Reliability of ion accumulation and growth components for selecting salt tolerant lines in large populations of rice

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Abstract

Ion accumulation and growth under salt stress were studied in two experiments in a rice mapping population derived from parents CO39 and Moroberekan with 4-fold differences in shoot Na⁺ accumulation. The 120 recombinant inbred lines (RILs) had up to 100-fold differences in Na⁺. Measurement of “salt tolerance” (growth of the RILs in 100 mM NaCl relative to controls after 42 days showed a 2-fold difference in “salt tolerance” between parents, with five RILs being more tolerant than the more tolerant parent CO39. The reliability of various traits for selecting salt tolerance in large populations was explored by measuring Na⁺, K⁺ and K⁺/Na⁺ ratios in leaf blades and sheaths after 7 or 21 days of exposure to 100 mM NaCl, and their correlation with various growth components and with leaf injury. The highest correlations were found for Na⁺ in the leaf blade on day 21 with injury at day 42 in both experiments (r=0.7). Earlier measurements of Na⁺ or of injury had lower correlations. The most sensitive growth components were tiller number per plant and shoot water content (water per g dry weight), and correlated significantly with Na⁺ and to a lesser extent with K⁺/Na⁺. These studies showed that exposure for at least 42 days may be needed to clearly demonstrate the beneficial effect of the trait for Na⁺ exclusion on growth under salinity.

Additional Keywords: salinity, screening, criteria, HKT transporter, tissue tolerance, sodium, potassium, *Oryza sativa*

1 **Introduction**

2 About 950 Mha of the world crop production area has become unsuitable for production due to
3 the problem of soil salinity and/or sodicity (Szabolcs 1989). Rice is one of the most sensitive
4 crops to salt stress (Lafitte *et al.* 2004) but, its cultivation is recommended during the process of
5 soil reclamation due to its high water requirement which helps leach salts into deeper zones
6 (Bhumbla and Abrol 1978). The world's food security demands that rice production must
7 increase from 600 to 800 million tonnes by the year 2025 (Green *et al.* 2005). Salt tolerance is a
8 complex trait and understanding its molecular basis is essential for breeding and transformation
9 in crop plants (Yeo and Flowers 1986; Chinnusamy *et al.* 2005). The promising field of marker-
10 assisted breeding can be a powerful tool to increase efficiency of selection for stress adaptation
11 in breeding programs (Witcombe *et al.* 2008). In rice, many efforts have been made in the past to
12 understand the genetics and physiology of salt tolerance but unfortunately progress is slow due to
13 the complex nature of this multigenic character. It could be partially because of low selection
14 efficiency for general agronomic traits and a lack of effective evaluation methods for assessing
15 salt tolerance among genotypes (Zeng *et al.* 2003).

16 The main adverse effects of salinity on plant growth are due to osmotic effects, ionic toxicity and
17 nutritional imbalances such as reduced K^+ uptake (Läuchli and Epstein 1990). High
18 concentrations of salt around roots affect water relations of the plant and may generate signals
19 which reduce cell expansion rates and stomatal conductance in shoot tissues (Davies and Zhang
20 1991; Munns 2002). If roots do not exclude most of the salt from the soil solution while taking
21 up water, Na^+ travelling in the xylem stream may accumulate in leaves to toxic levels causing
22 premature plant death (Munns 2005). Chloride (Cl^-) may accompany the Na^+ although it is less
23 likely to become toxic (Munns and Tester 2008). Potassium transport is usually decreased and
24 the K^+/Na^+ ratio in leaves can drop to critically low levels. Halophytes can use Na^+ and Cl^- for
25 internal osmotic adjustment to grow under saline conditions (Flowers *et al.* 1977), but
26 glycophytes mainly survive saline environments by excluding most Na^+ from the transpiration
27 stream (reviewed by Munns 2005). The success of this mechanism in glycophytes depends on
28 the balance between salt influx into the plant and its cellular compartmentation. Too little
29 accumulation of ions in leaves can lead to poor osmotic adjustment, while too much may result
30 in toxic concentrations of Na^+ and/or Cl^- in leaves or reproductive tissues (Flowers *et al.* 1986).

1 Exclusion of Na^+ from photosynthetically active leaves is a key strategy in plants to thrive under
2 salinity (Munns and Tester 2008; Yeo and Flowers, 1986), and the concentration of Na^+ in leaves
3 of crop plants has been used as one of the indicators of salinity tolerance (Ashraf and Harris
4 2004; Munns *et al.* 2006). Na^+ exclusion was proposed as a criterion for assessing salt tolerance
5 in wheat and rice (Munns *et al.* 2006; Yeo *et al.* 1990). Generally, salt tolerant lines of rice
6 maintain lower Na^+ in their leaves than salt sensitive lines when exposed to salt stress (Flowers
7 and Yeo 1981; Yeo *et al.* 1990; Lutts and Guerrir 1995). The decrease in K^+/Na^+ ratio may relate
8 directly to a decrease in yield in some conditions (Asch *et al.* 2000). More recently, Platten *et al.*
9 (2013) reported a strong correlation between leaf blade Na^+ concentration and salt tolerance (as
10 assessed by leaf injury symptoms) in the majority of accessions of two rice species.

11 Maintenance of a low net uptake of Na^+ from the soil is accomplished by membranes that have
12 low permeability to Na^+ . In rice and other cereals, Na^+ can enter through K^+ -selective cation
13 transporters such as the ‘class 2’ HKT transporters (high affinity potassium transporters) that
14 while having a high K/Na selectivity would allow uptake of Na^+ under conditions of high soil
15 NaCl (Garciadeblas *et al.* 2003; Horie *et al.* 2012). Na^+ may be effluxed from the cells and hence
16 to the soil by the $\text{SOS1 Na}^+/\text{H}$ antiporter (Shi *et al.* 2003) which has an ortholog in rice
17 (Martinez-Atienza 2007). The small fraction that enters the transpiration stream can be further
18 controlled by the ‘class 1’ HKT transporters which in wheat are suggested to reduce Na^+
19 transport to leaves by the retrieval from transpiration stream and deposition in xylem
20 parenchyma cells (James *et al.* 2006). In wheat, the transporter encoded by the candidate gene for
21 Nax1 , TmHKT1;4 , is responsible for retrieval of Na^+ from the transpiration stream for storage in
22 the leaf sheath tissue (Huang *et al.* 2006; James *et al.* 2006). The transporter encoded by the
23 candidate gene for Nax2 , TmHKT1;5 , plays a similar role, but functions primarily in root tissue
24 (Byrt *et al.* 2007; Munns *et al.* 2012). Targeted overexpression of the Arabidopsis HKT1;5
25 homolog, AtHKT1;1 , in Arabidopsis and rice has been shown to increase Na^+ exclusion from the
26 shoot (Moller *et al.* 2009; Plett *et al.* 2010). Cotsaftis *et al.* (2012) also suggested that retrieval of
27 Na^+ from the xylem transpiration stream in the root or leaf sheath is a possible mechanism to
28 maintain low Na^+ in leaf blades of rice, and analysed Na^+ exclusion in rice by 3D modeling of
29 HKT transporters (OsHKT1;5 and OsHKT1;4). They suggested that Na^+ accumulation in the leaf
30 blade is controlled by regulation of complex gene expression, alternate splicing and protein
31 structure of transporters under salt stress.

