+ content among TMT rice genotypes. Interestingly, the same rice accessions recorded the highest Ca^{2+} content (Table 1). This could be because of Na^+ entering nonselective cation channels in plants is strongly influenced by Ca^{2+} (Amtmann & Sanders, 1998). Contrarily, Grattan and Grieve (1999) reported that salinity stress reduces Ca^{2+} availability and mobility when the stress is due to Na^+. However, according to Gupta and Shaw (2021), Ca^{2+}

reduces Na^+ intake when the K^+/Na^+ ratio is higher in salt-tolerant rice compared to salt sensitive varieties. Wu and Wang (2012) concluded that Ca^{2+} regulates K^+/Na^+ in rice influencing selectivity for K^+ over Na^+ and reducing the Na^+ transportation in plants at low salinity. The multifaceted nature of salinity tolerance in plants at the physiological level could exhibit diverse relationship between tolerant traits (Liu *et al.,* 2022).

Table 2: Correlation of Na^+, K^+ and Ca^{2+}content with other parameters

Traits	r	P
Correlation between seedling shoot Ca^{2+} content and strength of tolerant level*	-0.331	0.000
Correlation between seedling Na^+ content and strength tolerant level	-0.267	0.003
Correlation between seedling shoot K^+ content and strength tolerant level	0.480	0.000
Correlation between seedling Na^+/K^+ and strength tolerant level	-0.430	0.000
Correlation between seedling Na^+ and K^+	-0.283	0.001
Correlation between seedling Na^+ and Ca^{2+}	0.624	0.000
Correlation between seedling K^+ and Ca^{2+}	-0.438	0.000

r = Pearson's correlation coefficient, p = Probability level, * Tolerant levels were indicated by an arbitrary scale.

The Na^+/K^+ ratio has an inverse correlation with salt tolerance in rice (Zhu *et al.,* 2001; Gao *et al.,* 2007), with higher ratios observed in salinity-susceptible rice accessions (Goudarzi & Pakniyat, 2008; Zhu *et al.,* 2001; Gao *et al.,* 2007). Salinity-tolerant plants heavily absorb K^+ to maintain a lower Na: K ratio under salinity stress (Ahmad *et al.,* 2007). This is supported by data showing that rice genotypes classified as TMT consistently have low Na^+ content and high K^+ content (Table 1). Ca^{2+} and K^+ show an inverse relationship in absorption (Table 2); Ca^{2+} uptake reduces K^+ uptake, and high K^+ absorption reduces Ca^{2+} uptake (Rengel, 1992). In natural conditions, Ca^{2+} levels in salinized water increase with daily evaporation due to selective precipitation of Ca^{2+} over Na^+ (Grattan & Grieve, 1999). These relationships of Ca^{2+} further extend with N and P (Grattan & Grieve, 1999). When studying germplasm with diverse genetic bases, such as traditional rice accessions, a single parameter may not be effective as different mechanisms of salinity tolerance may prevail. Plant responses to salt stress occur at the organismic, cellular, and molecular levels and are complex including maintaining ionic balance, adjusting osmotic conditions, scavenging reactive oxygen species (ROS), and ensuring nutritional equilibrium (Liu *et al.,* 2022). However, some studies recommend isolated parameters for salinity screening: Mahmood *et al*. (2009) have recommended shoot dry weight as a more reliable parameter than Na^+ content. Reduction of osmotic potential has been found to be the most sensitive parameter to distinguish tolerant plants from susceptible plants (Zhu *et al.,* 2001; Ahmad *et al.,* 2007).

CONCLUSION

Significant relationships were observed among Na^+, K^+, and Ca^{2+} and their impact on salinity tolerance in rice seedlings. Inverse correlations were found between the levels of Na^+, Ca^{2+}, and the Na^+/K^+ ratio with salinity tolerance. Conversely, a positive correlation was found between K^+ content and salinity tolerance in rice at the seedling stage. Na^+ and Ca^{2+} contents were higher, whereas K^+ content was lower in the stressed seedlings compared to the control group. It was evident that rice seedlings tend to accumulate K^+ and exclude Na^+ as a response to salinity stress. Na^+, K^+, and the Na:K ratio were potential parameters in a methodology for screening salinity tolerance in rice seedlings. However, Na^+ and K^+ content alone did not provide a comprehensive assessment of salinity tolerance. This conclusion is supported by the weak inverse correlations between Na^+ content and salinity tolerance (r=-0.267) and Na^+ and K^+ level (r=-0.283). Moreover, Ca^{2+} content was high in stressed seedlings and there was a strong positive correlation between Na^+ and Ca^{2+} contents (r=0.624) in seedlings grown under salinity stress. This suggests that both Ca^{2+} and Na^+ were taken up through physiological processes in response to salinity stress. A negative correlation was observed between Ca^{2+} and K^+ content, highlighting the influence of K^+ on the availability and mobilization of Ca^{2+}. The relationship between any two ions of Na^+, K^+, and Ca^{2+} in dry matter varied among salinity TMT genotypes suggesting genotype-specific ion balancing for salinity tolerance. This study reveals that while Na^+,