In a previous study, a recombinant inbred line (RIL) population from the cross between two parents CO39 and Moroberekan with differences in leaf Na⁺ concentration was chosen to look for QTLs for ion regulation, in the understanding that this would be useful in selecting for salt tolerance. CO39 has a 4-fold lower Na⁺ concentration in leaves than Moroberekan (Akhtar 2002; Haq et al. 2010). On day 7 of salt stress, two QTLs each for Na⁺ and K⁺/Na⁺ ratio in fully expanded leaf were identified on Chromosome 1 with high likelihood of odd (LOD) and explained up to 35 and 38 % of the total phenotypic variation in these traits, respectively (Haq *et al.* 2010). After 21 days stress same QTL for leaf Na⁺ was detected on Chromosome 1 (11.56 – 11.72 Mbp) with LOD of 9.8 and explaining 24% of the total phenotypic variation. QTLs were also found for Na⁺ accumulation in sheath after 21 days but not after 7 days salt stress (Haq *et al.* 2010). Fine mapping identified a chromosomal region that was most likely the same as the SKC1 QTL (Lin *et al.* 2004), later identified as the Na⁺ transporter *OsHKT1;5*, responsible for retrieval of Na⁺ in rice by being unloaded directly from the xylem sap (Ren *et al.* 2005).

In this paper, which presents results of two experiments with the same parents and population, the relation between leaf Na⁺ and degree of injury over time, and % biomass production was measured. As the biomass production of the two parents differed substantially in the absence of salt, the first experiment included a control treatment where the effects of salinity on tiller number and total biomass could be calculated, but only small population number could be handled. The second experiment included a larger population number (120 RILs) but with salt treatment only. Here, salt injury over time was measured (day 21 and 42) as well as Na⁺ in sheath and blade of leaf at earlier times to investigate the reliability of leaf injury in predicting future growth in saline conditions and in selection for salt tolerance. This allowed us to relate Na⁺ accumulation to injury, to speculate at what concentration sodium becomes toxic in rice, and to relate this to growth in saline conditions.

Materials and Methods

Plant material

The F₉ recombinant inbred line (RIL) mapping population employed in this study was developed at the International Rice Research Institute (IRRI), Philippines, from the cross

CO39×Moroberekan. The maternal parent variety CO39 is a salt tolerant lowland *Indica* cultivar with medium height having originated from India. Moroberekan is a salt sensitive, tropical upland *Japonica* variety of long stature having its origin in West Africa. The population is skewed towards the CO39 alleles (80%) (Champoux *et al.* 1995). In Study-1, a total of 32 RILs were included, whereas in Study-2, a larger set of 120 RILs was investigated for ion accumulation and growth attributes under salt stress.

Sowing of rice nursery

The rice seeds of RILs and parent varieties (CO39 and Moroberekan) were sown in P-84 plug trays (Desch Plantpak, Malden, UK) filled with John Innes Compost No. 1 (Corwen, Clwyd, UK) at Pen y Ffridd, Research Station, Bangor University, Wales, UK. These trays were kept on a bench in greenhouse (at 25 °C) and watered every day until transplantation. Twenty three days after seed sowing, healthy and uniform seedlings of each line (at the three leaf stage) were transplanted into 2 litre plastic pots (one seedling/pot in Study-1 and 2 seedlings/pot in Study-2) lined with horticultural fleece and filled with John Innes No. 2 Compost (Lawrence and Newell 1939).

Setup of flood bench system

Flood bench system was comprised of plastic tanks (80 cm×56.3 cm×32.5 cm) each containing 24 pots of two liters capacity. The water reservoirs each had a capacity of more than 200 litres of water provided with submersible electric pumps. Tanks with plant pots were placed on an iron bench, approximately one meter high from the greenhouse floor. Water reservoirs were placed on the floor underneath the tanks. Submersible electric pumps were placed in water reservoirs to pump water into tanks. There were two connections of the water to each tank, one for water to enter into the tank from the reservoir and the other for water to drain from the tank to the reservoir. Pots were placed into each flood bench tank in a randomized layout with three replications in Study-1 and two replicates in Study-2. The plants were flooded once a day for at least fifteen minutes, twice on hot days and also twice when plants were fully grown in size. Nutrient solution having Phostrogen plant food (Phostrogen, Corwen, Wales, U.K) @ 1.0 g L⁻¹ plus 0.5 ml L⁻¹ micro-nutrients (Hoagland and Arnon 1950) and 0.1 ml L⁻¹ sodium silicate were supplied to the plants with the irrigation solution. After establishment of seedlings (one week after transplantation, 28 days after sowing), salt stress was started in daily increments of 25 mM

and 1.25 mM for NaCl and CaCl₂ respectively to reach the final level of 100 mM NaCl + 5.0 mM CaCl₂ except for the controls. Salinity level was maintained in the solution; this was by recording the electrical conductivity (EC) with a portable waterproof conductivity meter (Hanna Instruments, U.K) in each reservoir twice a week. Water lost by transpiration and evaporation was replaced to keep the reservoir volume at 200 litres. Supplementary 400 Watt high pressure sodium vapour lamps were used to maintain a minimum photon flux density of photosynthetically active radiation (400 – 700 nm) of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ during the 16 hour photoperiod. The minimum temperature was maintained at 25°C during the photoperiod and 20°C during the dark period.

Parent varieties CO39, Moroberekan and 32 F₉ RILs were evaluated for salt tolerance under above growth control and salt conditions for Study-1, and 120 F₉ RILS in the salt conditions only for Study-2.

Measurements

In Study-1, after three weeks (day 21) of salt stress, the youngest fully expanded leaf was sampled from each plant for ionic analysis. The samples were washed with distilled water, dried with tissue paper and stored in labeled, 1.5 ml micro centrifuge tubes (Sigma Chemicals, UK) and stored at –20 °C in a commercial freezer for two weeks before sap extraction. After thawing, the tissues were crushed with a tapered end steel rod. Two pin holes were made in the base and cap of each eppendorf tube. Each tube was placed inside a second eppendorf tube and centrifuged at 8000g for 10 min. The sap was collected into the lower tube and remaining tissue left in the upper one was discarded (Gorham *et al.* 1997). The sap was either analyzed immediately or frozen for later analysis. Sodium and potassium ions were analyzed in diluted sap with a Jenway PFP-7 flame photometer (Bibby Scientific Limited, Stone, UK). The whole shoot of plants from both control and salt treatments were harvested on day 42 of stress, and tillers per plant, shoot fresh weight, and dry weight was recorded. NEED TO SAY AT WHAT TEMPERATURE AND FOR HOW LONG THEY WERE DRIED.

In Study-2, after 7 days of salt stress, one of each pair of seedlings from two replicates were harvested and two tissue samples were taken. These were (i) a fully expanded leaf and (ii) the combined leaf sheaths. Twenty-one days after reaching full salt stress, one tiller of the second

plant was harvested and samples were taken again from the fully expanded leaf and combined leaf sheath. The samples were washed briefly with distilled water, dried with tissue paper and stored in 1.5-cm³ micro centrifuge tubes at –20°C in the freezer for 1 week. Leaf sap was extracted from each sample and ions were determined as described earlier. A portable SPAD-502 Meter (Minolta, Japan) was used to record the chlorophyll in leaves. Each mean value is the average of nine SPAD observations from leaf 1, 2 and 3 (one leaf on each of three tillers) on main tillers of the same plant. The SPAD readings from each leaf were taken from the base, middle and tip, along the length of leaf to cover the whole (or the total) leaf area.

Leaf injury scoring

In both studies, leaf injury scoring in the saline treatment was done on days 21 (Score-1) and 42 (Score-2) on the top mature leaves on a scale of 1 to 4. The injury value 1 was assigned to leaves showing little damage, a value of 2 with leaves showing slight damage, a value of 3 where the leaf damage was moderate, and a value of 4 to leaves experiencing severe damage.

Results

Effects of salinity on growth and appearance of CO39, Moroberekan and RILs, and correlations between leaf injury and biomass production

In Study-1, the two parents and 32 RILs were grown in control and 100 mM NaCl for 42 days. The adverse effects of salinity on overall plant appearance and growth after 42 days at 100 mM NaCl were greater on Moroberekan than on CO39. Moroberekan had more vigorous vegetative growth than CO39, producing over double the biomass in the absence of salinity (Table 1), so the genetic differences in "salt tolerance" need to be expressed as a % of growth in the control conditions. Moroberekan produced a shoot fresh weight under salt stress that was only 23% of the controls, whereas CO39 had a fresh weight that was 42% of controls (Table 1). Six RILs had an even higher % shoot fresh weight than CO39 (Fig. 1a). Shoot dry weights showed a similar result (Suppl. Fig. 1a). The variation in shoot fresh and dry weight was highly significant ($p<0.01$) in treatments (saline and non-saline), RILs and Salinity×RIL interactions as is revealed

by the analysis of variance in Study-1 (Suppl. Table 1). Tillers were the same number (about 12) in both parent varieties in the control treatment, and so could be used to assess effects of salinity on growth in the absence of a control treatment. Under salt stress, the number of tillers plant⁻¹ was more in CO39 (9.0) than Moroberekan (4.7) on day 42 of exposure to salt stress (Table 1). Out of the 32 RILs, 17 had tillers plant⁻¹ greater than CO39 (Fig. 1b). Shoot water (g g⁻¹ DW) of parents and all RILs was reduced by salinity (Table 1), but the effect of Salinity×RILs interaction was not significant (p=0.07) (Suppl. Table 2).

In Study-1, leaf injury for Moroberekan was greater than CO39 on day 21 (Table 1). However there was no correlation between injury at day 21 and growth components on day 42 other than tiller number and shoot water content (Table 2) suggesting that the injury had until then little effect on biomass production, but might predict a decrease in growth rate. This was borne out by the finding that injury at day 42 correlated negatively with all growth components except for shoot dry weight (Table 2).

In Study-2, the parents and 120 RILs were grown in 100 mM NaCl for 42 days without a control treatment. On day 42, shoot fresh and dry weights of CO39 were 167 and 104% respectively higher than Moroberekan (Table 3). This confirms CO39 as the more salt tolerant cultivar as its biomass was less than Moroberekan in the absence of salinity. The results of the first study indicated that tiller number and shoot water can be used as an index of salt tolerance. The shoot water content was higher in CO39 (2.8 g g⁻¹ DW) than in Moroberekan (1.9 g g⁻¹ DW) and varied between 1.42 and 3.61 g g⁻¹ in RIL population (Table 3). Tillers plant⁻¹ were higher in CO39 (15.0) than Moroberekan (5.0). The number of tillers plant⁻¹ ranged between 4.5 and 16.5 in the RIL population (Table 3).

In Study-2, the overall appearance was more affected in Moroberekan than CO39 as shown by chlorophyll loss and injury scores (Table 3). The phenotypic differences in parental varieties and RILs in response to salt stress can be further seen in pictures taken on day 42 (Suppl. Fig. 2). Chlorophyll concentration in the upper leaves on day 42 was higher in CO39 than Moroberekan (39 versus 13 SPAD units), and ranged between 10 and 42 in the RIL population (Table 3). Injury measured on day 21 was higher in Moroberekan (3.7) than CO39 (1.4) and ranged between 1 and 3.8 for the RILs, and correlated significantly with tillers, shoot water, and even shoot fresh and dry weight (Table 4). Injury measured on day 42 did not have a much higher

score than on day 21 (Table 3) but the correlations with all growth components were higher than at the earlier time of measurement (Table 4).

Variation in ion accumulation in CO39, Moroberekan and RILs

In Study-1, Na⁺ accumulation in the blade of recently fully expanded leaves at day 21 was 4 fold lower in CO39 (15 mM in leaf sap) than Moroberekan (64 mM) in the saline treatment (Table 1). Under salinity treatment, three out of 32 RILs had Na⁺ concentration higher than Moroberekan), whereas in 14 RILs the Na⁺ concentration was even lower than CO39, showing transgressive segregation towards CO39, the low Na⁺ parent variety (Suppl. Fig. 1b). The analysis of variance revealed that treatments, RILs and Salinity×RILs interaction had highly significant ($p<0.01$) effects on Na⁺ concentrations of the leaf blades (Supplemental Table 1). Leaf K⁺ was very much higher in CO39 than Moroborekan, being 363 mM versus 263 mM in the control treatment, and only a little less in the salt treatment (Table 1). Under salinity, two out of 32 RILs had K⁺ concentration higher than CO39, whereas, six RILs had values of K⁺ concentration less than Moroberekan, showing an approximate normal frequency distribution for the above trait (Suppl. Fig. 3a). There was a highly significant negative correlation of Na⁺ with leaf K⁺ concentration and K⁺/Na⁺ on day 21 of salt stress (Table 2). CO39 showed about a nine-fold greater K⁺/Na⁺ ratio than Moroberekan under salinity stress (Table 1). Eleven out of 32 RILs maintained values of K⁺/Na⁺ ratio even greater than the high parent CO39 (Suppl. Fig. 3b). Differences in the K⁺/Na⁺ ratio of expanded leaves were highly significant ($p<0.01$) in the case of treatments, RILs and Salinity×RILs interaction (Suppl. Table 1). In summary, there was a significant increase in leaf Na⁺ under salinity (Table 1) but the decrease in K⁺ was not significant. K⁺ concentrations increased in many RILs after salt stress.