K^+, Ca^{2+}, and the Na^+/K^+ ratio are valuable parameters for evaluating salinity tolerance in rice seedlings, a holistic approach considering multiple factors is necessary to accurately assess the tolerance levels of different rice genotypes, as many other metabolic and physiological reactions influence salinity tolerance beyond ion balancing.

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Supplementary Table

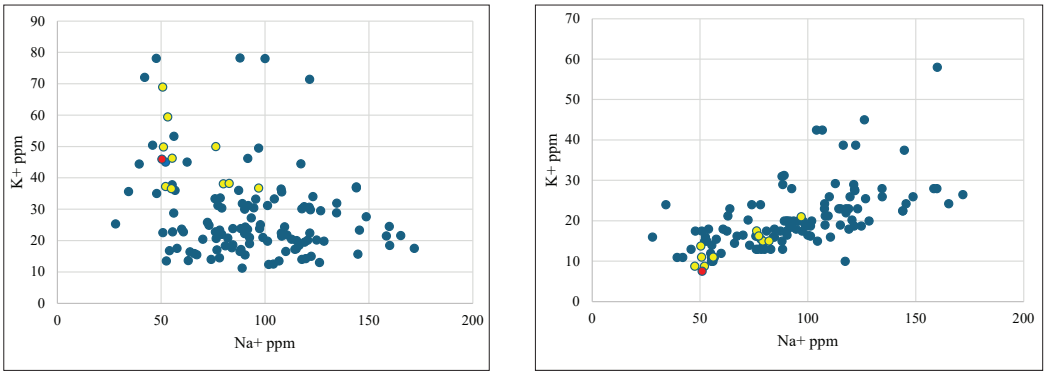
Supplementary Table 1: Na^+, K^+, Na:K ratio of control and salinity stressed rice seedlings.