In Study-2, both the leaf blade and leaf sheath Na⁺ concentrations were measured on day 7 and 21 of salt stress in the most recent fully expanded leaves. The leaf sheath had a much higher Na⁺ concentration than the leaf blade, especially at the later time of sampling of 21 days (Table 3, Fig. 4a). The increase over time of Na⁺ in the leaf blades was negligible for CO39 but large for Moroberekan (Fig. 4a). In the leaf sheath, the Na⁺ increased by 2-fold in CO39, and 3-fold in Moroberekan. K⁺ in the expanded leaf blades and sheaths was higher in CO39 than Moroberekan on 21 of salt stress (Table 3). On day 21 the K⁺ concentration in the leaf sheaths was even higher

in CO39 compared to its concentration on day 7 of salt stress (data not shown). With K^+/Na^+ ratios with increase in exposure time of salt stress from 7 to 21 days, there were reductions in both CO39 and Moroberekan; however the reductions were greater in Moroberekan than CO39 (Table 3 and Fig. 4b).

Correlations between ion accumulation, growth attributes and leaf injury

In Study-1, there was no correlation between leaf Na^+ measured on day 21 and injury scored on that day, but the correlation with injury scored on day 42 was highly significant ($r=0.689$) (Table 2, Fig. 2). This indicates that most of the leaf injury is due to accumulation of toxic concentrations of Na^+ , particularly in the fully expanded leaves. Leaf K^+ had a significant negative correlation with injury on day 42 ($r= -0.532$), suggesting that K^+ reduced the toxic effects of Na^+ by improving K^+/Na^+ ratio of leaves under salt stress (Table 1). Na^+ concentrations on day 21 were not significantly correlated with SFW, SDW, SFW%C or SDW%C, however, correlations with tillers $plant^{-1}$ and shoot water were significant (Table 2). Leaf K^+ showed positive and highly significant correlations with SFW, SDW and tillers $plant^{-1}$ but not when SFW or SDW was expressed as % of the controls (Table 2).

In Study-2, ion and injury measurements were made on day 7, as well as on day 21. There was no injury to leaves on day 7, injury started on day 21, and became severe on day 42 of salt stress, as shown in the photographs in Suppl. Fig. 2. Na^+ in the leaf blade on day 21 correlated with injury on that day ($r=0.35$) and more strongly with injury on day 42 ($r=0.71$). This indicates that Na^+ concentrations even when not yet toxic are predictive of subsequent injury. Likewise Na^+ on day 7 although low and not associated with injury on day 7 or day 21, was associated with injury on day 42 ($r=0.32$). Na^+ on day 7 was negatively correlated with all growth components, although the correlations were not as high as Na^+ on day 21 (Table 4). The correlation of K/Na with growth components was not as great as Na^+ (Table 4). It is therefore assumed that injury is mainly because of accumulation of Na^+ in leaves and had a significant effect on shoot fresh and dry weights under salinity stress.

Na^+ concentrations in the sheath had a similar relation to injury and growth components (Suppl. Table 2a; Fig. 7a) as did Na^+ in the leaf (Table 4). K^+ concentrations in the sheath at day 7 had similar relationships to growth components as did K^+ in the leaf, but on day 21 there were much

stronger and positive correlations with all growth components and stronger negative correlations with leaf injury (Suppl. Table 2a). The K^+/Na^+ ratio of leaf sheaths had a significant effect on the number of tillers, SPAD and SDW but not on SFW and shoot water on day 7 (Suppl. Table 2a; Fig. 7b). Similarly, the K^+/Na^+ ratio of leaf sheaths exerted significant and positive effects on all growth attributes except shoot water and leaf injury on day 21. The relationship was significant and negative with injury on day 42 (Suppl. Table 2a; Suppl. Fig.4b).

Discussion

Mechanisms of salt tolerance, and selectable traits

When plants are first exposed to salinity, the rapid reductions in growth are due to the osmotic effect of the salt concentration in the root medium which changes the water status of plants, and affects the young growing leaves via root signals. Later, the buildup of Na^+ to toxic levels in older leaves affects growth due to lack of photosynthate supply to growing tissues (Munns 1993; Munns 2002). This was described as a two-phase growth response to salinity (Munns 1993). In a prolonged study with various wheat genotypes differing in the ability to exclude Na^+ , differences in growth rates between genotypes were not observed until 20% of the leaves were injured, but by then growth rates were very much less than controls due to the osmotic effect of the salt. In the two experiments reported here, there was no sign of leaf injury by day 7, so Na^+ had not reached toxic levels by that time yet growth rates were already obviously severely reduced (data not shown). Only until day 21 in both studies was injury considerable, by which time biomass was already considerably affected. TANVEER – DID YOU HAVE OBSERVATIONS AT DAY 14???

The aim of this study was to compare different selectable traits for reliable detection of salinity tolerant lines of rice and in particular to quantify the relationships between Na^+ exclusion, leaf injury, and growth under salt stress. Plants would need to be grown for a long enough time to allow the accumulation of Na^+ to toxic concentrations to affect growth via reduction in photosynthate supply. This is especially difficult with large numbers of lines, and when comparing lines that differ in intrinsic biomass production in the absence of salinity. The CO39×Moroberekan RIL population was used alongside both parents. Although Moroberekan had a higher Na^+ accumulation, it was more vigorous and produced twice the biomass of CO39

over time – and so it was expected that there might be segregation for the several genes responsible for Na^+ exclusion with combinations of genes affecting dry weight production.

As a proxy for salt tolerance we measured the percent biomass production in saline versus non-saline control conditions after 42 days under 100 mM NaCl, and assessed the correlation of this trait with Na^+ and K^+ concentrations in leaves and with leaf injury for 32 RILs. For a larger number of RILs (120) we measured the biomass production and leaf injury in the presence of 100 mM NaCl (without controls – due to the unfeasibility of maintain plants growing at optimum growth rates for such a large number of genotypes) and assessed the relationship of leaf blade and sheath Na^+ and K^+ with injury. We also measured the tillers per plant⁻¹, chlorophyll concentration with a SPAD meter, and shoot water content on a dry weight basis. We have focused on recognizing traits which could be more useful for reliable selection from a large number of individuals with limited resources and time. We discuss the suitability of % SDW, % SFW, tillers plant⁻¹ as index of salt tolerance and a number of morpho-physiological traits (shoot water content, leaf injury scores, SPAD-chlorophyll, leaf blade and sheath Na^+ , K^+ and K^+/Na^+) for selecting salt tolerant germplasm in large populations of rice.

This study confirmed CO39 as a more salt tolerant rice cultivar than Moroberekan, and identified five of 32 RILs with salt tolerance (% control biomass) better than CO39. In addition, several RILs were shown in the second study as having even lower Na^+ accumulation than CO39, along with very high tillers plant⁻¹ and shoot biomass, indicating that these would have a greater salt tolerance (% control biomass).