PGRC No.	Name	Na^+ (ppm)		K^+ (ppm)		Na^+/K^+		Ca^{2+} (ppm)	
		Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
2203	*Dikwee*	16	101.2	128	19.8	0.13	5.11	10	16.25
2340	*Wedaheenati*	18.4	118.9	97.2	30.8	0.19	3.86	10	23
2349	*Massamba*	20	121.5	119.7	71.4	0.17	1.7	10	18.75
2866	***Randhunipagal***	**17.2**	**52.15**	**68.2**	**45**	**0.25**	**1.16**	**5**	**8.75**
3071	*Polayal*	16	134.4	84	28.8	0.19	4.67	10	28
3072	*Thanthiribalan*	17.6	55.5	87	22.8	0.2	2.43	7.5	14
3131	*Dahanala2014*	12.6	91.7	115.6	46.2	0.11	1.98	10	18.75
3132	*Heenati-309*	12	104.5	103.7	33.3	0.12	3.14	10	15
3136	*Pachchaiperumal2462-11*	12.8	90.2	109.8	22.2	0.12	4.06	10	20
3146	*Dewaredderi*	14.4	87.3	95.2	36	0.15	2.43	10	17.5
3158	*KaluBalawee*	16	104	73.4	12.5	0.22	8.32	10	42.5
3160	*Valihandiran*	26.5	121.3	152	30.6	0.17	3.96	10	29
3161	*Heenwee*	20.8	110	100.8	16.5	0.21	6.67	10	26
3170	*Sudubalawee*	19.6	106.75	48	13.5	0.41	7.91	5	42.5
3171	*Suduhetada*	24	112.75	49.4	20.4	0.49	5.53	5	29.25
3183	*Hathiel*	29.6	116.4	98	18.5	0.3	6.29	10	38.75
3191	*Heendikwee*	13.8	96.9	132.6	49.5	0.1	1.96	10	20
3194	*Katharamana*	16.8	90.35	71.4	15.4	0.24	5.87	10	16.5
3195	*Gallkatta*	34	122.2	134.1	15	0.25	8.15	10	38.75
3197	*Nanduheenati*	21.2	165.3	91.8	21.6	0.23	7.65	10	24.25
3200	*Kaluheenati*	19	91.6	63.9	23.55	0.3	3.89	10	20
3202	*Rambuttanwee*	19.2	120.5	119.4	20.1	0.16	6	10	20.25
3214	***Matholuwa***	**22**	**52.2**	**112**	**37.2**	**0.2**	**1.4**	**5**	**15**
3341	*Galpawee*	17.2	76.45	102	20.7	0.17	3.69	5	16.25
3383	*Eatsamba*	36.4	52.5	147.2	13.5	0.25	3.89	12.5	16
3387	*Kahatawee*	19.2	79.2	70.4	21.95	0.27	3.61	10	15
3388	***Moddaikaruppan***	**12.8**	**55.3**	**108**	**46.25**	**0.12**	**1.2**	**7.5**	**11**
3390	*Rathheenati*	15	45.85	108	50.4	0.14	0.91	7.5	13
3391	***Sinnakaruppan***	**25.2**	**97**	**129**	**36.7**	**0.2**	**2.64**	**10**	**21**
3394	*Muthusamba*	15.2	126.8	129	29.5	0.12	4.3	10	25.5
3397	***Suduheenati***	**26**	**47.6**	**97.5**	**78.1**	**0.27**	**0.61**	**5**	**8.75**
3407	*Dewaradderi*	19.8	95.55	131.4	33.25	0.15	2.87	7.5	20
3409	*BG 35-2*	18.4	90.5	100.8	24.3	0.18	3.72	7.5	20
3410	*BG 35-7*	13.2	78.1	139.2	14.5	0.09	5.39	5	24
3415	*BG 34-8*	30	70	100.5	20.45	0.3	3.42	10	16.5
3427	*Nauduwee*	10.8	82.05	126	20.8	0.09	3.94	5	15
3445	*Yakadawee*	8.4	88.4	97.2	17.15	0.09	5.15	5	29
3463	*Karayal*	25.5	57.6	123.2	17.5	0.21	3.29	7.5	15.5
3469	*SuduweeRatnapura*	16	50.8	105	22.5	0.15	2.26	10	17.5
3472	*Masuran*	20	117.7	112.8	14	0.18	8.41	5	22