Indica rice has the ability to exclude Na^+ to avoid toxic accumulation in leaves, and to absorb more K^+ to maintain the Na^+/K^+ balance in the shoot (Gregorio and Senadhira 1993). The salt tolerant Indica variety (CO39) in this study also has ability to exclude Na^+ from the shoot to a greater extent than the sensitive Japonica (Moroberekan) in which Na^+ levels were 4 fold more than those in Indica rice. Moreover, the tolerant Indica rice also had the ability to absorb more K^+ to maintain the Na^+/K^+ balance in the shoot.

The study showed that: Na^+ exclusion (low Na^+ accumulation in the leaf blades) was strongly associated with leaf injury, and is presumed to be the main cause of it. Na^+ accumulation was useful in identifying RILs with higher salt tolerance. Injury of top leaves was useful in the absence of growth rate measurements in relation to the growth in control conditions. However

1 there was no correlation between Na⁺ exclusion and growth even at day 42 in Study-1 although
2 there was in Study-2. Possibly if Study-1 was conducted for a longer period of time then the
3 same results as for Study-2 would have been found. It appears that plants might have grown
4 faster and differences in salt tolerance became more distinct in Study-2, as in Study 1 CO39
5 reached 17.5 g with 9 tillers under salt but in Study-2 it reached 29.5 g with 15 tillers. In
6 contrast, Moroborekan had the same tiller number (5) but a lower SFW in the second study
7 (Table 1). This illustrates the importance of experimental replication when assessing a
8 quantitative trait, as it is difficult to control all environmental variables within a glasshouse over
9 different seasons, and genotype x environment interactions are so strong.

10 Several earlier papers using a number of different rice cultivars or genotypes have shown a
11 correlation between genetic differences in the Na⁺ concentration in a leaf and salt tolerance as
12 expressed by % control biomass production (Haq *et al.* 2008), plant survival (Yeo and Flowers
13 1984; Yeo and Flowers 1986), or the degree of injury or leaf death under salinity stress (Yeo *et*
14 *al.* 1990; Gregorio *et al.* 1997).

15 The questions we addressed were: how useful are Na⁺ concentrations and their QTLs as traits for
16 selection for salt tolerance? What is the mechanism by which Na⁺ accumulation rate affects
17 growth rate and thereby yield? Can molecular markers for a QTL for Na⁺ be used to screen for
18 salt tolerance, in the absence of data on biomass production in saline versus non-saline
19 conditions?

20 To assess the level of leaf injury, scoring can be a useful and inexpensive indicator. The score
21 used here was 1-4, although 1-5 (Haq *et al.* 2010) and 1-9 (Platten *et al.* 2013) have been
22 successfully used. In the second experiment, chlorophyll concentration was measured with the
23 SPAD meter, which might be expected to give more precise quantitative results, but the
24 correlations between SPAD and the sensitive growth attributes and with Na⁺ were very similar to
25 those with injury (Table 4), suggesting that visual scores are a reliable substitute for chlorophyll
26 estimations.

27
28 ***What is causing the leaf injury?***

1 Significant positive correlations of Na⁺ with leaf injury scores in these studies indicates that Na⁺
2 buildup to a toxic level is the main cause of leaf senescence particularly of the older leaves at
3 later stages of salt stress. Lin *et al.* (2004) also correlate Na⁺ accumulation with leaf salt damage
4 under high salinity in rice.

5 On day 42 of salt stress there was large variation in salt injury to rice plants (Fig. 10a and 10b).
6 Moroberekan experienced more leaf injury than CO39 (Fig. 10a). This was most likely due to
7 toxic accumulation of Na in the leaf blade. In Study-1, most of the leaf damage was due to
8 accumulation of toxic levels of Na⁺ in the expanded leaf as indicated by the strong correlations
9 between Na⁺ and injury Score-2 (Fig. 3a and 9). The correlation of salinity-induced leaf injury
10 with whole-plant and leaf blade Na⁺ concentrations was high in nearly all accessions of *Oryza*
11 *sativa* and *Oryza glaberrima* (Platten *et al.* 2013).

12 There are two other explanations for the onset of leaf injury: that it was due to toxic
13 accumulation in the leaf sheath which reached a higher level first, or that it was due to the salt
14 around the roots ie osmotic stress.

15 Sheath Na⁺ concentrations were three times the leaf blade, and approached toxic concentrations
16 earlier in the leaf sheath. The high concentration of Na⁺ in sheath tissue than leaf blade of CO39
17 (salt tolerant variety) under salt stress may also be due to removal of Na⁺ from the transpiration
18 stream and its deposition in xylem parenchyma cells to reduce delivery into the shoots. Four
19 fold difference in Na⁺ accumulation between sheath and leaf blade in rice was also documented
20 by Yeo and Flowers (1982). The leaf sheath concentration was always higher on a tissue water
21 basis, and incidentally much higher again on a dry weight basis (the water of the sheath being
22 almost twice as high as the blade). It is possible that this leaf sheath concentration is responsible
23 for the onset of injury seen in the leaf blade. Platten et al (2013) found sheath:blade ratios of 2-3
24 depending on genotype. Based on the visual injury as seen in the photographs from Study-2,
25 when the conc. of Na⁺ in the expanded leaves remained below 20 mM and in sheath below 65
26 mM in about three weeks' time under salt stress there was no damage to leaves and leaves
27 remain health and green even after 42 days exposure. However when the Na⁺ in expanded leaves
28 accumulated above 50 mM and in sheath crossed 100 mM in about three weeks' time it caused a
29 significant damage to plant and injury was further increased with increase in Na⁺ accumulation.

Leaf injury might be promoted by the osmotic effects of the salt outside the roots, accelerating leaf senescence as it does water stress caused by dry soils. In Study-2, where Na^+ was measured at two times the correlation between injury and Na^+ of expanded leaves changed with time and it was higher on day 21 compared to correlation on day 7 of stress. This means under salt stress at least some time is required for Na^+ to accumulate in the expanded leaf and to affect growth, supporting the two-phase growth model under salinity put forward by Munns *et al* (1995). Therefore it can be assumed that much of the reduction in growth at earlier stage of stress may be due to osmotic and other effects.

Role of leaf K^+ in salt tolerance

It has been commonly observed that the accumulation of Na^+ in shoot tissues is often accompanied by a reduction in shoot K^+ concentrations, resulting in decreased K^+/Na^+ ratios (Asch *et al.* 2000). Salinity results in a reduction of leaf K^+ concentration, however, this reduction was less in CO39 compared with Moroberekan under salt stress. K^+ concentration was much higher (27%) in CO39, when compared with Moroberekan in non saline conditions (Table 1), and the K^+ was 28% higher in the leaves of CO39 than Moroberekan at 21 days of salt stress. Interestingly, In case of Study-2, again CO39 exhibited higher K^+ concentration in the sap of all tissue types sampled at both salt stresses, however, differences were more pronounced at 21 days than 7 days salt stress. This resulted in a higher K^+/Na^+ ratio in these studies (Suppl. Table 2). A good supply of K^+ to plants can minimize injurious effects of high Na^+ under salinity. Salt tolerant crop varieties also show special ability to maintain high K^+/Na^+ ratio in cell cytosol, when exposed to NaCl salinity (Carden *et al.* 2003; Golldack *et al.* 2003; Peng *et al.* 2004). Differences between susceptible Japonica and Indica rice types were highly significant for all growth parameters and shoot Na^+ but not for shoot K^+ concentration (Lee *et al.* 2003). Similarly, in Japonica rice shoot K^+ showed no relation to salt tolerance but tolerant Indica varieties maintained very high K^+ in the shoot (Lee and Senadhira 1996) supporting our results. Likewise, Akhtar (2002) also found negative and significant correlation between K^+ in the sap of leaf blade and SFW.

1 Partly the differences in measured Na⁺ ion concentrations were also caused by greater
2 dehydration of the leaves of Moroberekan, and this was almost entirely responsible for the
3 increase in leaf K⁺ concentrations (Akhtar 2002).

5 *Useful Morphological and Physiological traits for screening in rice*

6 The growth measurements or physiological traits depend on the objective of screening. If the
7 objective is to develop varieties for good yield on salt affected lands absolute yield or biomass at
8 anthesis is the appropriate criterion of selection. However, if the purpose of selection is to
9 identify the better sources of salt tolerance to further use in breeding programs by the breeders
10 then SDW % of control would be a more valuable index to use for screening against salinity.
11 However, this is not possible for large populations. Salt tolerance with a large population or
12 number of genotypes is not feasible because of the space required to grow plants in the control
13 treatment with enough space = light to give optimal growth and sufficient replications to produce
14 a meaningful statistical value. This study showed that for experiments when genotype numbers
15 preclude the growth in control conditions as well, and when growth in that condition varies
16 between parents and hence between genotypes, then tiller number and shoot water content are
17 sensitive traits. This of course would not be valid if the parents differed in tiller number in
18 control conditions. Then shoot water content, which reflects biomass as well as desiccation to
19 upper leaves caused by injury is valid. These traits are recommended as they reflect the growth
20 of the plant during the period of salinity and hence productive biomass that is related to yield.

21 However injury as a trait also has a place as it can be measured easily and non-destructively, on
22 the top 2-3 leaves either by visual scoring by chlorophyll concentration with a SPAD meter. It is
23 highly correlated with Na accumulation, and so is a useful trait for identifying QTLs for Na
24 exclusion. It also can be predictive on future biomass production.

25 An aspect which might have been neglected to some extent is the environmental factors during
26 the process of selection. The factors such as temperature, growth stage and humidity have wide
27 spread effects on salt tolerance of plants. They need to be considered as integral components of
28 the selection process. The use of marker assisted selection (MAS) also provides a great potential
29 for screening but depends on the availability of tightly linked markers having universal

applicability. The one major advantage of these markers over morpho-physiological markers is that they are not affected by the growth stage and the environmental conditions and are large in number.

In conclusion, this study confirmed that plants need to be grown for a lengthy period of time before genetic differences in salt tolerance appear, and before leaf injury is an indicator of salt tolerance. It showed that short-term exposure of even 21 days may not be enough to pick genetic differences, and that exposure of more than 42 days is required to get valuable information. We have also shown that much of the growth reduction, which was previously thought to be due to toxic ions, was due to the osmotic effects of the salt around the roots, supporting the two phase model of salt stress response. The study showed that high Na⁺ accumulation in the leaf blades was strongly associated with leaf injury and is presumed to be the main cause of it. Injury of top leaves was useful in predicting the ultimate salt tolerance of the RILs.

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Fig.1. (a) Frequency distribution for shoot fresh weight after 42 d in 100 mM NaCl as % of control for RIL population (n= 32) in Study-1. Normal curve for the distribution is also shown. The arrows indicate mean values for CO39 and Moroberekan. (b) Relationship between tiller's plant⁻¹ and SDW on day 42 in 100 mM NaCl. The red and blue circles indicate mean values for CO39 and Moroberekan respectively. r = Pearson's correlation coefficient (2- tailed)

Fig.2. Relationship of leaf injury (Score-2) measured on day 42 with leaf blade sap Na⁺ measured on day 21 of salt stress in Study-1. r = Pearson's correlation coefficient (2-tailed)

Fig.3. Relationships of leaf blade sap K/Na with tillers plant⁻¹ on day 21/42 of salt stress in Study-1. r = Pearson's correlation coefficient (2-tailed)

Fig.4. Change in (a) Na⁺ concentration in leaf blade and leaf sheath (b) K⁺/Na⁺ ratio of leaf blade and sheath sap on day 7 (open bars) and day 21 (black bars) in CO39 and Moroberekan under salt stress in Study-2 (Error bar shows SEM). Leaf and sheath K⁺ concentration values are in Table 3.

Fig.5. Relationship of (a) leaf blade sap Na^+ with Tillers plant⁻¹ and (b) Leaf blade sap K^+/Na^+ with tillers plant⁻¹ on day 21/42 of salt stress in Study-2. r = Pearson's correlation coefficient (2-tailed)

Fig. 6. Relationship of (a) SPAD (Chlorophyll concentration) with leaf blade sap Na^+ and (b) SPAD with leaf blade sap K^+/Na^+ on day 21/42 of salt stress in Study-2. r = Pearson's correlation coefficient (2-tailed)

Fig.7. Relationship of (a) SPAD (Chlorophyll concentration) with sheath sap Na^+ concentration and (b) SPAD with sheath sap K^+/Na^+ on day 21/42 of salt stress in Study-2. r = Pearson's correlation coefficient (2-tailed)

Table 1. Physiological and growth attributes of CO39, Moroberekan and population of 32 RILs grown in control (non-saline) conditions and in 100 mM NaCl for 42 days (Study-1). Growth was measured on day 42 for both control and saline treatments. For the saline treatment only, ions were measured on day 21 in the youngest fully expanded leaf, and injury scores measured on day 21 and day 42 on a scale of 1-4. Each value is mean of three replicates, except RILs where it is average of 102 plants

Traits	CO39		Moroberekan		RILs		RILs	
	Control	Saline	Control	Saline	Control	Range	Saline	Range
SFW (g)	42.1	17.5	89.8	19.5	70.6	22.8 – 185.5	21.1	8.7 – 32.5
SFW % of control	–	41.8	–	22.6	–	–	35.9	10.6 – 110.3
SDW (g)	6.8	4.3	15.2	4.6	12.6	3.79 – 35.1	5.1	2.0 – 9.0
SDW % of control	–	63.5	–	30.2	–	–	46.1	19.7 – 93.7
Tillers plant ⁻¹	11.7	9.0	12.0	4.67	11.7	3.0 – 28.0	9.4	3.0 – 18.0
Shoot water (g g ⁻¹ dw.)	5.2	3.1	4.9	3.2	4.8	3.0 – 6.8	3.2	2.1 – 4.1
Leaf Na ⁺ (mM) on day 21	1.4	14.6	1.1	63.9	1.2	0.9 – 3.0	30.5	2.7 – 136.2
Leaf K ⁺ (mM) on day 21	363	336	263	245	277	173 – 397	283	166 – 369
K ⁺ /Na ⁺ ratio on day 21	264.5	32.9	239.7	3.8	231.8	84.5 – 312.8	27.3	2.0 – 113.1
Score-1 on day 21	–	1.3	–	1.7	–	–	1.8	1.0 – 3.7
(Top leaves)								
Score-2 on day 42	–	2.0	–	2.7	–	–	2.5	1.0 – 4.0
(Top leaves)								

Table 2. Correlation matrix (Pearson's 2-tailed) for various traits of 32 RILs grown in saline (100 mM NaCl) conditions (Study-1).