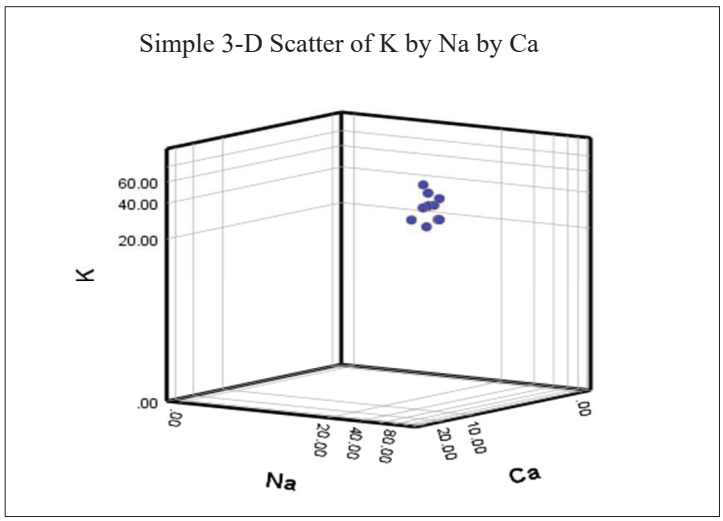
PGRC No.	Name	Na^+ (ppm)		K^+ (ppm)		Na^+/K^+		Ca^{2+} (ppm)	
		Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
3479	*KiriNaran*	20.8	62.5	102.6	45	0.2	1.39	5	17.5
3480	*Karayal*	16.8	124.8	100.8	20.2	0.17	6.18	7.5	18.75
3482	*Akuramboda*	24.5	76.1	149.6	22.8	0.16	3.34	5	13
3486	*Puwakmalatasamba*	19.2	88	133	16.5	0.14	5.33	10	31
3487	*Palasithari601*	20	53.2	99	59.4	0.2	0.9	5	15
3491	*Murungakayan304*	16.8	115.2	120.4	20	0.14	5.76	10	23
3506	*MI329*	23	63	138	13.6	0.17	4.63	10	21.25
3508	***Madaelgalle***	**15**	**79.95**	**126**	**38.1**	**0.12**	**2.1**	**7.5**	**13**
3550	***Bathkiri el***	**25**	**50.75**	**145**	**68.9**	**0.17**	**0.74**	**5**	**11**
3562	*Thunmarhamara*	21	72.3	126	25.8	0.17	2.8	10	20.25
3572	*Sudurusamba*	23.1	110.5	79.2	21.8	0.29	5.07	2.5	16
3573	*Pokkali*	16	91.85	159.6	31.2	0.1	2.94	10	18.5
3579	*kakiriatabalawee*	25	42	107.8	72.05	0.23	0.58	5	11
3591	*Mudukiriel*	26	63.9	133	16.4	0.2	3.9	10	23
3599	*Mahamawee*	24	145.5	57.6	23.35	0.42	6.23	10	24.25
3611	*Balakaharamana*	12	158.4	142.4	21.5	0.08	7.37	7.5	28
3613	***Lumbini***	**16**	**76.3**	**112**	**49.95**	**0.14**	**1.53**	**10**	**17.5**
3616	*Jamiswee*	16	94.6	81	30.45	0.2	3.11	12.5	18
3629	*Ruwanrathran*	18.4	28	98	25.3	0.19	1.11	7.5	16
3634	*Thavalu*	18.4	171.9	117.6	17.5	0.16	9.82	12.5	26.5
3639	***Polayal***	**16**	**56.15**	**61.6**	**53.25**	**0.26**	**1.05**	**5**	**11**
3641	*Heendikwee*	16	128.4	79.5	19.8	0.2	6.48	10	20
3642	*Kahatasamba*	16	66	141.2	15.85	0.11	4.16	7.5	14.5
3644	*Herath*	30	56.7	68	36	0.44	1.58	7.5	11
3645	*Muthumanikkam*	16	109.45	132.6	24.4	0.12	4.49	10	21.25
3646	*Indurukarayal*	27	88.3	67.2	23.85	0.4	3.7	7.5	13
3647	*Kalugires*	23	123.1	134	34	0.17	3.62	10	23
3650	*Madabaru*	22	73	116.2	24.9	0.19	2.93	5	14
3652	*Burumathavalu*	34.8	78.2	126	23.3	0.28	3.36	5	15.5
3656	*Kuruluthudu*	14.4	159.8	103.7	24.5	0.14	6.52	10	28
3662	*Mahasuduwwee*	16.8	90.2	79.2	30	0.21	3.01	5	17.5
3663	*Murunga*	22	74	105.6	14	0.21	5.29	7.5	24
3664	*Tissawee*	21.5	60.7	61.6	22.8	0.35	2.66	7.5	18
3665	*SuduKarayal*	22.2	75.9	79.2	33.35	0.28	2.28	7.5	16.25
3671	*Sudurusamba*	17.6	34.25	123.2	35.6	0.14	0.96	7.5	24
3672	***Mudaliwi***	**16.8**	**82.8**	**115.2**	**38.2**	**0.15**	**2.17**	**2.5**	**13**
3674	*Kirikara*	20	80.85	56.4	18.3	0.35	4.42	10	17.5
3676	*Denawee*	21.2	77.3	120	31.15	0.18	2.48	10	16.25
3679	*Kottakaram*	24	92.5	111.6	19	0.22	4.87	7.5	28
3683	*Mawee*	16.8	47.85	102.6	35	0.16	1.37	10	17.5
3684	*Rathkara*	24	93.4	67.6	27.2	0.36	3.43	10	20
3691	*Gunarathna*	34.3	54.95	92.4	36.5	0.37	1.51	5	12
3692	*Handiran*	12.8	79.2	140	30.4	0.09	2.61	10	13.75
3695	*Kahatasamba*	14	97.35	95	23.8	0.15	4.09	10	17.5