Na, K and K/Na= Leaf Na, K and ratio on day 21, SFW= Shoot fresh weight (g), SDW= Shoot dry weight (g), Tiller= Tillers Plant⁻¹, SW g g⁻¹ dw = Shoot water on dry weight basis, Score-1 & 2 = Leaf injury on day 21 and 42 on top leaves

Traits	Na ⁺	K ⁺	K ⁺ /Na ⁺	SFW	SDW	SDW%C	Tiller	SW g g ⁻¹	SFW%C	Score -1	Score -2
K ⁺	-0.415**	1.0									
K ⁺ /Na ⁺	-0.652**	0.519**	1.0								
SFW	-0.093 ^{NS}	0.346**	0.154 ^{NS}	1.0							
SDW	0.018 ^{NS}	0.372**	0.146 ^{NS}	0.938**	1.0						
SDW%C	-0.145 ^{NS}	0.096 ^{NS}	0.019 ^{NS}	0.362**	0.246*	1.0					
Tillers	-0.224*	0.520**	0.489**	0.610**	0.667**	0.288**	1.0				
SW g g ⁻¹ DW	-0.274**	-0.150 ^{NS}	-0.021 ^{NS}	-0.065 ^{NS}	-0.398**	0.199*	-0.333**	1.0			
SFW%C	-0.170 ^{NS}	0.079 ^{NS}	0.032 ^{NS}	0.395**	0.250*	0.983**	0.266**	0.273**	1.0		
Score -1	0.130 ^{NS}	0.012 ^{NS}	0.071 ^{NS}	0.095 ^{NS}	0.184 ^{NS}	-0.013 ^{NS}	-0.252*	-0.295**	-0.091 ^{NS}	1.0	
Score -2	0.689**	-0.532**	-0.638**	-0.307**	-0.191 ^{NS}	-0.231*	-0.332**	-0.271**	-0.242*	0.162 ^{NS}	1.0

*, ** = Correlation was significant at ≤ 0.05 and 0.01 levels, respectively, NS = non-significant

Table 3. Physiological and growth attributes of CO39, Moroberekan and population of 120 RILs grown in 100 mM NaCl for 42 days (Study-2).

Ions are given on day 21 in the youngest fully expanded leaf, and injury scores on day 21 and day 42 on a scale of 1-4. Each value is mean of two replicates, except RILs where it is average of 240 plants.

Traits	CO39	Moroberekan	RILs	RILs range
<i>Growth, ions and injury on day 21/42 of salt stress</i>				
Shoot fresh weight (g) on day 42	29.5 ± 1.16	11.04 ± 1.78	24.07 ± 0.61	9.6 – 40.9
Shoot dry weight (g) on day 42	7.78 ± 0.08	3.81 ± 0.12	6.47 ± 0.15	3.3 – 10.8
Tillers plant ⁻¹ on day 42	15.0 ± 1.15	5.0 ± 0.58	10.14 ± 0.28	4.5 – 16.5
SPAD on day 42	33.13 ± 0.32	13.33 ± 0.88	27.80 ± 0.63	10 – 41.8
Shoot water (g g ⁻¹ dw.)	2.80 ± 0.18	1.89 ± 0.42	2.71 ± 0.04	1.42 – 3.61
Na ⁺ in leaf blade	7.9 ± 2.1	67.4 ± 15.8	26.2 ± 3.0	2.1 – 159.4
Na ⁺ in sheath	57.6 ± 10.5	129.7 ± 15.2	75.3 ± 3.9	10.6 – 208.5
K ⁺ in leaf blade	317 ± 19	264 ± 30	298 ± 3	194 – 401
K ⁺ in sheath	303 ± 35	208 ± 15	258 ± 4	154 – 355
K ⁺ /Na ⁺ in leaf blade	47.5 ± 14.6	4.2 ± 0.6	36.2 ± 3.0	1.8 – 154.2
K ⁺ /Na ⁺ in sheath	5.7 ± 1.2	1.65 ± 0.2	6.6 ± 0.7	1.0 – 26.7
Injury Score-1 on day 21	1.4 ± 0.3	3.7 ± 0.5	1.7 ± 0.1	1.0 – 3.8
Injury Score-2 on day 42	2.4 ± 0.3	3.9 ± 0.0	2.3 ± 0.1	1.0 – 4.0

Each mean value is average of 2 repeats ± SEM, except RILs where it is average of 102/240 plants

Table 4. Correlation matrix for ion accumulation in leaf blade and sheath for 120 RILs on day 7 and 21, injury scores on day 21 (Score-1) and day 42 (Score-2) and growth traits on day 42 under salts stress (100 mM NaCl) (Study-2)

	LNa7	LK7	LK/Na7	LNa21	LK21	LK/Na21	Tillers	SPAD	SFW	SDW	WC g/g	Score-1
LNa7	1.0											
LK 7	-0.25**	1.0										
LK/Na7	-0.67**	0.40**	1.0									
LNa21	0.47**	-0.30**	-0.43**	1.0								
LK21	-0.01 ^{NS}	0.50**	0.06 ^{NS}	-0.19*	1.0							
LK/Na21	-0.47**	0.27**	0.73**	-0.57**	-0.09 ^{NS}	1.0						
Tillers	-0.24**	0.35**	0.25*	-0.56**	0.07 ^{NS}	0.47**	1.0					
SPAD	-0.32**	0.10 ^{NS}	0.27**	-0.72**	0.30**	0.44**	0.48**	1.0				
SFW	-0.27**	0.28**	0.19 ^{NS}	-0.63**	-0.13 ^{NS}	0.40**	0.62**	0.67**	1.0			
SDW	-0.21*	0.33**	0.22*	-0.48**	-0.08 ^{NS}	0.44**	0.62**	0.53**	0.91**	1.0		
SW g/g	-0.30**	0.02 ^{NS}	0.07 ^{NS}	-0.60**	-0.14 ^{NS}	0.12 ^{NS}	0.23**	0.57**	0.52**	0.17 ^{NS}	1.0	
Score-1	0.08 ^{NS}	0.06 ^{NS}	0.06 ^{NS}	0.35**	0.12 ^{NS}	-0.14 ^{NS}	-0.42**	-0.54**	-0.38**	-0.28**	-0.38**	1.0
Score-2	0.32**	-0.17 ^{NS}	-0.27**	0.71**	0.27*	-0.46**	-0.55**	-0.87**	-0.72**	-0.57**	-0.54**	0.55**

*, ** = Pearson correlation (2 tailed) significant ≤ 0.05 and 0.01 levels, respectively

NS = Non significant

LK7 = Leaf K⁺ concentration on day 7

LK/Na7 = Leaf K⁺/Na⁺ ratio on day 7

L Na21 = Leaf Na⁺ concentration on day 21

LK21 = Leaf K⁺ concentration on day 21

LK/Na21 = Leaf K⁺/Na⁺ ratio on day21

Supplementary Table 1

Mean squares of different traits studied in CO39×Moroborekan RILs (n=34) on day 21 (ions) or day 42 (growth) in saline and non-saline conditions (Study-1).

SOV	D.F	Leaf Na ⁺	Leaf K ⁺	K ⁺ /Na ⁺	SFW	SDW	Tillers Plant ⁻¹	SW g g ⁻¹ dw.
Salinity	1	43610.2**	2993.8**	2118399.1**	124715.3**	2835.1**	241.5**	128.8**
RILs	33	1089.4**	6307.4**	5292.8**	1408.8**	61.7**	71.9**	0.92**
Salinity×RILs	33	1079.5**	1654.9**	1523.1**	1203.9**	43.5**	21.8**	0.22 ^{NS}

*, ** = Means were significant at ≤ 0.05 and 0.01 levels, respectively

NS = Means difference was non-significant

Supplementary Table-2a

Correlation matrix for ion accumulation in the leaf sheath on day 7/21, growth traits on day 42 and injury scores on day 21 (Score-1) or day 42 (Score-2) under salt stress (100 mM NaCl) (Study-2)

	SNa7	SK7	SK/Na7	SNa21	SK21	SK/Na21
SNa7	1.0					
SK7	-0.31**	1.0				
SK/Na7	-0.71**	0.38**	1.0			
SNa21	0.64**	-0.28**	-0.55**	1.0		
SK21	-0.50**	0.34**	0.36**	-0.75**	1.0	
SK/Na21	-0.53**	0.30**	0.77**	-0.68**	0.52**	1.0
Tillers	-0.29**	0.28**	0.28**	-0.56**	0.64**	0.35**
SPAD	-0.31**	0.07 ^{NS}	0.29**	-0.65**	0.49**	0.32**
SFW	-0.33**	0.19 ^{NS}	0.17 ^{NS}	-0.59**	0.60**	0.38**
SDW	-0.32**	0.25*	0.27**	-0.53**	0.54**	0.47**
SW g/g	-0.19 ^{NS}	-0.02 ^{NS}	-0.08 ^{NS}	-0.39**	0.37**	0.01 ^{NS}
Score-1	0.14 ^{NS}	0.20*	-0.04 ^{NS}	0.26**	-0.27**	0.01 ^{NS}
Score-2	0.31**	-0.13 ^{NS}	-0.25**	0.66**	-0.51**	-0.28**

SNa7 = Sheath Na⁺ concentration on day 7

SK7 = Sheath K⁺ concentration on day 7

SK/Na7 = Sheath K⁺/Na⁺ ratio on day 7

SNa21 = Sheath Na⁺ concentration on day 21

SK21 = Sheath K⁺ concentration on day 21

SK/Na21 = Sheath K⁺/Na⁺ ratio on day 21

Supplementary. Table-2b

Correlation matrix for ion accumulation in the leaf blade versus the leaf sheath on day 7/21, under salt stress (100 mM NaCl) (Study-2)

	LNa7	LK7	LK/Na7	LNa21	LK21	LK/Na21	SNa7	SK7	SK/Na7	SNa21	SK21
LK 7	-0.25**	1.0									
LK/Na7	-0.67**	0.40**	1.0								
LNa21	0.47**	-0.30**	-0.43**	1.0							
LK21	-0.01 ^{NS}	0.50**	0.06 ^{NS}	0.19*	1.0						
LK/Na21	-0.47**	0.27**	0.73**	-0.57**	-0.09 ^{NS}	1.0					
SNa7	0.80**	-0.19**	-0.76**	0.43**	0.09 ^{NS}	-0.57**	1.0				
SK7	-0.36**	0.65**	0.55**	-0.30**	0.29**	0.40**	-0.31**	1.0			
SK/Na7	-0.46**	0.24*	0.75**	-0.34**	-0.10 ^{NS}	0.67**	-0.71**	0.38**	1.0		
SNa21	0.57**	-0.18 ^{NS}	-0.59**	0.79**	0.24**	-0.77**	0.64**	-0.28**	-0.55**	1.0	
SK21	-0.47**	0.31**	0.47**	-0.71**	0.02 ^{NS}	0.58**	-0.50**	0.34**	0.36**	-0.75**	1.0
SK/Na21	-0.38**	0.15 ^{NS}	0.60**	-0.40**	-0.16 ^{NS}	0.78**	-0.53**	0.30**	0.77**	-0.68**	0.52**

LK7 = Leaf K⁺ concentration on day 7

LK/Na7 = Leaf K⁺/Na⁺ ratio on day 7

L Na21 = Leaf Na⁺ concentration on day 21

LK21 = Leaf K⁺ concentration on day 21

LK/Na21 = Leaf K⁺/Na⁺ ratio on day 21

Suppl. Fig. 1. (a) Frequency distribution for shoot dry weight and (b) leaf blade Na⁺ under salt stress in RIL population (n = 32) in Study-1. The arrows indicate mean values for CO39 and Moroberekan

Suppl Fig. 2. (a) Frequency distribution for leaf blade K^+ and (b) leaf blade K^+/Na^+ in RIL population ($n = 32$) under saline treatment in Study-1. The arrows indicate mean values for CO39 and Moroberekan

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Suppl Fig. 3. Relationships of (a) SDW % of control with leaf blade Na⁺ in study-1 and (b) Shoot water with leaf blade Na⁺ in Study-2 on day 21/42 of salt stress. r = Pearson's correlation coefficient (2-tailed)

Suppl Fig. 4. Relationship of (a) Leaf injury Score-2 with Na^+ concentration in sap of leaf sheath and (b) Score-2 with K^+/Na^+ in sheath on day 21/42 of salt stress in Study-2. r = Pearson's correlation coefficient (2-tailed)

Supplemental Fig.8 Relationship of (a) SPAD (Chlorophyll) with Tillers plant⁻¹ and (b) SPAD with SDW (g) on day 21/42 of salt stress in Study-2. r = Pearson's correlation coefficient (2-tailed)

Supplemental Fig.9 ???. Relationship of leaf blade Na⁺ concentration with Score-2 on day 21/42 of salt stress in Study-2. r = Pearson's correlation coefficient (2-tailed)

a
b

Fig.10. Differential salinity leaf damage in (a) parent varieties (CO39 & Moroberekan) and (b) randomly selected two RILs after 42 days growth under salt stress in Study-2