PGRC No.	Name	Na^+ (ppm)		K^+ (ppm)		Na^+/K^+		Ca^{2+} (ppm)	
		Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
3701	*Pokkali*	12	144.75	107.2	15.65	0.11	9.25	12	37.5
3713	*Kalukanda*	19.2	148.75	128	27.6	0.15	5.39	10	26
3718	***MadaThawal***	**17.6**	**51.1**	**130**	**49.8**	**0.14**	**1.03**	**7.5**	**7.5**
3720	*Kirikara*	22.4	39.4	119.7	44.4	0.19	0.89	10	11
3721	*Manamalaya*	28	54	122.1	16.8	0.23	3.21	7.5	18
3724	*GodaHeenati*	11.4	100	136	78.05	0.08	1.28	10	16.5
3725	*Sivuruwee*	27	56	55.8	28.75	0.48	1.95	7.5	10
3726	*Dandumara*	22	126.25	142.4	13	0.15	9.71	10	45
3734	*KanniMurunga*	11.4	115.05	92.4	17.4	0.12	6.61	5	19
3735	*Welihandiran*	13.8	101.2	100.8	31.15	0.14	3.25	10	18.75
3744	*3744*	14.1	78.4	108	33.6	0.13	2.33	2.5	13
3756	*3756*	16	84.5	108	18.6	0.15	4.54	5	16
3881	*Pokkali*	15	134.5	120.4	31.9	0.12	4.22	7.5	26
3882	*Dostaraheenati*	15.2	117.7	114.8	30.15	0.13	3.9	10	23
3919	*Godawee*	12.6	88	39.6	78.2	0.32	1.13	10	15
3922	*Pokkali*	34.4	160	86.4	18.45	0.4	8.67	5	58
3932	*Suduheenati*	26.5	117.3	201.6	44.5	0.13	2.64	5	10
3982	*Kuruwee*	15.2	144	90	37.1	0.17	3.88	10	22.5
4145	*4145*	16.8	107.7	96.2	36.45	0.17	2.95	12.5	23
4159	*Podiwee*	15.2	114.4	67.2	17.2	0.23	6.65	10	23
4178	*Rathumadilla*	13.6	55.3	72	37.8	0.19	1.46	2.5	10
4402	*Haththepasdawasewee*	15.2	99	106.4	21	0.14	4.71	12.5	18.75
4726	*Gonabaru*	24.5	84	127	17.7	0.19	4.75	7.5	16
4732	*Horana wee*	23	107.8	106.4	22.4	0.22	4.81	10	24.25
4753	*Kombila*	21	101.8	134	12.4	0.16	8.21	10	20
4762	*Maharajah*	31.5	67.15	127	15.45	0.25	4.35	7.5	16.25
4770	*Molligoda*	16	119.6	105.6	14.25	0.15	8.39	10	26
4785	*Poovellai*	27.6	144	113.1	36.8	0.24	3.91	12.5	22.5
4809	*Suwandal*	19.6	119	139.2	19.5	0.14	6.1	10	18
4819	*Veliainellu*	13.2	50.4	105.6	45.9	0.13	1.1	10	13.75
4834	*Kallurundoivellai*	17.2	97.75	87	25.05	0.2	3.9	2.5	20
4841	*KoopanSivappu*	27.5	89	129	11.2	0.21	7.95	2.5	31.25
4852	*Nandumawee*	22.5	92.5	129.6	21	0.17	4.4	12.5	18.5
4858	*Potkilivan*	16	76.8	101.2	17	0.16	4.52	2.5	13
4992	*Rathuheenati*	16	89.1	90	31.8	0.18	2.8	10	20
5671	*Suduhandiran*	15	84.5	74.8	23.8	0.2	3.55	10	18
6179	*Gires*	14.4	59.85	177	23.65	0.08	2.53	2.5	12
6249	*6249*	16.8	108.05	144	35.5	0.12	3.04	10	21.25
6863	*Pappaku*	23	108	100.5	21.4	0.23	5.05	2.5	19

* Tolerant or moderately tolerant genotypes were in bold letters.



Supplementary figure 1A: Na^+, K^+ and Ca^{2+} contents of rice accessions under salinity stress. Red: Salinity tolerant *Randunipagal* accession, Yellow: Moderately tolerant rice accessions, Blue: Susceptible or Highly susceptible rice accessions.



Supplementary figure 1B: Na^+, K^+ and Ca^{2+} contents of rice salinity tolerant and moderately tolerant rice accessions under salinity stress in 3-D scatter plot.